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Abstract

Ochratoxin A (OTA), a widespread food contaminant, is considered as one of the most hazardous mycotoxins commonly occurring in beer. Herein, we describe a novel impedimetric aptasensor based on Pencil Graphite Electrodes (PGE) for the analysis of OTA. Taking advantage of its affordability and high active surface area, a PGE was modified with a specific aptamer, to achieve sensitive detection of OTA. This strategy relies on the target-induced increase in the electron transfer resistance (R_{ct}) upon OTA recognition using ferro/ferricyanide as a redox probe. With the addition of OTA, the formation of OTA–aptamer complex resulted in an increase of Rct. The change of R_{ct} strongly depends on OTA concentration, which is applied for mycotoxin quantification. An acceptable linear range was obtained from 0.1 to 2.0 ng.mL^{-1} with a very good limit of detection as low as 0,1 ng.mL^{-1}. This simple-to-design aptasensor was successfully tested in spiked beer samples, with OTA concentrations in the range of 0.4-1.6 ng.mL^{-1}, attesting a recovery percentage of 93.4 ± 6.6%. It also showed a high level of stability and reproducibility thanks to the electro-grafting method via in-situ generation of 4-carboxyphenyl diazonium salts. This PGE-based platform is a versatile approach for the design of low-cost aptasensors for food safety control.

Keywords

Electrochemical Aptasensor, Pencil Graphite Electrode, Electrochemical Impedance Spectroscopy, Diazonium Electrografting, Ochratoxin A.

1. Introduction

In order to improve human life, the progresses in technologies as well as the mass industrial production of novel materials have rapidly become a part of everyday life. As a consequence, various global health risks including the increase of pollutants and toxins in food industry have occurred (Nácher-Mestre *et al.*, 2015). For these reasons, a food contamination has been getting more attention and, nowadays, it represents one of the most important worldwide issues. The food commodities are often exposed to a variety of mycotoxins which are produced at inconvenient conditions, such as air humidity and regulation, temperature, time of storage etc. OTA belongs to the class of the most toxic species of mycotoxins which is produced by several fungi, such as *Penicillium verrucosum* and by several species of *Aspergillus* (Bui-Klimke & Wu, 2015).

The relatively high levels of OTA concentration are principally found in cereals, grape juice, coffee, nuts, spices, cocoa beans or wine. Because of the chemical stability of OTA and its long half-life in mammalian tissues, the contamination could be also occurred in pork and pig blood products as well as in beer beverages (Walker, 2002). Since almost all countries did not provide information on the contribution of food products, the overall intake values from OTA-contaminated food commodities were calculated, whereby cereals resulted as the main contributors (50%) followed by wine (13%), coffee (10%), spices (8%), others (6%), beer (5%), cocoa (4%), dried fruits (3%) and meat (1%) (Europea, 2002). According to The International Agency for Research on Cancer (IARC) (IARC, 1994) and The World Health Organization (WHO, 2007), OTA has been shown to be nephrotoxic, carcinogenic, teratogenic and immunotoxic to human and animal beings and causes kidney and liver tumors. The consumption of OTA-contaminated foods has a huge negative impact on population health hand must be controlled. The maximum levels of OTA concentration in a wide range of foodstuffs available in European countries have been established in 2002 by the Directorate-General Health and Consumer Protection (SANCO), responsible for EU legislation in the areas of food safety, safety of products, public health and consumer safety.

As beer products play an important role in social life, their consumption has reached to significantly high levels. For this reason, the continual control of OTA concentration in beer products is required. According to SANCO, the daily dietary intakes of OTA ranges from 0,01 ng/kg (Italy) to 0,14 ng/kg (Denmark) (Europea, 2002). The most commonly used analytical approaches for OTA detection are liquid chromatography (LC) with fluorescence

detectors (Appell *et al.*, 2018), gas chromatography with mass spectrometry (GC-MS) (Olsson *et al.*, 2002), thin layer chromatography (TLC) (Patterson & Roberts, 1979), or enzyme linked immunosorbent assay (ELISA) (Flajs *et al.*, 2009). However, electrochemical biosensors (EBs) represent appealing analytical tools, owing a good price/performance ratio and providing a relatively fast detection of target analyte, which make them great candidates for mycotoxins detection in food samples. Generally, these EBs consist of an ingenious combination of bioreceptor and signal transducer. Among different biorecognition elements (including enzymes, amino acids, nucleic acids, tissues, cells, etc.), aptamers are emerging synthetic biomolecules originated from in vitro selection (SELEX) (Blind & Blank, 2015) that offer several advantages in biomolecular recognition, including their high thermal and chemical stability, great affinity and specificity for a wide range of targets, and low-cost production (Labuda *et al.*, 2010). Aptamers are able to undergo a unique three-dimensional structure upon binding to target which provides a great flexibility during the development of aptamer-based EBs or E-aptasensors. They are often tagged with a redox label, such as methylene blue (Guo *et al.*, 2015) or ferrocene (Ferapontova *et al.*, 2008) in order to perform the electrochemical detection of analyte based on the measurement of a current signal generated from the oxidation/reduction of the redox label at a fixed/variable potential. The change in the current corresponds to the concentration of the analyte in the sample. On the other hand, the conformational changes of an aptamer sequence need to be performed in a relatively large area which ends in increasing the distance of the electro-active label from the transducer surface. Moreover, the process of aptamer labeling is quite expensive and the construction strategies of analysis are more complex and time-consuming because the labeling can decrease the activity of immobilized biomolecules (Daniels & Pourmand, 2007; Rhouati *et al.*, 2016). A better alternative may be to develop a label-free impedimetric aptasensor using the electrochemical impedance spectroscopy (EIS) as a detection technique. Heretofore, the EIS has become the most reported transduction approach because it allows performing a direct sensitive detection of target analyte via monitoring the interfacial reaction mechanisms and the dynamics of biomolecular interactions. Therefore, many impedimetric aptasensors have been developed to successfully detect a variety of mycotoxins in food samples (Chen *et al.*, 2015; Danesh *et al.*, 2018; Khan *et al.*, 2019; Mishra *et al.*, 2015; Ocaña & del Valle, 2018).

On the other hand, cheap pencil mines, commonly named Pencil Graphite Electrode (PGE), have been increasingly investigated as working electrodes for biosensing applications. These

available carbonaceous transducers show a great potential in developing EBs ensuring a reliable option in disposable biosensors (Torrinha *et al.*, 2018). Recent reviews evidenced that PGE paved the way to be considered as valuable alternative to other carbon-based electrodes thanks to their thin dimensions and negligible cost (David *et al.*, 2017). However, as far we know, a relevant part of published works refers to the immobilization of enzymes as bioreceptors. The application of PGE with aptamers is still limited and even non-existing in mycotoxins analysis field, predicting a new window of opportunities in research.

A crucial step throughout the development of electrochemical aptasensor is the optimization of aptamer immobilization strategy. One of the simplest immobilization methods is the physical adsorption of aptamer onto electrode surface via electrostatic interactions. However, a poor reproducibility and sensitivity can be achieved. Moreover, the physical hindrance of target analytes with a low molecular weight could be insufficient for observing the signal variations. For these reasons, the activation of the electrode surface by desired functional groups, according to their coupling partners, can be performed using various electro-grafting methods (Bélanger & Pinson, 2011; Jiang *et al.*, 2017; Yáñez-Sedeño *et al.*, 2018).

This work describes the development of a label-free electrochemical impedimetric aptasensor, based on PGEs for the rapid detection of OTA and its quantification in beer samples. Briefly, the PGE surface was electrografted with 4-carboxyphenyl (4-CP) groups via *in-situ* generated 4-ABA diazonium salts in order to immobilize NH_2-modified anti-OTA aptamers via amide coupling. The detection strategy is based on target-induced variation in charge transfer resistance, which is measurable using electrochemical impedance spectroscopy. Analysis is then performed by studying the change in electrical or physical properties of the PGE surface which depend solely on the affinity of interaction between OTA and its specific aptamer and thus the concentration of the mycotoxin in samples.

2. Experimental section

2.1 Reagents and materials

The amine modified aptamer of following sequence (Cruz-Aguado & Penner, 2008) was purchased from Microsynth (Balgach, Switzerland):

NH_2-5'-GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA-3'.

Sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), Magnesium chloride ($MgCl_2$), potassium chloride (KCl), sulfuric acid (H_2SO_4,98%), sodium chloride (NaCl), hydrochloric acid (HCl, 37%), acetic acid (CH_3COOH, 98%) ethanol (C_2H_6O, 98%), sodium nitrite ($NaNO_2$), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), 4-aminobenzoic acid, and ethanolamine (C_2H_7NO) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Aptamer solutions were prepared in binding buffer (BB, pH 7.4) containing 1 mM $MgCl_2$, 140 mM NaCl, 2.7 mM KCl, 0.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. Acetate buffer solution (ABS, 0,5 M) was used in the pretreatment of PGEs. All solutions were prepared in deionized Milli- Q water (Millipore, Bedford, MA, USA). OTA purchased from Merck-sigma Aldrich (Saint-Quentin-Fallavier, France) was firstly dissolved in methanol (5 mg. mL^{-1}) and subsequently diluted in BB.

2.2 Apparatus

All measurements were performed using the electrochemical workstation SP-150 from Bio-Logic Science Instruments (France), connected to the EC-Lab software V.11.25. A conventional three-electrode system was used, consisting of Ag/AgCl reference, PGE as working electrode (φ=0.5 mm) and platinum wire as counter electrode. The immersed contact area of graphite working electrodes in all measurement solutions corresponds to 1.5 cm in length. This specification is required to obtain reproducible results. In order to provide electrical conductivity between disposable graphite lead and lead holder (pencil) a conductive wire was soldered to the metallic part of the holder.

2.3 Design of the aptasensor

Before running the experiment, all the PGEs were uniformly cut to a 3 cm length then the useful surface of 1,5 cm was immerged into the electrochemical cell solution for pretreatment.

In a first step, PGE transducers were pretreated in the acetate buffer solution (ABS, pH 4.8, 20 mM NaCl contained) by applying 1.40 V for 30 s without stirring. After that, reproducible bare electrodes were carefully selected for further modifications, based on CV and EIS measurements in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution.

Subsequently, the diazotation process was achieved by mixing 4-aminobenzoic acid (4-ABA) diluted in in 0.5 M HCl with 1.43-fold excess of sodium nitrite. The solution was left to react for 10 minutes in a cooling bath for maximum generation of 4-carboxyphenyl (4-CP) diazonium salt. Afterward, the electrodeposition was carried out via linear sweep voltammetry (LSV) with potential ranging from 0.5 to -0.1 V and a scan rate of 50 $mV.s^{-1}$. LS voltammogram showed a cathodic peak of 4-CP diazonium salt at -0.2 V (See inset LSV graph in Fig. 1). In this method, the electrochemical *in-situ* reduction of a diazonium salt leads to the formation of aryl centered radicals after the spontaneous elimination of dinitrogen gas (N_2). They form a robust covalent bond with the carbonaceous electrode surface by using LSV (Khan *et al.*, 2019). This method allows to get a well-defined carboxyl-modified layer on the electrode surface with a high level of stability and reproducibility (Yáñez-Sedeño *et al.*, 2018). The terminal COOH groups of 4-CP-functionalized PGE surface, were then activated through EDC/NHS chemistry for the immobilization NH_2-modified OTA aptamer via covalent amide coupling, at an optimized concentration of 0.8µM.

A schematic representation of the stepwise design strategy of the developed impedimetric aptasensor is shown in Fig. 1. Such prepared aptasensors could be freshly used or stored at

Preferred place for Fig. 1

4°C for several days without significant loss of sensitivity and reproducibility of the PGE-modified platform.

2.4 Electrochemical measurements

All the modification steps were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The CV was carried out within potential range of 0.0 V to 0.5 V and scan rate of 100 $mV.s^{-1}$. The impedance spectra were recorded using a sinusoidal AC potential perturbation of 5 mV (rms), in the frequency range from 10^5 - 10^{-1} Hz, superimposed on a DC potential of 0.1 V. All measurements were performed in a

solution of 5 mM ferri/ferrocyanide redox indicator $[Fe(CN)_6]^{3-/4-}$ in BB, pH 7.4, as a background electrolyte.

In this work, we focused on the variation in electron transfer resistance (R_{ct}) to quantify the targeted mycotoxin in samples. In order to minimize the electrode to electrode variations and to obtain reproducible and independent results, relative and normalized signals were required. Accordingly, the Δratio parameter, subtracting the error due to inherent differences between bare electrodes, was calculated for all the PGEs as following:

$$\Delta Ratio = R_{ct\ (after\ OTA)} - R_{ct\ (bare)} / R_{ct\ (before\ OTA)} - R_{ct\ (bare)}$$

where $R_{ct\ (after\ OTA)}$ is the value obtained after incubation of OTA, $R_{ct\ (bare)}$is the charge transfer resistance of the bare electrode, and $R_{ct\ (before\ OTA)}$ is registered in blank BB before OTA detection.

2.5 Ochratoxin A aptasensing

A stock solution of OTA was prepared in methanol (5 $mg.mL^{-1}$) and subsequently diluted in binding buffer. For OTA aptasensing, selected concentrations ranging from 0.1 to 6.4 $ng.mL^{-1}$, including the maximum admissible level in beer samples of 2 $ng.mL^{-1}$, were tested. A small standard solutions volume of 200 µL was required for modified-PGEs incubation. Based on previous works published by our group ((Hayat et al., 2013), (Mishra et al., 2015)), an incubation time of 1h was sufficient to enable the aptamer's structure to switch upon OTA recognition. This time has been thus selected for OTA analysis. Afterward, the fabricated PGEs were thoroughly rinsed with BB to carry out electrochemical measurements.

2.6 Beer samples analysis

A rapid and simple pretreatment is required before real sample analysis. The beer samples were therefore prepared following a previously established protocol (Rhouati *et al.*, 2013). Briefly, three samples were spiked with known concentrations of OTA stock solutions freshly prepared in binding buffer. Then, all samples (spiked and blank) were cooled at 4°C for 30 min to prevent rapid foam formation, which may lead to sample overflow during the degassing step. This latter step is performed by mean of an ultrasonic bath for up to 1 h. The pH of prepared samples was then checked to be adjusted to 7.4 using BB if needed. Finally, the prepared beer samples were filtered through a syringe filter of 0.45 mm to minimize

potential matrix interferences. After pretreatment, samples are directly tested following the same protocol described for standard OTA solutions.

3. Results and discussion

3.1 Electrochemical characterization of the aptasensor

Electrochemical characterizations using CV and EIS were performed in the presence of

Preferred place for Fig. 2

$[Fe(CN)_6]^{3-/4-}$ redox indicator in order to investigate the electrode surface properties as well as to confirm the effectiveness of surface modification in each development step of the aptasensor.

3.1.1. Cyclic voltammetry characterization

The electron transfer at the interface electrode-electrolyte was studied after each step of surface modification and the CV results are presented in Fig. 2A. The sequential fabrication of aptasensor was characterized by monitoring the changes in the current response of a redox couple and the changes in the peak-to-peak separation. The electrochemical behavior of redox indicator on PGE surface showed a characteristic reversible system with the cathodic/anodic current peak ratio of approximately 1 and the peak-to-peak separation of 0.05 V.

The approximate specific surface area of bare PGE can be estimated according to Randles-Sevcik formula, $I_p = 2.69\times10^5\ D^{1/2}\ n^{3/2}\ A\ V^{1/2}\ C$ (where I_p is the redox peak current (A); n is the electron transfer number, $n(Fe(CN)_6^{3-/4-})=1$; C is the concentration of $Fe(CN)_6^{3-/4-}$) ($mol.cm^{-3}$); A is the surface area (cm^2); D is the diffusion coefficient of the electrochemical probe ($cm^2.s^{-1}$), $D_{25°C}=6.5\ 10^{-7} cm^2.s^{-1}$ and V is the scan rate ($0.1\ V.s^{-1}$)). Based on an average peak current of 70 µA, active area was found to be equal to 0.20 cm^2. This value is greatly superior to usual specific surface calculated using SPCEs (a maximum of 0.07 cm^2 for φ=3 mm).

After the electrografting of 4-CP groups, the PGE surface was completely modified and a stable and reproducible layer of COOH groups was formed. Due to the repulsive forces, the electron transfer between both negatively charged redox probe and electrode surface extremely decreased which resulted in the complete disappearing of both redox peaks of redox probe (Fig. 2A). After the activation of the COOH groups with EDC-NHS chemistry,

the reversibility of the system improved, allowing the current values of the cathodic and anodic peaks to increase. The aptamers were immobilized on pre-functionalized PGE surface via amide coupling, which resulted in the formation of a steric barrier for the electron transfer, decreasing thus the redox signal of $[Fe(CN)_6]^{3-/4-}$. After incubating the aptasensor in OTA solution, the aptamer underwent 3D conformational changes upon binding OTA. A decrease in CV response accompanied with an increase in peak-to-peak separation confirmed the interaction of aptamer sequence with OTA, forming a G-quadruplex (Badie *et al.*, 2017) that blocks further the electron transfer.

3.1.2. Characterization of the aptasensor using EIS

Electrochemical impedance spectroscopy is a powerful tool for studying the electrochemical behavior of modified electrode surfaces. Therefore, the stepwise development of our OTA aptasensor was investigated using EIS (Fig. 2B). The impedance spectra of bare electrode consisted of a semicircle portion, whose diameter corresponds to the electron transfer resistance and a linear portion, which represents a diffusion-controlled process. It is well-known that this behavior fits to Randles theoretical circuit. After the *in-situ* generation of 4-CP diazonium salts with further carboxyl groups fixation on PGE surface, the resistance significantly increased. A formed organic layer of 4-CPs acted as an electrostatic barrier which repealed $[Fe(CN)_6]^{3-/4-}$anions and hindered the electron transfer between redox probe and PGE surface. The resistance value increased from 30 Ω for bare electrode to 8 kΩ for 4-CP modified electrode. The second modification step was the immobilization of aptamer. For this purpose, the COOH groups on PGE surface were activated with EDC-NHS chemistry and the aptamer was immobilized through amide coupling between NH_2 groups of the aptamer sequences and pre-activated COO^- groups of the 4-CP layer. Comparing to the R_{ct} value of the 4-CP layer, the resistance of the aptamer-modified layer significantly decreased after the aptamer immobilization. Such electrochemical behavior of modified electrode could be explained by successful covering of pre-activated COO^- groups with NH_2-terminated aptamers thus, the electrostatic repulsion between COO^- groups and $[Fe(CN)_6]^{3-/4-}$ anions decreased. After the incubation of aptasensor in OTA solution, the R_{ct} value significantly increased. In this case, the aptamer sequence underwent the conformational changes and formed a G-quadruplex upon binding OTA. This formed 3D structure blocked the molecular gates and did not allow ferri/ferrocyanide to freely reach the electrode surface due to the electrostatic repulsion.

3.2 Optimization of aptamer immobilization

The most crucial step through the development of the aptasensor is to optimize experimental conditions for the bioreceptor immobilization onto the transducer surface, in order to achieve an aptasensing platform with a high level of stability and reproducibility. For this purpose, after obtaining a stable electrografted layer of 4-CPs, the maximum concentration of aptamer immobilized on PGE surface was optimized. Variation in the Δ_{Ratio} values was obtained in the concentration range from 0.05 µM to 1.20 µM incubating PGE in aptamer solutions for 120 min. It was observed that the saturation level of immobilized aptamer was reached in case of 0.80 µM aptamer (as illustrated in Fig. S2(A)). Moreover, different incubation times of aptamer were tested in order to achieve the maximum value of fixed aptamers. The Δ_{Ratio} values were calculated for several time intervals ranging from 30 min to 240 min. A period of 120 min as an optimized incubation time of aptamer was used (Fig. S2(B)).

Preferred place for Fig. 3

3.3 Impedimetric detection of OTA

The APT/4-CP/PGE surface, modified with specific anti-OTA aptamers, was exposed to various concentrations of OTA for 120 min in order to evaluate the analytical performances of developed aptasensors. The OTA concentration ranged from 0.1 ng/mL to 6.4 ng/mL and the PGE surface resistance was measured using EIS. In order to calculate a reliable estimation of increase in impedance, Δ_{Ratio} is calculated for all the aptasensors (n=3 *i.e.* different electrodes developed in triplicate).

After the incubation of aptasensors in OTA solution, a significant increase in the diameter of Nyquist circle was observed, thus, the calculated Δ_{Ratio} values was found to be directly proportional to increasing OTA concentration (Fig. 3A). A linear response of aptasensors upon binding OTA was found in the concentration range up to 1.6 ng/mL (Fig. 3B-inset). It exhibited a promising LOD of 0.1 $ng.mL^{-1}$, commonly based on the standard deviation of the baseline signal and a LOQ equal to 0.32 $ng.mL^{-1}$. According to the linear calibration curve slope, the aptasensor sensitivity is 0.33 $ng.mL^{-1}$.

These results suggest that the formation of aptamer/OTA complex greatly hinders the electron transfer between $[Fe(CN)_6]^{3-/4-}$ and PGE surface due to aptamer rearrangement into G-

287 quadruplex structure upon recognition, which leads to the proportional increase of Rct values
288 with OTA concentration. Furthermore, OTA molecule has carboxyl and phenolic groups
289 which evidently contribute to the complex negative charges, present at the PGE surface upon
290 recognition. We believe that both electrostatic as well as steric blocking effects have
291 contributed to the increase of electron transfer resistance.

292 3.4 Aptasensor selectivity

293 Because selectivity is a critical feature of biosensing tools, control experiments were

Preferred place for Fig. 4

294 performed using OTA and its metabolite OTB as interfering analyte, since the latter belongs
295 to the same mycotoxin group. For that purpose, the 4-CP/PGEs were incubated for 120 min
296 using four different concentrations (0.4, 0.8, 1.6 and 3.2 $ng.mL^{-1}$) of OTA and OTB. As
297 illustrated in Fig. 4, the Δ_{Ratio} values obtained upon binding OTB did not significantly
298 increase comparing to those of OTA. These results confirmed a high level of selectivity of
299 prepared aptasensor toward OTA against low interferences of chemically similar mycotoxins.

300 3.5 Analysis of real samples

Preferred place for Table 1

301 In order to evaluate the feasibility and reliability of developed aptasensor, the beer samples
302 were spiked with OTA at three different concentrations which had been selected considering
303 the linear part of the calibration plot (0.4, 0.8 and 1.6 $ng.mL^{-1}$). The matrix effects were
304 reduced by 5-fold dilution of the samples in BB. As summarized in table 1, the determined
305 concentration values relatively corresponded to the spiked ones with a high level of recovery
306 percentage (93.4 ± 6.6%) which confirms the analytical capability of the aptasensor as a
307 sensing tool for OTA detection in beer industry.

308 3.6 Comparison with previous work

The analytical performances of our electrochemical aptasensor are compared to those of other recently reported aptasensing assays using the same aptamer sequence for OTA detection. This comparison is summarized in the table 2 below. It is worth mentioning that this work describes the first fabrication of an OTA aptasensor based on a pencil graphite electrode, which showed competitive analytical performances to SPCE-based aptasensors, widely studied for mycotoxins detection as low-cost carbonaceous transducers.

The comparison revealed that PGE-based aptasensor offers comparable analytical performances to most of the developed SPCE-based platforms. Although wider dynamic ranges and lower LODs are described in some previous works, we believe that this platform offers the best cost / efficiency ratio without the need to integrate nanomaterials or to label aptamers as is the case of reported aptasensors in (Rivas et al., 2015) and (Catanante et al., 2016) respectively. This would greatly reduce fabrication costs for a large-scale production. Regarding specific beer analysis, our group reported a previous study on SPCE modification with binary diazonium salt for the same detection purpose (Hayat et al., 2013). Albeit more sensitive, this multi-step carbon activation seems to be time-consuming while exhibiting almost the same modification yields in terms of coverage rate (96% *Vs* 98% in this work).

On the other hand, we demonstrate that using disposable PGE instead of other low-cost carbonaceous electrodes for mycotoxins E-aptasensing is also practicable for real sample analysis. This approach could be interesting to develop affordable affinity biosensors in any laboratory equipped only with electrochemical instrumentation, since PGE are widely available in every-day market with a variety of graphite compositions.

Preferred place for Table 2

Conclusion

In this project, we describe a novel label-free aptasensor based on Electrochemical Impedance Spectroscopy for the direct detection of ochratoxin A, starting for the first time from low-cost pencil graphite electrodes. This strategy relies on the target-induced increase in the electron transfer resistance using ferro/ferricyanide as a redox probe. The EIS has been demonstrated to be a sensitive method for the control of resistance changes on the electrode surface, during both the stepwise fabrication process and upon target recognition. This developed tool allows OTA detection at a relatively low concentration level of 0.10 $ng.mL^{-1}$ in standard solutions and 0.40 $ng.mL^{-1}$ in beer samples. Obtained analytical performances are in good agreement with standards prescribed by the EU regulation for food safety. The aptasensor showed an excellent recovery rate of 93.4 ± 6.6 % in beer samples even at trace levels of the mycotoxin without requiring a complex pretreatment protocol. It also showed a high level of stability and reproducibility thanks to the electro-grafting method via in-situ generation of diazonium salts. Interestingly, the PGE-based system showed a greater sensitivity than previously used screen-printed carbon electrodes (SPCE), owing to an extended active surface area and thus a high loading capacity of aptamers. Moreover, the electrochemical aptasensor has been challenged against OTB as a chemically similar metabolite to prove its good selectivity toward OTA. Therefore, this versatile approach opens up the path for designing highly-sensitive and cost-effective aptasensors for the detection of many fungal toxins by changing simply the specific aptamers.

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Conflict of interest

The authors declare no conflict of interest.

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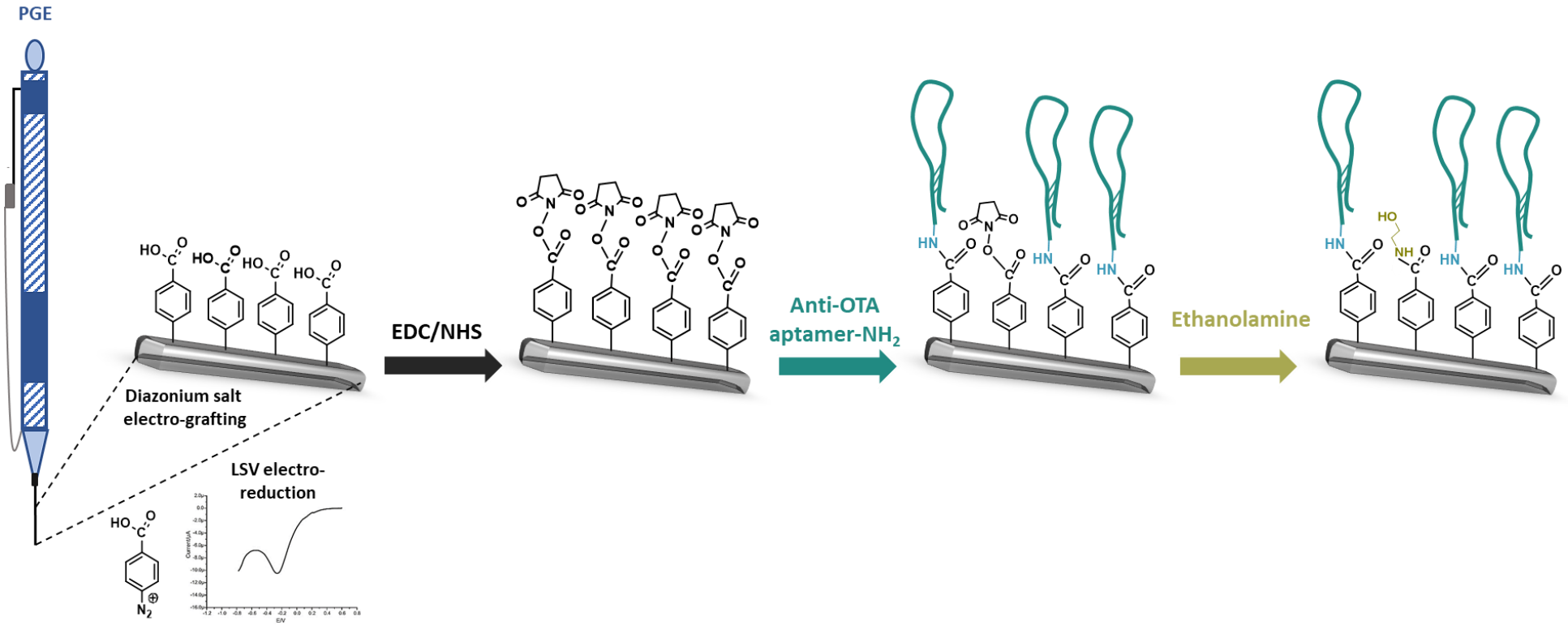


Fig. 1 Stepwise fabrication process of the PGE-based aptasensor for OTA detection

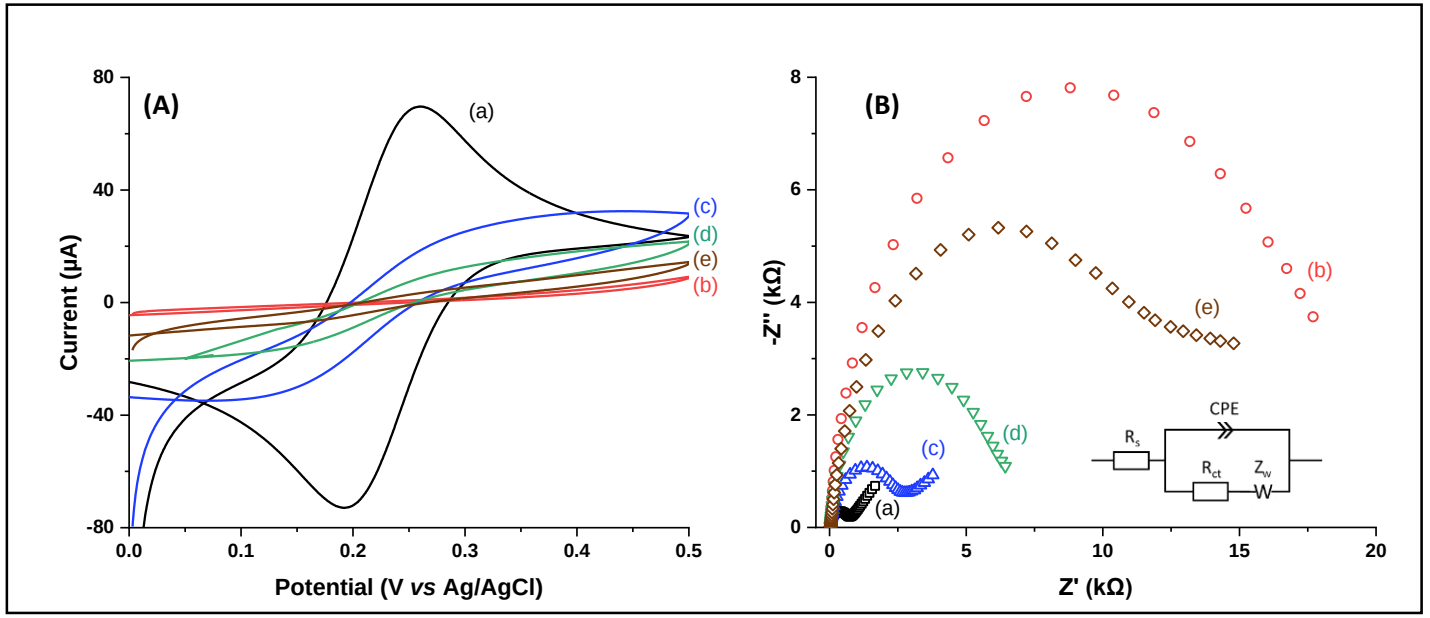


Fig. 2 (A) Cyclic voltammograms after each modification step, performed in 5 mM $[Fe(CN)_6]^{3-/4-}$ at 100 $mV.s^{-1}$ **(B)** Corresponding Nyquist plot of EIS analysis at an applied potential of 0.23 V (Vs Ag/AgCl reference electrode) using a frequency range of 10.0 kHz to to 0.1 Hz and an AC amplitude of 10 mV. The inset represents the equivalent electric Randles circuit applied to fit the EIS. (a) bare PGE (b) 4-CP-modified PGE (c) activation of COOH groups on PGE (d) immobilization of 0.8 µM aptamer (e) after the incubation of aptasensor in 0.1 $ng.mL^{-1}$ OTA.

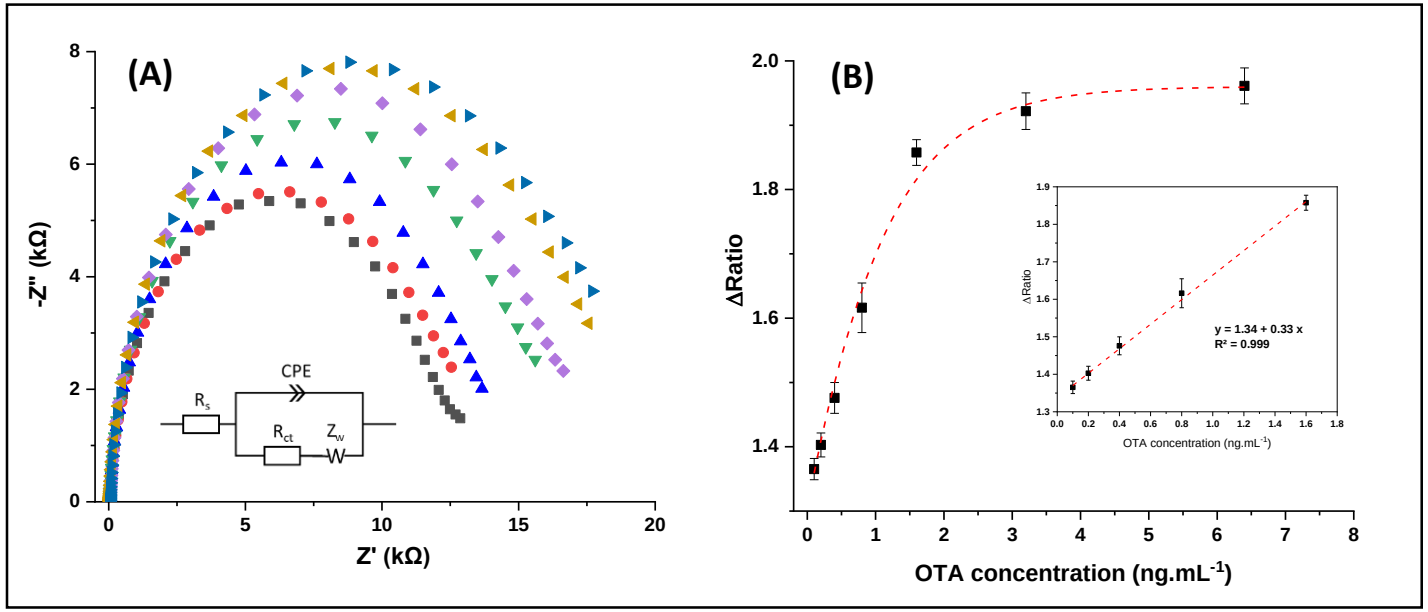


Fig. 3 (A) Nyquist plots of anti-OTA APT modified PGEs with different concentrations of OTA (ng.mL^{-1}): (a) 0.1 (b) 0.2 (c) 0.4 (d) 0.8 (e) 1.6 (f) 3.2 (g) 6.4 **(B)** Variations in Δratio values with increasing concentration of OTA from 0.1 ng.mL^{-1} to 6.4 ng/mL after the incubation time of 60 min with a linear response of aptasensor to increasing OTA concentration as an inset.

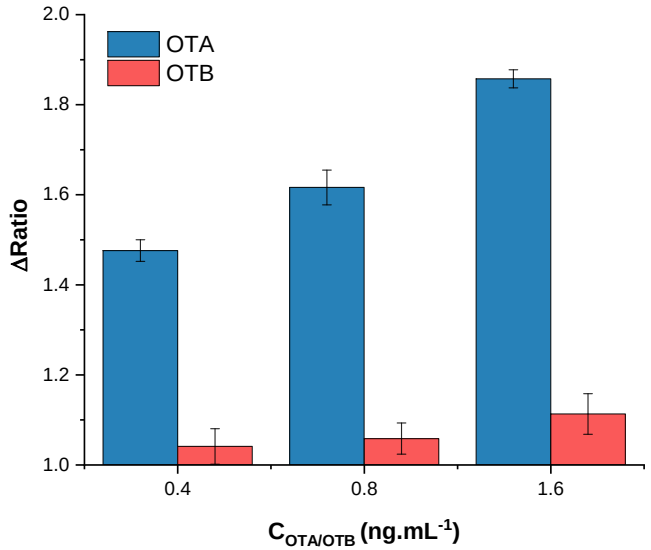


Fig. 4 Selectivity histograms presenting the calculated Δ_{Ratio} values obtained for different concentrations of OTA and OTB.

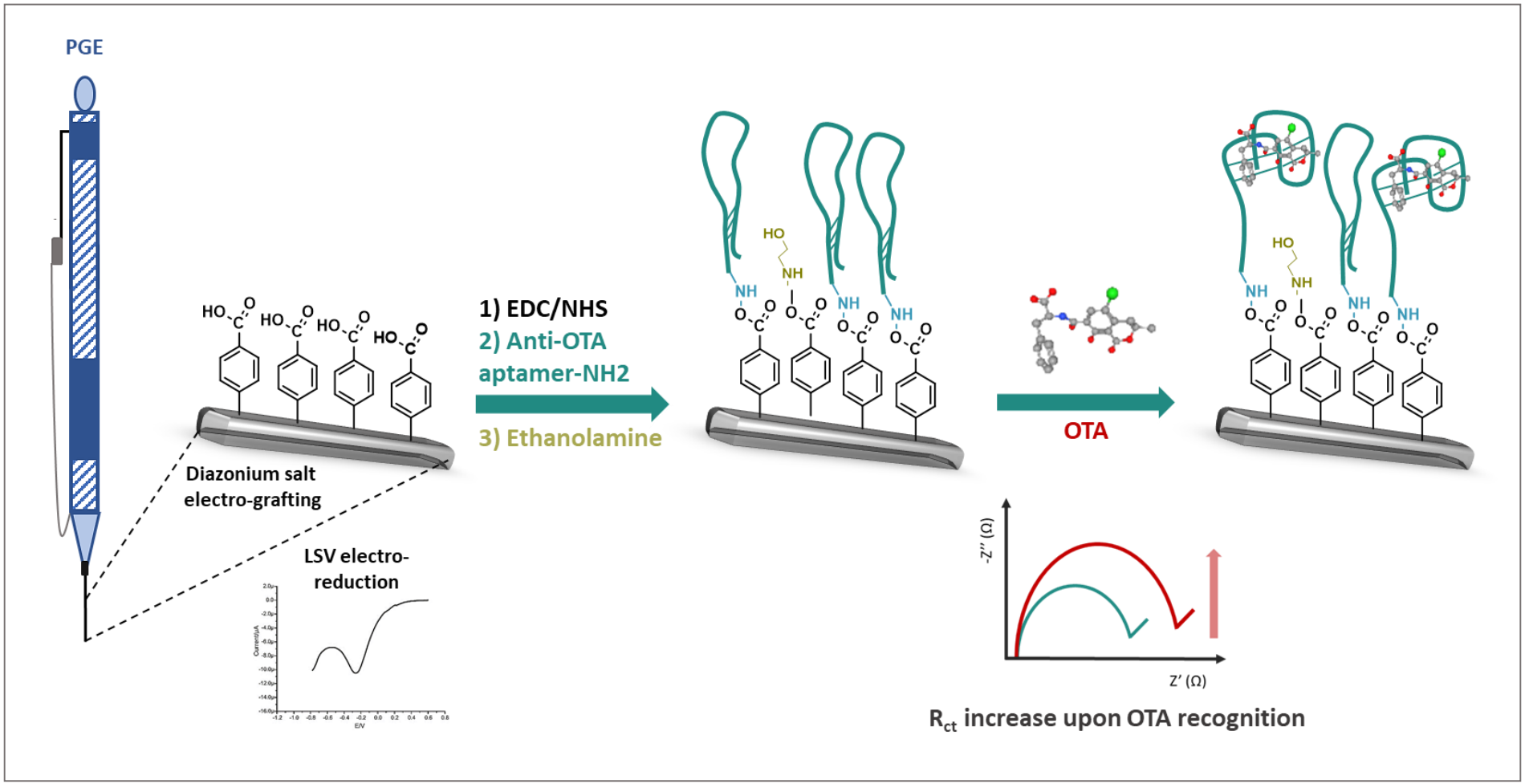
Table 1 Recoveries of OTA in spiked beer samples

Spiked OTA ($ng.mL^{-1}$)	**Determined OTA ($ng.mL^{-1}$)**	**RSD (%)**	**Recovery (%)**	**Relative error (%)**
0.4	0.37	3.49	**92.10**	7.90
0.8	0.75	3.95	**94.12**	5.88
1.6	1.50	4.95	**94.03**	5.96

Table 2 Comparison of the current work with some previously reported electrochemical aptasensors for OTA detection using SPCEs

Transducer	Modification	Aptamer coupling	Detection method	Dynamic range & LOD (ppb)	Real sample	Ref.
SPCE	4-((trimethylsilyl)ethynyl) benzene and p-nitrobenzene via 2-step diazonium salt electro-grafting followed by a deprotection step	Click chemistry using azido-aptamer	EIS	0.00125 - 0.5; 0.00025	Beer	(Hayat et al., 2013)
	4-ABA via diazonium salt electro-grafting	Amide coupling	EIS	0.150 - 2.5; 0.150	Cocoa beans	(Mishra et al., 2015)
	Polythionine and iridium oxide nanoparticles	Electrostatic interaction	EIS	0.004 - 40.4; 0.006	Wine	(Rivas et al., 2015)
	Hexamethylene diamine electrodeposition	Amide coupling of MB*-tagged aptamer	DPV	0.010 - 5.0; 0.010	Cocoa beans	(Catanante et al., 2016)
	Polythiophene-3-carboxylic acid	Amide coupling	EIS	0.125 – 20; 0.125	Coffee	(Zejli et al., 2018)
PGE	4-ABA via diazonium salt electro-grafting	Amide coupling	EIS	0.1 – 6.4; 0.100	Beer	***This work***

* Methylene blue



Graphical abstract : Modification of Pencil Graphite Electrode for the development of an impedimetric aptasensor for OTA detection