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DNAzyme-based biosensor for Cu^{2+} ion by combining hybridization chain reaction with fluorescence resonance energy transfer technique

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ABSTRACT

A novel signal amplification strategy based on Cu^{2+}-dependent DNAzyme was developed for sensing Cu^{2+} ion by combining hybridization chain reaction (HCR) with fluorescence resonance energy transfer (FRET) technique. In the presence of Cu^{2+} ion, the substrate strands of Cu^{2+}-dependent DNAzyme immobilized on magnetic beads were specifically cleaved and released. The released strands initiated the HCR process of hairpin H1 and H2 labeled with FAM as the donor and TAMRA as the acceptor, respectively. Long nicked dsDNA structures were self-assembled to bring the donor and the acceptor in close proximity, resulting in a FRET process. The relative ratio of fluorescent intensities of the acceptor and donor was used to quantitatively detect Cu^{2+} ion with a limit of detection of 0.5 nmol L^{-1}. This proposed biosensor was applied to detect Cu^{2+} ion in tap water with satisfactory results.

1. Introduction

Copper ion (Cu^{2+}), one of the essential micronutrient elements for human life, is a necessary cofactor or structural component of numerous enzymes [1]. However, a high concentration of Cu^{2+} ion in biological or environmental system is toxic to human health [2,3]. Thus, the development of a simple and sensitive method to monitor Cu^{2+} ion is of great importance to human health and environmental science. Traditional analytical methods, such as atomic absorption spectroscopy [4], and inductively coupled plasma mass spectroscopy [5], have been used as the most common techniques for the high sensitive Cu^{2+} ion detection. However, these methods usually involve sophisticated instruments, elaborate sample pretreatment and professional training to operation, which limit their widespread application. Therefore, the development of alternative approaches for the sensitive, selective and convenient detection of Cu^{2+} ion in the environmental and biological samples is in great demand.

DNAzyme, a kind of catalytic DNA molecule isolated through in vitro selection process or systematic evolution of ligands by exponential enrichment [6], can catalyze many chemical reactions in the presence of specific cofactors including inorganic, organic and biomolecules, and even cells or viruses. Due to its facile preparation and outstanding stability under harsh conditions Cu^{2+}-dependent DNAzyme isolated by Breaker [7] have been used as an ideal recognition platform for Cu^{2+} ion [8]. Therefore, many biosensors for Cu^{2+} ion have been designed by using colorimetric method [9,10], dynamic light scattering technique [11], electrochemical method [12,13], fluorescent method [8,14–23]. Among them, fluorescent biosensor is particularly attractive due to its high sensitivity, easy readout, low sample volume, simple operation and feasibility of quantification.

Hybridization chain reaction (HCR), which is first introduced by Dirk and Pierce [24], is an enzyme-free amplification technique where DNA self-assembly is triggered by an initiator DNA and leads to the formation of long nicked double-stranded DNA structures at mild conditions. Nowadays, HCR has become a fascinating and effective amplification technique to construct various sensing platforms for the detection of DNA [25], microRNA [26], protein [27], small molecule [28], cell [29], et al. Recently, our group has utilized HCR technique with G-quadruplex to design a colorimetric sensor for Hg^{2+} ion [30], fluorescent biosensors for ATP [31] and HIV DNA [32]. To further extend our interesting in the distinct features of HCR for enzyme-free signal amplification, herein we proposed a novel DNAzyme-based biosensor for Cu^{2+} ion by combining HCR with FRET techniques. This biosensor mainly consisted of Cu^{2+}-dependent DNAzyme and two hairpin probes, which were labeled with carboxyfluorescein (FAM) as the donor and tetramethylrhodamine (TAMRA) as the acceptor, respectively. Cu^{2+} ion initiated the catalytic reaction to hydrolytically cleave the substrate strands in Cu^{2+}-dependent

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DNAzyme. Subsequently, the cleaved substrate strands as an initiator triggered the HCR process to form long nicked dsDNA structures. The donor and the acceptor were brought in close proximity, resulting in a FRET process between them. The relative fluorescent intensities of the acceptor and donor was used to quantitatively detect Cu^{2+} ion.

2. Experimental

2.1. Chemical and materials

Sybr Green I (SG I) and streptavidin functionalized Dynabeads magnetic beads (1.0 μm in diameter, 10 mg mL^{-1}) were purchased from Beijing Search Biotech Co., Ltd (Beijing, China). Sodium ascorbate (AR) was purchased from Aladdin. $Cu(NO_3)_2$ was bought from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China). Tris(hydroxymethyl) aminomethane (Tris, BR), and Na_2HPO_4, NaH_2PO_4, NaCl were bought from Sinopharm Group Co., Ltd. (Shanghai, China). HCl (AR) was bought from Hengyang Kaixin Chemical Reagent Co., Ltd. (Hengyang, China) Ethylenediaminetetraacetic acid disodium salt (EDTA, AR) was obtained from Guangdong Shantou Xinning Chemical factory (Shantou, China). The enzyme strand and biotin-modified substrate strand of Cu^{2+}-dependent DNAzyme, and the fluorophore-labeled hairpin probes (H1 and H2) were synthesized and HPLC-purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). These sequences of the oligonucleotides were listed as follows:

Substrate strand (S): 5′-ACA TAT GCT TCT TTC TAA TAC GGC TTA CCA GAG ATT TTT T-3′-biotin;

Enzyme strand (E): 5′-AAA TCC TCT GGT AAG CCT GGG CCT CTT TCT TTT TAA GAA AGA AC-3′;

Initiator strand (I): 5′-ACA TAT GCT TCT TTC TAA TAC G-3′;.

Hairpin DNA (H1): 5′-CGT ATT AGA AAG CAT ATG TAA CTT(FAM) CCC ATA TGC TTC TTT CT-3′;

Hairpin DNA (H2): 5′-ACA TAT GCT TCT TTC TAA TAC GAG AAA GAA GCA T(TAMRA)AT GGG AAG TT-3′.

The stock solutions (10.0 μmol L^{-1}) of oligonucleotides were prepared by a phosphate buffer A (500 mmol L^{-1} NaCl, 50 mmol L^{-1} Na_2HPO_4, pH 7.4). Other metal ion solutions were prepared from nitrate salts. Other chemicals are analytical grade and were used without further purification. The solutions were prepared using ultrapure water which was purified by Millipore Milli-Q (18 MΩ cm).

2.2. Immobilization of Cu^{2+}-dependent DNAzyme on magnetic beads

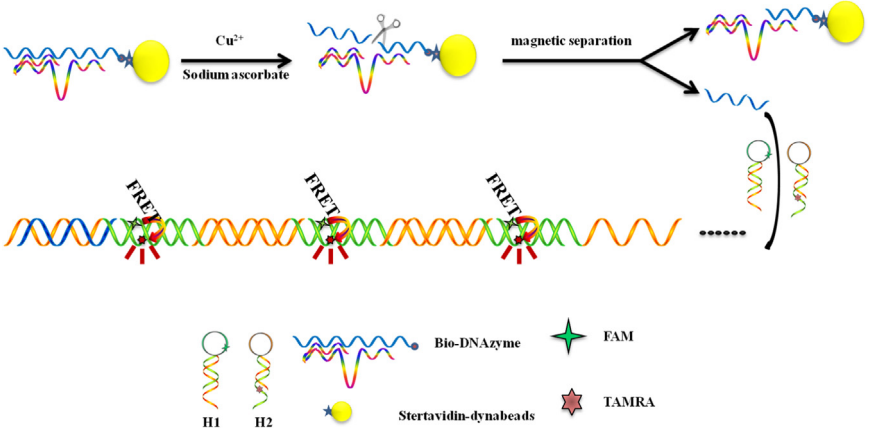
Enzyme strand (10.0 μmol L^{-1}) and substrate strand (10.0 μmol L^{-1}) were mixed at 1.5:1 in volume and the mixture was heated to 95 °C for 2 min and cooled to room temperature (25 °C) at least for 4 h to form stable Cu^{2+}-dependent DNAzyme. The Cu^{2+}-dependent DNAzyme was stored in the refrigerator (4 °C) before use.

Before magnetic beads were taken out of the stock vial, the vial was vortexed to ensure the magnetic beads were homogenously dispersed. 8.0 μL magnetic beads (10 mg mL^{-1}) were washed three times by 50 μL 1 × bind and wash (B&W) buffer (10 mmol L^{-1} Tris–HCl, pH 7.5, 1.0 mol L^{-1} NaCl) to remove the preservative and were dispersed in 10 μL 2 × B&W buffer (10 mmol L^{-1} Tris–HCl, pH 7.5, 2.0 mol L^{-1} NaCl). Then 5 μL Cu^{2+}-dependent DNAzyme was added and the mixture was incubated at 30 °C for 60 min with gentle shaking to immobilize Cu^{2+}-dependent DNAzyme on magnetic beads. The Cu^{2+}-dependent DNAzyme-immobilized magnetic beads were separated and washed six times by 10 μL 1 × B&W buffer to remove the unbound Cu^{2+}-dependent DNAzyme and excess enzyme strand in the supernatant with the help of a magnet and then redispersed in 8 μL phosphate buffer B (50 mmol L^{-1} NaCl, 50 mmol L^{-1} Na_2HPO_4, pH 7.4) for further use.

2.3. Detection of target Cu^{2+} ion

Before detection of Cu^{2+} ion, fluorophore-labeled hairpin probes H1 and H2 were respectively heated to 95 °C for 2 min and then allowed to cool down to room temperature (25 °C) at least for 4 h to form hairpin structure. The annealed hairpin probes were stored in the refrigerator (4 °C) before use.

8 μL Cu^{2+}-dependent DNAzyme-immobilized magnetic beads, 5 μL ascorbic acid (5 mmol L^{-1}), and 1 μL Cu^{2+} ion of different concentration were added to 86 μL phosphate buffer B and the mixture was incubated at 25 °C for 30 min to cleave the Cu^{2+}-dependent DNAzyme. After separation by a magnet, the supernatant (50 μL) was shifted and added into 244 μL Tris–HCl buffer (10 mmol L^{-1}, pH 7.5, 1 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA). Then, 3 μL H1 (10 μmol L^{-1}) and 3 μL H2 (10 μmol L^{-1}) were added, the mixture was incubated at 25 °C for 60 min. The fluorescent spectra between 490 nm and 650 nm were recorded under the excitation of 480 nm by a Cary Eclipse fluorospectrophotometer (Varian, USA). The ratios of fluorescent intensities at 580 nm and 520 nm ($I_{F(580)}/I_{F(520)}$) were used to quantitatively detect Cu^{2+} ion.



Scheme 1. Schematic illustration of Cu^{2+}-dependent DNAzyme-based fluorescent biosensor by combining the HCR with FRET technique.

3. Results and discussion

3.1. Principle of Cu^{2+} ion detection

The principle of this biosensor for sensing Cu^{2+} ion was shown in Scheme 1. The enzyme strand and biotin-modified substrate strand were hybridized to form the Cu^{2+}-dependent DNAzyme. Then the DNAzyme was immobilized on the magnetic beads via the biotin-streptavidin interaction. At the presence of target Cu^{2+} ion, the substrate strand was irreversibly cleaved at the cleavage site (the guanine base). The cleaved piece at the 5′-end of the substrate strand was released due to the decreased affinity to the enzyme strand. The un-cleaved Cu^{2+}-dependent DNAzyme was magnetically separated from the solution to decrease the background signal. The cleaved substrate strand as the initiator was then mixed with the hairpins H1 and H2, which were labeled with FAM as the donor and TAMRA as the acceptor respectively, and initiated the HCR process to form the nicked double-helix structure. In this state, FAM and TAMRA were brought into close proximity, resulting in the occurrence of FRET. Thus, the fluorescent emission of FAM (at 520 nm) decreased and that of TAMRA (at 580 nm) increased simultaneously. By means of ratiometric measurement, Cu^{2+} ion could be quantitatively detected. In the absence of Cu^{2+} ion, the substrate strand still hybridized steadily with the enzyme strand and the Cu^{2+}-dependent DNAzyme was magnetically separated from the solution. No initiator strand triggered the HCR process, no FRET occurred.

To support the Cu^{2+}-dependent cleavage of DNAzyme, SG I was used to indicate the structure conversion of DNAzyme in the presence of various concentrations of Cu^{2+} ion. SG I is a weakly fluorescent agent in aqueous media, while it can emit bright fluorescence after intercalating the double-stranded DNA of DNAzyme. After addition of Cu^{2+} ion, a significant decrease in fluorescence intensity was observed with the increase of Cu^{2+} ion concentration (Fig. S1), which indicated that the substrate strands of Cu^{2+}-dependent DNAzyme were cleaved in the presence of Cu^{2+} ion and the cleaved substrate strands were released into the solution due to the decreased affinity for the enzyme strands. To support the occurrence of HCR, gel electrophoresis was used to verify the HCR. The cleaved substrate strands (the portion of substrate strand in the 5′) were used as the initiator to trigger the HCR process. As shown in Figure S2, there were only low molecular weight bands in lane 8 (only H1) and lane 7 (H1/H2), indicating no HCR occurred in absence of initiator strand. Once the initiator strand was introduced (lanes 2–6, H1/H2/ initiator strand), smears of high molecular weight bands were observed, which indicated that initiator strand triggered the HCR process between H1 and H2 to form long nicked dsDNA structures.

3.2. Optimization of experimental conditions

In order to obtain a highly sensitive response for sensing Cu^{2+} ion, the optimization of experimental conditions was essential. Sodium ascorbate was reported to react with Cu^{2+} ion to generate Cu^{+} ion which then participated in the consequent reaction of cleaving the substrate strands of DNAzyme [14,20]. As shown in Fig. 1, the ratios of fluorescent intensities at 580 nm and 520 nm ($I_{F(580)}/I_{F(520)}$) increased with the concentration of sodium ascorbate form 0–250 μmol L^{-1}, but decreased when further increasing the concentration of sodium ascorbate. Therefore, the optimal concentration of sodium ascorbate was chosen at 250 μmol L^{-1}. The composition of the buffer also played an important role in the sensitivity of biosensor. Ionic strong not only can affect the stability of hybridization and catalytic activity of DNAzyme, but also the HCR process and the result of FRET. In this work, NaCl was used to regulate the ionic strength of buffer solution. As Cu^{2+}-dependent

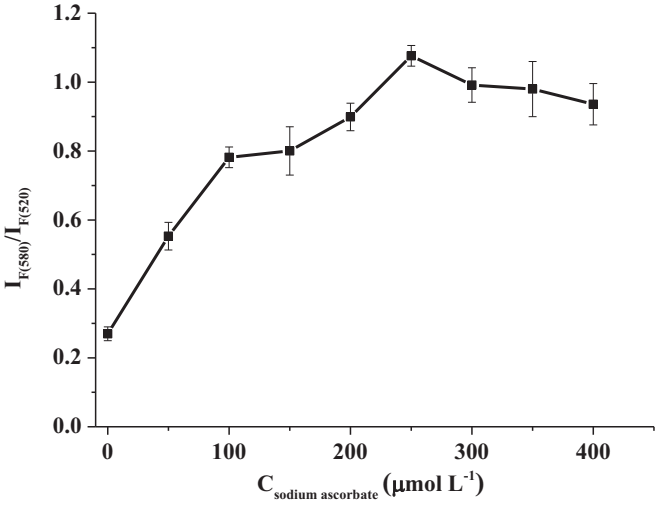


Fig. 1. Effect of different concentrations of sodium ascorbic on the cleavage of Cu^{2+}-dependent DNAzyme. (DNAzyme: 60 nmol L^{-1}, Cu^{2+} ion: 100 nmol L^{-1}, H1: 100 nmol L^{-1}, H2: 100 nmol L^{-1}).

DNAzyme contains a DNA triplex, its stability is not as high as that with normal Watson-Crick base pairs. To ensure the stability of Cu^{2+}-dependent DNAzyme, high ionic strength (500 mmol L^{-1} NaCl) in buffer solution is necessary [20]. While in the presence of Cu^{2+} ion and sodium ascorbate, the substrate was cleaved and released in the solution and the enzyme strands still immobilized on magnetic beads via the biotin-streptavidin interaction. Therefore, buffer B (50 mmol L^{-1} PBS, 50 mmol L^{-1} NaCl, pH 7.4) with relative low ionic strength was chose as the reaction solution [20]. However, to make sure the stability of the hairpins and reduce the background interference, high ionic strength (1 mol L^{-1} NaCl) was chose for the subsequent HCR. Besides, as Cu^{2+} ion is a paramagnetic metal ion with intrinsic fluorescence quenching property [14], the FRET result may be affected by the residual Cu^{2+} ion. Therefore, EDTA was added to chelate with Cu^{2+} ion after the HCR process. As shown in Fig. 2, initiator strand could trigger the HCR process and induce the occurrence of FRET in the coexistence of Cu^{2+} ion, the FRET efficiency was enhanced at the presence of 1 mmol L^{-1} EDTA.

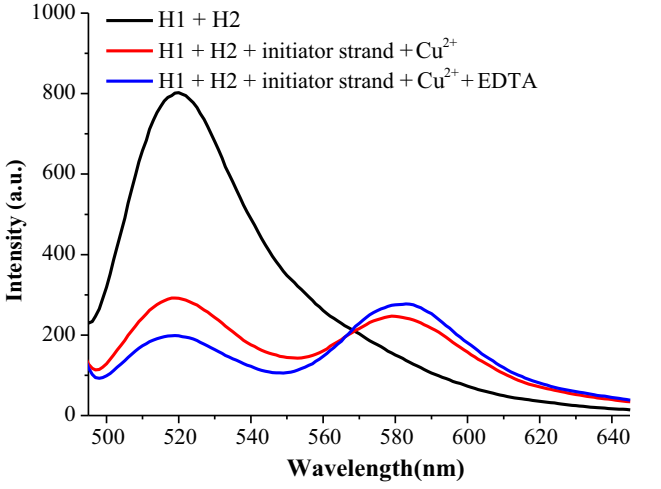


Fig. 2. Effect of EDTA on the FRET efficiency during the HCR process. (Cu^{2+} ion: 100 nmol L^{-1}, H1: 100 nmol L^{-1}, H2: 100 nmol L^{-1}, EDTA: 1 mmol L^{-1}, initiator strand: 10 nmol L^{-1}).

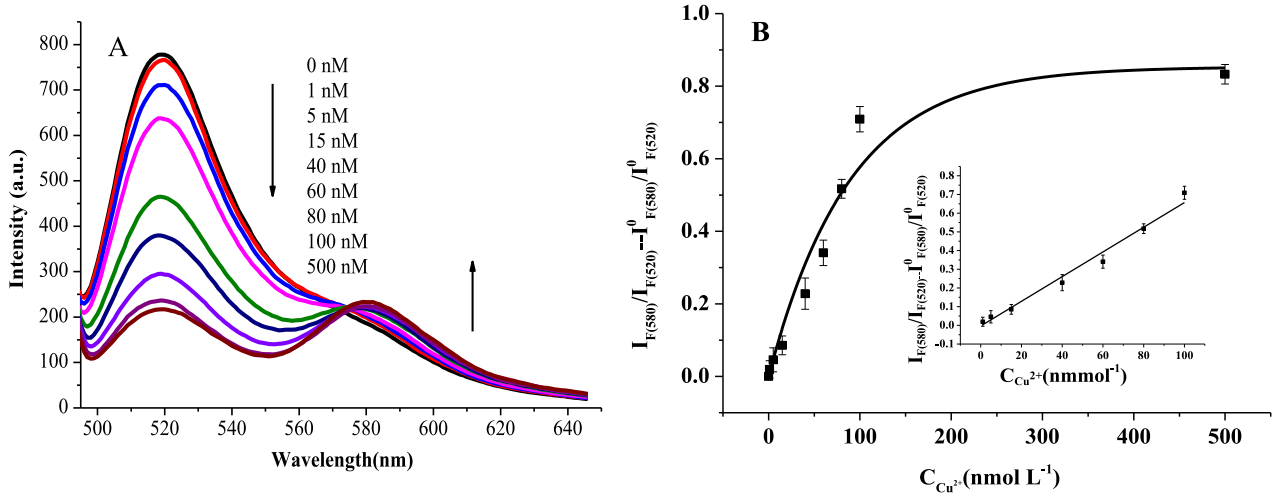


Fig. 3. (A) Fluorescence spectra of detection solution in the presence of various concentration of Cu^{2+} ion. (B) Plot of $I_{F(580)}/I_{F(520)}$ - $I°_{F(580)}/I°_{F(520)}$ vs Cu^{2+} ion concentration. Inset is the calibration curve. The error bars indicated the standard deviations of three experiments.

3.3. Sensitivity and selectivity

The fluorescent spectra toward different concentrations of Cu^{2+} ion were measured under the optimal conditions. As shown in Fig. 3A, the fluorescent intensity of FAM at 520 nm decreased and that of TAMRA at 580 nm increased with the increasing concentration of Cu^{2+} ion due to the HCR process, resulting from the FRET from the donor FAM to the acceptor TAMRA. Fig. 3B showed the change of relative fluorescent intensities of TAMRA at 580 nm and FAM at 520 nm ($I_{F(580)}/I_{F(520)}$–$I°_{F(580)}/I°_{F(520)}$) increased over the Cu^{2+} ion concentration of 0–500 nmol L^{-1}. The calibration curve (inset of Fig. 3B) indicated that there was a good linear relationship between the relative fluorescent intensities ($I_{F(580)}/I_{F(520)}$–$I°_{F(580)}/I°_{F(520)}$, y) and the concentration of Cu^{2+} ion (x) over the range of 1–100 nmol L^{-1}. The regression equation was listed as following:

$$y = 0.0067x - 0.0102$$

Regression coefficient (R) of the regression equation was 0.989, which was larger than the critical value (0.951) with 99.9% confidence level. The detection limit was about 0.5 nmol L^{-1} ($3\sigma/K$, in which σ is the standard deviation for the blank solution, and K is the slope of the calibration curve).

The specificity of the biosensor was tested by measuring and comparing the response of Cu^{2+} ion with other metal ions, such as Ca^{2+}, Cd^{2+}, Hg^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{3+}, K^{+}, respectively. As shown in Fig. 4, the concentrations of Ca^{2+}, Cd^{2+}, Hg^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{3+}, K^{+} were fifty folds higher than that of Cu^{2+} ion while yielded a significantly lower fluorescent response. These results revealed that this proposed biosensor exhibited a good specificity to discriminate Cu^{2+} ion.

3.4. Application

To demonstrate the feasibility of the biosensor for direct quantitation of Cu^{2+} ion in real samples, the analysis of Cu^{2+} ion in tap water was carried out. However, there was no response signal for the concentration of Cu^{2+} ions in the tap water samples were lower than the limit of detection of this sensor. Therefore, recovery experiments were performed by spiking different concentrations of Cu^{2+} ions into the tap water and measured according to the detection method. The results were listed in Table 1, indicating that this biosensor was feasible to analyze Cu^{2+} ion in practical samples.

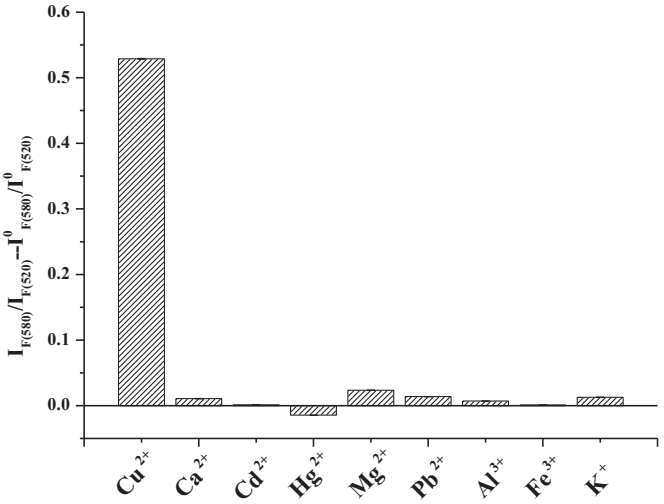


Fig. 4. Fluorescent response for Cu^{2+} ion and other interfering ions. (Cu^{2+} ion: 80 nmol L^{-1}, Ca^{2+}, Cd^{2+}, Hg^{2+}, Mg^{2+}, Pb^{2+}, Al^{3+}, Fe^{3+}, K^{+} ion all in 4 μmol L^{-1}).

Table 1
Analysis of Cu^{2+} ion in tap water.

Tap water	Added (nmol L^{-1})	Found (nmol L^{-1}) Mean ± SD[a]	Recovery (%)
Sample 1	40.0	41.20 ± 0.2	103
Sample 2	60.0	55.55 ± 0.7	92.6
Sample 3	80.0	80.59 ± 0.3	100.7

[a] SD was the standard derivation of three measurements.

4. Conclusions

In summary, we proposed a novel DNAzyme-based biosensor for Cu^{2+} ion by combining the HCR with FRET technique. The biosensor could be used to quantitatively detect Cu^{2+} ion with a limit of detection of 0.5 nmol L^{-1}. The utilization of Cu^{2+}-dependent DNAzyme enhanced the specificity of the biosensor for Cu^{2+} ion. Importantly, the ratiometric measurement by FRET technique could reduce the influence of those external factors in practical application such as excitation source fluctuation and probe concentration. Therefore, this biosensor shows a potential application for Cu^{2+} ion in practical environmental screening and bioassays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.04.057.

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