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Label-free colorimetric biosensing of copper(II) ions with unimolecular self-cleaving deoxyribozymes and unmodified gold nanoparticle probes

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Abstract

Using unimolecular copper(II)-dependent self-cleaving deoxyribozymes (DNAzymes), a label-free colorimetric biosensor for copper(II) ions (Cu^{2+}) has been developed based on the sequence-length-dependent adsorption of single-stranded deoxyribonucleic acid (ssDNA) on unmodified gold nanoparticles (AuNPs). In the presence of Cu^{2+}, the Cu^{2+}-dependent DNAzyme could be self-cleaved into short ssDNA fragments. The cleaved short ssDNA could adsorb rapidly onto the surface of the AuNPs. This enhanced the stability of the AuNPs against salt-induced aggregation, and thus the solution color remained red. In the absence of Cu^{2+}, however, uncleaved long ssDNA adsorbed relatively slowly onto the AuNPs and upon the addition of salt, the electrostatic repulsion between the AuNPs was screened, resulting in aggregation of the AuNPs which produced a red-to-blue color change. Thus, Cu^{2+} detection could be realized by monitoring the color change of the AuNPs. The calibration curve showed that the absorption ratio values at 520 and 620 nm increased linearly over the Cu^{2+} concentration range of 0.625–15 μM, with a limit of detection of 290 nM. The other environmentally relevant metal ions did not interfere with the determination of Cu^{2+}. Subsequently, the assay was employed to determine Cu^{2+} in several water samples, and the results were satisfactory. It is expected that the present colorimetric strategy will be possibly extended to the detection of cofactors of other *in vitro*-selected unimolecular self-cleaving DNAzymes, such as amino acids, nucleic acids, metal ions and small organic molecules.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Deoxyribozymes (DNAzymes) are a class of catalytic nucleic acids selected by the systematic evolution of the ligand by the exponential enrichment (SELEX) process. Similarly to protein enzymes or ribozymes, DNAzymes can catalyze many chemical and biological transformations, and some of the reactions require specific cofactors such as amino acids, nucleic acids, metal ions and small organic molecules, which make it a novel platform for developing highly selective biosensors. Up until now, many electrochemical [1–3] and optical techniques [4–11] for biosensors based on DNAzymes have been reported, and among them, simple colorimetric biosensors require minimal instrumentation, allow on-site, real-time detection, and thus are commonly used for routine analysis. Metallic nanoparticles,

especially gold nanoparticles (AuNPs), have been recently emerging as important colorimetric reporters, because such nanoparticles possess high extinction coefficients, allowing sensitive colorimetric detection with minimal material consumption [12]. More importantly, their surface plasmon resonance (SPR) absorption is strongly distance-dependent [13]. Well-dispersed AuNPs having interparticle distances substantially greater than their average particle diameter appear red, whereas the color of the aggregated AuNPs changes to blue as the interparticle distance drops below the average particle diameter. However, it is difficult to control the distance between AuNPs precisely. DNAzyme, on the other hand, provides such a control due to DNA's programmable nature [14, 15]. Thus, the functionalization of AuNPs with DNAzyme provides a possibility for selective colorimetric sensing and has recently been used to detect various analytes, such as Pb^{2+} [4, 9, 16] and adenosine [5]. However, they mostly still involve complicated thiol-modification to DNAzyme and/or its substrate strands at the 3′ or 5′ end so that the DNA strand can bind to the surface of AuNPs through the alkane thiol group. The modification of AuNPs with thiol-containing DNAzyme and/or its substrate strands requires rather time-consuming steps and the synthesis of thiol-labeled DNA is elaborate and expensive. Thus, the development of modification-free AuNP colorimetric biosensors to simplify the detection process is important and attractive.

In 2004, Rothberg *et al* reported a DNA colorimetric biosensor based on the fact that unfolded single-stranded deoxyribonucleic acid (ssDNA) could be adsorbed onto the citrate-protected AuNPs, while double-stranded deoxyribonucleic acid (dsDNA) could not [17]. Based on this phenomenon, Dong's group [9] and Lu's group [16] recently reported lead(II) (Pb^{2+}) ion detection with Pb^{2+}-dependent RNA-cleaving DNAzyme and unmodified AuNP probes. They firstly hybridized the Pb^{2+}-dependent DNAzyme and its substrate with a molar ratio of 1:1 in order to form a duplex precisely. Then, in the presence of Pb^{2+}, the hybridized duplex could cleave the substrate strand and release the ssDNA fragments. The ssDNA could be adsorbed on the AuNPs thus protecting the AuNPs from aggregation under high-salt conditions. On the other hand, the duplex could remain in the absence of Pb^{2+}, which could not adsorb on the AuNPs and thus could not stabilize the AuNPs under the same conditions. These approaches do offer a general means of DNAzyme-based biosensing of cofactors with unmodified AuNPs probes. However, the need for a hybridization process of two oligomers (DNAzyme and its substrate) makes this strategy laborious. Additionally, Wang *et al* [16] previously reported that the hybridization process of two oligomers by annealing of the DNAzyme and its substrate strands with a molar ratio of 1:1 to form a duplex precisely is very important for sensitive detection of cofactors because very small numbers of unhybridized ssDNAs can still stabilize AuNPs and increase the background.

In 2009, Nie *et al* reported the property of the sequence-length-dependent adsorption of unimolecular ssDNA on the citrate-protected AuNPs [18]. Unlike the conventional unmodified AuNPs colorimetric assay based on conformation change from unfolded ssDNA conformation to rigid dsDNA [17], they focused more attention on the effect of sequence length of unimolecular ssDNA on the adsorption rate of ssDNA onto AuNPs, which is a potential mechanism for sensing but largely neglected. Then they also exploited this rationale for colorimetric assay of enzymatic cleavage and oxidative damage of ssDNA. Herein, based on this phenomenon reported by Nie *et al* [18], we extended the concept to the colorimetric biosensing of copper(II) (Cu^{2+}) ions by using Cu^{2+}-dependent DNA-cleaving DNAzyme as the target recognition element and unmodified AuNPs as probes. This design employed unimolecular ssDNA containing DNAzyme and substrate strands, and thus eliminated the hybridization procedure between DNAzyme and substrate strands and facilitated Cu^{2+} ion detection.

2. Experimental methods

2.1. Reagents and chemicals

Hydrogen tetrachloroaurate(III) tetrahydrate was purchased from Acros Organics (New Jersey, USA). Trisodium citrate and sodium chloride were obtained from Beijing Chemical Reagent Company (Beijing, China). L-ascorbic acid was obtained from Sigma-Aldrich (Milwaukee, WI, USA). 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was purchased from Shanghai Lizhudongfeng Biotechnology Co., Ltd (Shanghai, China). The 46-mer Cu^{2+}-dependent self-cleaving DNA oligonucleotide (5′-GAATTCTAATACGACTC AGAATGAGTCTGGGCCTCTTTTTAAGAAC-3′) was obtained from Sangon Biotechnology Co. Ltd (Shanghai, China). Copper(II) sulfate was obtained from Beijing Yili Fine Chemical Co., Ltd (Beijing, China). Before use, the oligonucleotide sample was dissolved in 10 mM HEPES buffer of pH 7.4. The concentration of the oligonucleotide was determined by measuring the 260 nm UV absorbance. Unless specified otherwise, the other reagents and chemicals were at least analytical reagent grade. The water used throughout all experiments was purified with a Milli-Q system (Millipore, Bedford, MA) to a specific resistance of >18 MΩ cm.

2.2. Synthesis of AuNPs

All glassware employed in these preparations was cleaned in a bath of freshly prepared aqua regia (three parts hydrochloric acid, one part nitric acid), rinsed thoroughly in water, and oven-dried prior to use. AuNPs were synthesized using the reported method [19]. A 250 ml aqueous solution of 1 mM hydrogen tetrachloroaurate(III) tetrahydrate was brought to a vigorous boil while being stirred in a round-bottom flask fitted with a reflux condenser, and then 38.8 mM of trisodium citrate (25.0 ml) was rapidly added to the solution. The solution was boiled for another 15 min, during which time its color changed from pale-yellow to wine-red. This solution was cooled to room temperature with continuous stirring. The resulting wine-red solution was stored in a refrigerator at 4 °C before use.

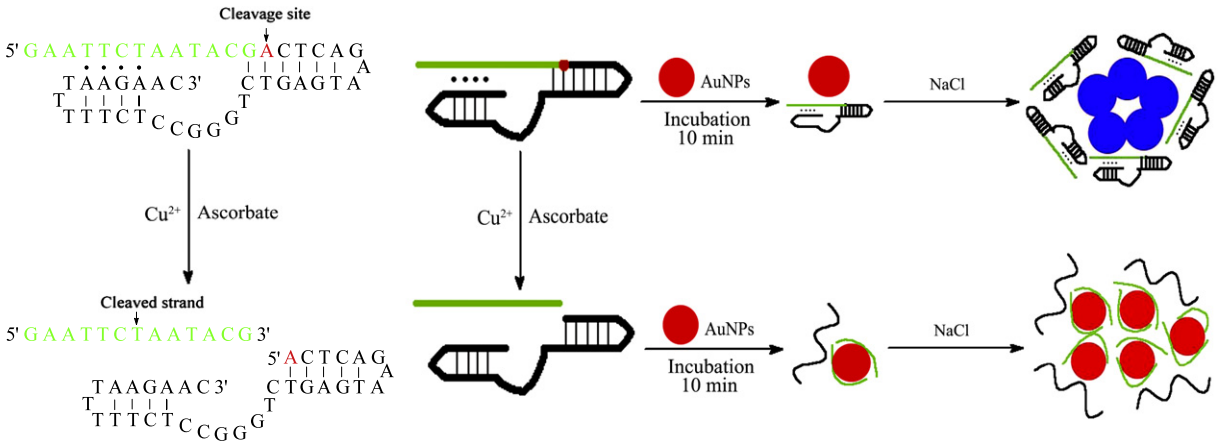

Figure 1. Left: the secondary structure of 46-mer Cu^{2+}-dependent self-cleaving DNAzyme. After copper-induced cleavage, 13-mer ssDNA fragments are released which can be adsorbed onto the AuNP surface. Right: schematic representation of AuNPs colorimetric strategy for Cu^{2+} detection. The copper-cleaved/uncleaved complex and AuNPs are incubated for 10 min, and then mixed with NaCl. The AuNPs aggregate in the absence of copper but remain dispersed in the presence of copper.

2.3. Instrumentation

UV–visible absorption spectra were recorded on a Cary 50 UV–visible spectrometer (Varian, USA) using quartz cuvettes with an optical path length of 1 cm at room temperature. The pH was measured with a Model 420 A pH meter (Orion). TEM measurements were made on a Hitachi H-8100 transmission electron microscope operated at an accelerating voltage of 200 kV.

2.4. General procedure of colorimetric determination of Cu^{2+}

The reaction mixture (40 μl) containing 4 μM Cu^{2+}-dependent self-cleaving DNAzyme, 10 μM ascorbic acid, buffer A (50 mM HEPES (pH 7.0), 0.5 M NaCl, 0.5 M KCl), and an appropriate concentration of Cu^{2+} was incubated in a 0.6 ml microcentrifuge tube for 1 h. After that, 35 μl of the reaction solution was transferred into a 1.5 ml microcentrifuge tube, and then 185 μl of water, and 120 μl of AuNPs were micropipetted into this solution to give a volume of 340 μl. After incubation for 10 min, 60 μl of 1 M NaCl was added immediately to give a final volume of 400 μl. A photograph was taken with a Canon IXUS 110 IS digital camera, or the UV–visible absorption spectrum was recorded with respect to water. All assays were herein performed at room temperature.

2.5. Selectivity and interference measurements for Cu^{2+}

To investigate the selectivity of the system to Cu^{2+} over various other metal ions, the following metal ions were used: Cu^{2+}, Cd^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Pb^{2+}, Hg^{2+}. An appropriate volume of 5 mM various metal ion solution (final concentration is 15 μM) was mixed with 4 μM of Cu^{2+}-dependent self-cleaving DNAzyme in the buffer solution (50 mM HEPES (pH 7.0), 0.5 M NaCl, 0.5 M KCl, 10 μM ascorbic acid) in the absence or presence of 15 μM Cu^{2+} and then analyzed as described under the general procedure.

3. Results and discussion

3.1. Principle for the biosensing system

The mechanism for the Cu^{2+} biosensor is shown in figure 1. As can be seen in figure 1, the unimolecular Cu^{2+}-dependent DNA-cleaving DNAzyme reported by Breaker *et al* was 46-mer ssDNA and possessed a complex secondary structure [20]. It consisted of two stem-loops that are interspersed with three single-stranded domains. The 5′-portion of the DNAzyme bound stem located at the end of the 3′-region through formation of a DNA triplex. In the presence of Cu^{2+}, the DNAzyme could be irreversibly self-cleaved at the cleavage site (the adenine in red), releasing the 13-mer cleaved strand (in green). The biosensor system also contained 10 μM ascorbic acid because it could significantly enhance the cleavage rate [20]. The uncleaved and cleaved ssDNA could be adsorbed onto AuNPs via base-dependent binding and electrostatic attraction with a sequence-length-dependent rate [18]. Uncleaved long ssDNA was adsorbed relatively slowly onto AuNPs in a controllable time. (Based on Nie's experiments [18], the incubation of 10 min is herein enough to efficiently differentiate the uncleaved and cleaved ssDNA with various lengths.) After the addition of salt, the electrostatic repulsion between AuNPs was screened under high-salt conditions, resulting in the aggregation of AuNPs which produced a red-to-blue color change. However, in the presence of Cu^{2+}, the cleaved short ssDNA could be adsorbed rapidly onto the surface of AuNPs. This enhanced the stability of AuNPs against salt-induced aggregation, and thus the solution color remained red.

3.2. Colorimetric biosensing of Cu^{2+}

Figure 2 shows a typical colorimetric detection of Cu^{2+} using Cu^{2+}-dependent DNA-cleaving DNAzyme and unmodified AuNPs. DNA self-cleavage was conducted in a reaction mixture (40 μl) containing 4 μM Cu^{2+}-dependent DNAzyme, 10 μM ascorbate, buffer A (50 mM HEPES (pH 7.0), 0.5 M

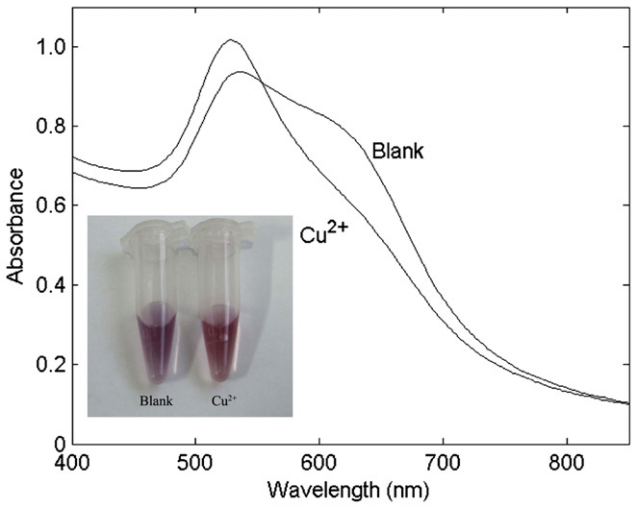

Figure 2. UV–visible absorption spectra of the ssDNA/AuNPs solution in the absence (blank) or presence of 15 μM Cu^{2+} after the addition of 150 mM NaCl. Inset: the corresponding color change of ssDNA/AuNPs solution in the absence or presence of 15 μM Cu^{2+}.

NaCl, 0.5 M KCl) and an appropriate concentration of Cu^{2+} at room temperature. After incubation for 1 h, 35 μl of the mixture was transferred into the AuNP solution. NaCl was added 10 min later. The AuNP solution with uncleaved ssDNA changed from red to blue as soon as the NaCl was added, whereas that with short ssDNA fragments after the addition of Cu^{2+} to the DNAzyme remained red (inset in figure 2). Correspondingly, a surface plasmon resonance (SPR) absorption band of the AuNPs solution with uncleaved ssDNA at about 520 nm with a shoulder band at about 620 nm was observed. As for the cleaved ssDNA/AuNPs/NaCl solution in the presence of Cu^{2+}, only one characteristic peak located at 520 nm appeared. Direct evidence for Cu^{2+}-stimulated dispersion of the AuNPs could be further supported by transmission electron microscopy (TEM) measurements that revealed aggregation of nanoparticles in the absence of Cu^{2+} (figure 3(a)) and individual NPs in the presence of Cu^{2+} (figure 3(b)). Thus, through this color change phenomenon, the cleavage of the DNAzyme could be directly detected with the naked eye, realizing the detection of Cu^{2+} conveniently. In addition, we investigated the kinetic process of the system in the absence or presence of Cu^{2+} by monitoring the absorption ratio value at 520 and 620 nm ($A_{520\ \mathrm{nm}}/A_{620\ \mathrm{nm}}$). As can be seen in figure 4(a), the $A_{520\ \mathrm{nm}}/A_{620\ \mathrm{nm}}$ values sharply decreased and remained stable after 4 min in the absence or presence of Cu^{2+}. Figure 4(b) clearly shows that the net absorption ratio value ($\Delta A_{520\ \mathrm{nm}}/A_{620\ \mathrm{nm}}$) in the absence and presence of Cu^{2+} remarkably decreased with the increase of time. Thus, on the basis of higher sensitivity, we collected the UV–visible absorption spectra immediately after the addition of NaCl.

3.3. Sensitivity and selectivity for the Cu^{2+} biosensor

To determine the sensitivity of the biosensor, the UV–visible spectra of the ssDNA/AuNPs solution in the presence of various concentrations of Cu^{2+} after the addition of NaCl were

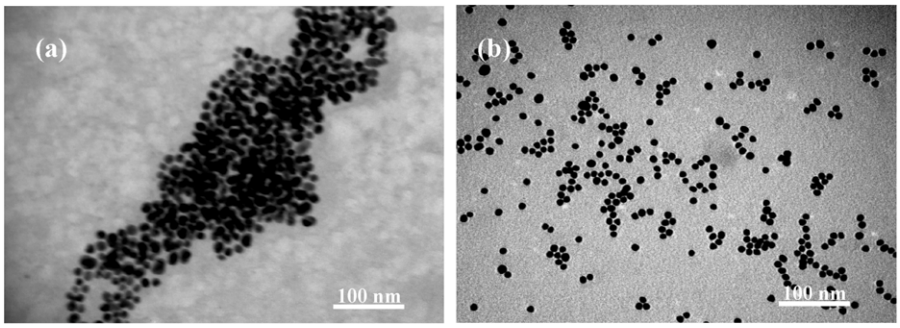

Figure 3. TEM images of ssDNA/AuNPs solution in the absence (a) or presence (b) of 15 μM Cu^{2+} after the addition of 150 mM NaCl.

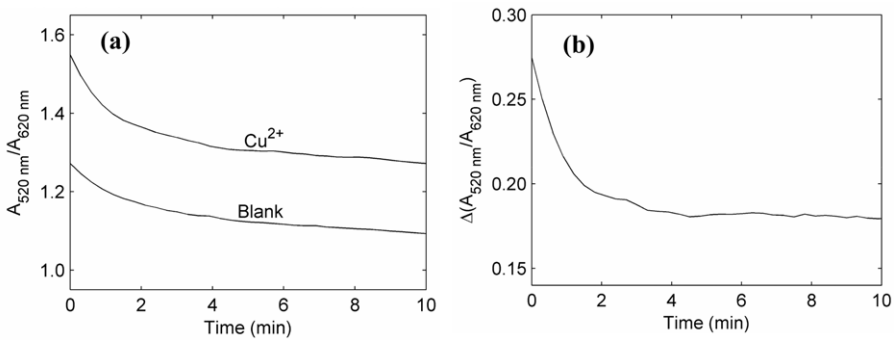

Figure 4. (a) Absorption ratio value at 520 and 620 nm ($A_{520\ \mathrm{nm}}/A_{620\ \mathrm{nm}}$) in the absence (blank) or presence of Cu^{2+} versus time. (b) Net absorption ratio value ($\Delta A_{520\ \mathrm{nm}}/A_{620\ \mathrm{nm}}$) in the absence and presence of Cu^{2+} as a function of time.

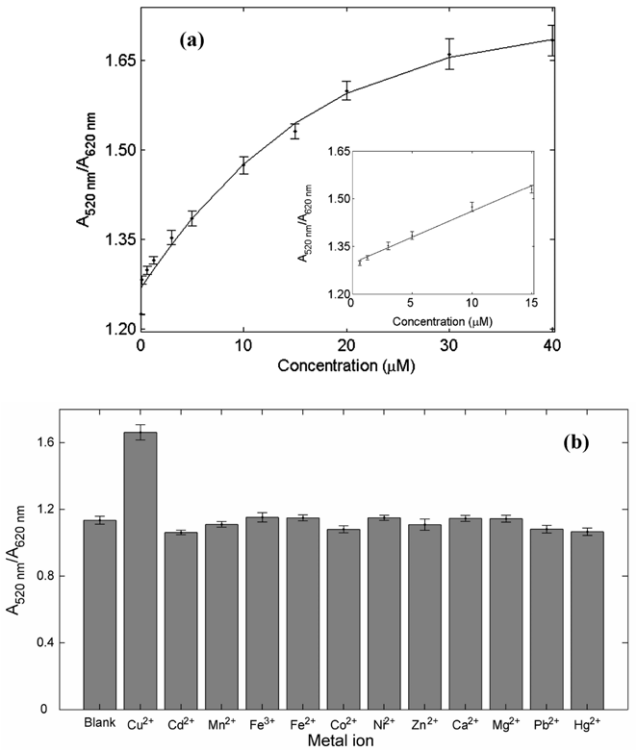

Figure 5. (a) Plot of the absorption ratio ($A_{520\ nm}/A_{620\ nm}$) against Cu^{2+} concentration. Inset: derived calibration curve. (b) Selectivity study corresponding to the analysis of Cu^{2+} by the proposed method. The concentration of all the metal ions was 15 μM.

recorded. The absorption ratio value ($A_{520\ nm}/A_{620\ nm}$) versus the concentration of Cu^{2+} (0–40 μM) is plotted in figure 5(a). It was clearly observed that the $A_{520\ nm}/A_{620\ nm}$ value gradually increased with the increase of the concentration of Cu^{2+}. The absorption ratio could be fitted as the equation of $A_{520\ nm}/A_{620\ nm} = 1.30 + 0.0163c$ (μM) over the range of 0.625–15 μM (figure 5(a) inset) with the correlation coefficient of 0.994, and the detection limit was determined to be 290 nM (3σ/slope), which compared well with those achieved by the most sensitive analytical methods based on fluorescent dyes [21, 22]. The US Environmental Protection Agency (EPA) and the World Health Organization (WHO) defined the Cu^{2+} maximum contamination level in drinking water to be 20 μM and 30 μM, respectively, which are well within the biosensor dynamic range.

To test the selectivity of the biosensor, different environmentally relevant metal ions, including Cu^{2+}, Cd^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Pb^{2+}, Hg^{2+} were added to the biosensor solution separately. As indicated in figure 5(b), the absorption ratio values ($A_{520\ nm}/A_{620\ nm}$) upon the addition of various other metal ions were similar to that given by the blank, and the $A_{520\ nm}/A_{620\ nm}$ values in the presence of various metal ions caused less than 7% variations relative to that given by the blank, suggesting that the biosensor had nearly no response to various other metal ions. However, Cu^{2+} gave a remarkable enhanced absorption ratio at the identical concentration, demonstrating that the biosensor had good selectivity towards Cu^{2+}. Additionally, the detection of Cu^{2+} in the presence of various interfering metal ions gave the absorption ratio value ($A_{520\ nm}/A_{620\ nm}$) close to that obtained exclusively in the presence of Cu^{2+}, and the variations in the $A_{520\ nm}/A_{620\ nm}$ values in the presence of Cu^{2+} and various other metal ions were less than 5% relative to that given exclusively in the presence of Cu^{2+} (data not shown). This further indicated a negligible influence of these substances on our sensor system to detect Cu^{2+}.

Table 1. Determination of Cu^{2+} in water samples using the proposed procedure.

Samples	Added (μM)	Mean found[a] (μM)	Mean recovery[b] (%)	RSD[c] (%)
1	1.00	1.01	101	2.5
2	5.00	5.08	102	0.6
3	7.00	6.93	99	0.8

[a] Mean concentration of three replicates.
[b] Mean recovery (%) = 100 × ($c_{mean\ found}/c_{added}$).
[c] Relative standard deviation of three determinations.

3.4. Application: determination of Cu^{2+} in lake water samples

The applications of the proposed method were evaluated for determination of Cu^{2+} in lake water samples (South Lake, Changchun, China). The lake water samples collected were filtered through a 0.22 μm membrane, then centrifuged for 15 min at 14 000 rpm. Then, we employed an inductively coupled plasma-mass spectrometry (ICP-MS) technique to evaluate the level of copper(II) in lake water, and the results showed that the concentration of Cu^{2+} was 6.01 nM, far less than the detection limit given by the proposed method. Thus, the as-prepared water samples were spiked with Cu^{2+} at different concentration levels and then analyzed with the proposed method. The results are summarized in table 1. As can be observed in table 1, the mean recoveries of such samples were around 100%. The results revealed the potential application of this Cu^{2+} biosensor in real samples.

4. Conclusion

By using *in vitro*-selected unimolecular Cu^{2+}-dependent self-cleaving DNAzyme as the target recognition element and unmodified AuNPs as probes, a simple and cost-effective assay for the sensitive and selective colorimetric detection of Cu^{2+} ion was developed originally. In the presence of Cu^{2+}, Cu^{2+}-dependent DNAzyme could be self-cleaved into short ssDNA fragments. Then, the dynamic effect of the adsorption of ssDNA with various lengths onto AuNPs and the length-dependent stability of ssDNA/AuNPs solution against salt-induced aggregation could be exploited for biosensing of Cu^{2+}. This colorimetric biosensing strategy provides a general platform for sensing the cofactors of other *in vitro*-selected unimolecular self-cleaving DNAzymes, such as amino acids, nucleic acids, metal ions and small organic molecules. Further work towards this direction is underway.

Acknowledgments

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