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An enzyme-free and label-free assay for copper(II) ion detection based on self-assembled DNA concatamers and Sybr Green I†

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An enzyme-free and label-free fluorescence turn on biosensor for amplified copper(II) ion (Cu^{2+}) detection has been constructed based on self-assembled DNA concatamers and Sybr Green I. This assay is simple, inexpensive and sensitive, enabling quantitative detection of as low as 12.8 pM Cu^{2+}.

Introduction

Heavy-metal ion pollution is a major environment problem that has attracted more and more attention in recent years. Considerable efforts have been devoted to the detection of heavy-metal ions due to their high toxicity towards the ecosystem and human health. Copper(II) ion (Cu^{2+}), as one of the heavy-metal ions, is also an important micronutrient element for human life.[1] The reason lies in the fact that Cu^{2+} is an essential element required as a cofactor and/or structural component of numerous enzymes and proteins needed in metabolic processes. However, lower and higher concentrations of Cu^{2+} can cause adverse health effects. For instance, a lower level of Cu^{2+} can affect the activity of enzymes and cell metabolism,[2] while a higher level of Cu^{2+} may cause gastrointestinal disturbance and liver or kidney damage, and loss of cognition in the elderly and individuals with Wilson's disease.[3,4]

Classical approaches for the detection of Cu^{2+}, such as graphite furnace atomic absorption spectrometry[5] (AAS) and inductive coupled plasma atomic emission spectroscopy (ICP-AES),[6] have been used as standard procedures. However, the requirements of costly instruments and highly trained personnel prevent their use in many laboratories. Some new methods and technologies, including use of Cu^{2+}-dependent DNA ligation DNAzyme,[7] DNAzyme catalytic beacon sensors,[8] fluorescent anisotropy sensors,[9] dynamic light scattering techniques,[10] lateral flow nucleic acid biosensors,[11] DNA cleavage-dependent graphene-quenched DNAzyme[12] and G-quadruplex DNAzyme based methods,[13] have been developed in recent years. Although these methods are effective, the use of DNA ligase and fluorophore labeled oligonucleotides is not only expensive but also increases the complexity of the assay. Use of the lateral flow biosensor is a semi-quantitative method for Cu^{2+} detection. Hence, the development of an inexpensive and sensitive method for quantitation of the concentration of Cu^{2+} is urgently needed.

One way to obtain an inexpensive and sensitive method is through signal amplification without using enzyme and fluorophore labeled oligonucleotides. Yin and co-workers have constructed a dual-DNAzyme unimolecular probe for colorimetric detection of Cu^{2+},[14] and the detection limit is 1 μM, which is lower than the maximum level (20 μM) of Cu^{2+} in drinking water permitted by the U.S. Environmental Protection Agency (EPA).[15] An even higher sensitivity is required if the method for Cu^{2+} detection is to be used in clinical or environmental samples. In 2010, Xia and co-workers reported a signal amplification assay for sensitive detection of DNA (100 fM) based on a super-sandwich structure without using any protease, and it only required a label-free signal probe (SP) and a methylene blue modified SP.[16] Herein, we developed an enzyme-free and label-free biosensor by introducing DNA self-assembled concatamers and a double-stranded DNA (dsDNA) binding dye Sybr Green I (SG) as a signal reporter for Cu^{2+} detection.

SG, a commonly used fluorescent DNA binding dye, preferentially binds to dsDNA over single-stranded DNA (ssDNA).[17] It has been successfully used in DNA quality evaluation and quantification technologies, such as agarose gel electrophoresis[18] and real-time PCR.[19] When SG intercalates and binds to the minor groove of dsDNA, its fluorescence intensity increases dramatically. However, SG shows very weak fluorescence upon binding to ssDNA through electrostatic interaction.[20] In

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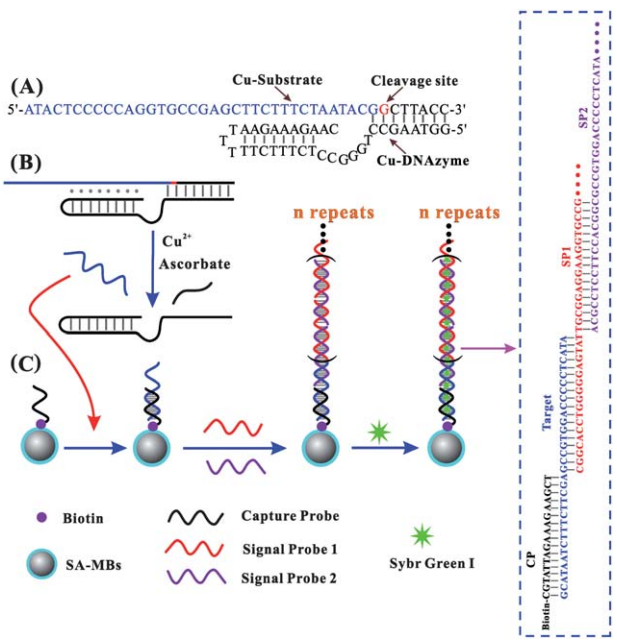
addition, the intensity of the fluorescence signal varies depending on the amount of dsDNA present.

We employed the Cu^{2+}-specific DNAzyme as the molecular recognition element for the detection of Cu^{2+}. The substrate is elongated at the 5′-end to 42 nt without changes to the original DNAzyme; the sequence of the new substrate is shown in Scheme 1(A). Also, we designed two auxiliary probes, named SP1 and SP2. DNA sequences used in the experiment are as follows. The sequence of the target DNA is 5′-ATA CTC CCC CAG GTG CCG **AGC TTC TTT CTA ATA CG**-3′. The sequence of the biotinylated capture probe (CP) is 5′-biotin-**CGT ATT AGA AAG AAG CT**-3′. The sequence of SP1 is 5′-CGG CAC CTG GGG GAG TAT *TGC GGA GGA AGG TGC CG*-3′. The sequence of SP2 is 5′-ATA CTC CCC CAG GTG CCG *CGG CAC CTT CCT CCG CA*-3′. The CP is complementary to the bold domain at the 3′ end of the target DNA. The underlined domain at the 5′ end of SP1 can hybridize with the underlined domain at the 5′ end of SP2. The italic domain at the 3′ end of SP1 can hybridize with the italic domain at the 3′ end of SP2. As a result, SP1 (SP2) can hybridize with two different domains of SP2 (SP1). In addition, the underlined domain of SP1 is complementary to the underlined domain at the 5′ end of the target DNA. Thus, even a single target DNA molecule can trigger a cascade of hybridization events between SP1 and SP2 to form a long dsDNA concatamer.

To fabricate the enzyme-free and label-free fluorescent biosensor for Cu^{2+} detection based on self-assembled DNA concatamers, the Cu^{2+}-specific DNAzyme strand was first hybridized with the substrate strand to form Cu^{2+}-specific DNAzyme. In the presence of Cu^{2+}, the substrate is irreversibly cleaved at the cleavage site (the guanine in red). The cleaved piece (in blue) at the 5′-end of the substrate is released due to decreased affinity to the enzyme. During this process, the released strand can act as a catalyst to trigger the self-assembly of SP1 and SP2 on the surface of MBs. After magnetic separation, SG was added to bind to the long dsDNA concatamers and the fluorescence spectra of the solution were recorded with the aid of a fluorospectrophotometer. In the absence of Cu^{2+}, there is no cleavage of the Cu-substrate and it still hybridizes steadily with Cu-DNAzyme. In this case, the target DNA cannot be released and trigger the self-assembly of SP1 and SP2, and there is no dsDNA on the surface of MBs after magnetic separation. As SG stains ssDNA with much lower intensity than dsDNA, only very weak fluorescence can be detected.



Scheme 1 (A) Secondary structure of the Cu^{2+}-specific DNAzyme. (B) The substrate is cleaved into two fragments in the presence of Cu^{2+}. The cleavage site is indicated in red (G). (C) The cleaved DNA strand (indicated in blue) is used as a catalyst to trigger the self-assembly of SP1 and SP2 on the surface of magnetic beads (MBs). After magnetic separation, SG was added to stain the dsDNA, the fluorescence spectra were recorded using a fluorospectrophotometer.

Experimental

Chemicals and materials

Carboxylated magnetic beads (MBs) with a diameter of 4–5 μm were purchased from BaseLine ChromTech Research Centre. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC or EDAC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Aladdin Chemistry (Shanghai, China). 2-Morpholino-ethanesulfonic acid (MES) was purchased from Qingdao MDBio Biotech. Copper(II) chloride dihydrate and other metal ions were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Human serum, $HAuCl_4$ and trisodium citrate dehydrate were purchased from Sigma-Aldrich (St Louis, USA). All buffer solutions used in this study were prepared in our lab. Other chemicals were purchased from standard commercial sources and were of analytical grade purity. Oligonucleotides purified by HPLC were synthesized by Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China).

Preparation of SA coated magnetic beads (SA-MBs)

Carboxylated MBs (50 μL (1% w/v)) were functionalized with SA (50 μL, 1 mg mL^{-1}). Briefly, EDC (20 mg mL^{-1}, 20 μL) and NHS (10 mg mL^{-1}, 20 μL) dissolved in pre-cooled MES buffer (25 mM, pH 5) were successively added into MB solution and shaken gently for 30 minutes at room temperature (RT) to activate the carboxyl groups. After washing three times with MES buffer, SA was added and shaken gently for 2 hours. Excess activated carboxyl groups on the surface of MBs were blocked with Tris–BSA buffer (50 mM Tris·HCl, 0.1% Tween-20, 0.5% BSA, pH 7.4) for 30 minutes. After magnetic separation, the supernatant was aspirated and discarded. The SA-MB pellets were resuspended in Tris–BSA buffer and stored at 4 °C before use.

Preparation of capture DNA coated MBs (DNA-MBs)

Biotinylated capture DNA was added into SA-MB solution with a final concentration of 1 μM, and shaken gently for 30 min at RT. The capture DNA functionalized MBs were magnetically washed three times with Tris–BSA buffer and stored at 4 °C before use.

Analytical procedure

A 1 μM DNAzyme substrate strand was first incubated with a 5 μM DNAzyme strand in 10× saline-sodium citrate (10× SSC, pH 7.0)

at 90 °C for 2 min to dissociate any intermolecular interaction, and gradually cooled to RT for 1 h to form the DNAzyme. Cu^{2+} with various concentrations was then added into the resulting DNAzyme solution. To promote the cleavage, 50 μM ascorbate was added to each reaction and incubated for 60 min at room temperature to allow the maximum cleavage of the DNAzyme substrate strand at the G position. Block DNA and DNA-MBs were then added simultaneously, and the mixture was shaken gently for 30 min at RT. After washing three times with 2× SSC, SP1 and SP2 were added with a final concentration of 1 μM to form long dsDNA concatamers. Finally, SG was added and the fluorescence spectra were collected with a fluorospectrophotometer.

Safety considerations

Because most of the tested heavy metal ions are toxic and have deleterious effects on human health, all experiments involving heavy metal ions should be performed with protective gloves. The waste solutions that contain heavy metal ions should be collectively reclaimed to avoid polluting the environment.

Results and discussion

Optimization of the experimental conditions

Although excess Cu-DNAzyme strands were used in the experiment, there was still free Cu-substrate which could hybridize with the capture DNA on the MBs. As a result, fluorescence can be detected in the absence of Cu^{2+}. In order to minimize the background fluorescence, a block DNA probe was used to hybridize with the free Cu-substrate first. The sequence of block DNA is the same as capture DNA but the former is not biotinylated (5′-CGT ATT AGA AAG AAG CT-3′). Different concentrations of block DNA (0, 500 pM, 1 nM, 5 nM, 10 nM and 20 nM) were tested. As shown in Fig. 1, the background fluorescence decreased with increase of the block DNA concentration. Their corresponding fluorescence responses were further confirmed by recording the intensity at the maximum emission peak. When the block DNA concentration reached 10 nM or higher, the fluorescence intensity became the lowest. Therefore, 10 nM block DNA was selected as the optimal concentration in the current study.

The fluorescence intensity was closely correlated with the concentration of SP1 and SP2 or the hybridization time of the self-assembly. So the optimal concentration of signal probes and the hybridization time were further investigated. As shown in Fig. 2A, the fluorescence intensity increases gradually as the signal probe concentration increases up to 1 μM within 45 min in the presence of 200 nM Cu^{2+}. However, the intensity does not increase significantly up to a signal probe concentration of 1.5 μM. Both SP1 and SP2 were of the same concentration. In addition, the effect of hybridization time was also examined. As shown in Fig. 2B, the fluorescence intensity increases as the hybridization time changed from 0 to 45 min for detection of 200 nM Cu^{2+} and almost remained at a constant level after that. Hence, 1 μM and 45 min were chosen respectively in this study as the optimum signal probe concentration and hybridization time for self-assembly.

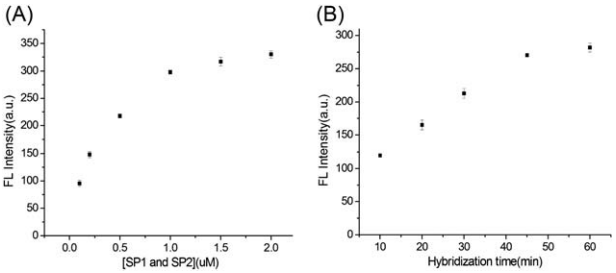


Fig. 2 Effect of (A) the concentration and (B) the hybridization time of SP1 and SP2 on the fluorescence intensity for detection of 200 μM Cu^{2+}. The fluorescence spectra were recorded using a fluorospectrophotometer. The concentrations of Cu-substrate and Cu-DNAzyme were 100 nM and 500 nM, respectively. The error bars represent the standard deviation of three independent measurements.

Detection of Cu^{2+} under optimal experimental conditions

To evaluate the sensitivity and dynamic range of the enzyme-free and label-free fluorescent biosensor, different concentrations of Cu^{2+} (10 μM, 1 μM, 200 nM, 50 nM, 2 nM, 100 pM, 20 pM, 0 pM) were examined simultaneously. As shown in Fig. 3A, in the absence of Cu^{2+}, the solution showed a very weak fluorescence. In the presence of Cu^{2+}, the fluorescence of the solution increased with increasing Cu^{2+} concentration. Their corresponding fluorescence intensities at the maximum emission peak were also recorded. The resulting calibration curve showed that the fluorescence intensities are proportional to the logarithm of Cu^{2+} concentration in the range of 20 pM to 1 μM (Fig. 3B). The limit of detection was 12.8 pM, which is calculated based on triple standard deviation from the mean of blanks.

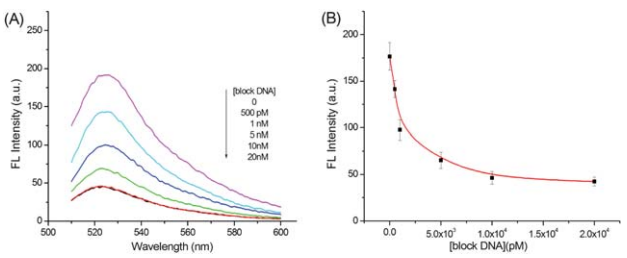


Fig. 1 (A) The fluorescence spectra of solutions with different concentrations of block DNA. (B) Plots of fluorescence intensity *vs.* the concentration of block DNA. The fluorescence spectra were recorded using a fluorospectrophotometer. The concentrations of Cu-substrate and Cu-DNAzyme were 100 nM and 500 nM, respectively. The error bars represent the standard deviation of three independent measurements.

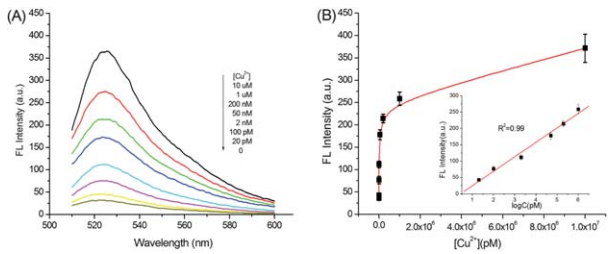


Fig. 3 (A) Fluorescence spectra of solutions with different concentrations of Cu^{2+}. (B) Plots of fluorescence intensity *vs.* concentration of Cu^{2+}. Inset: calibration curve of fluorescence intensity *vs.* the logarithm of Cu^{2+} concentration. The error bars represent the standard deviation of three independent measurements.

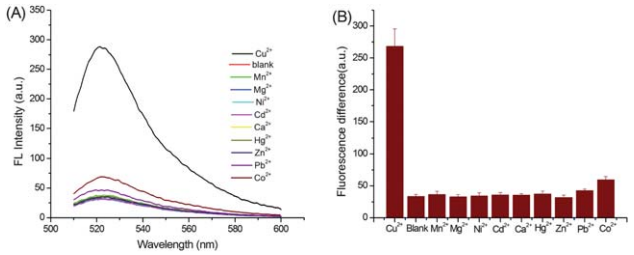


Fig. 4 (A) Fluorescence spectra of solutions in the absence (blank) or presence of Cu^{2+} (1 μM) and other competing metal ions (each 10 μM). (B) Difference in fluorescence intensity between the blank and solutions containing different metal ions. The error bars represent the standard deviation of three independent measurements.

Compared to previously reported colorimetric,[7,10] fluorescence anisotropy,[9] lateral flow nucleic acid[11] and graphene-quenched DNAzyme biosensors[12] for Cu^{2+} detection, this new detection strategy developed here possesses much higher sensitivity.

Such an attractive detection limit of our assay can be primarily attributed to the continuous self-assembled long dsDNA concatamers in the presence of Cu^{2+}. The detection limit of our strategy (12.8 pM) is six orders of magnitude lower than the EPA limit of Cu^{2+} in drinkable water (20 μM). Therefore, this assay is promising for monitoring of Cu^{2+} with its superior detection limit and wide dynamic range.

Specificity of the assay

To validate the specificity of the fluorescent biosensor for Cu^{2+} analysis, several other metal ions, including Mn^{2+}, Mg^{2+}, Ni^{2+}, Cd^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, and Co^{2+}, were tested. As shown in Fig. 4, there is no obvious increased fluorescence in the presence of 10 μM of other metal ions except for the slightly higher fluorescence with Pb^{2+} and Co^{2+} than that of the blank, while 1 μM Cu^{2+} can produce much higher fluorescence. These results indicate that our strategy exhibits high specificity for the detection of Cu^{2+}.

The practical application of the fluorescent biosensor was demonstrated by applying it to detect Cu^{2+} in tap water and human serum samples. Recovery experiments were performed by spiking different concentrations of Cu^{2+} into tap water and human serum, respectively. The detection results are shown in Table S1 (ESI†). Satisfactory recovery rates in tap water (between 86.9% and 104%) and human serum (between 80% and 105.8%) for Cu^{2+} detection were obtained, which confirmed that the proposed biosensor was applicable for practical Cu^{2+} detection in water and human serum samples.

Conclusion

In summary, we have successfully constructed an enzyme-free and label-free fluorescence turn on biosensor for Cu^{2+} detection. The reaction mechanism is based on the cleavage of Cu^{2+}-specific DNAzyme, the released target DNA triggering the formation of dsDNA concatamers and using SG as a signal reporter. In comparison with previously reported colorimetric and fluorescent-labeled methods for Cu^{2+} detection, the whole reaction process does not need any protein enzyme and fluorescent-labeled DNA, making the system more simple and cost-effective. The detection limit for Cu^{2+} is 12.8 pM under optimal conditions. In addition, this assay is generally applicable for other metal ions based DNAzymes, such as Pb^{2+}, Hg^{2+} and UO_2^{2+} based DNAzymes.[21–23]

Acknowledgements

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