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Colorimetric Detection of Copper Ion Based on Click Chemistry

Lingwen Zeng, Zhiyuan Fang and Yunbo Wang

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Abstract

Two colorimetric assays, lateral flow biosensor (LFB) and hemin/G-Quadruplex DNAzyme-based colorimetric assay, were developed for the detection of copper ion based on click chemistry. Two single-strand DNA (ssDNA) with azide- and alkyne-modified at 3' and 5' separately can be linked by the Cu^+ -catalyzed click chemistry. For hemin/G-Quadruplex DNAzyme-based assay, the two ssDNA fragments linked by Cu^+ -catalyzed click chemistry could form a complete G-rich sequence that severed as a horse-radish peroxidase. In the presence of hemin and K^+ , the colorless substrate tetramethyl benzidine (TMB) is catalyzed into a colored product by the G-rich sequence. The concentration of Cu^{2+} can then be quantitatively analyzed by measuring the color density. For the LFB assay, the two ligated ssDNA fragments could form a sandwich complex between an ssDNA fragment immobilized on gold nanoparticles and another ssDNA fragment on test zone of a biosensor, respectively. The biosensor enables visual detection of copper ion with excellent specificity. In comparison with conventional methods, the present assays are simpler to operate and more cost-effective to use, and so have great potential in point-of-care diagnosis and environmental monitoring.

Keywords: colorimetric detection, lateral flow biosensor, click chemistry, copper ion detection

1. Introduction

Cu^{2+} is one of the important cofactors or structural components for numerous enzymes needed in metabolic processes, and therefore, an essential micronutrient for human life. However, high intracellular concentration of Cu^{2+} can cause adverse health effects, such as liver damage, gastrointestinal disturbance, and neurodegenerative diseases including

Alzheimer, Menkes, and Wilson diseases [1, 2]. The maximum contamination concentration for Cu^{2+} in drinking water set by the U.S. Environmental Protection Agency (EPA) is 20 mM [3]. Therefore, sensitive and specific analytical methods for the monitoring and detection of Cu^{2+} pollution in environment and food are imperative.

The standard methods for Cu^{2+} determination include graphite furnace atomic absorption spectrometry (AAS) [4, 5] and inductively coupled plasma atomic emission spectroscopy (ICPAES) [6]. These methods have advantages of high sensitivity and repeatability, which enable the analysis of a variety of samples. However, the shortcomings of these techniques are that they require expensive instruments and experienced operators. Recently, a variety of methods based on Cu^{2+} -specific DNAzyme have been developed for Cu^{2+} detection [7–11]. Although these methods are effective, the reaction of Cu^{2+} -dependent DNA-cleaving DNAzyme needs strict conditions, these methods are not suitable for real sample analysis. Furthermore, the use of fluorophore-labeled oligonucleotides is not only expensive but also increases the complexity of the operation.

Cu^+ -catalyzed click chemistry refers to the [3 + 2] cycloaddition reaction between an azide group and an alkyne group at room temperature in aqueous solution, which finally results in the formation of a five-membered triazole ring [12]. The reactivity of in situ-generated copper(I) acetylides renders this reaction with high efficiency and selectivity. The applications of click chemistry allow the modification of a wide range of biomolecules. Since click reaction acts in a quick, irreversible, and water uninteruptible manner, it has widely been used in biomedical fields, such as drug discovery [13, 14], bioconjugations [15], and imaging [16]. Due to the ability of adding probes into target molecules, extensive research was carried out for the detection of biomolecules and disease diagnosis based on click chemistry, including viral infection [17], carbohydrate detection [18], enzyme [19], single nucleotide polymorphism [20] and ions [21].

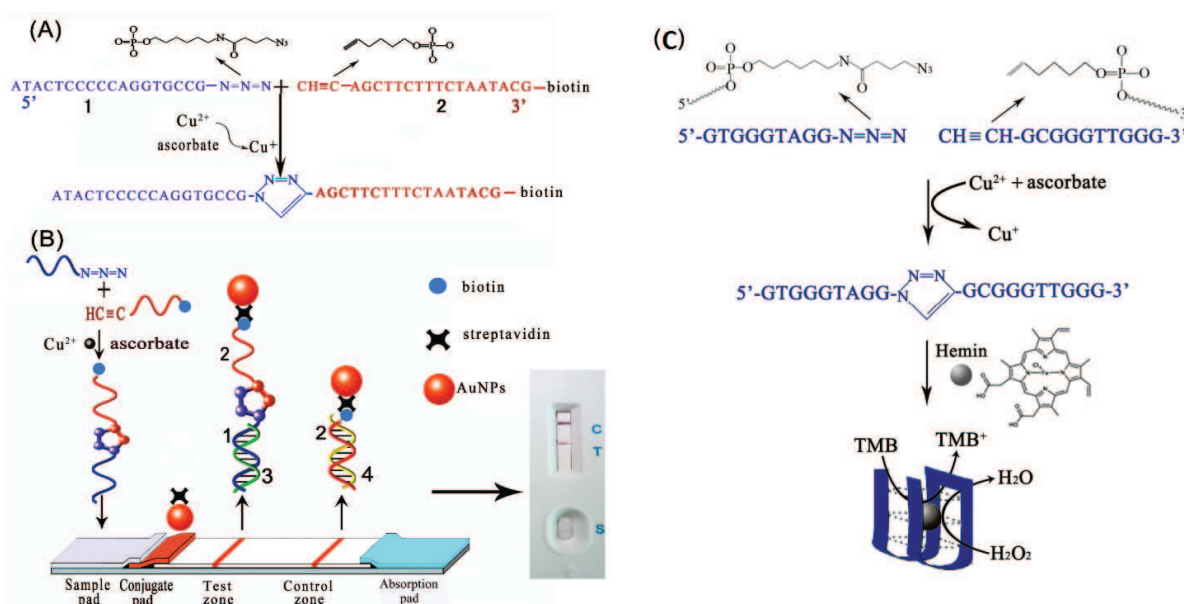


Figure 1. Principle of the biosensor for Cu^{2+} detection. (A) The formation of the ligation product of azide-DNA and alkyne/biotin-DNA based on Cu^+ -promoted click chemistry. (B) The product is analyzed by a lateral flow biosensor. (C) Hemin/G-quadruplex HRP-mimicking DNAzyme formed by the catalyzation of click chemistry. The self-assembly of hemin/G-quadruplex catalyzed its colorless substrate TMB into a colored product (adapted from [22, 23]).

In this chapter, two simple methods for Cu^{2+} detection based on Cu^+ -catalyzed click chemistry by the reduction of Cu^{2+} in the presence of sodium ascorbate were proposed. **Figure 1** shows a lateral flow biosensor based on Cu^+ -catalyzed click chemistry [22, 23]. The two biosensors have the following features: (1) easy to use and operate; (2) no requirement for bulky and costly apparatus; (3) high sensitivity; and (4) excellent selectivity to other metal ions. Details of the methods, procedures, and results are presented below. A section on Summary and Future Directions is given at the end of the chapter.

2. Materials

The materials used in this project and their suppliers are listed below:

- Sodium ascorbate: Shanghai Aladdin Reagent Co., Ltd. (Shanghai, China).
- Ammonium peroxodisulfate (AP), urea, copper(II) chloride dihydrate, potassium chloride, and other metal ions: Guangzhou Chemical Reagent Factory (Guangzhou, China).
- SYBR Green II, hemin, dimethylsulfoxide (DMSO), bovine serum albumin (BSA), and human serum: Sigma-Aldrich (St. Louis, USA).
- 3, 3', 5, 5'-tetramethyl benzidine hydrochloride dihydrate (TMB-2HCl): Cellway-Lab (Luoyang, China).
- Phosphate buffer solution (PBS): Genetimes Technology, Inc. (Shanghai, China).
- Tris-base, boric acid, ethylene diamine tetraacetic acid (EDTA), Triton X-100, and hydrogen peroxide (H_2O_2): Guangzhou WhiGa Technology Ltd. (Guangzhou, China).
- Oligonucleotides purified by HPLC: Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).
- Rinsing buffer: 1% BSA and 8% sucrose containing 0.05% NaN_3 . Rinsing buffer was stored at 4°C until use.
- Sample pad buffer: 0.01 M PBS pH 7.8 containing 0.5% BSA, 5% Triton X-100, and 0.05% NaN_3 . Sample pad buffer was stored at 4°C .
- Loading buffer: $4\times$ SSC containing 0.1% Triton X-100.
- Adhesive plate: PVC plate, obtained from Vinostech, Shanghai, China.
- Nitrocellulose membrane: AE99, 25 mm \times 30 cm, capillary rate: 140 ± 40 s, thickness: 145 ± 20 μm , obtained from GE Healthcare, Shanghai, China.
- Fiberglass, absorbent paper: Shanghai Jie Ning Biotechnology, Shanghai, China.
- Dispenser: XYZ dispenser HM3030, obtained from Shanghai Kinbio, Shanghai, China.
- Cutter: Automatic Strip Cutter ZQ2000, obtained from Shanghai Kinbio, Shanghai, China.
- Strip reader: Test Strip Reader DT2032, obtained from Shanghai Kinbio, Shanghai, China.

Probe name	Sequence (5' → 3')
Azide-DNA	ATACTCCCCCAGGTGCCG
Alkyne/biotin-DNA	AGCTTCTTTCTAATACG
Control zone-DNA	CGTATTAGAAAGAAGCTCGTATTAGAAAGAAGCT
Test zone-DNA	CGGCACCTGGGGGAGTATCGGCACCTGGGGGAGTAT

Table 1. DNA probes used in this assay.

All buffer solutions used in this study were prepared in our lab. Other chemicals were purchased from standard commercial sources and the sequences of the oligonucleotides are listed in **Table 1**.

3. Lateral flow biosensor detection of Cu²⁺

3.1. Preparation of streptavidin-modified gold nanoparticle (AuNP-SA) conjugates

AuNPs with 15 nm were prepared as follows: heat 100 mL of 0.01% H₂AuCl₄ to boil and add 4 mL of 1% trisodium citrate immediately in a 500-mL round-bottom flask with rapid stirring. The solution was boiled for additional 10 min after its color turned red. After then, it was cooled to room temperature with gentle stirring.

AuNP-SA conjugates were prepared as follows: pH of 1 mL AuNP solution was adjusted by adding 4 µL of 0.1 M K₂CO₃, followed by adding 10 mg (46 µL) SA. The mixture was incubated at room temperature for 1 h with gentle shaking. AuNP was blocked with 1% BSA. The mixture was incubated at room temperature for another 1 h with gentle shaking. After blocking, AuNP was collected and rinsed three times with rinsing buffer (12,103 rpm, 20 min). The red pellet was resuspended in 50 mL of rinsing buffer and then stored at 4°C until use.

3.2. Construction of lateral flow biosensor

The biosensor was constructed as follows:

- The sample pad (17 mm × 30 cm) was prepared by soaking a glass fiber pad in sample pad buffer. The pad was then dried and stored in a low-humidity chamber at room temperature;
- The conjugate pad (8 mm × 30 cm) was prepared by dispensing AuNP-SA solution (10 µL cm⁻¹) onto the fiberglass using a dispenser. The pad was dried at room temperature for 12 h and stored at room temperature in low humidity until use;
- Test zone and control zone were prepared by dispensing 30 µL control zone DNA (100 µM) and 30 µL of test zone DNA (100 µM) onto the nitrocellulose membrane simultaneously by a lateral flow dispenser. There should be 5 mm distance between the test zone and the control zone. The nitrocellulose membrane was exposed to ultraviolet light for 15 min to

immobilize DNA. The membrane was then dried in low humidity at room temperature. It was stored in ziplock bag with desiccant at room temperature.

- Lateral flow biosensor was assembled by adhesion of the four components onto a plastic adhesive backing (60 mm × 30 cm). Each component had a part of 2 mm overlapped. The backing along with four components was then cut into 0.4-cm wide strips using a paper cutter.

3.3. Detection of Cu^{2+} using the lateral flow biosensor

- Azide-DNA and alkyne/biotin-DNA were mixed at the same mole ratio (10 μM , 2 μL) in 14 μL of PBS buffer, followed by adding 2 μL of Cu^{2+} solution.
- Test without Cu^{2+} was used as control.

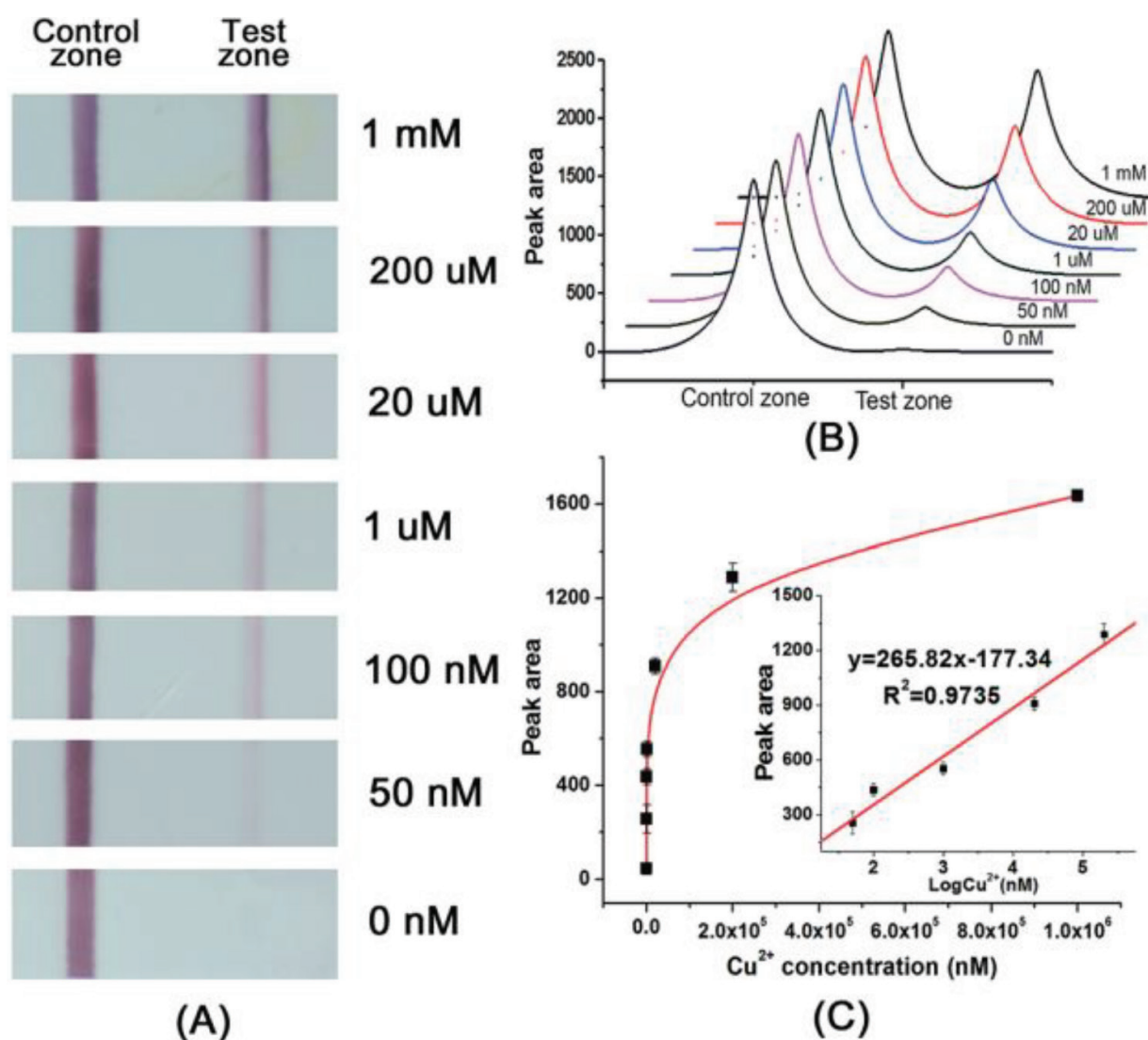


Figure 2. Sensitivity of the assay. (A) Typical photo images of the strip. (B) Recorded corresponding optical intensities of the strip. (C) Plots of the optical intensities of red bands on the test zone vs. different concentrations of Cu^{2+} . Inset: calibration curve of the optical intensities of red bands on the test zone vs. the log value of Cu^{2+} concentration. The error bars represent the standard deviation of three independent measurements (reproduced with permission from [22]).

- Sodium ascorbate (2 μL) was added to a final concentration of 600 mM to accelerate the reaction between azide and alkyne group.
- Shake gently at room temperature for 45 min.
- After the reaction, 4 μL of loading buffer was added and mixed with the above mixture. It was then loaded onto the sample pad. Results could be observed visually in 15 min, and the optical intensities of test zone and control zone were recorded using the strip reader.

3.4. Results of biosensor for Cu^{2+} detection

As shown in **Figure 2**, visible test zone could be observed in the presence of 100 nM Cu^{2+} , which was determined as the limit of detection (LOD) for visual detection. The linear range between optical intensities and logarithm of Cu^{2+} concentration was 50 nM to 200 mM with a linear equation of optical intensity = $265.82x - 177.3$ ($R^2 = 0.9735$).

4. Colorimetric detection of Cu^{2+}

The detection was based on the hemin/G-quadruplex HRP-mimicking DNAzyme produced by Cu(+)-catalyzed click chemistry between azide- and alkyne-modified short G-rich sequences. The intact G-quadruplex can catalyze its colorless substrate TMB into a colored product, indicating the content of copper ion.

4.1. Preparation of chemicals

- Azide-modified G1 and alkyne-modified G2 were mixed at a mole ratio of 1:1 in reaction buffer at a final concentration of 1 μM .
- Cu^{2+} with various concentrations was added. Control experiment (in the absence of Cu^{2+}) was conducted under the same condition.
- Sodium ascorbate (2 μL) was added to a final concentration of 100 μM to accelerate the reaction between azide and alkyne group.
- Shake the mixture gently for 2 h at RT.
- After the reaction, add 1 μM hemin and 100 mM KCl, continued to shake for 20 min in order to form the hemin/G-quadruplex HRP-mimicking DNAzyme in aqueous solution.
- Equal volume (30 μL) of 0.5% (w/v) H_2O_2 and 0.05% (w/v) TMB were added to DNA-hemin mixture.
- 40 μL of 2 M H_2SO_4 was added to stop the reaction in 15 min, transferred solution into a 96-well microtiter plate. Record the OD values at 450 nm in each well using the microplate reader.

4.2. Results of colorimetric Cu^{2+} detection

The color intensity of the solution in each well increased with increasing Cu^{2+} concentration from 100 nM to 200 μM . In the absence of Cu^{2+} , the color of the solution had almost no change and

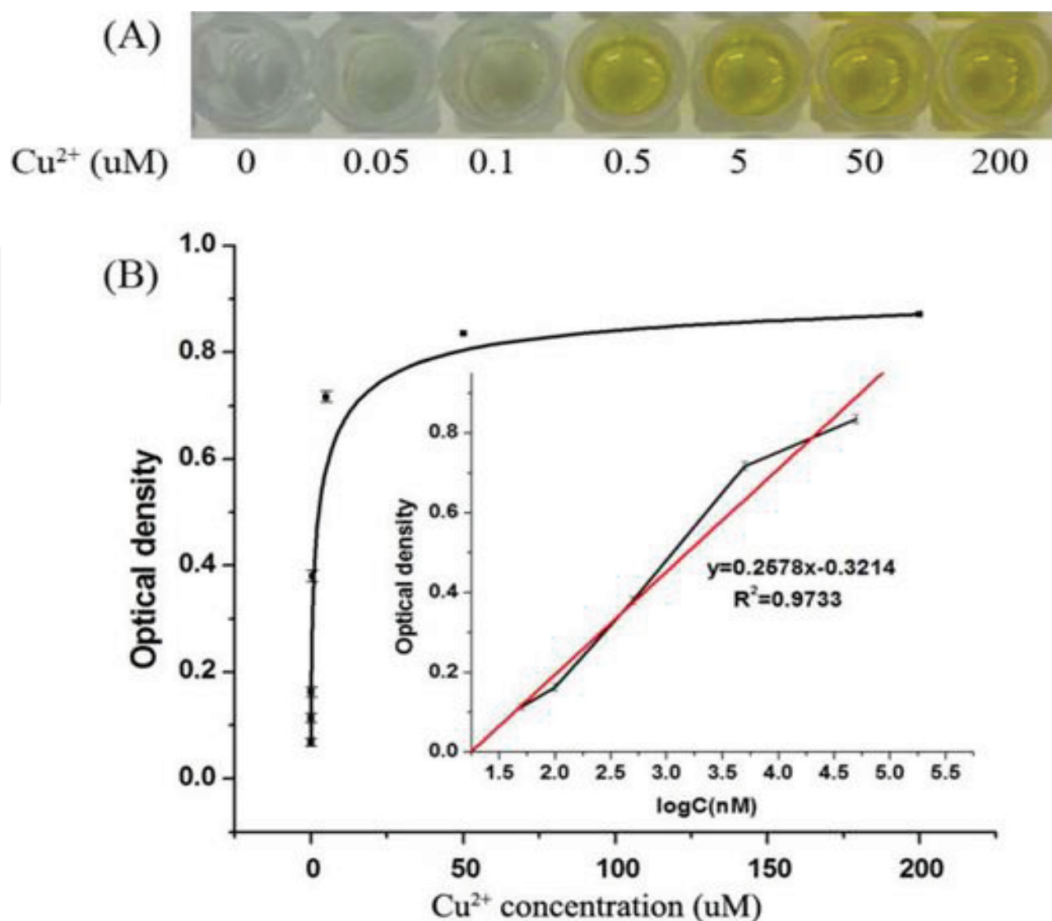


Figure 3. The sensitivity for Cu^{2+} detection based on click chemistry and hemin/G-quadruplex DNAzyme. (A) Photo images of the color intensity with different concentrations of Cu^{2+} (0, 100 nM, 200 nM, 500 nM, 1 μM , 10 μM , 100 μM , and 200 μM). (B) Plots of the OD value at 450 nm vs. Cu^{2+} concentration. Insert: calibration curve of OD value at 450 nm vs. the logarithm of Cu^{2+} concentration. The error bars represent the standard deviation of three independent measurements (reproduced with permission from [23]).

was still colorless. Calibration curve shows that the OD value is proportional to the logarithm of Cu^{2+} concentration in the range of 0.05–50 μM with a linear equation of OD value = $0.2578(\log \text{Cu}^{2+}) - 0.3214$ ($R^2 = 0.9733$). Using this equation, the limit of detection (LOD) of 5.9 nM was calculated based on triple standard deviation plus the mean of blanks [23] (Figure 3).

5. Summary and future directions

In summary, we have successfully constructed two sensors for rapid detection of copper ion based on click chemistry. Separated elements of probes were constructed at the presence of copper ion, which mediated the following color detection. The present methods are independent of complex operations and costly apparatus, while high sensitivity is achieved. The LOD of visual and colorimetric assay are 100 nM and 5.9 nM, respectively, which are much lower than 20 mM defined by EPA limit of Cu^{2+} in drinking water. In the future, the development of one-step process of microfluidic devices that integrate click chemistry and color development would provide even more convenient and more sensitive assays.

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