Development of a label-free electrochemical aptasensor based on diazonium electrodeposition: Application to cadmium detection in water

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Abstract:

In this study we have developed a new aptasensor for cadmium (Cd^{2+}) detection in water. Gold electrode surface has been chemically modified by electrochemical reduction of diazonium salt (CMA) with carboxylic acid outward from the surface. This was used for amino-modified cadmium aptamer immobilization through carbodiimide reaction. Chemical surface modification was characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). This latter was also used for Cd^{2+} detection. The aptasensor has exhibited a good linear relationship between the logarithm of the Cd^{2+} concentration and the impedance changes in the range from 10^{-3} to 10^{-9} M with a correlation R^2 of 0.9954. A high sensitivity was obtained with a low limit of detection (LOD) of $2.75*10^{-10}$M. Moreover, the developed aptasensor showed a high selectivity towards Cd^{2+} when compared to other interferences such as Hg^{2+}, Pb^{2+} and Zn^{2+}. The developed aptasensor presents a simple and sensitive approach for Cd^{2+}detection in aqueous solutions with application for trace Cd^{2+} detection in spring water samples.

Keywords: Label-free detection, aptasensor, diazonium electrografting, gold electrode, cadmium detection.

Introduction

Recently, pollution caused by heavy metals is becoming more and more severe. Heavy metals are naturally occurring elements characterized by high atomic weight and density, which is at least five times greater than that of water [1]. The distribution of heavy metals; like Arsenic, Cadmium, Chromium, Lead, and Mercury in the environment is a result of their multiple applications, and raising concerns about their potential effects on human health and environment in most regions around the world, because of their high degree of toxicity [2]. Cadmium (Cd^{2+}) is a toxic heavy metal whose presence in the environment is mainly issued from human activities [3]. It is produced in the manufacture of batteries and pigments which can permeate, soil and water supply. Thereby, exposing the general population to low Cd^{2+}doses can affect a variety of cellular mechanisms such as proliferation, apoptosis, differentiation, cell signalling and gene expression. For these reasons, Cadmium has been classified as carcinogenic to humans by the World Health Organization (WHO) even at low doses, the maximum limit is 0.003 $mg.L^{-1}$. Indeed, it has been reported that exposure to this ion plays a particular role in prostate cancer[4, 5].[4]. Numerous analytical methods traditionally used for detection of Cadmium (II) ions have been widely reviewed in the literature; like atomic absorption/emission spectroscopy (AAS/AES) [6, 7], ultraviolet-visible spectrometry [8], Square-wave anodic stripping voltammetry [9] and atomic fluorescence spectrometry (AFS) [10]. Despite their performance, most of these methods are often tedious, time consuming and require great amounts of solvents, reagents and sophisticated apparatus. Therefore, inexpensive, sensitive and selective techniques are very desirable for cadmium monitoring.

Biosensors are considered as very promising tools for sensitive, rapid and low cost monitoring of wide range of analytes in different fields of applications. A wide range of biomolecules or chemical species have been used in the literature as sensitive layer for chemical or biosensors manufacturing[11]. Aptamers are considered as bio-inspired receptors which have gained great interest since their discovery in 1990 [12].

Aptamers are RNA or DNA molecules with specific 3D structures, selected by *in vitro* selection process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [13]. Due to their unique conformations, aptamers can bind a wide range of targets such as small molecules, proteins, and even whole cells. Their advantageous characteristics, such as high stability, simple chemical synthesis, possibility of modification, made them strong competitors of antibodies in molecular analysis [14-16]. They constitute thus

good candidates as effective probes for chemical sensors. Several aptasensors have been developed in the literature for Cadmium ions detection. Hamid et *al*[17] have developed an electrochemical cadmium sensor using a DNA aptamer, where the sensing mechanism was based on target binding-induced conformational change. Here, magnetic beads were used as immobilization support for the aptamer. Despite the biosensor selectivity toward Cd^{2+}, the detection range [250-1000 nM] of this electrochemical sensor was still rather narrow. In another report, Guo et al. have developed a new colorimetric method for sensing Cd^{2+} by using unmodified AuNPs [18]. Here, the linear detection range [5-40 µM] of this method was narrow and could not be used for the detection of Cd^{2+} in various types of samples.

The critical point in aptasensor fabrication technology remains in the immobilization of active biomolecules onto the transducer surface. Several immobilization techniques can be adopted according to the biomolecules architecture and the desired application. For instance, direct attachment of bioreceptors to gold electrodes has been reported, by using 3' or 5' thiol-labelled aptamers self-assembly through gold-sulfur bond interaction by forming ordered single-carrier membrane[19, 20]. However, this technique suffers from non-specific adsorption (random adsorption) and cannot be applied on other materials. Attachment of aptamers onto sensor surfaces based on hybridization with partially complementary oligonucleotides has been also adopted [20]. Nevertheless, annealing and hybridization steps are required, enduring thus the experimental conditions. Covalent attachment to chemically-modified sensor surfaces is a promising method based on the interaction of a labelling group and a chemically functionalized surface resulting in a layer of ordered film of aptamers. Chemical covalent attachment may increase the specificity and selectivity of the aptasensing strategy [20].

In this study we have chosen an amino-modified aptamer for Cd^{2+} detection that we immobilized on screed printed gold electrode through diazonium salt chemistry. To the best of our knowledge, this is the first report describing an electrochemical aptasensor exploring diazonium-coupling reaction mechanism for Cd^{2+} determination. To achieve that, Cd^{2+}aptamer was immobilized onto gold electrodes through 4-carboxymethyl aryl diazonium (CMA) molecules which are electrochemically reduced on the electrode surface. Unlike conventional grafting methods, diazonium salt-based electrochemical deposition involves simple reagents without isolation and purification steps [21-24]. Gold working electrodes (WE) have been characterized with cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) before and after WE chemical surface modification and after aptamer immobilization. EIS was then used to monitor the extent of binding between Cadmium and its specific capture aptamer.

Experimental

Chemicals and Reagents

Cadmium (II) nitrate [Cd (NO_3)$_2$],4-aminophenylacetic acid (4-carboxymethylaniline CMA), Sodium nitrite ($NaNO_2$), isopropyl alcohol (IPA) 96%, N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) were obtained from Sigma–Aldrich, France. Phosphate buffer (NaH_2PO_4.H2O ; $Na_2HPO_4.2H_2O$) and KCl solution containing ferricyanide K_3 [$Fe(CN)_6$] and ferrocyanide K_4 [$Fe(CN)_6$]) were used as supporting electrolytes. Cadmium aptamer was synthesized and purchased by Microsynth (Switzerland). The specific sequence of the aptamer was as follows: (5' amino-modified):5'-ACC GAC CGT GCT GGA CTC TGG ACT GTT GTG GTA TTA TTT TTG GTT GTG CAG TAT GAG CGA GCG TTG CG-3' [25].

Electrochemical analysis

The electrochemical experiments were carried out with a potentiostat (Biologic EC-Lab SP-300). The electrochemical measurements were performed in PBS with pH 7.4 at room temperature in a conventional electrochemical glass cell containing three-electrode system [26].

Cyclic Voltammetry (CV) measurements were used before and after chemical gold surface modifications. CV analysis were performed in 5 mM of $[Fe(CN)_6]^{3-/4-}$ solution within a potential range of (- 0.2V to + 0.6V) and a scan rate of 80 $mV.s^{-1}$.

Electrochemical impedance spectroscopy (EIS) was used to study the barrier properties of the modified electrode [27, 28]. The EIS measurements were conducted at a potential of 128 mV on the Gold WEs over a frequency range between 100 mHz and 200 kHz. The data of EIS were fitted using Randles equivalent circuit model. The modelling of the obtained EIS data was made by the EC-Lab software using the Randomize + Simplex method. Here, randomize was stopped on100 000 iterations and the fit stopped on 5000 iterations.

Real sample analysis

In order to validate and evaluate the accuracy and applicability of the our aptasensor, analyses were carried out on a real river water which is polluted by public work wastes and containing nitrate and cadmium [29, 30]. This river is crossed by a volcanic water source from Hammam Essalihine valley (khenchela, Algeria) which is rich in minerals, such as bicarbonates, sulphates, calcium and magnesium. We have made sampling from the river

which contains cadmium and other potential interferences. Real samples were tested by EIS combined with standard addition method [31]. This method consists of adding known amounts of a standard solution to a sample of interest with unknown concentrations. Then, the analytical signals of standard solutions are recorded in response to each addition [31]. In brief, a constant volume (2.5mL) of water real sample was added to each flask of 25mL. Then in the first flask, a 22.5mL of PBS were added to the 2.5mL of real samples in order to reach a final volume of 25 mL and this flask was called **Level 1.** The standard solution containing the analyte was then added in increasing volumes: 0.25, 2.5 and 5 mL, implying the addition of 10, 100 and 200 nM of Cd^{2+} to the subsequent flasks 2, 3 and 4. Finally theses flasks were made up to the volume of 25 mL with PBS giving thus **Level 2**, **Level 3** and **Level 4,** respectively.

Results and discussion

Electrochemical aptasensor preparation

Fig.1 illustrates the detection principle of Cd^{2+} by using the electrochemical aptasensor. Cd^{2+}-aptamer immobilization was achieved through the covalent linkage between amino groups of the aptamer and the abundant reactive carboxylic groups on the gold surface. The electrode surface was first modified by deposition of a diazonium salt film, and activation of the terminal carboxylic groups with EDC/NHS. Then, the aptamer was linked to the gold surface via its activated carboxylic groups. In the presence of Cd^{2+}, a variation in the aptamer conformation takes place from a random coil structure to an aptamer–Cd^{2+} complex [32, 33]. This aptamer-target interaction hampers the electron transfer, enhancing thus the surface resistance which is proportional to cadmium concentration in the sample.

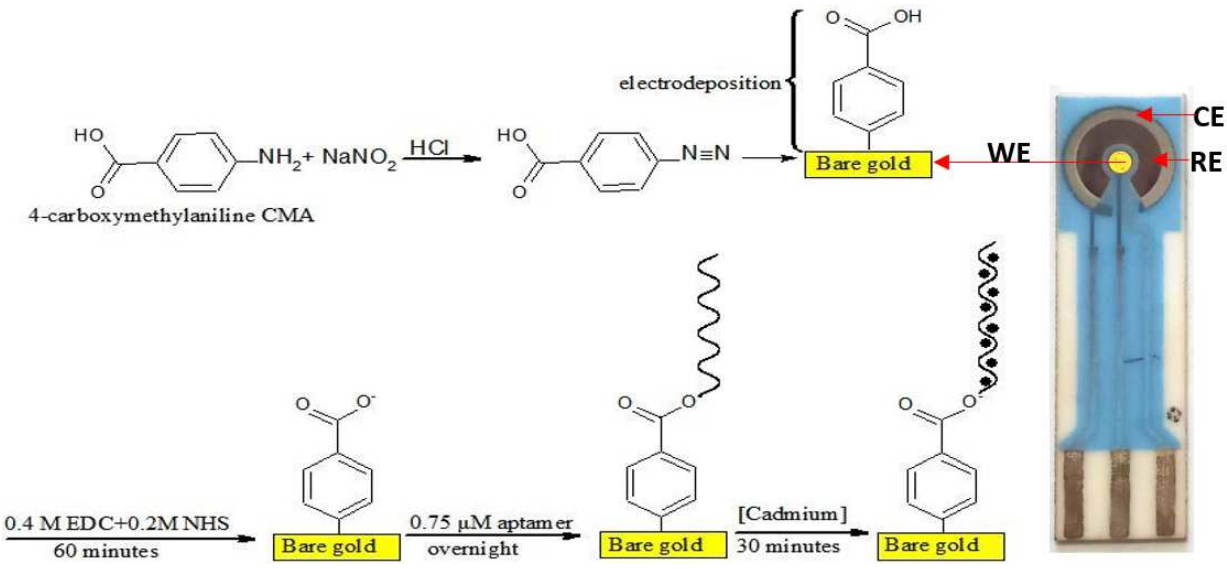


Fig. 1 Schematic illustration of gold WE chemical surface modification by CMA followed by aptamer and immobilization for Cadmium detection[32, 33]using integrated RE and CE.

Characterization of the functionalized gold surface

Cyclic voltammetry studies

For the Cyclic Voltammetry studies, **Fig.2A** shows the different cyclic voltammograms of the construction steps of this aptasensor. The oxidation-reduction peaks of the redox couple are visible for the bare Gold WE. **Fig.2A (a)** showing a pair of quasi-reversible peaks characteristic of the oxidation-reduction with cathodic and anodic current peak ratio of approximately one and peak-to-peak separation of 0,1V.Then, a significant decrease in the current **Fig.2A (b)** has been observed after the electrochemical grafting of diazonium salt **Fig.2B.** The electrochemical reduction of the diazonium salt was generated *in situ* by a reaction of CMA with $NaNO_2$ in a solution of HCl, with a scanning speed of 80 $mV.s^{-1}$, and within a potential range of (-0.2 to -1V). In the first cycle, the current variation showed a reduction wave localized at -0.6V. This current corresponds to the reduction of the diazonium salt in solution which leads to the formation of a radical amine. Afterwards, we noticed from the second scan cycle that the reduction current decreases remarkably. This can be attributed to the decrease of the electron transfer rate that was created by the CMA blocking layer. This was confirmed by a significant decrease in the current **Fig.2A (b)**.After aptamer immobilization, CV peaks at the electrode have significantly increased compared to CMA modified gold WE electrode **Fig.2A(c)**.This implies that most of the surface of the electrode was covered with aptamer and demonstrates successful aptamer immobilization. Then, the modified gold WE was incubated in a PBS solution containing 1nM cadmium Cd^{2+}during 30mn followed by CV measurement **Fig.2A (d)**. Here we have again a decrease of CV peaks which means that the Cd^{2+}have been successfully adsorbed onto the gold WE surface. The Cd^{2+} adsorption prevents or decreases the transfer of electrons between gold WE surface and the redox couples in solution due to the opening of the rod portion of aptamer and formation of the complex Cd^{2+}- aptamer

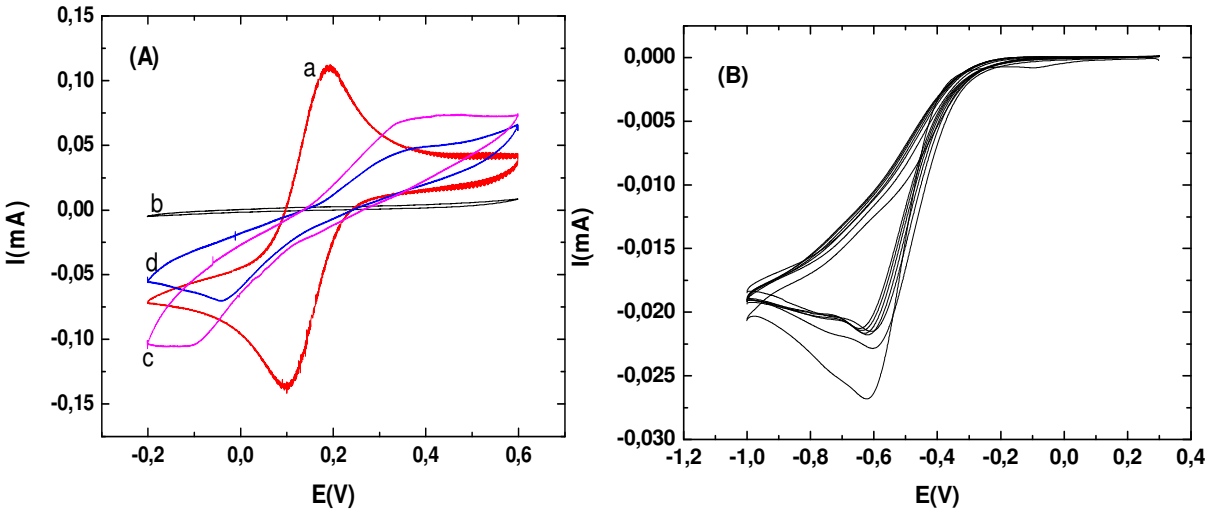


Fig. 2 (A) Cyclic voltammograms of (a) bare gold WE (b) after CMA deposition(c) after aptamer immobilization and (d) after the detection for 10^{-9}M of Cd^{2+} solution.(B) Cyclic voltammograms for electrodeposition of CMA for repetitive potential cycles from -0.2V to −1V with a scan rate of 0.8V.s^{-1}

Electrochemical impedance spectroscopy studies

Impedance spectra were employed to assess the electrochemical behaviour of the aptasensor fabrication and realized in ferric potassium ferrocyanide solution. The Nyquist plot semi-circles allow to assess the transfer charge resistance R_{ct} generated by any modification on the gold WEs surface. The more the surface is passivated, the higher is the resistance R_{ct} value **Fig.3**. Here, the first Nyquist plot semi-circle **Fig.3 (a)** corresponds to the bare gold which presents negligible resistance R_{ct}. Then, the formation of an organic layer of CMA onto the gold WE surface has increased the R_{ct} to 25 290 Ω **Fig.3 (b)**. This was due to the CMA layer which decreases the electron transfer. Subsequently, the surface carboxylic groups were activated using carbodiimide chemistry to allow the Cd^{2+}-aptamer immobilization through the covalent linkage between amino groups of aptamer and the abundant reactive carboxylic groups of CMA. Aptamers immobilization has induced a strong increase in the semi-circular diameter, with an R_{ct} of 28 096 Ω **Fig. 3(c).** Finally, the developed aptasensor was tested for Cadmium detection by incubating the aptasensor in Cd^{2+} solution at 10^{-9}Mfor 30 min. Here we observed an increase of R_{ct} up to 42 537 Ω **Fig.3(d)** due to the conformational changes induced by aptamer switching from a random coil structure to an aptamer–target complex[32, 33].

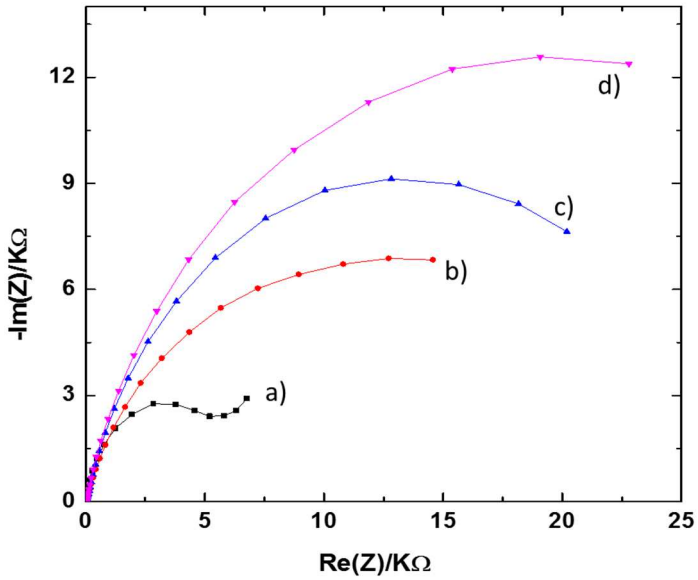


Fig. 3: Nyquist diagrams of the bare gold electrode (■), the electrochemically grafted CMA (●), after deposition of the aptamer (▲) and after incubation of Aptamer/CMA/AU in 10^{-9}M Cd^{2+} (▼) in the presence of 5 mM of $[Fe(CN)_6]^{3-/4-}$solution in a frequency range of 100 mHz - 200 kHz. The potential polarization used for these measurements was optimized at 128 mV.

Optimization of aptamer concentration

The aptamer concentration determines its final density onto the gold WE surface [34]. The optimization of aptamer concentration is a crucial step which affects the Cd^{2+}ions detection and the analytical performance of the aptasensor. Therefore, three different concentrations of aptamer (0.25, 0.5 and 0.75 µM) were tested within the range of 10^{-10} to 10^{-3}M of cadmium by using EIS. Fig. 4 shows the variation of R_{ct} as function of Cd^{2+} concentration for the three aptamer concentrations. As it can be observed, when using the aptamer concentration of 0.25 µM, the Rct did not change significantly, to the weak complexation of the aptamer with its target. This aptamer concentration generated a low signal with a slope of 1.744 kΩ (R^2= 0.927). The aptamer concentration of 0,5µM resulted a narrow range of linearity with a slope of 3.891 kΩ (R^2=0.911). Finally, by using the aptamer concentration of 0.75 µM, the signal was improved and the analytical response of the aptasensor was linear within the range of 10^{-9} to 10^{-4} M with a LOD of $2.75*10^{-10}$M. In addition, the sensitivity increased to 4.683 kΩ when compared to that obtained with the lower concentrations.

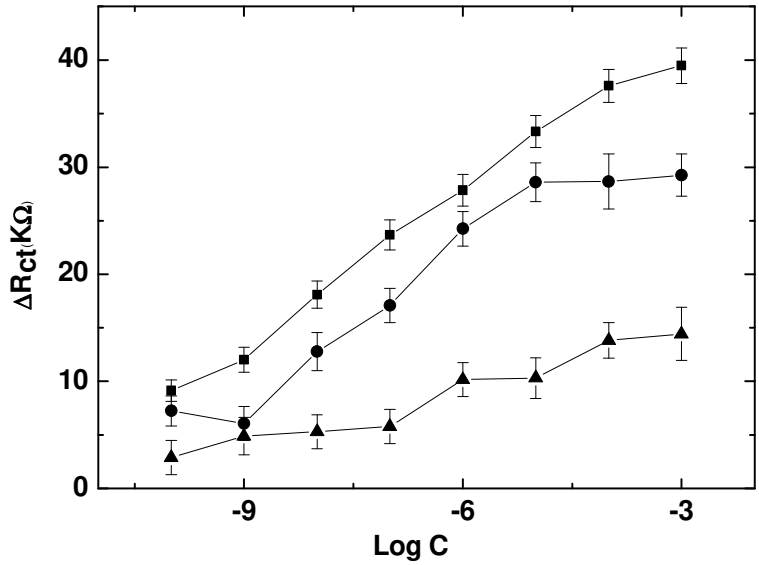
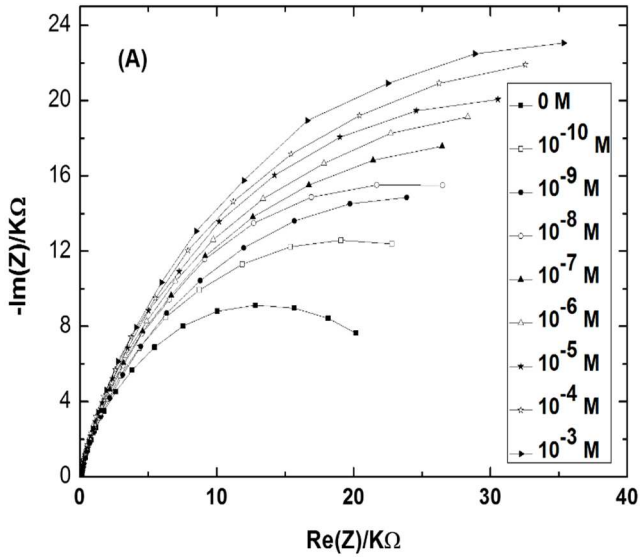


Fig. 4 Optimization of Cd^{2+}-aptamer concentration by EIS; (▲) 0.25 µM, (●) 0.5 µM and (■) 0.75µM of aptamer.

Analytical performance of the aptasensor

Under the optimal conditions, the aptasensor was used for cadmium detection in PBS buffer using EIS. To achieve that, the modified aptamer gold WE surface was incubated with different concentrations of cadmium for 30 minutes. Experimental results showed a significant increase in the diameter of Nyquist plot semi-circles by increasing Cd^{2+} concentration **Fig. 5A.** The first Nyquist plot semi-circle corresponds to the aptamer-modified gold WE before the addition of cadmium. After incubation of aptasensor with increasing concentrations of the target, the Nyquist plot semi-circles continue to increase indicating the interaction between cadmium and its specific aptamer.



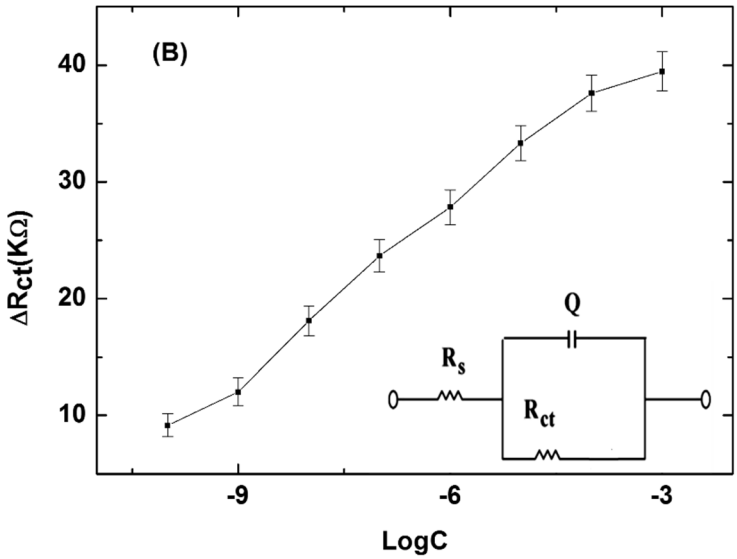


Fig.5: **(A)** Nyquist plots of Cd^{2+}-aptamer-Gold WE surface incubated with increasing Cd^{2+} concentrations ranging from 10^{-3} to 10^{-10} M, **(B)** sensitivity of the aptasensor by normalization of the data towards the varying Cadmium concentrations. Inset: equivalent circuit used for the EIS Nyquist plot semi-circles fitting.

The transfer charge resistance R_{ct} was extracted from Nyquist plot semi-circles by using the equivalent circuit in Figure 5B Inset [21]. Where Rs represents the solution resistance, Rct; the charge-transfer resistance and Q1; the constant phase element (an equivalent model of double-layer capacitance). The real part of impedance (Re(Z)) increased as function of cadmium concentration. The linear regression equation was normalized to $\Delta R_{ct} = R_{ct}$ (aptamer-cadmium) – Rct (aptamer) as a function logarithm of Cadmium concentration. Rct (aptamer-cadmium) is the value of the electron transfer resistance after Cd^{2+} binding to the immobilized aptamer on the modified electrode surface. Rct (aptamer) represents the value of the electron transfer resistance after the aptamer immobilization on the CMA-modified electrode surface. The resistance of the aptasensor exhibited a linear correlation to Cadmium concentration through the range of (10^{-10}-10^{-4}M), with a good correlation coefficient of (R^2 = 0. 9954) and a sensitivity of 4.683kΩ per cadmium concentration decade. The limit of detection (LOD) of the aptasensor is the concentration of cadmium corresponding to the 3 times "s/m" ratio, where "s" is the standard deviation of the blank impedance signal (three replicates) and "m" is the slope of the related calibration curve [35]. Here, the calculated LOD was $2.75*10^{-10}$M.The detection limit obtained in this work was lower the detection limits of Cd^{2+}aptamer strategies and other methods **(Table 1).**

Table 1: Comparison of other sensors with different detection methods for the determination of Cd^{2+}.

Detection methods	**Linear range (nM)**	**Limit of detection (LOD) (nM)**	**Refs.**
Fluorescent aptasensor	0 to 1000	40	[36]
Fluorescent aptasensor	7.19 to 200	2.15	[37]
Colorimetric aptasensor	10 to 400	4.6	[25]

Electrochemical aptasensor	1to1000	0.337	[38]
Electrochemical aptasensor	250 to 1000	92	[17]
Electrochemical aptasensor	0.1to10^6	0.27	This work

The reproducibility of the developed aptasensor was tested with interassay precision. The interassay precision was evaluated by incubating three electrodes, independently prepared under the same experimental conditions with a same cadmium concentration of 10^{-6} M. A relative standard deviation from of 4.49% was calculated, indicating acceptable aptasensor reproducibility.

For the specificity study, the **Fig.6** depicts the impedance changes after incubation with Cd^{2+} and other heavy metals such as Hg^{2+}, Pb^{2+} and Zn^{2+}during 30 minutes. Three concentrations have been tested for each analyte; 10^{-8}, 10^{-7} and 10^{-6}M. At this stage, the interactivity of the aptasensor and cadmium detection was performed only in PBS buffer. As it can be observed in (Fig.6); the impedance responses show clearly that the aptasensor was highly selective to cadmium as compared to the other tested heavy metals. No significant signals have been recorded, even in the presence of Pb^{2+} and Zn^{2+}. However, a slight cross-reactivity was noted with Hg^{2+},it may be attributed to the special T–Hg–T mismatched base pair between Hg^{2+} and Cd^{2+}- aptamer[39-41].These results show that the selected aptamer immobilized on the electrode surface maintains its affinity and specificity in reaction with the cadmium, giving significant Δ Rct increase. This may be considered as a validation of the proposed aptasensor selectivity, because it allows the discrimination between the tested heavy metals into the entire selected range.

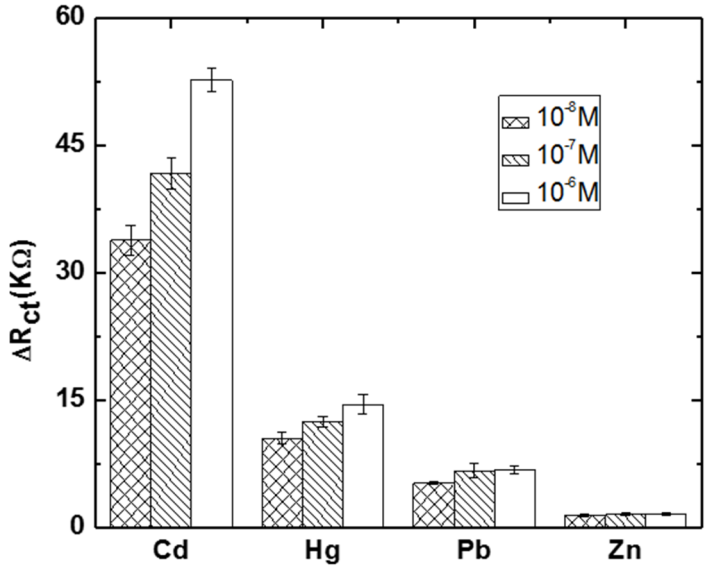


Fig. 6 Selectivity studies of the aptasensor with Hg, Pb and Zn.

Application in water samples by the standard addition method

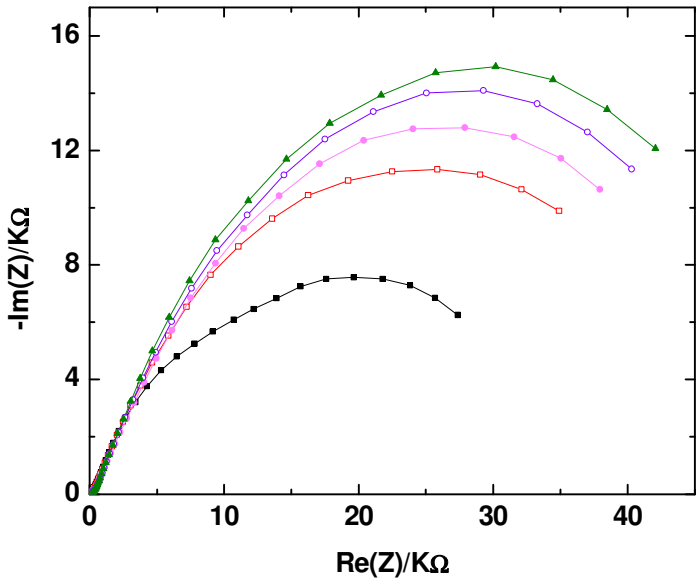
The developed aptasensor was applied for the detection of Cadmium in water samples of Hammam Essalihine valley using standard addition method as described previously. Four flasks containing the real water samples were prepared as mentioned in experimental, and then analysed by EIS measurements **Fig.7** depicts the first Nyquist plot corresponding to the aptamer-modified gold surface before cadmium addition, while the second one is attributed to the aptasensor after being incubated in the first flask (**level 1**) for 30 min. A shift in the semi-circle from the first one was observed, it was explained by the increase of resistance provided from aptamer reaction with cadmium. The second flask, corresponding to **level 2**, showed the same behaviour as the first flask. The analysis of the other flasks was performed in the same parameters. An increase in the semi-circular diameter was noticed and was absolutely explained by the increase of cadmium amount in the flasks. In order to determine the concentration of Cd^{2+} in the real sample, slope (m) and y-intercept (b) of the resulting calibration curve (R^2 = 0.987) were used, and the unknown Cd^{2+} concentration of real sample was estimated to be $4.29.10^{-8}$M.

Table 2: Determination of Cd2+ by proposed aptasensors and atomic absorption spectroscopy (AAS) in water samples and recovery study of the aptasensor for determining cadmium

Ion in sample	AAS(C)	The aptasensor(Cs)	Recovery (%)
Cd2+	$4.76*10^{-8}$M	4.29*10-8M	90.12

Recovery% = Cs/C×100%[42]

Aiming to validate our aptasensor, the tested sample was also analyzed by atomic adsorption spectroscopy (AAS) which is a reference method usually used for Cadmium detection[6, 7] . The obtained results are presented in (Table2). They are in a good agreement with those obtained by the standard addition method.



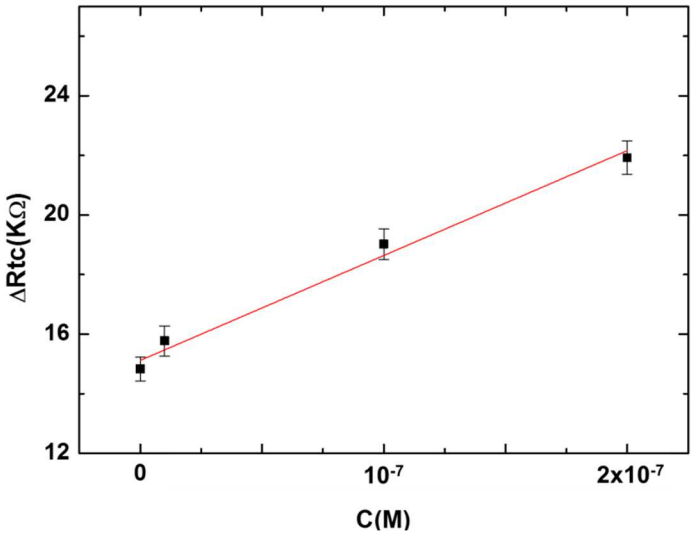


Fig. 7 (A) Nyquist impedance plots obtained from the performance application on a real sample. (■) PBS; (□) Level 1 (corresponding to an addition of 0 M); (●) Level 2 (addition of 10^{-8}M); (○) Level 3 (addition of 10^{-7}M) and (▲) Level 4 (addition of $2*10^{-7}$M). (B) Sensitivity curve used to calculate the concentration of analyte in the unknown sample by the standard addition method. Error bars represent the relative standard deviation on the measurements performed in triplicate.

Conclusion

In summary, we have developed an electrochemical aptasensor for Cadmium detection. The aptamer was covalently immobilized onto gold WE through CMA molecules. The studied aptasensor showed a linear relationship between the impedance changes and the logarithm of Cd^{2+}concentration in a broad range from 10^{-3} to 10^{-10} M, with a LOD about $2.75*10^{-10}$M. Indeed, the present aptasensor displays excellent sensitivity and selectivity, in addition to a good reproducibility. Moreover, the aptasensor was highly selective to cadmium in the presence of other interferences, such as Hg^{2+}, Pb^{2+} and Zn^{2+}. In order to prove the method applicability, the constructed aptamer sensor has been employed to detect cadmium in real spring water samples with high accuracy. These promising results show that the developed method could be an excellent alternative for traditional techniques because of its accuracy, reliability, simplicity and low cost. This miniaturized sensor could be used wide wearable devices in rivers or directly in spring water sources. In addition, the working electrode could be functionalized by other aptamers to allow the concomitant determination of several heavy metals.

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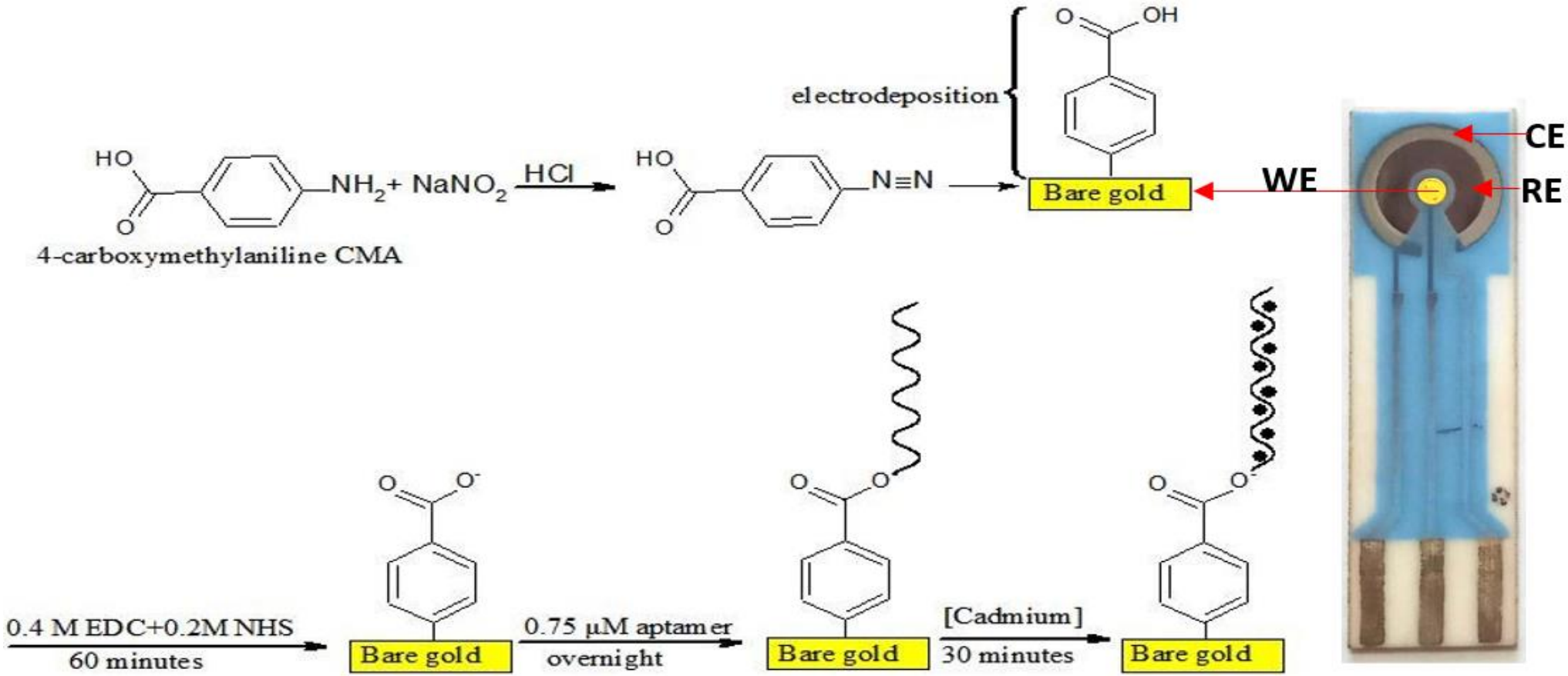
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