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Dual signal amplification strategy for high-sensitivity detection of copper species in bio-samples with a tunable dynamic range†

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A facile and sensitive method with a tunable dynamic range has been proposed for the detection of Cu^{2+} based on the self-cleavage of Cu^{2+}-specific DNAzyme and the Cu^{2+}-based inhibition of HRP activity, and this method was applied to evaluate the copper species in healthy people and WD patients.

Copper is an essential micronutrient for living organisms, and it plays a crucial role in metabolic and immune regulation.[1] Approximately 85–95% of copper intake is transported to the liver and combined with α2 globulin to form ceruloplasmin (Cp), known as ceruloplasmin-bound copper; the remainder is loosely bound to albumin or amino acids, which is called free copper or exchangeable copper (CuEXC).[2] Excess copper, particularly in its free form, can induce oxidative stress due to the formation of hydroxyl radicals and is implicated in liver or kidney damage[3] and severe neurodegenerative diseases, such as Wilson's disease (WD), Alzheimer's disease and Parkinson's disease.[4,5] Therefore, monitoring the level of copper species in bio-samples is of great significance in the diagnosis and follow-up examination of relevant diseases. At present, CuEXC is roughly calculated by the following formulas:[6] CuEXC = serum total copper (CuT, mg L^{-1}) − 0.3 × Cp (mg L^{-1})/100 or CuEXC = CuT (μM) − 0.049 × Cp (mg L^{-1}). Nevertheless, negative results can be found in about 20% of the patients. In addition, CuEXC and CuT can be directly determined by conventional techniques, including atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS).[7,8] These methods can achieve excellent performance in terms of sensitivity and specificity, but they require sophisticated instruments, complicated sample pretreatment and highly trained personnel. All of these shortcomings have restricted their applications. Thus, to meet clinical demands, it is essential to develop a facile, reliable and low-cost method for the determination of copper species in bio-samples.

The vigorous development of copper ion (Cu^{2+}) biosensors provides a potential way to address this challenge.[9–11] Cu^{2+}-dependent DNAzyme (Cu-DNAzyme) consists of a catalytic sequence (Cu-Enzy) and a cleavage substrate sequence (Cu-Sub). Cu-Enzy exhibits catalytic activity upon the introduction of Cu^{2+} and irreversibly cleaves Cu-Sub at the cleavage site, which makes it an ideal recognition platform for generating Cu^{2+} sensors. Compared to antibodies, Cu-DNAzyme has unique advantages of facile preparation and outstanding stability. Hence, since the discovery of Cu-DNAzyme, a series of fluorescent Cu^{2+}-sensors have been designed.[12,13] The past few years have witnessed great progress in Cu^{2+} detection based on Cu-DNAzyme, such as electrochemical analysis,[14] colorimetric tests,[15] and label-free visual assays[16] for Cu^{2+}. Nevertheless, practical applications of these methods have rarely been shown in bio-samples. This is mainly because the homogeneous fluorescent or colorimetric detection strategy used in a majority of sensors can suffer from interference in complex bio-samples and give false positive or negative signals. In addition, the limit of detection (LOD) is another factor in the determination of trace copper in bio-samples. To overcome these drawbacks, chemiluminescence (CL) with a low background signal and high sensitivity has attracted considerable attention.[17] Horseradish peroxidase (HRP) is a common catalytic enzyme in CL systems, and Cu^{2+} can bind to HRP and inhibit its activity in the presence of ascorbic acid.[18]

Therefore, we proposed a dual signal amplification strategy by combining Cu-DNAzyme self-cleavage and HRP activity inhibition to improve the detection sensitivity. Magnetic nanoparticles (MNPs), which have excellent rapid separation and enrichment performance under an external magnetic field,[19] were used as carriers of Cu-DNAzyme. MNPs-SA were first functionalized with Cu-Sub to form MNPs@Cu-Sub, and this incomplete probe could

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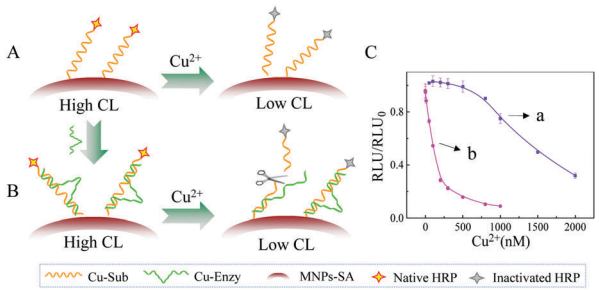

Scheme 1 The scheme of the dual signal amplification strategy for high-sensitivity chemiluminescent detection of Cu^{2+} (A and B) and C is the Cu^{2+} response curves of MNPs@Cu-Sub (a) and MNPs@Cu-Sub@Cu-Enzy (b).

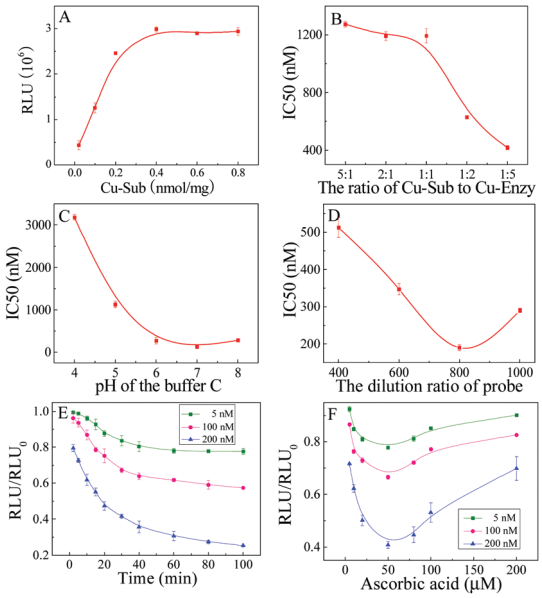

Fig. 1 Single factor optimization of the addition amount of Cu-Sub to 1 mg of MNPs (A); the Cu-Sub to Cu-Enzy ratio (B); the pH of the reaction buffer (C); the probe concentration (D); the reaction time (E); and the concentration of ascorbic acid (F). Error bars represent standard deviation (n = 3).

also respond to Cu^{2+} because Cu^{2+} is reduced to Cu^{+} in the presence of ascorbic acid and binds to HRP, which inhibits the activity of HRP (Scheme 1A). Next, Cu-Enzy was introduced to form a stable secondary structure with Cu-Sub *via* Watson–Crick base pairing. Thus, the completed MNPs@Cu-Sub@Cu-Enzy probe was constructed (Scheme 1B). In the absence of Cu^{2+}, the structure of the Cu-DNAzyme complex on the surface of MNPs remained stable, and HRP maintained its natural conformation; thus, the CL intensity (RLU) of luminol-H_2O_2 was extremely high. When Cu^{2+} was added, the substrate was cleaved and released the fragments marked with HRP due to the decreased affinity for the Cu-Enzy, resulting in a decrease in HRP amount; additionally, the HRP activity was inhibited by Cu^{2+}. The activity inhibition and amount reduction of HRP result in a dramatic decrease in the RLU by a Cu^{2+} concentration-dependent manner.

To verify whether the decrease in RLU results from the self-cleavage of Cu-DNAzyme and the inhibition of HRP activity, the Cu^{2+} response curves of MNPs@Cu-Sub and MNPs@Cu-Sub@Cu-Enzy were plotted (Scheme 1C), respectively. The RLU decrease can be observed in both of them. The difference is that the latter is more sensitive to low concentrations of Cu^{2+}, while the former inhibited RLU only in the presence of higher concentrations of Cu^{2+}. This phenomenon demonstrated that the designed probe combined the self-cleavage of Cu-DNAzyme and the inhibition of HRP activity to produce dual signal amplification. Compared to other reported sensing methods, the strategy proposed in this study is more suitable to detect trace Cu^{2+} due to its higher sensitivity.

The probe synthesis and reaction conditions were optimized to further improve the sensitivity. For probe synthesis, the amount of Cu-Sub added to 1 mg of MNPs and the ratio of Cu-Sub to Cu-Enzy were investigated (Fig. 1A and B). The RLU first increased and then remained constant with the increased amount of Cu-Sub, suggesting that the Cu-Sub on the MNP surface was saturated due to the limited biotin binding sites when 0.4 nmol mg^{-1} Cu-Sub was added. The probe with a Cu-Sub to Cu-Enzy ratio of 1 : 5 was most sensitive to Cu^{2+}, providing the lowest half inhibitory concentration (IC_{50}) of Cu^{2+}, which was attributed to a more complete substrate hybridization. Further increases in Cu-Enzy were not explored due to the cost of material consumption. The Cu-DNAzyme-based probe was effective in neutral pH (Fig. 1C), because an appropriate pH is essential for maintaining the structure and activity of DNAzyme and HRP, highly acidic or alkaline environments will destroy the DNAzyme complex by reducing the solubility of DNA molecules while also affecting the catalytic activity of HRP. Since the probe will aggregate at high concentration, a low IC_{50} corresponding to a dilution ratio of 800 (Fig. 1D) was used in the experiment. Besides, ascorbic acid is another key factor in this method, the RLU/RLU_0 was decreased upon increasing the ascorbic acid concentration from 0 to 50 μM, and then increased (Fig. 1E) where the RLU and RLU_0 are the CL intensities in the presence and absence of Cu^{2+}, respectively. This result is due to Cu^{2+} being reduced to Cu^{+}, which significantly improved the reaction rate. Furthermore, the time-dependent RLU/RLU_0 can be observed, thus reaching equilibrium at approximately 40 min (Fig. 1F). Accordingly, the probe was diluted 800 times and reacted for 40 min in buffer C at pH 7.0 containing 50 μM ascorbic acid in the following expriments.

For various samples, the desired concentration of the same target analyte may be different; thus, a detection method with a tunable dynamic range is more suitable for practical applications. For example, the reference value of CuT is 10.9–21.8 μM, while the CuEXC is 5–15% of the CuT, and the urinary copper (CuU) levels of patients with copper metabolic disorders are even more diverse depending on the de-copper therapy.[20] Thus, a detection method with a μM level dynamic range would be ideal for measuring CuT, but not for detecting CuEXC or CuU. To achieve a tunable dynamic range that can be adapted for different detection requirements, we explored whether the ionic strength of the reaction buffer can be used as a tunable parameter. The structure of the Cu-DNAzyme complex contains a DNA triplex, which causes its stability to decrease compared to normal Watson–Crick base pairs. Therefore, high ionic strength buffer was used to form stable Cu-DNAzyme complexes. However,

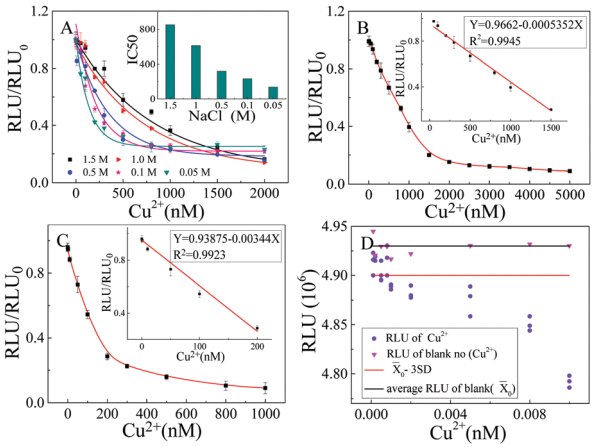

Fig. 2 The effect of ionic strength on dynamic range (A); the calibration curve of Cu^{2+} determination in buffer C with 1.5 M NaCl (B) and the calibration curve and limit of detection of Cu^{2+} determination in buffer C with 0.05 M NaCl (C and D). Error bars represent the standard deviation (n = 3).

the cleavage reaction of DNAzyme is sensitive to ionic strength due to its effects on the catalytic activity of Cu-Enzy. We hypothesized that a higher concentration of Cu^{2+} should be needed to achieve the same RLU inhibition rate at a higher ionic strength with the same reaction time. To verify this hypothesis, the proposed method was used for Cu^{2+} sensing in buffer C with different ionic strengths (Fig. 2A), which were regulated by adding NaCl. The IC_{50} gradually decreased with the decrease of NaCl, thus reaching its minimum with 0.05 M NaCl. This phenomenon can be explained by the following two factors. First, the cleavage activity of Cu-Enzy on Cu-Sub is gradually inhibited as the concentration of NaCl increased. On the other hand, ionic strength may partially affect the suspension stability of the MNPs. High concentrations of NaCl can cause MNPs to aggregate, thereby reducing the chance of contact between Cu-DNAzyme and Cu^{2+} due to the reduced surface area of MNPs. Consequently, a tunable dynamic range can be achieved with different ionic strengths. Furthermore, because the activities of Cu-Enzy and HRP were sensitive to pH, it is also possible to further tune the dynamic range by changing the pH of buffer C.

Under optimal conditions, the linear ranges at different ionic strengths were investigated (Fig. 2B and C). A good linear correlation was observed between the RLU/RLU_0 and the concentration of Cu^{2+} over the range of 50–1500 nM at 1.5 M NaCl, while it changes to 0.01–200 nM when the concentration of NaCl was reduced to 0.05 M. Thus, the linear range of this method can be easily adjusted by the ionic strength of buffer C to accommodate accurate detection in different situations. Moreover, the LOD in 0.05 M NaCl was determined experimentally (Fig. 2D). The 8 blank samples were detected to obtain $\bar{X}_0 - 3SD$, and the LOD was regarded as the Cu^{2+} concentration corresponding to $RLU < \bar{X}_0 - 3SD$. Therefore, the measured LOD of this method is 1.0 pM. The recoveries and relative standard deviations (RSD) were set out to evaluate the accuracy and precision of this method. As displayed in Table S5 (ESI†), the intra- and inter-assay recoveries were 90.0–111.5% and 87.0–116.0%, with the corresponding RSDs of 2.75–9.52% and 4.17–12.57% (n = 6).

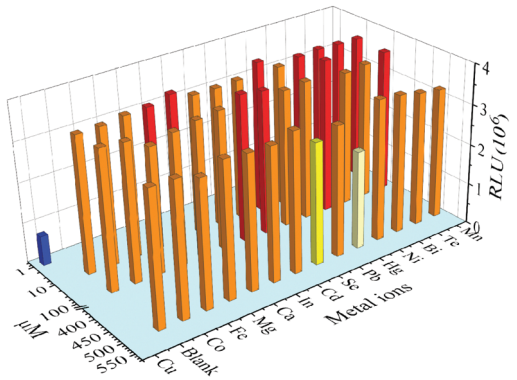

Fig. 3 The selectivity of the proposed method. The concentration of Cu^{2+} was 0.1 μM, and the concentrations of other 13 competing metal ions were 10, 100 and 500 μM.

These results revealed the reliability of the dual signal amplification sensing platform. The selectivity was investigated by 13 common competing metal ions at three concentrations. This result (Fig. 3) revealed the excellent selectivity for Cu^{2+} over Mn^{2+}, Te^{2+}, Bi^{3+}, Ni^{2+}, Pb^{2+}, Se^{2+}, In^{3+}, Ca^{2+}, Mg^{2+}, and Co^{2+}: the RLU values upon the addition of these metal ions are similar to the RLU given by the blank. A slight reduction in RLU was observed in the presence of 500 μM Hg^{2+} and Cd^{2+}, but the extent of this reduction was much lower than that with 0.1 μM Cu^{2+}. However, in bio-sample analysis, Hg^{2+} and Cd^{2+} are unlikely to interfere because they are not present in such a high concentration. Consequently, the influence of these common metal ions on the detection of copper species in bio-samples is negligible.

WD is a recessive inherited disease arising from copper homeostasis dysfunction caused by mutations in the ATP7B coding gene.[21] CuT, CuEXC, relative exchangeable copper (REC = CuEXC/CuT) and CuU are the conventional clinical evaluations in WD diagnosis and follow-up examination.[22,23] To validate the clinical feasibility of this method, serum and urine samples from healthy subjects and patients with WD receiving treatment were analyzed by this method and AAS. A good correlation (Fig. 4) between the proposed method and AAS can be observed with a correlation coefficient of 0.990. Additionally, a paired sample t-test also showed no significant

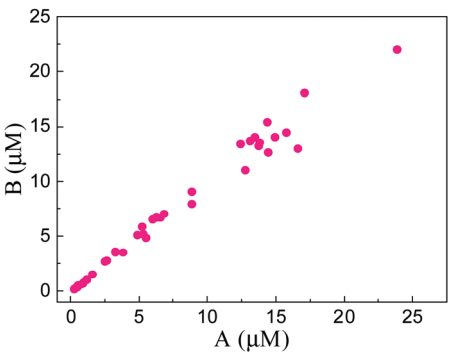

Fig. 4 The correlation analysis of the results of bio-samples obtained by the proposed method and AAS (n = 38). (A and B are the results from AAS and the proposed method, respectively).

Table 1 Results of copper species in bio-samples detected by the proposed method

Samples	CuT (μM)	CuEXC (μM)	REC (%)	CuU (μM)
Healthy subjects (n = 20)	15.48 ± 3.24	1.40 ± 0.55	9.21 ± 2.77	0.62 ± 0.32
Treated patients with WD (n = 18)	5.16 ± 2.79[a]	1.38 ± 0.70	32.18 ± 5.90[a]	7.37 ± 4.69[a]

[a] Represent the statistical difference (α = 0.01).

difference ($P > 0.01$) for the two methods in the detection of copper species in bio-samples. This proved that this method with high accuracy can be used for the effective detection of copper species in real samples.

The reference ranges of CuT, CuEXC, REC and CuU obtained by our established method are shown in Table 1. The independent sample t-test indicated that there was no significant difference in CuEXC ($P > 0.01$) between normal subjects and treated WD patients, but significant differences can be observed in CuT, REC and CuU ($P < 0.01$). These data reflected the changes in copper-related indicators during the WD treatment to a certain extent. In the course of copper-chelation therapy, excess CuEXC is chelated and excreted in urine, thereby reducing CuEXC to a normal level, which led to a much higher CuU in patients with WD than in healthy people. As WD is a rare disease, the samples of preliminarily diagnosed patients were missing, but we can infer that the CuEXC of WD patients is much higher than the normal reference value, although further experiments are required for verification. Nonetheless, the REC in patients with WD was still higher than that in healthy people due to the decrease in CuT, which contributes to the Cp synthesis disorders.

In conclusion, we have developed a dual signal amplification probe based on Cu-DNAzyme self-cleavage and HRP activity inhibition to realize high-sensitivity determination of copper species in bio-samples with a tunable dynamic range. Under the optimal conditions, the detection limit was 1.0 pM, which represents one of the most sensitive Cu^{2+} sensors (Table S6, ESI†). In addition, the method exhibited a good linear relationship of Cu^{2+} with high accuracy and precision. Additionally, this method was more suitable for real sample detection due to the tunable dynamic range, which can be modified simply by adjusting the concentration of NaCl in the reaction buffer. The detection of bio-samples showed that the CuT, CuEXC, REC and CuU of healthy people were 15.48 ± 3.24 μM, 1.40 ± 0.55 μM, (9.21 ± 2.77)% and 0.62 ± 0.32 μM, respectively. After treatment of WD, the CuEXC was decreased to a normal level, while the REC was still higher, and the difference was statistically significant ($P < 0.01$). Therefore, the proposed method exhibits satisfactory feasibility for the detection of copper in real samples because of the high specificity of Cu-DNAzyme cleavage, suggesting that it has promising potential for applications in clinical diagnosis and the follow-up examination of copper metabolism-related diseases. Moreover, the assay was conducted in 96-well plates and does not require trained personnel or complex and expensive instruments, allowing it to be used in remote hospitals where diagnostic facilities are limited.

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Conflicts of interest

There are no conflicts to declare.

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