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Hemin/G-quadruplex-based DNAzyme concatamers for *in situ* amplified impedimetric sensing of copper(II) ion coupling with DNAzyme-catalyzed precipitation strategy

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ABSTRACT

A new signal-amplification strategy based on copper(II) (Cu^{2+})-dependent DNAzyme was developed for sensitive impedimetric biosensing of Cu^{2+} in aqueous solution by coupling with target-induced formation of hemin/G-quadruplex-based DNAzyme and enzymatic catalytic precipitation technique. Initially, the target analyte cleaved the Cu^{2+}-specific DNAzyme to generate an initiator strand on the sensor. Thereafter, the initiator strand underwent an unbiased strand-displacement reaction between hairpin probes in turn to construct a nicked double-helix, accompanying the formation of hemin/G-quadruplex DNAzyme on the long duplex DNA. The newly formed DNAzyme could oxidize the 4-chloro-1-naphthol (4-CN) to produce an insoluble precipitation on the sensor, thus resulting in a local alteration of the conductivity. Under the optimal conditions, the resistance increased with the increasing Cu^{2+} in the sample, and exhibited a wide dynamic working range from 0.1 pM to 5.0 nM with a detection limit of 60 fM. The methodology also displayed a high selectivity for Cu^{2+} relative to other potentially interfering ions owing to the highly specific Cu^{2+}-dependent DNAzyme, and was applicable for monitoring Cu^{2+} in real river samples. Thus, our strategy has a good potential in the environment surveys.

1. Introduction

Heavy metal contaminations have seriously threatened human living environment and health, which were associated with exposure to lead, copper, cadmium, mercury and arsenic (Gumpu et al., 2015; Fu et al., 2013). Copper (Cu^{2+}) ion is an essential micronutrient element as a cofactor and/or structural component to support the biological metabolic activities of numerous enzymes and proteins (Verwilst et al., 2015; Hosseini et al., 2015). Unfavorably, excess copper intake to human body may cause some adverse afflictions, *e.g.*, liver or kidney damage, gastrointestinal disturbance, and degrading memory in the elderly or individuals with Wilson's disease (Mihai et al., 2015). Recently, different methods and strategies based on various signal-generation principles have been developed for the determination of copper ion, *e.g.*, by using atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS), fluorescence spectroscopy, and voltammetric or colorimetric assay (Cao et al., 2015; Foroushani et al., 2015; Ndokoye et al., 2014; Park et al., 2014; Jin and Han, 2014; Ding et al., 2014). Unfortunately, this process is relatively expensive, time-consuming or multiple labels. To keep pace with expectations in future point-of-care testing, there is the request for more flexible, yet highly sensitive, quantitative, and easy-to-use methods.

Electrochemical detection protocols are suitable for sensor miniaturization and automation because of their high sensitivity, low cost and low-power requirements (Liu et al., 2015; Bathany et al., 2015). For example, Li et al. constructed a highly sensitive and doubly orientated selective molecularly imprinted voltammetric sensor for the detection of Cu^{2+} (Li et al., 2015). Zhang et al. developed a new ratiometric electrochemical biosensor for monitoring of Cu^{2+} in a rat brain by coupling with a specific molecule (Zhang et al., 2015). Recent research has looked to develop innovative and powerful monitoring platform for Cu^{2+} to simplify the assay process while preserving the essential benefits in sensitivity (Su and Aprahamian, 2014; Kumar et al., 2014). DNAzyme, a kind of catalytic DNA molecule, can catalyze many chemical reactions. Similar to protein enzymes, DNAzymes depend on specific metal ions (*e.g.*, Pb^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and Mg^{2+}) or neutral molecules as cofactors, and show high catalytic hydrolytic cleavage

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activities toward certain substrates (Ren et al., 2015; Hou et al., 2015). Cu^{2+}-dependent DNAzyme accounts for specific cleavage site (the guanine) of the substrate in DNAzyme/substrate complex, which can make it as an ideal recognition platform for monitoring Cu^{2+} resulting from unique advantages, *e.g.*, facile preparation and outstanding stability under harsh conditions (Yin et al., 2009; Li et al., 2013).

Except for the cofactor-dependent DNAzymes, some G-quadruplex DNA aptamers have been found to strongly bind hemin to form the DNAzymes with peroxidase-like activity towards H_2O_2-mediated oxidation (Xu et al., 2013). Zeng and coworkers utilized the hemin-G-quadruplex-based DNAzyme to catalyze the oxidation of luminal by H_2O_2 for the detection of DNA methyltransferase (Zeng et al., 2013). Kaneko et al. used electrochemical method for high-throughout quantitative screening of peroxidase-mimicking DNAzymes on a microarray (Kaneko et al., 2013). Recently, our group found that hemin-based DNAzyme could catalyze the oxidation of 4-choloro-1-naphthol (4-CN) to produce an insoluble benzo-4-chlorohexadienone product (Hou et al., 2014). To this end, our motivation in this work is to integrate Cu^{2+}-dependent DNAzyme with hemin-based DNAzyme together for the development of impedimetric Cu^{2+} sensor by coupling with enzymatic biocatalytic precipitation.

Inspiringly, DNA concatamer, one of linear polymeric structures that was arisen by self-association of short DNA fragments through specific reactions (*e.g.*, hybridization chain reaction, HCR), has attracted increasing attention in molecular biology and DNA diagnostics (Filippov et al., 2009; Tang et al., 2012; Zhang et al., 2012). The alternative signal amplification approach of functional nucleic acid nanostructures recognizing the target to trigger an autonomous cross-opening process has been put forward on the detection of metal ions in environmental samples. The Willner's group presented an enzyme-free sensing platform of two functional hairpins self-assembly cross-opening to yields polymer DNA wires upon the recognition of Mg^{2+}-dependent DNAzyme (Wang et al., 2011). Herein, we report the proof-of-concept of a novel and label-free impedimetric DNA sensor for ultrasensitive detection of Cu^{2+} in aqueous solution on Cu^{2+}-dependent DNAzyme by coupling with target-induced self-assembly formation of hemin-based DNAzyme concatamers (Scheme 1). The signal is amplified through the formed DNAzyme concatamers toward catalytic precipitation of 4-CN. The system mainly consists of Cu^{2+}-dependent DNAzyme and two hairpin probes. The autonomous cross-opening hairpin probes can yield plentiful hemin/G-quadruplex DNAzyme concatamers upon recognizing the target analyte, which can oxidize the 4-CN to form an insoluble product with the help of H_2O_2. The insoluble product can be used as the electrical barrier to block the interfacial electron transfer, thereby efficiently causing the change in the impedimetric signal. The detectable signal directly relies on the concentration of target Cu^{2+} in the sample. By monitoring the change in the resistance, we can exactly calculate the level of target analyte.

2. Experimental

2.1. Reagents and chemicals

The catalytic strand and substrate strand for the Cu^{2+}-dependent DNAzyme were purchased from Dingguo Biotech. Co., Ltd. (Beijing, China), which was designed referring to the literature (Ge et al., 2013). Hairpins DNA (H1 and H2) containing hemin-binding aptamer were designed according to the literature (Shimron et al., 2012). The sequences of the oligonucleotides were listed as follows:

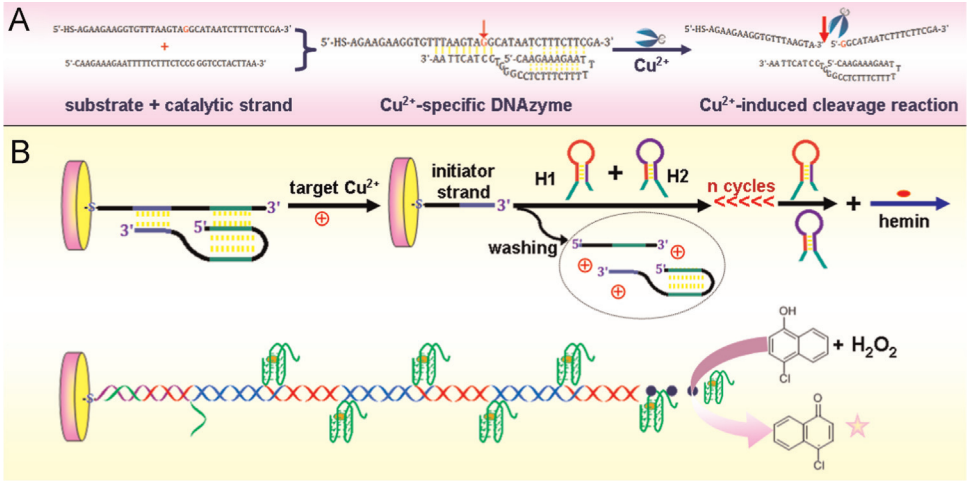
Substrate strand for Cu^{2+}-specific DNAzyme (S1): 5′-HS-AGAAGAAGGTGTTTAAGTAGGCATAA TCTTTCTTCGA-3′

Catalytic strand for Cu^{2+}-specific DNAzyme (S2): 5′-CAA-GAAAGAATTTTTCTTTCTCCGGGTCCT ACTTAA-3′

Hairpin DNA (H1): 5′-AGGGCGGGTGGGTGTTTAAGTTGGA-GAATTGTACTTAAACACCTTCTT CTTGGGT-3′

Hairpin DNA (H2): 5′-TGGGTCAATTCTCCAACTTAAACTAGAA-GAAGGTGTTTAAGTTGGGTA GGGCGGG-3′

All oligonucleotides stock solutions (100 μM) in this work were prepared into phosphate-buffered saline (PBS, 10 mM, pH 7.4) solution. 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) was obtained from Sigma-Aldrich. $CuCl_2$, KCl, NaCl, $CdCl_2$, $Ni(NO_3)_2$, $Pb(NO_3)_2$, $MgCl_2$, $Zn(NO_3)_2$, $CaCl_2$, $FeCl_3$ and $CoCl_2$ of analytical grade were acquired from standard commercial sources and used without further purification or treatment. 4-chloro-1-naphthol (4-CN) was from Sinopharm Chem. Re. Co., Ltd. (Shanghai, China). Ultrapure water used in this study was obtained from a Milli-Q water purifying system (resistivity ≥ 18.2 MΩ cm^{-1}, Milli-Q, Millipore). Hemin was purchased from Aladdin (Shanghai, China). A hemin stock solution (5.0 mM) was prepared in dimethyl sulphoxide (DMSO) and stored in the dark at −20 °C.



Scheme 1. Schematic illustration of (A) Cu^{2+}-cleaved substrate strand of the immobilized DNAzyme on the electrode, and (B) Cu^{2+}-specific DNAzyme-based impedimetric sensor by coupling with target-induced hybridization chain reaction between hairpin H1 and hairpin H2 accompanying formation of hemin-based DNAzyme concatamer and enzymatic catalytic precipitation technique.

2.2. Preparation of Cu^{2+}-dependent DNAzyme-modified electrode

Prior to modification, Cu^{2+}-dependent DNAzyme was synthesized on the basis of the reaction between catalytic strand and substrate strand. A mixture containing catalytic strand and substrate strand at an equimolar level was initially dispersed into 50 mM HEPES buffer (pH 7.0) containing 1.5 M NaCl, and then incubated for 100 min at 37 °C (note: Before incubation, the mixture should be heated to 80 °C for 2 min and cooled to 37 °C naturally in order to dissociate the possibly formed duplex DNA). During this process, the stable Cu^{2+}-specific DNAzyme was formed.

Next, a gold electrode (2 mm in diameter) was polished with 0.3 μm and 0.05 μm alumina, sonicated in ethanol and ultrapure water for 5 min each, and dried in nitrogen. The electrode was further cleaned by a series of oxidative and reductive scans in a 0.5 M H_2SO_4 solution for 5 times with the potential range from 0 to 2 V. After thorough rinsing with distilled water, the gold electrode was incubated with the above-prepared Cu^{2+}-specific DNAzyme for 30 min. During the process, the Cu^{2+}-specific DNAzyme was immobilized onto the gold electrode by the Au–S bond. Following that, the as-prepared DNA sensor was used for the detection of Cu^{2+}.

2.3. Impedimetric measurement toward target Cu^{2+}

Scheme 1 gives the measurement process of the Cu^{2+}-specific DNAzyme-coated electrode toward target Cu^{2+} by coupling target-induced hybridization chain reaction accompanying the formation of DNAzyme concatamers with enzymatic biocatalytic precipitation. All electrochemical measurements in this study were carried out on an AutoLab μAUTIII.FRA2.v electrochemical workstation (Eco Chemie, Netherlands) with a conventional three-electrode system including a gold working electrode, a platinum foil auxiliary electrode and an Ag/AgCl reference electrode. The assay was implemented as follows: (i) Target-induced cleavage of Cu^{2+}-specific DNAzyme by dropping 5.0 μL of Cu^{2+} standard at an expectative concentration on the as-prepared DNA sensor (Reaction: 60 min at 37 °C) to cleave the Cu^{2+}-specific DNAzyme; (ii) DNA-based hybridization chain reaction by immersing the resulting DNA sensor in a mixture containing 1.0 μM hairpin H1 and 1.0 μM hairpin H2 (Incubation: 120 min at 37 °C) to form a long nicked duplex DNA; (iii) Hemin-based DNAzyme formation by dipping the above-obtained electrode into 0.2 mM hemin (Reaction: 30 min at 37 °C) to form hemin/G-quadruplex DNAzyme concatamer; (iv) Enzymatic biocatalytic precipitation by dipping the modified electrode into a precipitation solution containing 1.0 mM 4-CN and 0.15 mM H_2O_2 (Reaction: 10 min at 37 °C); and (v) Impedimetric measurement in 0.01 M PBS (pH 7.4) containing 2.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl (Frequency: $10^{-2}-10^{6}$ Hz; potential: 220 mV; alternating voltage: 5 mV). After each step, the sensor was washed twice with pH 7.0 HEPES buffer. Nyquist plots (Z_{re} *vs* Z_{im}) were drawn to analyze the impedance results and calculated relative to zero analyte.

2.4. Gel electrophoresis

The experimental products were analyzed by a 2.0 wt% agarose gel. DNA sample was carried out in 0.5 × tris-borate-EDTA (TBE) (pH 8.0) as the running buffer loading at 100 V for 60 min. After being stained for 20 min with ethidium bromide, and destained for 10 min in distilled water, the gel was photographed by an Image Master VDS-CL (Amersham Biosciences).

3. Results and discussion

3.1. Construction and characterizations of impedimetric Cu^{2+} sensor

The impedimetric DNA sensor for the detection of Cu^{2+} is schematically illustrated in Scheme 1. Initially, Cu^{2+}-denpendant DNAzyme is covalently conjugated onto the cleaned gold electrode through the reaction (*i.e.*, Au–S bond) between gold and thiol group. Upon target Cu^{2+} introduction, the substrate strand of the immobilized DNAzyme on the electrode is catalytically cleaved by the Cu^{2+} analyte, resulting in the dissociation of catalytic strand or partial substrate strand from the electrode. In this case, the residual single-strand DNA on the electrode can be used as the initiator strand to pair with the partial bases at the sticky end of hairpin H1, which undergoes an unbiased strand-displacement reaction to open the hairpin. The newly exposed partial sticky nucleates at the sticky of probe H2 and opens the hairpin to expose a sticky at probe H2, which opens the hairpin DNA structure in sequence and propagate a chain reaction of hybridization events between two alternating hairpins to form a nicked double-helix. The unpaired hemin-based DNAzyme aptamers form the free branches on the DNA helix for the conjugation of the following hemin. In this way, numerous hemin-based DNAzyme complexes can concatamerized through the double-helix DNA. Upon addition of 4-CN and H_2O_2, the formed DNAzyme concatamers can catalyze the oxidation of 4-CN to produce an insoluble benzo-4-chlorohexadienone precipitation on the electrode, thus resulting in a local alteration of the conductivity for the development of impedimetric Cu^{2+} sensing strategy.

To realize our design, one precondition for the development of impedimetric Cu^{2+} sensor was that hairpins H1 and H2 should readily react with Cu^{2+}-specific DNAzyme in the presence of target analyte to form a long nicked double-helix DNA. To clarify this point, we used gel electrophoresis to investigate the Cu^{2+}-specific DNAzyme before and after reaction with hairpins H1 and H2 when the DNAzyme was cleaved by target Cu^{2+} (Fig. 1A). Lane 2 shows gel electrophoresis image of our designed Cu^{2+}-specific DNAzyme in the presence of 0.1-μM Cu^{2+}, and one relatively wide band was observed. The reason might be the fact that partial DNAzyme molecules were cleaved by target Cu^{2+}, and exhibited a gradient base number. When the cleaved DNAzyme molecules were reacted with 0.1-μM hairpin H1 and 0.1-μM hairpin H2, significantly, a long and wide band was appeared (lane 3), indicating the formation of different-length double-strand DNA. The results further indicated that the cleaved DNA by target analyte on Cu^{2+}-specific DNAzyme could cause chain hybridization reaction between hairpins H1 and H2. As control test, we also investigated whether hairpins H1 and H2 could non-specifically induce hybridization chain reaction in the absence of target Cu^{2+}. As indicated from lane 4, the resulting gel electrophoresis image was almost the same as that of Cu^{2+}-specific DNAzyme (lane 2). Thus, the long nicked duplex DNA could not be formed in the absence of target Cu^{2+} even if hairpins H1 and H2 were simultaneous present.

Logically, another concern arises to whether the formed long nicked double-helix could cause the change of the detectable impedimetric signal upon addition of hemin, 4-CN and H_2O_2. As so, the assembly and assay procedure of the impedimetric sensor toward Cu^{2+} was characterized after each step by using electrochemical impedance spectroscopy (EIS) in 0.01 M PBS (pH 7.4) containing 2.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl (Fig. 1B). It can be seen that the bare gold electrode exhibited a vary small resistance (eT) (close to a straight line) that was characteristic of a diffusional limiting step of the electrochemical process (curve 'a'). When the Cu^{2+}-specific DNAzyme was immobilized onto the cleaned gold electrode, the resistance (eT) increased ($R_{eT} \approx 151\ \Omega$, curve 'b'), indicating that the negatively charged phosphoric skeletons at

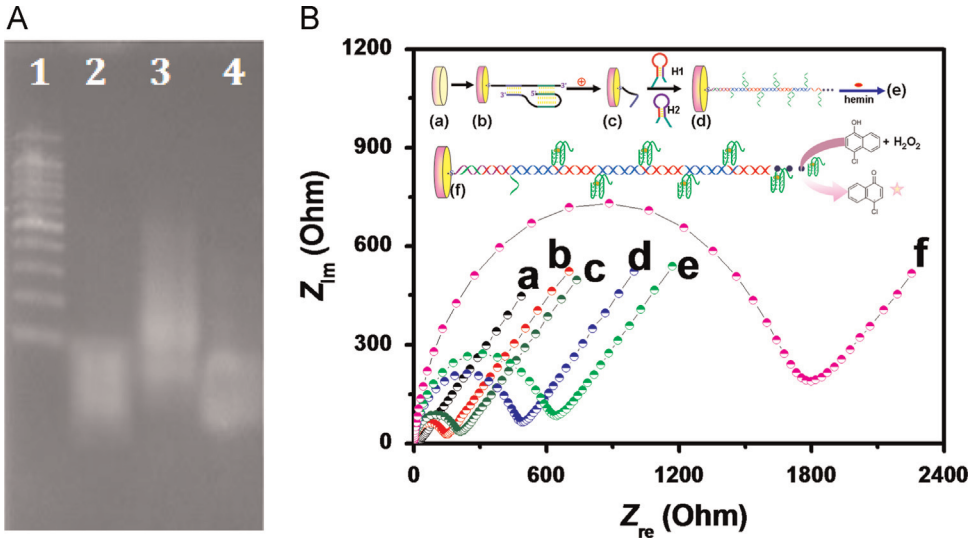
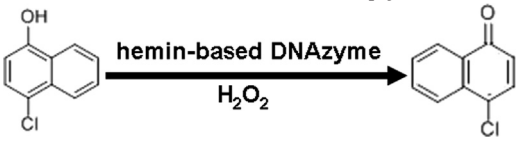


Fig. 1. (A) Gel electrophoresis [lane 1: DNA marker, lane 2: Cu^{2+}-specific DNAzyme+target Cu^{2+}, lane 3: Cu^{2+}-specific DNAzyme+target Cu^{2+}+(H1+H2), lane 4, Cu^{2+}-specific DNAzyme+(H1+H2)]. (B) Nyquist diagrams for (a) bare gold electrode, (b) electrode 'a'+Cu^{2+}-specific DNAzyme, (c) electrode 'b'+1.0 pM Cu^{2+}, (d) electrode 'c'+(H1+H2), (e) electrode 'd'+hemin, and (f) electrode 'e'+4-CN+H_2O_2 in 10 mM PBS (pH 7.4) containing 2.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl with the range from 10^{-2} Hz to 10^6 Hz at an alternate voltage of 5 mV.

DNAzyme hindered the electron transfer of $[Fe(CN)_6]^{4/3-}$ in the solution. Deceptively, the resistance increased again when the immobilized DNAzyme was cleaved by 1.0 pM Cu^{2+} ($R_{eT} \approx 221\ \Omega$, curve 'c'). We speculated that the residual short single-strand DNA molecules after cleavage fell to the electrode due to the decrease in the inflexibility relative to double-strand DNA. Further, the resistance gradually increased after the resulting electrode after cleavage was reacted with H1+H2 (curve 'd') and hemin (curve 'e') in turn. After the formed DNAzyme concatamer reacted with 4-CN and H_2O_2, importantly, the resistance heavily increased (curve 'f') relative to curve 'e'. The increased resistance mainly derived from DNAzyme concatamer toward the oxidation of 4-CN to generate an insoluble precipitation product (benzo-4-chloroheexadienone) coated on the electrode. The mechanism of enzymatic biocatalytic oxidation toward 4-CN could be simply summarized as follows:



On the basis of the impedimetric investigation, we ensured that the developed strategy could be employed for quantitative monitoring of target Cu^{2+} concentration.

3.2. *Comparative study of differently sensing strategies toward tARGET Cu^{2+}*

To demonstrate the amplification efficiency of DNAzyme concatamer toward the developed impedimetric Cu^{2+} sensing strategy, a comparative study by using different signal-transduction tags, *e.g.*, Cu^{2+}-specific DNAzyme+hemin, Cu^{2+}-specific DNAzyme+H1/H2+hemin, and Cu^{2+}-specific DNAzyme+$(H1+H2)_n$+hemin ('*n*' represents one *n* cycle), was carried out for the detection of 1.0 pM Cu^{2+} (used as an example) after enzymatic catalytic precipitation in the presence of 4-CN and H_2O_2 (Fig. 2). The comparison was evaluated on the basis of the change in the resistance relative to background signal (*i.e.*, Cu^{2+}-specific DNAzyme-modified electrode) (curve'a'). As seen from curves 'b–c', the strategies of using Cu^{2+}-specific DNAzyme+hemin (curve 'b') or Cu^{2+}-specific DNAzyme+H1/H2+hemin (curve 'c') only exhibited

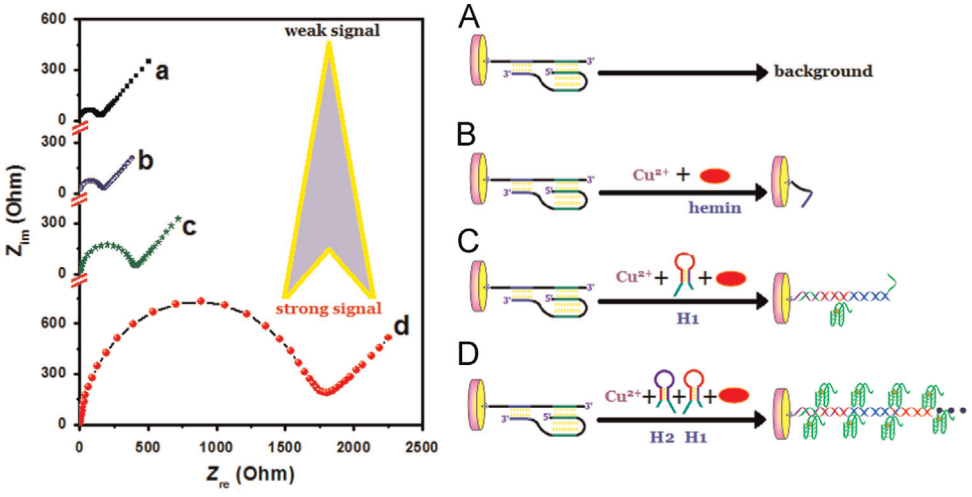


Fig. 2. Comparison of Nyquist diagrams for differently sensing strategies toward 1.0 pM target Cu^{2+}: (a) Cu^{2+}-specific DNAzyme-modified electrode, (b) sensor 'a'+hemin, (c) sensor 'a'+H1/H2+hemin, and (d) sensor 'a'+$(H1+H2)_n$+hemin after catalytic precipitation with 4-CN and H_2O_2 in 10 mM PBS (pH 7.4) containing 2.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl.

a low increase (221 Ω or 409 Ω) in the resistance, respectively. Significantly, a large increase in the resistance (1799 Ω) was achieved by using Cu^{2+}-specific DNAzyme+$(H1+H2)_n$+hemin (*i.e.*, DNAzyme concatamer) (curve 'd'). Relative to background signal (plots 'a'), introduction of hairpins H1 and H2 could cause a 1061 ± 21% signal increase of the impedimetric sensor, while the signal only increase 43 ± 3.2% and 164 ± 6.7% in the absence of H1 or H2. The phenomenon could be explained as follows: (i) the short single-strand DNA after cleavage could induce the progression of hybridization chain reaction between hairpin H1 and hairpin H2, thus resulting in the formation of numerous hemin-based DNAzyme molecules on the sensor; (ii) the formed hemin-G-quadruplex DNAzyme could act as the peroxidase-mimicking enzyme, which could catalyze the oxidation of 4-CN in the presence of H_2O_2 to produce an insoluble product; and (iii) the precipitation of the insoluble product generated by the accumulated DNAzyme concatamer could heavily block the electron transfer process. Therefore, DNAzyme concatamer could be used for *in situ* amplified signal of the impedimetric sensor for the determination of Cu^{2+}.

3.3. Optimization of experimental conditions

To achieve a good impedimetric response, some experimental conditions for the development of the impedimetric sensor should be studied. Initially, we investigated the effect of hybridization time between catalytic strand and substrate strand for the synthesis of Cu^{2+}-specific DNAzyme on the impedimetric signal (1.0 pM Cu^{2+} used in this case). As shown in Fig. 3A, the resistance increased with the increasing reaction time, and tended to level off after 100 min. A longer hybridization time did not significantly cause the change in the resistance. So, 100 min was selected for the preparation of Cu^{2+}-specific DNAzyme. At this condition, we also evaluated the effect of the cleavage time between target analyte and Cu^{2+}-specific DNAzyme on the impedimetric signal. Similar to the phenomenon with Fig. 3A, the resistance reached to the equilibrium after ~60 min. To save the assay time, 60 min was chosen as the cleavage time for Cu^{2+} detection.

In this work, the signal mainly originated from the formed DNAzyme concatamer toward the catalytic oxidation of 4-CN. Hence, the reaction time for HCR and the assembly time of hemin within the G-quardruplex were very crucial. Too short time might be unfavorable for the formation of DNAzyme concatamer, thereby decreasing the sensitivity of the impedimetric sensor. Usually, it takes some time for the strand hybridization and formation of the hemin–aptamer complex. As shown in Fig. 3C and D, the maximum resistances were achieved after 120 min and 30 min for HCR and the formation of hemin-based DNAzyme, respectively. Therefore, 120 min and 30 min were employed for hybridization chain reaction and the assembly of hemin with the aptamer.

3.4. Analytical performance

By virtue of the optimal conditions, the sensitivity and quantitative range of the impedimetric sensor were evaluated on Cu^{2+}-specific DNAzyme-modified electrode toward Cu^{2+} standards based on hemin-DNAzyme concatamer amplification strategy. The assay was carried out by using electrochemical impedance spectroscopy after catalytic precipitation in the presence of 4-CN and H_2O_2. As shown in Fig. 4A, the resistance increased with the increasing Cu^{2+} concentration from 0.1 pM to 5.0 nM in the sample. Two linear relationships between the resistance and Cu^{2+} level were obtained within the range of 0.1 pM – 5.0 nM (*i.e.*, 0.1–100 pM and 0.1–5.0 nM) (Fig. 4B). The linear regression equations were ΔR_{eT} $(\Omega)=261.77\times$ $\mathrm{Ln}C_{[Cu2+]}+1549.4$ (pM) ($R^2=0.9997$, $n=15$) for 0.1–100 pM and $\Delta R_{eT}(\Omega)=1233.4\times$ $\mathrm{Ln}C_{[Cu2+]}-2604.6$ (pM) ($R^2=0.9866$, $n=18$) for 0.1–5.0 nM, respectively. The detection limit (LOD) was 0.06 pM estimated at the signal-to-noise ratio of 3. The sensitivity was obviously comparable with those of ratiometric electrochemical biosensor (80.2 nM) (Zhang et al., 2015), ligation DNAzyme-induced magnetic nanoparticle assembly (2.8 nM) (Yin et al., 2014), dual-DNAzyme unimolecular probe-based colorimetric detection (1.0 μM) (Yin et al., 2009), molecularly imprinted electrochemical sensor (42.4 pM) (Li et al., 2015),

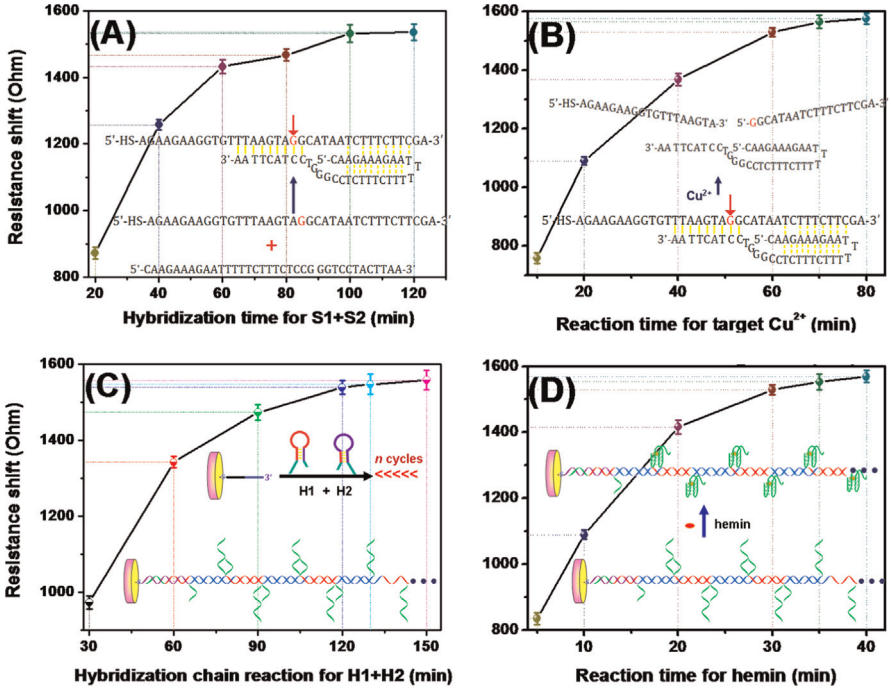


Fig. 3. The effects of (A) hybridization time between substrate strand and catalytic strand for the preparation of Cu^{2+}-specific DNAzyme, (B) cleavage time between target Cu^{2+} and Cu^{2+}-specific DNAzyme, (C) hybridization chain reaction time between H1 and H2, and (D) reaction time of hemin with hemin-based DNAzyme aptamer on the resistance responses of Cu^{2+} impedimetric sensor (1.0 pM Cu^{2+} used in this case).

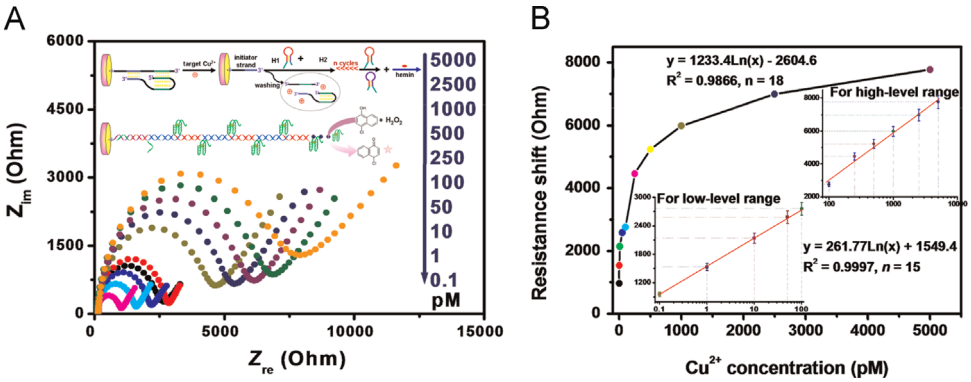


Fig. 4. (A) Nyquist diagrams for the developed impedimetric sensor toward various-concentration Cu^{2+} standards on Cu^{2+}-specific DNAzyme-modified electrode by coupling with hemin-based DNAzyme concatamers with enzymatic catalytic precipitation in 10 mM PBS (pH 7.4) containing 2.0 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl, and (B) the corresponding calibration plots (log *C vs.* ΔR_{eT}) from 0.1 pM to 5.0 nM (error bars: SD, $n=3$).

methyl-naphthyl substituted cyclam film-based impedimetric sensor (10 nM) (Mefteh et al., 2015), and TiO_2 sphere-based impedimetric sensor (4.29 pM) (Kang et al., 2015). Such a high sensitivity might be ascribed to the formed peroxidase-mimicking DNAzyme concatamer and enzymatic biocatalytic precipitation.

The reproducibility and precision of the impedimetric sensor were evaluated by repeatedly assaying 1.0 pM Cu^{2+}, using identical batches of the sensors. Experimental results indicated the relative standard deviation (RSD) of the intra-assay by using the same-batch sensors was 8.5% ($n=7$). The sensor-to-sensor reproducibility was estimated in the response to 1.0 pM Cu^{2+} with 20 different-batch sensors. This series yielded a mean resistance of $1687 \pm 13.3\ \Omega$ ($n=7$) and a RSD of 9.1%. So, the precision and reproducibility of the impedimetric sensor was acceptable. When being stored at 4 °C, the impedimetric sensor could retain 98.7% of initial response after one week.

The specificity of the impedimetric sensor was evaluated by challenging the system against other metal ions, *e.g.* Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ag^{+}, Pb^{2+} and Fe^{3+}. As seen from Fig. 5A, none of them could interfere with Cu^{2+} even at a 10000000-fold higher concentration compared with background signal. Such an excellent selectivity might be attributed to the high-specificity Cu^{2+}-dependent DNAzyme, which could be specifically cleaved to generate the initiator strand for the development of hybridization chain reaction. Hence the developed impedimetric sensing platform could be used for specific detection of target Cu^{2+}.

To further elucidate the analytical reliability and applicable potential of the impedimetric sensor for testing of real samples, Cu^{2+} standards with different concentrations were initially spiked into drinking water, and then these samples were monitored by using our developed Cu^{2+} sensor. The obtained results by our strategy (0.24 nM, 3.76 nM, 24.5 pM, 0.87 nM, 1.45 nM, 2.83 nM and 4.76 nM for samples 1–7, respectively) were compared with the reference values (0.26 nM, 3.57 nM, 22.3 pM, 0.91 nM, 1.43 nM, 2.91 nM and 4.67 nM toward the above-mentioned analytes, respectively) obtained from inductively coupled plasma mass spectrometry (ICP-MS). Comparison of the experimental results obtained from the sensor with those of ICP-MS was performed *via* the use of a least-squares regression method (Fig. 5B). The regression line was fitted to $y=0.9702x+35.121$ ($n=21$, $R^2=0.9982$). As seen from the regression line, the slope was close to ideal"1', revealing a good agreement between both analytical methods for the determination of Cu^{2+}. Therefore, the electrochemical sensor could be considered as an optional strategy for Cu^{2+} detection in the complicated samples.

4. Conclusions

In the present paper, we introduce a convenient and feasible impedimetric sensing protocol for highly sensitive monitoring of Cu^{2+} in aqueous solution on Cu^{2+}-specific DNAzyme-modified electrode based on DNA-based hybridization chain reaction accompanying the formation of peroxidase-like DNAzyme concatamer and enzymatic biocatalytic precipitation scheme. Utilization of Cu^{2+}-dependent DNAzyme enhanced the specificity of the impedimetric sensor to some extent. DNAzyme concatamer and enzymatic catalytic precipitation were used for the amplification of the detectable signal, thus lowering the detection of limit and enhancing the sensitivity of the impedimetric sensor. Importantly, some possible challenges by our designed strategy can be realized for the detection of other metal ions (*e.g.*, Pb^{2+}, Zn^{2+}, Hg^{2+}, and Mg^{2+}) or DNA fragments by controlling the immobilized biomolecules, thereby representing a versatile scheme.

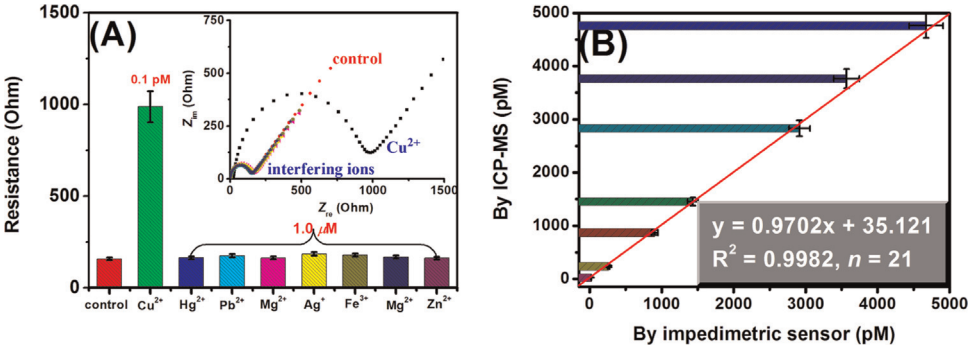


Fig. 5. (A) The specificity of the impedimetric sensor against 0.1 pM Cu^{2+}, 1.0 μM Hg^{2+}, 1.0 μM Mg^{2+}, 1.0 μM Mn^{2+}, 1.0 μM Zn^{2+}, 1.0 μM Ag^{+}, 1.0 μM Pb^{2+} and 1.0 μM Fe^{3+} (Inset: the corresponding Nyquist diagrams), and (B) comparison of the assayed results for spiking drinking water samples between the impedimetric Cu^{2+} sensor and the referenced ICP-MS method.

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