A Simple Colorimetric Assay for Sensitive Cu\(^{2+}\) Detection Based on the Glutathione-Mediated Etching of MnO\(_2\) Nanosheets

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In this paper, we developed a quick, economical and sensitive colorimetric strategy for copper ions (Cu\(^{2+}\)) quantification via the redox response of MnO\(_2\) nanosheets with glutathione (GSH). This reaction consumed MnO\(_2\) nanosheets, which acted as a catalyst for the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to a blue product (oxTMB). In the presence of Cu\(^{2+}\), the GSH was catalyzed to GSSG (oxidized glutathione), and the solution changed from colorless to deep blue. Under the optimum conditions, the absorption signal of the oxidized product (oxTMB) became proportional to Cu\(^{2+}\) concentration in the range from 10 to 300 nM with a detection limit of 6.9 nM. This detection system showed high specificity for Cu\(^{2+}\). Moreover, the system has been efficaciously implemented for Cu\(^{2+}\) detection in actual tap water samples. The layered-nanostructures of MnO\(_2\) nanosheets make it possess high chemical and thermal stability. TMB can be quickly oxidized within 10 min by the catalyzing of MnO\(_2\) nanosheets with high oxidase-like activity. There is no need of expensive reagents, additional H\(_2\)O\(_2\) and complicated modification processes during the colorimetric assay. Therefore, the strategy primarily based on MnO\(_2\) nanosheets is promising for real-time, rapid and highly sensitive detection of Cu\(^{2+}\) under practical conditions.

Keywords: colorimetric system, MnO\(_2\) mimetic enzyme, copper ions, rapid detection, water sample

INTRODUCTION

Copper is an essential microelement for the human body and an important component of human proteins and enzymes. Lack of copper ions (Cu\(^{2+}\)) will hinder the physiological activities of human body and easily cause various diseases (Scheiber et al., 2013; Chowdhury et al., 2018). On the other hand, copper is a heavy metal widely discovered in the environment, and excessive copper will produce severe toxic effects on humans, plants and microorganisms (Lee et al., 2016; Zhou et al., 2020). With the extensive utility of copper in industry and agriculture, it has become one of the main pollutants of social concern. Therefore, developing sensitive strategies to detect Cu\(^{2+}\) in environmental and biological samples is essential.

The detection techniques of Cu\(^{2+}\) have been greatly developed, such as atomic absorption spectroscopy (AAS) (Lima et al., 2012), inductively coupled plasma mass spectrometry (ICP-MS) (Dai et al., 2012; Khan et al., 2014), electrochemical techniques (Flavel et al., 2011; Zhu et al., 2017), fluorescence methods (Lan et al., 2010; Zhang et al., 2014; Zhang et al., 2020) and colorimetric assays...
Some of these methods suffer from the drawbacks of requirements for large instruments, special operators, complex sample pretreatment process, or poor reproducibility and reliability, which limits their on-side rapid detection and practical application (Du et al., 2014; Wang et al., 2019a). Among them, colorimetric analysis is concerned because of its simple and quick operation, low cost, visualization and no need for professional operators, which makes it widely applied.

Colorimetric assays are generally based on peroxidase or some nanomaterials as the mimic enzyme to initiate chromogenic reactions that produce colored compounds (Jangi et al., 2020; Wang et al., 2020; Xia et al., 2021). Many chromogenic reactions are carried out via catalyzing the peroxidase substrates, usually TMB or 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Tang et al., 2016). These reactions generally need additional hydrogen peroxide as the oxidant, which complicates the operation and increase the background signal. Strict time-controlling is necessary to improve the signal to noise ratio. There is also some other special color reactions, such as the etching of gold nanorods showing various color changes (Lin et al., 2016), the pink-blue transition caused by the aggregation of gold nanoparticles (Deng et al., 2013), and other methods generating colored compounds (Yin et al., 2015). Several colorimetric assays based on the nanomaterials of gold, silver and platinum nanoparticle have been developed for sensitive detection of Cu$^{2+}$ (Ma et al., 2011; Le et al., 2014; Wang et al., 2017). The scarcity, high cost and easily poisoned of these precious noble metals, have hindered their large-scale use in practical colorimetric system (Kuo et al., 2014; Asif et al., 2017b). As a result, the creation of more economical and environment-friendly materials for colorimetric detection of Cu$^{2+}$ is of significant importance.

In current years, some non-precious metals oxides-based nanosheets and nanosphere has been synthesized for electrochemical detection of various pollutants, such as MnO$_2$, CuO and graphene (Asif et al., 2017a; Asif et al., 2017b; Asif et al., 2018; Asif et al., 2019a; Aziz et al., 2019). MnO$_2$ nanosheets (NSs) have obtained high-level interest as a brand new type of two-dimensional (2D) nanomaterial (Chen et al., 2019). MnO$_2$ nanosheet is a kind of graphene-like nanomaterial, which can be used as a DNA nanocarrier to construct ratiometric fluorescence biosensors to detect miRNA and living cell imaging (Wang et al., 2019a). Due to its large particular sensing area, high chemical durability and inherent oxidative enzyme mimic activity, MnO$_2$ NSs are widely utilized in biosensors. The strong oxidation ability of MnO$_2$ NSs makes it great potential to constitute a colorimetric strategy for practical applications. Also, MnO$_2$ has excellent ion exchange and redox capabilities that can be widely used in supercapacitors, batteries and catalysis. It has been identified as the most promising electrode material for electrochemical energy storage systems, depending on its high density, high purity and sufficient electrochemical activity (Guo et al., 2019). some MnO$_2$ NSs based material has been synthesized for photoelectrochemical detection of Cu$^{2+}$ (Hammami et al., 2021), electrochemiluminescence detection of glutathione (Gao et al., 2016), fluorescence detection of Fe$^{2+}$ (Jiang et al., 2023), ascorbic acid (Xu et al., 2017), and glutathione (Wang et al., 2016), colorimetric detect of glutathione (Ge et al., 2019), acetylcholinesterase (Yan et al., 2017) and chlorothalnonil (Sheng et al., 2020). Nevertheless, the colorimetric assays based on MnO$_2$ NSs for Cu$^{2+}$ detection are very rare.

Herein, based on the catalytic oxidation activity of MnO$_2$ NSs, an easy, speedy and economic colorimetric assay is established for the sensitive determination of Cu$^{2+}$. TMB can be quickly oxidized by MnO$_2$ NSs to form blue oxTMB without the need for H$_2$O$_2$. After interaction with reduced glutathione (GSH), the MnO$_2$ NSs are dissolved, which reduce the formation amount of blue oxTMB, so the mixture remains colorless. When Cu$^{2+}$ ions are introduced into the reaction mixture, Cu$^{2+}$ catalyzes the formation of GSSG (oxidized glutathione) from GSH and thus inhibits the decomposition of MnO$_2$ NSs. As a result, the color of the mixture changes from colorless to blue. According to these results, a simple colorimetric method for Cu$^{2+}$ was established. Moreover, this method has high sensitivity and selectivity for the determination of Cu$^{2+}$. The applicability of the assay in practical samples was also investigated.

**METHODS AND MATERIALS**

**Materials and Reagents**

Tetramethylammonium hydroxide (TMAOH), cysteine (Cys), ascorbic acid (AA), uric acid (UA) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Bovine serum albumin (BSA) and L-glutathione (GSH) were received from Sigma-Aldrich (St. Louis, Missouri, United States ). Manganese chloride tetrahydrate (MnCl$_2$·4H$_2$O), hydrogen peroxide (H$_2$O$_2$, 30 wt%), sodium hydroxide (NaOH), copper sulfate (CuSO$_4$·5H$_2$O), acetic acid (HAc), and ethanol were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). TMB substrate solution was obtained from Beyotime Biotechnology (Shanghai, China). All of the reagents used were analytical grades and were used directly (without further treatment). All the solutions were prepared from ultrapure water produced by a Milli-Q system.

**Apparatus and Instrumentation**

UV-Vis absorption spectra had been measured on the Cary-300 UV-Vis spectrophotometer (Agilent, United States ). X-ray photoelectron spectroscopy (XPS) spectra were measured on the ESCALAB MKIIx-ray photoelectron spectrometer (Thermo Fisher Scientific, United States ). Morphology of MnO$_2$ NSs was analyzed by Nova NanoSEM 230 field-emission scanning electron microscopy (FEI, United States ). Transmission electron microscope (TEM) photographs of MnO$_2$ NSs were obtained on the JEM-2100 transmission electron microscope (JEOL, Japan). The level of metal ions in practical water samples was evaluated by an X SERIES II inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Fisher Scientific, United States ).

**Synthesis of MnO$_2$ NSs**

MnO$_2$ NSs were prepared according to the previous literature (Liu et al., 2017). Briefly, the mixture composed of 4.4 mL (25 wt
concentrations of Cu\textsuperscript{2+} solutions and reacted for 5 min. Then, 0.5 mM GSH solution was mixed with 40 μHg\textsuperscript{2+}, and Ag\textsuperscript{+}. 1.0 water and waste water samples.

oxidation product (oxTMB) from colorless substrate TMB, which concentration of other metal ions was 10 M, pH quickly added into 10 mL MnCl\textsubscript{2}.4H\textsubscript{2}O solution (0.3 M) within further use.

take away particulate impurity. Then different concentrations of Frontiers in Chemistry | www.frontiersin.org December 2021 | Volume 9 | Article 812503

RESULTS AND DISCUSSION

The Principle of Colorimetric Determination of Cu\textsuperscript{2+}

The strategy of Cu\textsuperscript{2+} colorimetric assay based on MnO\textsubscript{2} NSs is described in Scheme 1. Due to its intrinsic oxidase-like activity, the synthesized MnO\textsubscript{2} NSs can catalyze the formation of blue oxidation product (oxTMB) from colorless substrate TMB, which possesses an absorption peak at 650 nm. No additional H\textsubscript{2}O\textsubscript{2} is added during this process. As an antioxidant, GSH can react with MnO\textsubscript{2} NSs in the way shown in Eq. 1, resulting in the reduction and decomposition of MnO\textsubscript{2} NSs. The released Mn\textsuperscript{2+} loses the catalytic ability, thus the chromogenic reaction of TMB is inhibited. In the presence of Cu\textsuperscript{2+}, Cu\textsuperscript{2+} can catalyze GSH to GSSG (Tang et al., 2016), resulting in less decomposition of MnO\textsubscript{2} NSs and more generation of oxTMB. As a result, the color of the solution turns blue. Cu\textsuperscript{2+} concentration is linearly related to the absorption value of TMB oxidation products. Thus, a sensitive “turn on” Cu\textsuperscript{2+} detection scheme is established.

\[
\text{MnO}_2 + 2\text{GSH} + 2\text{H}^+ \rightarrow \text{Mn}^{2+} + \text{GSSG} + 2\text{H}_2\text{O} \quad (1)
\]

Feasibility of the Cu\textsuperscript{2+} Colorimetric Assay

Some control experiments were performed to confirm the feasibility of the colorimetric assay. As shown in Figure 1A, the characteristic peak (380 nm) of MnO\textsubscript{2} NSs gradually decreased with the increasing GSH concentration, indicating that MnO\textsubscript{2} NSs have been decomposed by GSH. Without GSH, the TMB oxidation process can be promoted by MnO\textsubscript{2} NSs, generating colored solution and significant absorption signal (Figure 1B, curve a). After interaction with GSH, the MnO\textsubscript{2} NSs was decomposed, and TMB oxidation was inhibited, which resulted in colorless solution and a great decrease of absorption signal (Figure 1B, curve b). Through the catalysis of Cu\textsuperscript{2+}, GSH was oxidized to GSSG, which inhibited MnO\textsubscript{2} NSs dissociation. Thus the absorption signal was enhanced (Figure 1B, curve c). According to these results, we concluded that Cu\textsuperscript{2+} could be quantified by measuring the absorption values of TMB oxidation products at 650 nm.

Characterization of MnO\textsubscript{2} NSs

After interaction with TMAOH, Mn\textsuperscript{2+} was oxidized to Mn\textsuperscript{4+} by H\textsubscript{2}O\textsubscript{2} to form bulk MnO\textsubscript{2}. By treating it with BSA under ultrasonication, MnO\textsubscript{2} NSs with good dispersibility, biocompatibility and high peroxidase activity were obtained, as previously described (Liu et al., 2012). The morphology of MnO\textsubscript{2} NSs was analyzed by scanning electron microscopy (SEM). As displayed in Supplementary Figure S1, the obtained MnO\textsubscript{2} NSs presented an obvious sheet-like morphology and well dispersed in water. As the TEM image (Figure 2A) shown, the obtained MnO\textsubscript{2} NSs exhibit characteristic 2-D layered architecture with partial folds/wrinkles, which contribute to the large specific surface area and good dispersibility.

The UV-Vis absorption spectra in Figure 2B shows that MnO\textsubscript{2} NSs have a wide absorption spectra in the 250–500 nm range with a maximum absorption band at 380 nm, which can be ascribed to a d-d jump of Mn\textsuperscript{4+}. This is in agreement with the optical properties of MnO\textsubscript{2} NSs reported previously (Liu et al., 2012). Furthermore, with the increase of MnO\textsubscript{2} NSs, the absorption intensity at 380 nm increased.

Chemical and elemental compositions of MnO\textsubscript{2} NSs were analyzed by XPS. The wide scan spectrum results are presented in Figure 2C. Four main peaks centered at 647.2, 528.7, 401.6 and 284.7 eV can be ascribed to Mn 2p, O 1s, N 1s and C 1s (Liu et al., 2012; Asif et al., 2019b). Figure 3 shows the high-resolution XPS spectra of Mn 2p and O 1s. From Figure 3A, two strong characteristic peaks located at 652.9 and 641.2 eV emerge in the Mn 2p\textsubscript{1/2} and Mn 2p\textsubscript{3/2} core-level spectra, corresponding to the presence of Mn\textsuperscript{4+} moieties (Itihäär et al., 2021). In Figure 3B, the peak at 528.7 eV is caused by O 1s. The O 1s
spectrum contains two sub energy states in Figure 3B and these peaks can be assigned to Mn–O–Mn and Mn–O–H (Liu et al., 2012; Asif et al., 2022; Aziz et al., 2022). This indicates that the acquisition of pure MnO₂ and the oxidation valence of Mn is +4 (Saha and Pal, 2014). These results indicate the successful synthesis of MnO₂ NSs.

**Optimization of the Experimental Conditions**

In order to improve the sensitivity of the Cu²⁺ detection system, we further optimized some important experimental conditions, including pH, reaction temperature, the dosage of TMB substrate solution and GSH concentration. At first, the optimal pH of the reaction system was studied. HAc-NaAc buffer solutions with different pH were added to the reaction system. Supplementary Figure S2 shows the absorption values at different pH in the presence and absence of Cu²⁺. In the presence of 250 nM Cu²⁺, the absorption increment increases sharply in the pH range of 3.5–5.0. While the pH reaches 5.5, the increment of absorption decreases instead. As the pH continues to rise, the absorption increment returns to a slow upward trend. It is determined that the absorption increment is maximal when the pH value is 5.0. Therefore, the pH was selected at 5.0 for further test.

We also studied the influence of temperature on Cu²⁺ sensing systems. The absorption values at different temperatures in the presence and absence of Cu²⁺ are shown in Supplementary Figure S3. In the range of 25–45°C, the increase of temperature is beneficial to the reaction, and the absorbance difference of the final solution presents a steady rise trend. When the temperature reaches 45°C, the absorbance difference reached the maximum. Once the temperature is above 45°C, the temperature has a negative impact on this system. In order to obtain good experimental results, the colorimetric Cu²⁺ detection was carried out at the temperature of 45°C.

Subsequently, it was found that the amount of TMB substrate solution was related to the absorption intensity of the reaction solution at 650 nm. As Supplementary Figure S4 shown, the absorption value gradually increases with the increase of TMB amount and tends to stable when the amount of TMB is more than 100 μL. Therefore, the dosage of TMB substrate solution was selected as 100 μL.

The concentration of GSH used to decompose MnO₂ NSs is critical. In order to achieve higher detection sensitivity, it was also optimized. As shown in Supplementary Figure S5A, the absorption value of the solution gradually decreases with the increase of GSH concentration in the reaction system. The solution color also changes from blue to colorless. These results indicated that MnO₂ NSs were decomposed into Mn²⁺ by GSH, which inhibited the production of blue oxTMB. Meanwhile, there was a good linear relationship between the absorbance and the concentration of GSH from 0 to 18.75 μM (Supplementary Figure S5B). Considering the sensitivity and
practical conditions of the system, the colorimetric assay was performed by selecting 17.5 μM GSH.

**Sensitivity, Reproducibility and Stability Investigation of Cu²⁺ Assay**

The sensitivity of the colorimetric assay was verified by adding different concentrations of Cu²⁺ to the solution at the optimal conditions. The recorded UV-vis absorption spectra of the system and the calibration curve established by absorption values of different concentrations of Cu²⁺ at 650 nm are presented in Figure 4. The results showed that with the increase of Cu²⁺ concentration, absorption value exhibited a rising trend and the color of the solution became deeper (Figure 4A). The absorption value has a good linear relationship with Cu²⁺ concentration from 10 to 300 nM, and
the correlation coefficient is 0.997 (Figure 4B). Cu$^{2+}$ detection limit (LOD) was estimated to about 6.9 nM (3σ). Because of the hazardous effects of high levels of Cu$^{2+}$, the US Environmental Protection Agency stipulates that the maximum level of Cu$^{2+}$ allowed in drinking water was about 20 μM (U.S.E.P. Agency, 2012). Thus, this colorimetric assay is suitable for the detection of low concentration Cu$^{2+}$ in the environment.

Compared with other reported methods in Table 1, this strategy could achieve a lower detection limit and a wider detection range. Besides, the characteristic of the novel colorimetric assay, such as simple operation (no additional H$_2$O$_2$ was required), low toxicity, and low cost (without the need of precious metal or nucleic acid), makes it great potential in high sensitivity and rapid test of Cu$^{2+}$ in drinking water and environment.

The reproducibility of the colorimetric sensor was evaluated by intra-assay. Five parallel measurements have been performed to detect 100 nM Cu$^{2+}$ at the same conditions. The obtained relative standard deviation (RSD) was calculated to be 2.9%. The long-term stability of the colorimetric sensor was also investigated. The MnO$_2$ NSs were stored in dark under 4°C when it is not used. After stored for more than 1 month, the Cu$^{2+}$ induced absorption response could retain 96% of its original value. These results indicate that the colorimetric sensor shows good repeatability and stability.

**Interference Study**

To investigate the specificity of colorimetric determination of Cu$^{2+}$, series interference experiments were also carried out. Various metal ions including Fe$^{3+}$, Ni$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, K$^+$, Zn$^{2+}$, Mn$^{2+}$, Pb$^{2+}$, Na$^+$, Hg$^{2+}$, Ag$^+$ and common compounds with redox properties were studied. Under the same conditions, 1.0 μM Cu$^{2+}$ or other metal ions with ten times the concentration of Cu$^{2+}$ were added to the system to collect the absorption signal. Although Ag$^+$ and Hg$^{2+}$ could give rise to measurable absorbance signals (Supplementary Figure S6) in the concentration of 10 μM, the interference is small when they are at the same concentration as Cu$^{2+}$ (1.0 μM) (Supplementary Figure S7), and they will not significantly affect the detection of Cu$^{2+}$ at this concentration in the environmental system. In addition, KBr (1.25 mM) and KCl (12.5 mM) were used as masking agents to eliminate the interference of Hg$^{2+}$ and Ag$^+$. It could be seen in Figure 5 that with the coexistence of KBr and KCl, the colorimetric assay showed high specificity for Cu$^{2+}$. At the same time, other metal ions with concentration ten times of Cu$^{2+}$ made no difference in the sensitivity of Cu$^{2+}$ analysis. Common redox substances, such as 100 μM of Cys, AA, UA and 1.0 mg/mL of BSA had no significant effect on the detection of Cu$^{2+}$ (Supplementary Figure S7). It is worth noting that the selectivity can be observed with the naked eye. Hence, the specific detection of Cu$^{2+}$ can be achieved by the established colorimetric assay.

**Detection of Cu$^{2+}$ in Real Samples**

To verify the applicability of this method in practical samples and complex sample matrix, Cu$^{2+}$ spiked tap water (collected from the laboratory) and waste water (collected from a point source suspected to discharge metal ions by Longyan Rare Earths LTD.) were prepared for the test. The practical samples were settled overnight at room temperature, and then the impurities were removed by filtration with a 0.45 μm filter membrane. For comparison, ICP-MS was also used to evaluate the Cu$^{2+}$ level in these samples. The initial concentration of Cu$^{2+}$ detected by ICP-MS in tap water and waste water is 3.5 and 235.2 nM, respectively. The analytical performances of the proposed colorimetric assay for the detection of Cu$^{2+}$ from waste water is in good agreement with the results obtained by ICP-MS with RSD value of 1.74% (Supplementary Table S1). This result indicated the good accuracy of the proposed colorimetric assay for the analysis of Cu$^{2+}$ in real samples. After dilution, series of Cu$^{2+}$ with different concentrations were added into the pretreated water samples and detected by this colorimetric method. The detection results are listed in Supplementary Table S1. The recovery of standard Cu$^{2+}$ spiked samples is between 95.2 and 105.7%, with RSD $< 3.24\%$ (n = 3). The results illustrate that this colorimetric method is promising for applying efficient quantitative detection of Cu$^{2+}$ in actual samples with high sensitivity.

**CONCLUSION**

To summarize, we developed a novel colorimetric method to detect Cu$^{2+}$ rapidly and effectively. The basic principle of the
method is that Cu$^{2+}$ can catalyze GSH to GSSG, thus inhibiting the decomposition of the single-layer MnO$_2$ NSs into Mn$^{2+}$ by GSH to achieve Cu$^{2+}$ detection. Under the optimal experimental conditions, the colorimetric system exhibits highly sensitive, broad linear range, low detection limit and rapid analysis. In addition, the experimental results showed that this method is expected to be used for the determination of Cu$^{2+}$ in practical conditions. Compared to natural enzymes, the layered nanostructures of 2D MnO$_2$ NSs makes themselves possess higher chemical and thermal stability. It should be noted that the TMB can be quickly oxidized by MnO$_2$ NSs with high oxidase-like activity within 10 min. Importantly, the colorimetric assay does not require any expensive reagents, additional H$_2$O$_2$ or complicated modification processes and can complete the detection under mild conditions in a short time, which is expected to be applied to the detection of Cu$^{2+}$ in other practical situations. It is worth mentioning that combining with Cu-contained nanomaterials, this colorimetric method can provide a novel general sensing strategy for the indirect detection of a variety of analytes.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.
AUTHOR CONTRIBUTIONS
ST and YW designed this study. QL carried out the experiment, data acquisition, and data analysis. JH provided assistance for data acquisition and manuscript editing. ST and QL drafted the manuscript. WC provided contribution suggestions of the manuscript. FA, HX, HS, and WY helped to revise the manuscript. All authors contributed to the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL
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