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Label-free selective impedimetric detection of Cu^{2+} ions using catalytic DNA

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Copper is an essential element for regulation of many biological processes, however, in excess it is considered to be toxic for human health. This metal is frequently accompanied by other elements such as cadmium, nickel and lead. Thus, developing a selective and simple method for determination of copper in a matrix containing other heavy metal ions is of great importance. In this work, a novel selective method for copper detection was developed using electrodes modified with the DNAzyme capturing Cu^{2+} ions. The DNAzyme reconstituted with copper catalyzes oxidation of ascorbic acid leading to the build-up and adsorption of oxidation products on the electrode surface and produces changes in the interfacial properties of the electrode. The increase in the interfacial electron-transfer resistance is probed with electrochemical impedance spectroscopy (EIS) in the presence of the reversible redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ as a marker. The DNAzyme based biosensor combines excellent selectivity against other heavy metal ions with sufficient sensitivity to Cu^{2+} in the range of 6.5–40 μM.

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Introduction

Copper is a redox-active nutrient required in a number of electron transfer processes of many biological reactions.[1] However, at elevated concentrations it has been considered to be toxic for human health. Short-term exposure to an excess of copper can cause gastrointestinal disturbance, while long-term exposure causes liver or kidney damage, neurodegenerative diseases, *etc.*[2] The World Health Organization (WHO) as well as the U.S. Environmental Protection Agency (EPA) have set the limit of copper in drinking water to be 2 mg L^{-1} (32 μM) and 1.3 ppm (20 μM) respectively.[3] Therefore, the development of a sensitive, selective, comparatively inexpensive and portable sensor for Cu^{2+} ion detection is of great importance.

Various analytical methods for the detection of copper ions such as atomic absorption/emission spectroscopy,[4,5] inductively coupled plasma mass spectroscopy (ICP-MS),[6,7] and capillary electrophoresis[8] provide reliable and accurate results, but require expensive and sophisticated instrumentation and complicated sample pretreatment. Many electrochemical sensors such as ion-selective electrodes,[9–12] and voltammetric[13,14] and electrochemiluminescence sensors[15,16] are simple and relatively inexpensive, however, they suffer from low reproducibility and have high detection limits and low selectivity. Metal-selective fluorescent chemosensors have attracted intense attention during the past few decades, however, they need complicated organic synthesis and surface modifications; they require expensive instruments as well.[17–20]

Recently DNAzyme-based assays have attracted considerable interest for the detection of various analytes, including heavy metal ions. DNAzymes (deoxyribozymes, catalytic DNAs, DNA enzymes) are DNA molecules that exhibit different catalytic activities such as RNA cleavage, ligation, phosphorylation and porphyrin metallation.[21] DNAzymes cleaving RNA in the presence of different divalent ion metals have been reported.[22–26] Considering a DNAzyme a promising platform for an application in biosensing various attempts were made for the detection of target DNA,[27,28] or ion metals such as lead,[29] uranyl ions,[30] and mercury.[31] So far, only a few DNAzyme based sensors for copper ions have been reported.[32–34] The design of these DNAzyme probes involves labeling of the oligonucleotide by fluorogenic molecules. In this paper we report a novel label-free electrochemical DNAzyme based biosensor for the detection of copper ions using avidin–graphite epoxy composite electrodes.[35] Electrochemical impedance spectroscopy (EIS) is a powerful and sensitive technique for following biosensing events that take place at the surface of the electrode. Any tiny disturbance at the interface would lead to impedimetric changes.[36] This method is of general use in laboratories and has already been extensively studied and applied for amperometric, enzymatic, immune and genosensing assays.[37,38] The combination of benefits of DNAzyme and electrochemical impedance spectroscopy allowed selective detection of copper with a detection limit of 6.5 μM.

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Experimental

Apparatus

AC impedance measurements were performed with an IM6e Impedance Measurement Unit (BAS-Zahner, Kronach, Germany) and Autolab PGStat 20 (Metrohm Autolab B.V, Utrecht, The Netherlands). Thales (BAS-Zahner) and FRA (Metrohm Autolab) software, respectively, were used for data acquisition and control of the experiments. A conventional three-electrode system was used to perform impedance measurements: an avidin–graphite epoxy composite (Av–GEC) electrode as a working electrode, an Ag/AgCl electrode as a reference electrode and a platinum-ring auxiliary electrode (Crison 52-67 1, Barcelona, Spain) as a counter electrode. Temperature-controlled incubations were done using an Eppendorf Thermomixer 5436.

Chemicals

Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, ascorbic acid, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium hydroxide, potassium chloride, sodium chloride, avidin, and copper(II) chloride were purchased from Sigma-Aldrich (Spain). DNA phosphoramidites, biotin-CE phosphoramidite, spacer-CE phosphoramidite 18 and other reagents required for solid phase oligonucleotide synthesis were purchased from Link Technologies (UK). Oligonucleotide purification cartridges (OPC) and solvents for DNA purification were purchased from Applied Biosystems (Spain). DNAzyme used in this study was prepared by automated solid-phase chemical synthesis (Applied Biosystems 3400 DNA Synthesizer) and purified with oligonucleotide purification cartridges (OPC), its purity was further analyzed by MALDI-TOF mass spectroscopy. The synthesized DNAzyme has the following sequence: 5′-biotin-$(-O-CH_2-CH_2-O-)_6$ TTC TAA TAC GAT TTA GAA TAA ATC TGG GCC TCT TTT TAA GAA C-3′, where $(-O-CH_2-CH_2-O-)_6$ corresponds to spacer-CE phosphoramidite 18. Solid-state electrodes (Av–GECs) were prepared using 50 μm particle size graphite powder (Merck, Darmstadt, Germany), avidin (Sigma-Aldrich) and Epotek H77 resin and its corresponding hardener (both from Epoxy Technology, Billerica, MA, USA). All reactions were conducted in the buffer containing 50 mM HEPES, pH 7.0, 0.5 M KCl, 0.5 M NaCl. All water used in the experiments was of nanopure grade (from Millipore Milli-Q system).

Experimental

Electrode preparation procedure

Avidin–graphite epoxy composite (Av–GEC) electrodes, used for easy immobilization of biotinylated DNAzyme, were prepared using a PVC tube body (6 mm i.d.) and a small copper disk soldered at the end of an electrical connector. The working surface is an epoxy-graphite conductive composite, formed by a mixture of graphite (18%), avidin (2%) and epoxy resin (80%), deposited on the cavity of the plastic body.[39,40] The composite material was cured at 40 °C for 7 days. Before each use, the electrode surface was moistened with MilliQ water and then thoroughly smoothed with abrasive sandpaper and finally with alumina paper (polishing strips 301044-001, Orion) in order to obtain a reproducible electrochemical surface. The electrodes were then washed twice for 10 minutes in the working buffer at room temperature while stirring.

Immobilization of DNAzyme probe

An Av–GEC electrode was immersed in 160 μL of the desired concentration of biotinylated DNAzyme in 50 mM HEPES, pH 7.0, 0.5 M KCl, and 0.5 M NaCl. After incubation for 1 h at RT while stirring, the electrodes were washed twice for 10 minutes in the working buffer at room temperature.

Reaction of DNAzyme with copper

After the immobilization of DNAzyme the electrode was immersed for 20 minutes in the solution of the desired concentration of $CuCl_2$ and 30 μM concentration of ascorbic acid in the working buffer at RT while stirring. The electrodes were then washed twice for 10 minutes in the working buffer at room temperature.

Impedimetric detection

Impedance spectra were recorded between 50 kHz and 0.05 Hz, at a sinusoidal voltage perturbation of 10 mV amplitude and a sampling rate of 10 points per decade above 66 Hz and 5 points per decade at the lower range. The experiments were carried out under open circuit potential conditions in an unstirred solution of 0.1 M PBS buffer solution containing a 0.01 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1 : 1) mixture, used as a redox marker. The impedance spectra were plotted in the form of complex plane diagrams (Nyquist plots, $-Z_{im}$ *vs.* Z_{re}) and fitted to a theoretical curve corresponding to the equivalent circuit with Z_{view} software (Scribner Associates Inc., USA). The equivalent circuit was formed by one resistor/capacitor element in series with a resistance. The chi-square goodness of fit was calculated for each fitting by the FRA software employed (Eco Chemie, the Netherlands). In all cases impedance data were recorded in the following order after each of above described steps. In order to compare the results obtained from the different electrodes used, and to obtain independent and reproducible results, relative signals are needed.[39] Thus, the Δ_{ratio} value was defined according to the following equations:

$$\Delta_{ratio} = \Delta_s/\Delta_p$$

$$\Delta_s = R_{ct(DNAzyme-AA-Cu)} - R_{ct(electrode-buffer)}$$

$$\Delta_p = R_{ct(DNAzyme)} - R_{ct(electrode-buffer)}$$

where $R_{ct(DNAzyme-AA-Cu)}$ was the electron transfer resistance value measured after incubation with ascorbic acid and copper; $R_{ct(DNAzyme)}$ was the electron transfer resistance value measured after DNAzyme immobilization on the electrode, and $R_{ct(electrode-buffer)}$ was the electron transfer resistance of the blank electrode and buffer.

Results and discussion

The analytical procedure for biosensing consists of the immobilization of the biotinylated DNAzyme onto the surface of the avidin modified GEC electrodes, followed by binding of copper ions to the –CTGGGCC– section of DNA. The reconstituted DNAzyme enhances the rate of ascorbic acid oxidation by oxygen. The accumulated oxidation products insulate the surface of Av–GEC electrodes, changing their interfacial properties, followed by electrochemical impedance spectroscopy (EIS) in the presence of the reversible redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ as a marker as depicted in Fig. 1.

Optimization of electrode surface

First, the concentration of DNAzyme immobilized onto the Av–GEC electrode surface was optimized in order to achieve the maximum surface coverage. For this purpose, increasing concentrations of DNAzyme were used to carry out the immobilization, evaluating the changes in the Δ_p caused by the DNA layer. The curve of adsorption of DNAzyme on the electrode surface is shown on Fig. 2. It can be observed that the difference in resistance (Δ_p) asymptotically approaches its maximum value starting from 0.2 μM of DNAzyme concentration. This is due to the formation of a chemical bond between the biotin moiety of DNAzyme and avidin on the electrode surface, which followed a Langmuir isotherm. The concentration of DNAzyme equal to 0.2 μM was chosen as the optimal concentration.

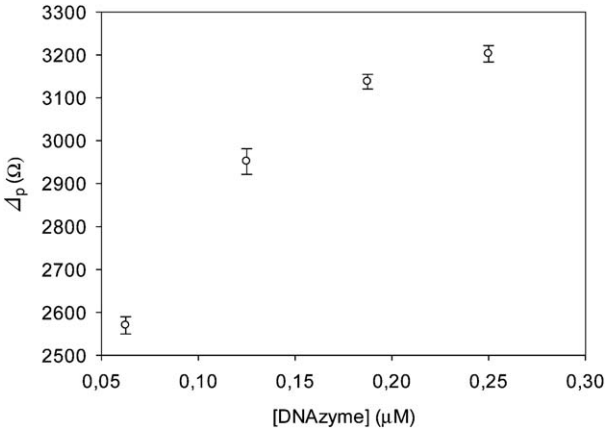

Fig. 2 Optimization of the concentration of DNAzyme placed onto the electrode. Uncertainty values corresponding to replicated experiments ($n = 5$).

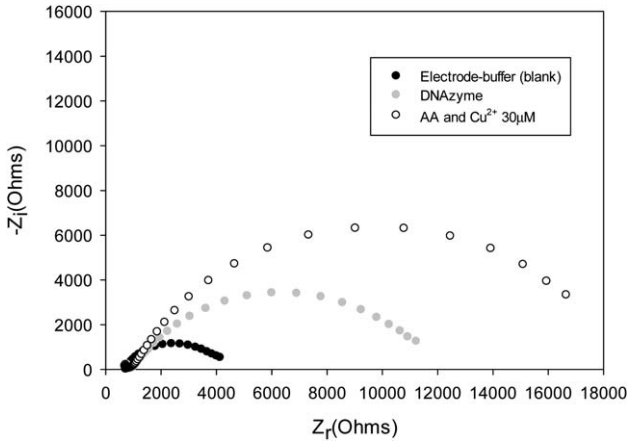

Fig. 3 Nyquist diagram of: (a) electrode–buffer ●, (b) DNAzyme, and (c) ascorbic acid and Cu^{2+} 30 μM.

Detection of Cu^{2+}

After the optimization of DNAzyme concentration, and following the above mentioned experimental protocol for the detection of copper, the biosensor response was initially evaluated. Cu^{2+} ions captured by the layer of DNAzyme significantly enhance the rate of ascorbic acid oxidation[41,42] in vicinity of the electrode, consequently the electrode surface is partially blocked due to the adsorption of oxidation products on it, resulting in an increase in the interfacial electron-transfer resistance. Representative Nyquist plots of DNAzyme in the absence and presence of Cu^{2+} are shown in Fig. 3. As can be seen, resistance R_{ct} between the electrode surface and the solution is increased. This fact is due to the effect of the kinetics of the electron transfer redox marker $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ which is delayed at the interface of the electrode, mainly caused by steric hindrance and electrostatic repulsion presented by the oxidized products formed.

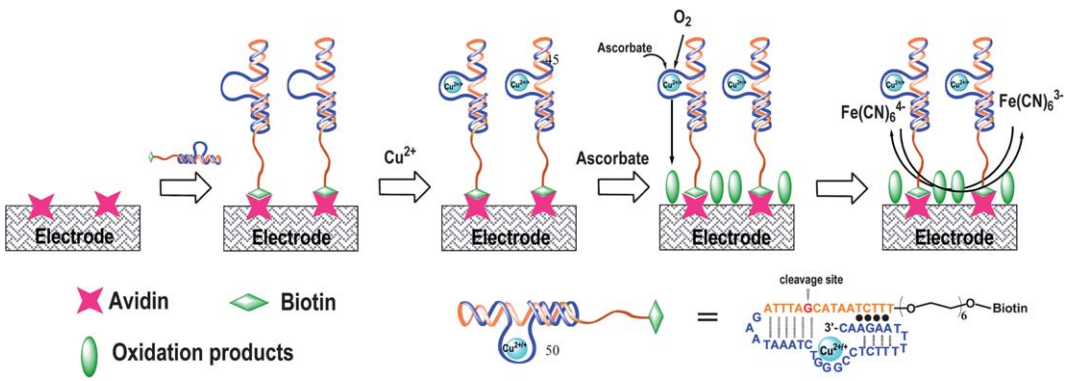

Fig. 1 Schematic illustration of an impedimetric DNAzyme sensor for Cu^{2+}.

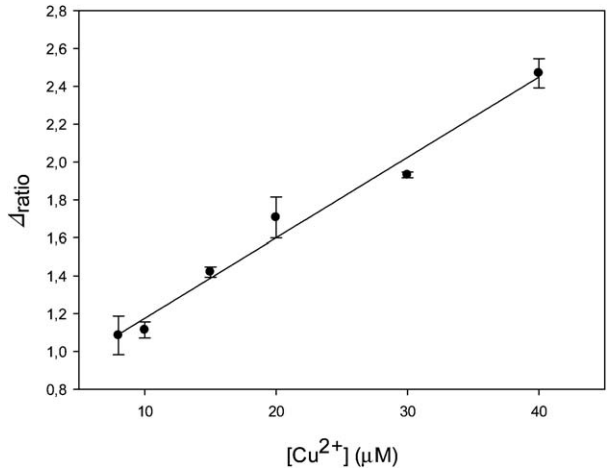

Fig. 4 Calibration curve, relative signal *versus* Cu^{2+} concentration. Uncertainty values corresponding to replicated experiments ($n = 5$).

Sensitivity of DNAzyme based sensor

To evaluate the sensitivity of the assay, new experiments with solutions containing different amounts of copper were performed and the calibration curve was built. Fig. 4 shows the evolution of the Δ_{ratio} relative signal, as extracted from the Nyquist diagrams, for the calibration of the copper sensor. To evaluate the linear range and detection limit of this DNAzyme-based copper sensor, the calibration curve was built, representing the analytical signal expressed as Δ_{ratio} *vs.* copper concentration (Fig. 4). As the copper concentration rises, the interfacial electron transfer resistance between the electrode surface and the solution also increases up to 40 μM of Cu^{2+}. As can be seen, a linear trend is obtained with a linear range from 10 μM to 40 μM for Cu^{2+}. From the least squares fitting, sensitivity appeared to be of 4×10^{-2} μM^{-1} and the detection limit of 6.5 μM. The repeatability of the method showed a relative standard deviation (%RSD) between 2 and 6%, obtained from a series of 5 replicates carried out for each standard in the linear range.

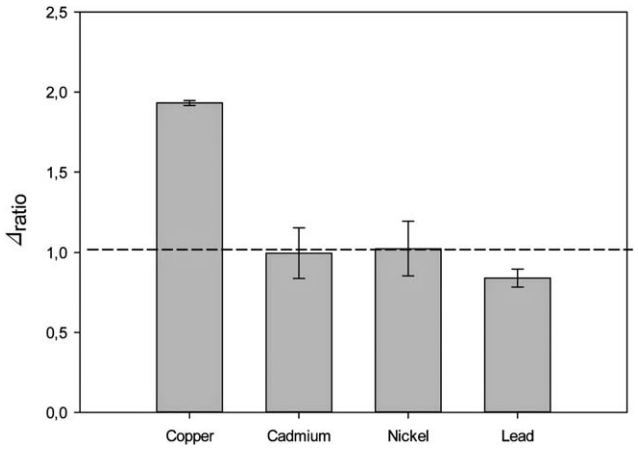

Fig. 5 Impedimetric response obtained with different metals assayed at the same concentration (30 μM). Uncertainty values corresponding to replicated experiments ($n = 5$).

Selectivity of DNAzyme based sensor

Next, the selectivity of the assay for the detection of Cu^{2+} over other divalent metal ions, frequently found together with copper, such as nickel, cadmium and lead was investigated. All concentrations tested were 30 μM. As can be seen from Fig. 5 only in the presence of copper, the primary species, does the biosensor give a significant response, so the interference with other metal ions can be considered negligible.

Conclusions

In conclusion, we have described a simple biosensor for the determination of copper(II) based on its DNAzyme immobilized by a strong non-covalent avidin–biotin interaction on the electrode surface. Upon reconstitution with Cu^{2+} this DNAzyme catalyzes oxidation of ascorbic acid with oxygen, leading to blocking of the electrode surface by the reaction products. The immobilization step, and the catalytic event inhibiting the electron-transfer kinetics of the redox probe at the electrode interface, can be monitored by labeless electrochemical impedance spectroscopy. The DNAzyme biosensor shows a linear response range of 10 μM to 40 μM Cu^{2+} and a detection limit of 6.5 μM. Interference to some related metals can be considered negligible. Advantages of the reported label-free method for Cu^{2+} detection over other conventional techniques are its simplicity and high selectivity.

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