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A DNAzyme-based Electrochemical Impedance Biosensor for Highly Sensitive Detection of Cu^{2+} Ions in Aqueous Solution

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We developed a simple, label-free, DNAzyme-based electrochemical impedance sensor for highly sensitive detection of Cu^{2+} ions in aqueous solution. A complex of Cu^{2+}-dependent DNAzyme and its corresponding substrate was immobilized on the gold electrode surface though Au-S bond. In the presence of Cu^{2+} ions, the cleavage of DNAzyme resulted in a significant change in the charge transfer resistance signal intensity. The resistance signal change correlated with the concentration of Cu^{2+} ions. The proposed sensor showed excellent sensitivity and selectivity. The limit of detection of this sensor is 5 nM, which is far below the limit of Cu^{2+} ions (～20 μM) mandated by United States Environmental Protection Agency (EPA). A series of metal ions, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cr^{3+}, Pb^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}, have little interference with the detection of Cu^{2+} ions.

Keywords: Electrochemical sensor; Cu^{2+}; DNAzyme; Electrochemical impedance spectroscopy (EIS)

1. INTRODUCTION

Copper is widely used in chemical industries. It can be released into the environment through various routs. Although trace amounts of Cu^{2+} is biologically essential for organisms, excess Cu^{2+} intake to organism body can cause some diseases, such as gastrointestinal disturbance, kidney damage, and the inactivation of enzyme system [1-3]. Therefore, the development of accurate and reliable methods for the detection of Cu^{2+} ions is highly desirable for environmental monitoring and medical diagnostics.

For this purpose, different methods have been developed to routinely determine Cu^{2+} ions in various samples with high sensitivity, including atomic absorption spectroscopy (AAS) [4], inductively coupled plasma mass spectrometry (ICP-MS) [5,6], fluorescence spectroscopy [7-9], and electrochemical assay [10-14]. However, some of these methods could not fulfill the need in real-time

and on-site monitoring of Cu^{2+} ions. Considering the intrinsic fluorescence quenching properties of Cu^{2+}, electrochemical approach offers considerable potential as an attractive alternative to develop sensitive and portable sensing systems for the detection of Cu^{2+} ions because of its low cost, fast response, portability, and simplicity of construction. These electrochemical sensors are prepared by immobilizing high sensitive and selective agents (e.g. molecularly imprinted polymer [15], polymer [16-17], nanocomposite [18-20], small organic molecules [21-23], and DNA [24]) on the electrodes to recognize Cu^{2+} ions. Hence, modification of electrochemical electrode with biological elements that specifically interacts Cu^{2+} ions has attracted much attention in monitoring Cu^{2+} ions.

DNAzymes, a new category of DNA molecules with catalytic properties of enzyme, have been proved to be very promising candidates as highly selective recognition elements for heavy metal ions due to their inherent advantages such as easy of synthesis, relatively high stability, and fast reaction rates [25]. These DNAzyme are isolated by *in vitro* selection using heavy metal ions (Pb^{2+}, Cu^{2+}, Zn^{2+}, UO_2^{2+}, etc.) as cofactors, thus, they have high specificity for heavy metal ions [26-31]. Recently, DNAzyme-based fluorescent sensors have been reported for the detection of Cu^{2+} ions [32-34].

Here we reported a simple, label-free, DNAzyme-based electrochemical impedance sensor for the detection of Cu^{2+} ions in aqueous solution. In this assay, the complex of Cu-specific DNAzyme and its substrate is used to construct sensitive sensing interfaces for Cu^{2+} ions. We apply electrochemical impedance spectroscopy (EIS) to follow the charge transfer resistance properties of the electrode interface. $[Fe(CN)_6]^{3-/4-}$, as an electrochemical reporter system, provides a signal proportional to the change of charge transfer resistance at electrode surfaces. Based on this strategy, a label-free, highly sensitive, electrical detection of Cu^{2+} ions could be achieved.

2. EXPERIMENTAL

2.1 Materials and chemicals

All oligonucleotides were synthesized and purified by Integrated DNA Technologies (Coralville, IA, USA). Their sequences were shown as follow:

Cu^{2+} DNAzyme (Cu-Enz): 5'-GGTAAGCCTGGGCCTCTTTCTTTTTAAGAAAGAAC-3'

Corresponding substrate (Cu-Sub): 5'-SH-$(CH_2)_6T_{10}$AGCTTCTTTCTAATACGrCTTACC-3'

Tris (hydroxymethyl) aminomethane, $CuCl_2$ and other metal ions were purchased from Sigma-Aldrich. Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) and 6-mercaptohexanol (MCH) were obtained from Sinopharm Chemical Reagent (Shanghai, China). All commercially available reagents were used as received without further purification. All solutions were prepared with ultrapure water (18.2 MQ cm) from a Millipore Milli-Q system.

2.2 Apparatus

Cyclic voltammetry (CV) and EIS were performed on electrochemical analyzer (CHI660D, CHI Instruments, shanghai, China). Quartz crystal microbalance (QCM) was carried out on CHI420a. The gel images were taken with Bio-Rad ChemiDoc MP imaging system.

2.3 Preparation of the complex/MCH modified gold electrode

A gold electrode (2 mm in diameter, CHI Instruments) was polished with α-Al_2O_3 suspensions (1.0, 0.3 and 0.05 μm in diameter) on a polishing microcloth and sonicated in ethanol and ultrapure water for 3 min, respectively. To further clean the electrode, a series of oxidation and reduction cycles were performed in 1.0 M NaOH and 0.5 M H_2SO_4 solution, respectively. Finally, the gold electrode was rinsed with ultrapure water and dried in gentle stream high-purity nitrogen.

To form the complex of Cu-Sub and Cu-Enz, 1 μM Cu-Sub was hybridized with 1 μM Cu-Enz for 30 min in 50 mM Tris-HAc buffer (500 mM NaCl, pH 8.2) at 60℃ and slowly cooled down to room temperature. Prior to immobilization on the clean gold electrode, the complex solution was incubated with 10 μM TCEP for 1 h to reduce disulfide bonds. The clean gold electrode was then immersed in the reduced complex solution for 16 h in the dark. The functionalized surface was passivated with 1 mM 6-mercaptohexanol for 30 min at room temperature. The modified electrode was used for electrochemical detection of Cu^{2+} ions as an electrochemical impedance sensor.

2.4 Gel electrophoresis assay

The cleavage reactions of the complex (1 μM) were performed in 10 mM Tris-HCl (pH 7.4, 50 mM NaCl) in the presence of different concentrations of Cu^{2+} ions. The cleaved products were separated from the substrate by a polyacrylamide (PAGE) gel (20% acrylamide, 19:1 acrylamide/bisacrylamide). The electrophoresis was carried out in 1×tris-borate-EDTA (TBE) buffer (90 mM Tris, 90 mM boric acid, and 10 mM EDTA, pH 8.0) at 180 V for 3 h. The gels were silver-stained.

2.5 Electrochemical measurement

A conventional three-electrode system consisting of the complex-modified gold electrode, an Ag/AgCl (3 $mol \cdot L^{-1}$) reference electrode, and a platinum counter electrode was used for all electrochemical measurements. CV was carried out in 1 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl from -0.2 to 0.6 V at a rate of 100 mV/s. EIS was performed in 1 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. The parameter of EIS was set as follow: the bias potential was 0.21 V (vs Ag/AgCl), the amplitude was 5.0 mV, and the EIS was recorded in the frequency range of 10^5 Hz to 1 Hz. For the detection of Cu^{2+} ions, the complex-modified electrode was incubated with different concentrations target Cu^{2+} in the 50 mM Tris-HAc buffer solution (500 mM NaCl, pH 8.2) for 6 min at room temperature. After each treatment, the electrode was rinsed with buffer solution, and then EIS was performed. Quartz crystal microbalance (QCM) equipped with an AT-cut 7.995 MHz piezoelectric quartz crystal (13.7 mm in diameter). Prior to modification, the quartz crystal was cleaned with a piranha solution (30% H_2O_2 and 70% concentrated H_2SO_4) for 5 min, rinsed thoroughly with deionized water and dried with nitrogen gas. The immobilization of Cu-Sub and Cu-Enz complex was the same as that of gold electrode.

3. RESULTS AND DISCUSSION

3.1 sensing mechanism and Sensor fabrication

Electrochemical impedance spectroscopy (EIS) is a powerful and sensitive technique to probe interfacial property at the electrode surface. Due to its label-free, cost-effective property, EIS is increasingly employed for sensing biorecognition events at the surface of electrode [35-36]. Based on this strategy, we developed a new DNAzyme-based EIS sensor for Cu^{2+} ions. The principle of the proposed is illustrated in Figure1.

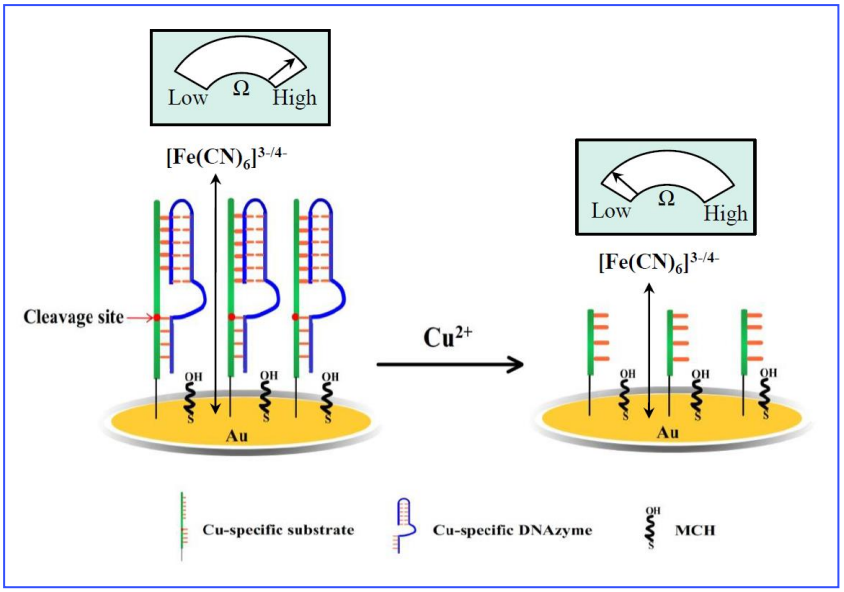
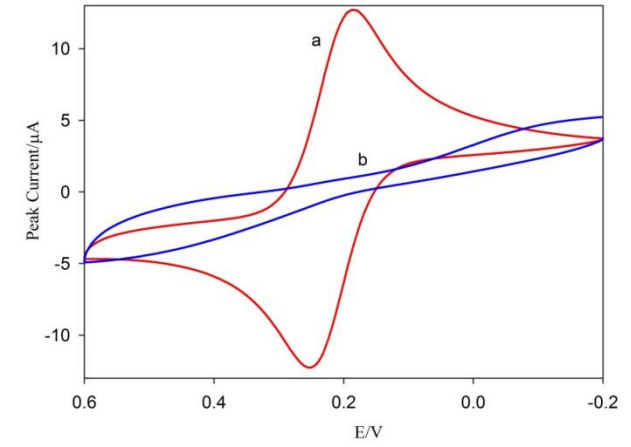


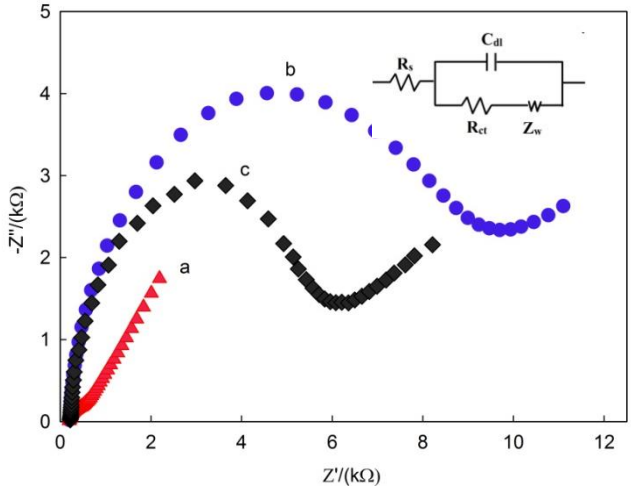
Figure 1. Schematic illustration of DNAzyme-based electrochemical impedance sensor for the detection of Cu^{2+} ions.

We employed an in vitro selected Cu-specific DNAzyme (Cu-Enz)/substrate (Cu-Sub) with minor modification to fabricate the sensor [34]. The 5'-end of Cu-Sub was modified with a thiol group for its immobilization on the surface of the gold electrode. The thiolated Cu-Sub was first hybridized with Cu-Enz, thereby forming the complex of Cu-Enz and Cu-Sub. The resulting complex was tethered to the surface of the gold electrode. And then, the assembly surface of electrode was treated with MCH, which blocked the unmodified sites of the electrode surface and prevented the nonspecific absorption of the excess complex [24]. The assembly process was characterized by cyclic voltammetry (CV) using $[Fe (CN)_6]^{3-/4-}$ as an electrochemical probe, and the results were showed in Figure 2A.

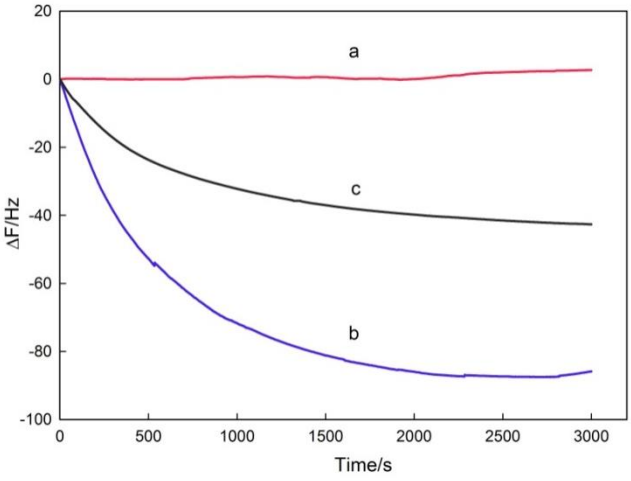
Figure.2A showed CV of $[Fe (CN)_6]^{3-/4-}$ on different modified electrodes. For the bare gold electrode, a pair of well-defined Re-Ox peaks was observed (Figure.2A, curve a). After modification with the complex and 6-mercaptohexanol (Figure.2A, curve b), the electrode showed the remarkable decrease of the current intensity and the separation between the cathodic and anodic peak of $[Fe(CN)_6]^{3-/4-}$.



A



B



C

Figure 2. (A) Cyclic voltammetry of bare gold electrode (curve a) and complex/MCH modified gold electrode (curve b); (B) Nyquist plots and (C) Time-dependent frequency changes of different electrodes. (a) Bare gold electrode; (b) Complex/MCH modified electrode; (c) Complex/MCH modified electrode after interaction with 10 μM Cu^{2+} ions.

The suppression of voltammetric response resulted from the fact that the assembled DNA complex on the electrode formed a compact negatively charged layer due to its phosphate backbones, thereby preventing the negatively charged redox probe $[Fe(CN)_6]^{3-/4-}$ from reaching the gold electrode. The result indicated that the sensing interface of the proposed sensor was successfully fabricated. Many reported immobilized sensors suffer from time-consuming and multistep processing during the fabrication or detection procedure [37-38]. Compared to these sensors, our sensor is simple, label-free and does not require lengthy handling procedures. Furthermore, our procedure can be used for direct assay detection of Cu^{2+} after sensor preparation, and the sensor can be prepared ahead of time and stored.

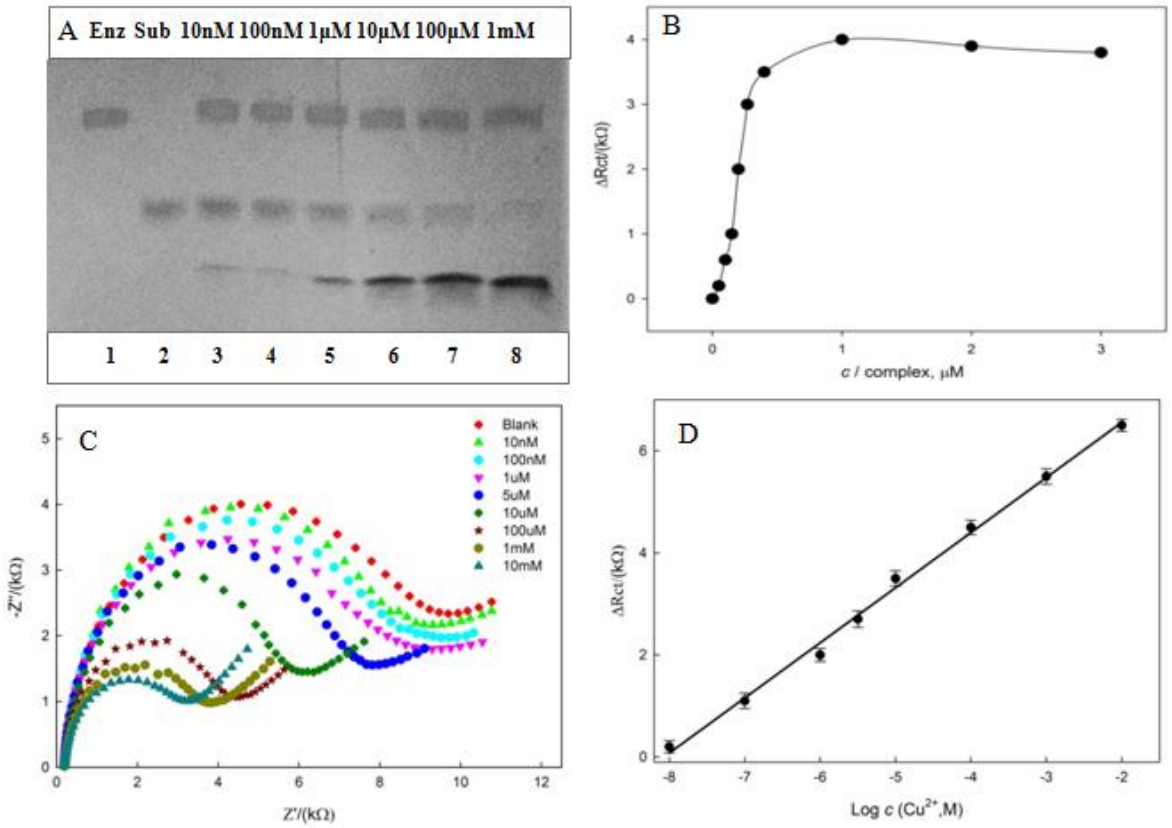


Figure 3. (A) 20% denaturing PAGE analysis. lane 1: Cu^{2+}-specific DNAzyme; lane 2: Cu^{2+}-specific substrate; lane 3-8: treatments of the complex with different concentrations of Cu^{2+} ions; (B) The effect of the complex concentration during the sensor fabrication on the EIS signal change; (C) Electrochemical impedance spectroscopy of the proposed sensor after incubation with different concentrations of Cu^{2+} ions; (D) The linear relationship between the R_{ct} change against logarithmic Cu^{2+} concentration. Error bars are standard deviations of three repetitive experiments.

To understand the charge transfer resistance at the sensor surface, EIS was carried out to record the interfacial information of the assembled electrodes. Figure.2B showed the electrochemical

impedance spectra of the sensor at different stages of development had classical Nyquist diagrams, which consist of a semicircle in high-frequency regions and a linear part in low-frequency regions [39-40]. The R_{ct} value is obtained by the equivalent circuit (Figure2B, inset).The bare gold electrode exhibited a very low R_{ct} (0.48 kΩ, Figure.2B, curve a). When the DNA complex was immobilized on the electrode, R_{ct} increased to 10.4 kΩ (Figure.2B, curve b), which was attributed to the formation of the compact negatively charge layer of the complex on the gold electrode surface, thus confirming the successful assembly of DNAzyme-based EIS sensors. To test the feasibility of the sensor, the assembled electrode was challenged with 10 μM Cu^{2+} ions. The complex was cleaved off the surface of electrode by Cu^{2+} ions at the cleavage site, and resulted in a drastic decrease of R_{ct} from10.4 kΩ to 5.96 kΩ (Figure.2B, curve c). To further verify that the significant change of R_{ct} was indeed from the cleavage of the DNAzyme complex, we used quartz crystal microbalance (QCM) to demonstrate the process. The frequency of the piezoelectric crystal is controlled by the mass of the crystal. Any decrease in the mass of the crystal will be accompanied by an increase in the resonance frequency of the crystal [41]. Treatment of the sensor with 10 μM Cu^{2+} ions resulted in a frequency increase ($\triangle f$ =43 Hz, Figure.2C, curve c). Visual evidence of the cleavage process was observed in the SDS-PAGE assay (Figure.3A). These results clearly indicated that it is feasible for the sensor to detect Cu^{2+} ions based on EIS.

3.2 EIS measurements of Cu^{2+} ions

The surface density of the complex on the electrode is a crucial parameter for the EIS signal change. Varying the complex concentrations during the preparation of sensor can regulate the surface density of the complex. To optimize the EIS signal change, we investigated the performance of the complex-modified electrode, which prepared with different concentrations of the complex, in the absence and presence of 10 μM Cu^{2+} ions. The change of R_{ct} ($^{\triangle}R_{ct}$) was used to characterize the charge change of electrode surface. As shown in Figure.3B, $^{\triangle}R_{ct}$ gradually increased with the increase of the complex concentrations, and reached the maximization at 1 μM. Therefore, 1 μM complex was chosen as the optimal concentration for the preparation of the sensor.

Under the optimal conditions, the detection of Cu^{2+} ions at different concentrations was performed by EIS. Figure.3C illustrated that R_{ct} decreased gradually with the increase of the concentration of Cu^{2+} ions. $^{\triangle}R_{ct}$ exhibited a linear relationship with the logarithm of Cu^{2+}concentrations in the range from 10 nM to 10 mM (Figure.3D). The regression equation was $^{\triangle}R_{ct}$ =8.717+1.08 X (R^2=0.996), where X was logarithm of Cu^{2+} concentrations, R was the regression coefficient. The limit of detection (LOD) was estimated to be 5 nM (S/N=3), which is far lower than the US EPA defined toxicity level for Cu^{2+} in drinking water (20μM) . As shown in Table1, although the sensing property of the proposed sensor is not the best comparing to some methods reported previously, the obtained sensing performance is good enough for practical application.

Table 1. Comparison of the major characteristics of DNAzyme-based literature methods used for Cu^{2+}detection.

Methods	Materials	Dynamic range (μM)	LOD (nM)	References
Fluorescent detection/DNAzyme	6-Carboxyfluorescein	0.1~0.5	35	34
Fluorescent detection/DNAzyme	Microarray	0.01~100	10	42
Fluorescent detection/DNAzyme	Quantum-dot	0.001~0.05	0.5	33
Colorimetric detection/DNAzyme	Horseradish peroxidase	0.05~1.2	5.9	43
Colorimetric detection/DNAzyme	Gold nanoparticle	0.001~0.02	0.47	44
Electrochemical detection / DNAzyme	Avidin–graphite epoxy composite	10~40	6500	45
Electrochemical detection / DNAzyme	gold nanoclusters	0.0001~0.4	0.0725	46
Electrochemical detection / DNAzyme	-	0.01~10000	5	This work

3.3 Selectivity of the DNAzyme-based EIS sensor

To evaluate the selectivity of the sensor, we challenged it with the same concentration (10 mM) of other metal ions, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cr^{3+}, Pb^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}. As shown in Figure.4, Only Cu^{2+} ions yielded a significantly increased $^{\triangle}R_{ct}$ relative to the blank, whereas no obvious $^{\triangle}R_{ct}$ increase was observed for other metal ions. The high selectivity of this sensor is attributed to the specific recognition between Cu-Enz and Cu^{2+} ions.

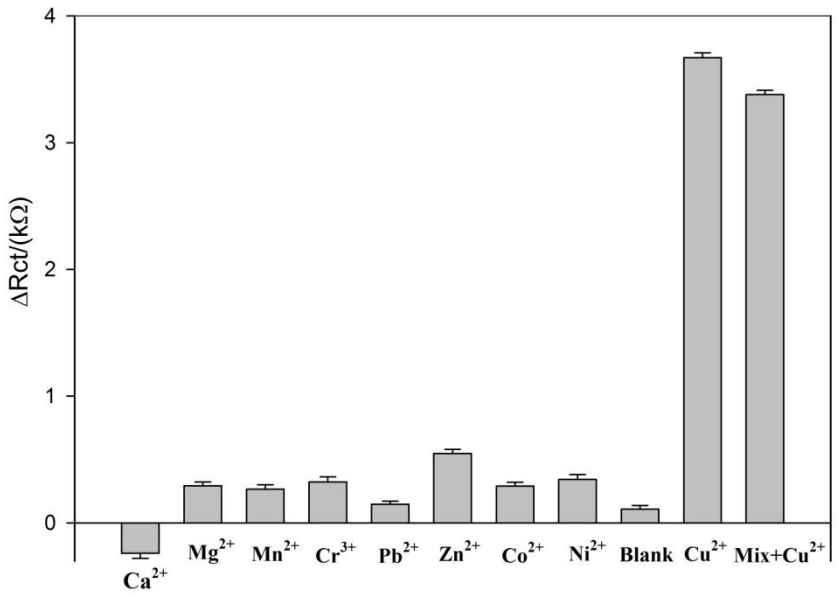


Figure 4. Selectivity of the sensor for Cu^{2+} determination. The R_{ct} change between the blank and other metal ions. Conditions: 10 μM Cu^{2+}, 100 μM other metal ions. Error bars are standard deviations of three repetitive experiments.

3.4 EIS measurements of real water samples

Table 2. Comparison of determination of Cu^{2+} ions in real samples by using the proposed sensor and ICP-MS (*n*=3). `

Samples	Blank (μM)	Cu^{2+}spiked (μM)	The proposed sensor		ICP-MS	
			Cu^{2+} founded (μM)	Recovery (%)	Cu^{2+}founded (μM)	Recovery (%)
Tap water	[a] 0.0067±0.0021	0.1	0.11±0.0064	103.3	0.105±0.0029	98.3
		1	0.98±0.047	97.3	0.99±0.034	98.3
		10	10.06±0.17	100.5	10.86±0.14	108.5
River water	0.0234±0.0016	0.5	0.57±0.077	109.3	0.55±0.063	105.3
		5	4.92±0.16	97.9	5.36±0.05	106.7
		50	49.92±0.49	99.8	52.52±0.19	104.9

[a] mean value±SD

To further explore the application of the sensor, the EIS determination of Cu^{2+} ions was carried out with the proposed sensor in tap water and river water samples using the standard addition method. The results listed in Table 2. Recovery was used to evaluate the accuracy of the developed sensor, and ICP-MS was employed as a reference method. The recoveries of this sensor were in the range of 97.3 ～109.3%, which was good agreement with that of ICP-MS. The results indicated that the sensor was valid for the real sample analysis.

4. CONCLUSIONS

We designed a DNAzyme-based electrochemical sensor for the determination of Cu^{2+} ions. This sensor is simple, label-free, and does not require the sample to be processed. The sensor combined the sensitive property of EIS with the high ion-specific DNAzyme. The sensor showed the good analytical performance, such as wide linear range, low limit of detection, and satisfactory selectivity. The proposed sensor will have broad applications in environmental protection and medical diagnostics.

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