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Enhancing antibiotic detection via an aptasensor: the case of ciprofloxacin

Eva Hobeika[1*], Joseph Saab[1], Souheil Hallit[2,4,5], Isaac-Aaron Morales Frias[3], Nicole Jaffrezic-Renault[3] and Abdelhamid Errachid[3]

Abstract

The need for fast, efficient, and cost-effective test systems for antibiotics is surging, to control resistant bacterial strains. Electrochemical biosensors offer a good alternative to routine laboratory-bound analytical methods. These biosensors are portable, suitable for in-field analysis and biocompatible for detection of small biomolecules. The aim of this work is the ciprofloxacin active pharmaceutical ingredient since resistance of bacteria to this antibiotic is reportedly increasing worldwide, especially in Lebanon where hospitalization bills are no longer affordable. So, the target is ciprofloxacin detection, a fluoroquinolone antibiotic, on screen-printed electrodes. Following diazonium salt, also known as carboxymethylaniline (CMA) deposition, a ciprofloxacin oligonucleotide was incubated on the electrode. This aptamer acts as an anchor for the ciprofloxacin molecule, allowing the latter's attachment to the electrode and its quantification. Electrochemical characterization, through cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) allowed for deposition of molecules on electrodes and confirmation that an electrochemical change took place. Scanning Electron Microscope images are used to confirm conformational changes on the surface of electrodes. Impedance results reported a limit of detection of LOD = 3 nM, a dynamic range from 10 nM to 100 µM, and reproducibility of results between two aptasensors to be 10%. Moreover, impedimetric sensor specificity evaluation was through the effect of interfering compounds tobramycin, ofloxacin, norfloxacin and ceftriaxone, on the aptasensor's response. Based on available literature, this LOD level reached allows for the detection of ciprofloxacin via a portable potentiostat in environmental (wastewater, food), biological (urine, saliva) and pharmaceutical samples (efficient market withdrawal of counterfeit medications from pharmaceutical storage facilities).

Highlights

- An aptasensor was developed for ciprofloxacin detection on screen-printed carbon electrodes (SPCEs), connected to a portable potentiostat. The use of a portable potentiostat is not, in itself, a new technique. The focus and novelty of this study is the combination of antibiotic detection and quantification using an electrochemical approach that saves time. Another unique aspect is the use of graphene electrodes. Our study carries out a comparison between the behavior of SPCE and graphene electrodes.
- The innovative part relies on the use of diazonium salt for the immobilization of the aptamer.

*Correspondence:
Eva Hobeika
eva.hobeika@hotmail.com
Full list of author information is available at the end of the article

- DNA aptamer, a custom amino-modified ciprofloxacin oligonucleotide, acts as a bioreceptor for the ciprofloxacin reference pharmaceutical standards. Using DNA aptamer increased the selectivity of the sensor to ciprofloxacin, in specific. The combination of DNA aptamer to the aptasensor reduced analysis time.
- This combination, instead of a single entity on its own, provided a decrease in analysis time when compared to conventional chromatographic techniques.
- A relatively low, 3 nM detection limit was recorded without additional costly nanomaterials.
- Materials used in this protocol, namely CMA, NHS, EDC and ethanolamine resulted in a synergistic effect, favoring the attachment of ciprofloxacin molecule on the screen-printed electrodes.
- Impedimetric selectivity was observed in the presence of four structurally similar antibiotic compounds.

Keywords Ciprofloxacin, Aptasensor, Antibiotic resistance, Portable potentiostat, Electrochemical aptamer biosensor, Screen-printed carbon electrodes

Introduction

The World Health Organization (WHO) declared antibiotic resistance a "major threat to public health". 300 million people worldwide are expected to die prematurely due to antibiotic resistance over the next 35 years [1]. The main cause of antibiotic resistance is too much antibiotic use [2]. Some bacteria die when antibiotics are consumed, but resistant bacteria can survive and even multiply. The overuse of antibiotics makes resistant more common bacteria. Antibiotics can be used voluntarily as medicines for treatments or involuntarily when eating [3] antibiotic-contaminated substances. Then the control of consumable substances is highly necessary.

Each year in the European Union (EU) alone, over 25,000 people die from infections caused by antibiotic-resistant bacteria [1]. Healthcare costs of antimicrobial resistance (AMR) are soaring worldwide. Regionally in Lebanon, a recent paper published in 2021 evaluates these costs in Lebanese hospitals [4]. An increase in resistant bacteria versus susceptible ones causes an increase in healthcare and community-associated infections. Thereby, causing an increase in patient bills. Longer hospital stays are recorded at 2.69 days vs 2.2 days, and higher hospital charges 1807 vs 889$ [4]. So, controlling the AMR problem globally can alleviate unnecessary charges and damages caused by the resistance of certain bacterial strains to normal courses of antibiotics.

Electrochemical biosensors are highly portable and biocompatible for small biomolecule detection, thus, making their research gain popularity and applications feasible since the 60 s [5–10]. Electrochemistry is the main driver of biosensor applications since it permits sensitive, specific, and affordable detection of molecules [11]. Compared to conventional analytical methods (high-performance liquid chromatography, gas chromatography, mass spectrometry, etc.), biosensors use wearable and low-cost instrumentation and do not require any sample preparation. By decreasing the limits of detection and quantification, up to nanogram concentrations can be measured using biosensors, versus only microliter ranges by conventional techniques. Faster and more accurate detection times are recorded via biosensors, leading to overall savings in materials and a better project budget [12]. The impact on the environment is better while using biosensors [13]. Although spectrometry techniques are selective, the instrumentation has a high-power consumption rate and is expensive to maintain [14].

To prevent further spreading, fast, efficient, and cost-effective test systems are therefore important to control food [15] for potential contamination with antibiotics. Biosensors as test systems have already proven themselves in various areas such as food quality control [16].

Electrochemical sensors and biosensors face many challenges before replacing standard analysis methods, the potential of screen-printed electrodes is increasingly exploited, and more applications are anticipated to advance toward commercial analytical tools [17].

Aptamers are short deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) with a length of 25 to 100 nucleotides [18]. Like antibodies, they can bind their target molecules with high affinity and specificity by forming complex three-dimensional binding pockets [19] that often almost completely enclose their specific target. Combining aptamer technology and biosensors allows for point-of-care detection [16]. In the field, a portable potentiostat can be used by connecting it to a smartphone [20]. Already prepared in the laboratory, electrodes can be instantly used to detect the sample at hand (urine, saliva, water, milk etc.) to quantify the antibiotic in these biological and environmental samples.

This study sheds light on detecting ciprofloxacin antibiotic (Fig. 1) in pharmaceutical/ environmental samples. Belonging to the second generation of fluoroquinolones class [21], ciprofloxacin is a broad-spectrum antibiotic targeting both Gram-negative and Gram-positive

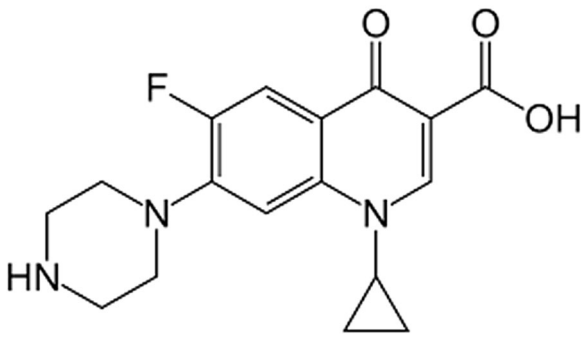

Fig. 1 Molecular structure of ciprofloxacin antibiotic [27]

pathogens [22]. Therapeutically, ciprofloxacin treats skeletal system infections (including joints) [23], infectious digestive system infections (such as abdominal and gastric inflammations) [24], lower and upper-respiratory tract and dermatological infections [25] as well as the very common urinary tract infections [26].

The quantification of ciprofloxacin is based on a biosensor approach, whereby an aptamer-based electrochemical sensor is used. Aptamer technology is currently the electrochemical detection method of choice, compared to the cumbersome antibody-antigen technique.

Fluoroquinolones is the class of medication targeted in this study. They have an average molecular weight, which is enough for electrochemical detection. Amine sites on the ciprofloxacin molecule can interact with the treated electrode surfaces. In incubation order, the aptamer sequence, containing an amino group addition at the 5' end, is immobilized on the CMA-modified electrode surface. In its turn, ciprofloxacin pharmaceutical standard (ciprofloxacin molecule) is immobilized on the amino-modified ciprofloxacin aptamer sequence. Thus, the amine organic group of ciprofloxacin molecule offers a chemical bonding advantage, since it forms stable bonds when immobilized on the carboxymethylaniline-modified [28] electrode surface. The impedimetric detection of ciprofloxacin was obtained with a detection limit in the nanometric range.

Materials and methods

Materials

Chemical compounds

Sodium nitrite ($NaNO_2$), potassium chloride (KCl), sterile water, phosphate buffered saline (PBS) tablets, absolute ethanol (99.8%), 4-aminophenylacetic acid (carboxymethylaniline (CMA) $C_8H_9NO_2$), hydrochloric acid (37% HCl), 1-(3-dimethylaminopropyl)−3-ethylcarbodiimide hydrochloride (EDC) ($C_8H_{18}ClN_3$), N-hydroxysuccinimide (NHS) ($C_4H_5NO_3$), MES (2-(N-morpholinoethanesulfonic acid), potassium hexacyanoferrate (II) trihydrate ($K_3FeCN_6.3H_2O$), potassium hexacyanoferrate (III) (K_4FeCN_6) and ethanolamine (C_2H_7NO) were purchased from Sigma Aldrich (France). These reagents are of analytical grade, used at room temperature.

DNA aptamer

The deoxyribonucleic acid (DNA) aptamer of Ciprofloxacin was purchased from Microsynth, Switzerland. It is modified by adding an amine at the 5′ end of the oligonucleotide sequence. An additional purification step was requested, due to the high chain length of the sequence (98 nt). The purification method performed in this case is PAGE purification (PolyAcrylamide Gel Electrophoresis). Hence the biotechnology company sent the oligonucleotide (aptamer) sequence in its purified form. This final sequence, similar to the ciprofloxacin oligonucleotide (aptamer) previously described in published references [29], is as follows:

5′-(NH_2 modified) ATA CCA GCT TAT TCA ATT GCA GGG TAT CTG AGG CTT GAT CTA CTA AAT GTC GTG GGG CAT TGC TAT TGG CGT TGA TAC GTA CAA TCG TAA GTT AG-3′.

Sterile water (commercial grade, purchased from Sigma-Aldrich France) is the solution used to prepare aliquots of the aptamer. Sterile water is an appropriate solvent for the aptamer solution because it is purified from RNA-ase and protease, making it suitable to be used as a solvent for a biological entity. It does not interfere with the oligonucleotide's DNA sequence, nor does it decrease its activity. Offering a biological advantage for solvent preparation.

Ciprofloxacin hydrochloride pharmaceutical secondary standard was purchased from Sigma-Aldrich (France). The range of concentrations, proposed for this standard, is based on electrochemical works [30] published for ciprofloxacin detection in pharmaceutical dosage form.

Instrumentation

Electrochemical scans, spectra and plots were obtained through a portable potentiostat analyzer (*Palmsens Sensit Smart*™, smartphone potentiostat). The detection was performed on screen-printed carbon electrodes (SPCEs). These easy-to-use carbon (graphite) screen-printed electrodes (SPEs) were provided by ItalSens IS-C™, having a flexible polyester support material. Their disc working electrode (WE) diameter is 3 mm.

Gii-sense® GF graphene electrodes were obtained from Integrated Graphene Ltd. The working electrode diameter area was 4 mm, with a 1 mm space between the counter electrode surrounding it in a C format. To complete the system, the Ag/AgCl reference electrode

is placed below the working electrode, and as a continuation of the counter electrode, maintaining 1 mm of separation.

Online databases search, in the domains of PubMed, Google Scholar and ScienceDirect, yields only around 81 published research articles (throughout the world) that reported ciprofloxacin detection on graphene aptasensor. Hence, a not-so-common approach is presented in this study.

Mettler Toledo FE20/EL20 pH-meter was used for adjusting the solution pH value. Scanning Electron Microscope, FEI Quanta FEG 250 SEM (University of Lyon 1–France), evaluated modifications on the electrodes' surface.

Methods

Preparation of electrodes

Pre-treatment of the screen-printed electrode Screen-printed carbon electrodes (SPCE) were pre-treated with 0.1 M KCl, under $E_{dc} = 1.0$ V, for 90 s to activate the pseudo-reference microelectrode [Ag form activated into Ag/Cl reference microelectrode (RE)], as recommended by the provider.

Electrodeposition of CMA (4-aminophenylacetic acid) The carboxymethylaniline (CMA) solution was prepared with a targeted 15 mM concentration, appropriate for this study's purpose. After preparation of the CMA solution, deposition of CMA on the screen-printed electrode were done by immersing the electrode in CMA solution. Cyclic voltammetry was applied for 15 cycles, after which electrodeposition was confirmed. Noting that the potential range is between − 1.2 V to 0.005 V, with the range being ± 0.005 V, while the scan rate is 0.05 V/s.

Activation of the carboxylic function To activate the carboxylic group, 0.1 M of N-Hydroxysuccinimide (NHS) and 0.4 M of 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC) solution was dissolved in 1 mL MES buffer [2-(N-morpholinoethanesulfonic acid)]. Screen-printed electrodes functionalized by CMA are incubated for an hour in this solution, at room temperature (22 °C). Electrodes are subsequently rinsed and carefully dried under a compressed air stream.

Incubation of aptamer on CMA activated electrode

Aptamer incubation The aptamer purchased from the biotechnology company was considered as stock solution. Dilutions of this stock were made to provide smaller volumes for incubation, and more aliquots to repeat the measurements. These aliquots were the result of aptamer stock solution dilutions in nuclease-free (sterile water) purchased from Sigma-Aldrich. A range of concentrations from 0.5 to 5 µM was assayed. The optimal concentration chosen was 2 µM.

20 µL of the prepared diluted aptamer solution was pipetted and placed on the working electrode. The small volume of aptamer solution, being placed directly on the SPE's working electrode, forfeits the requirement for agitation during incubation. Three different aptamer incubation times were tested: 1, 1.5 and 3 h, all under the same temperature conditions; at room temperature (22 °C). The best electrochemical response was recorded for the 3 h' time. Thus, the aptamer incubation time was 3 h, also backed by the study published by Hu et al. [29].

Ethanolamine blocking Following aptamer incubation, the electrode was rinsed with sterile (nuclease-free) water. Then, this electrode was immersed in an Eppendorf tube containing ethanolamine, for 30 min incubation time. It was then rinsed again with sterile water. Ethanolamine solution was purchased as analytical grade reagent (≥ 99%). From this reagent bottle, 2 µL of ethanolamine were pipetted and added to an Eppendorf tube, along with 1998 µL of sterile water.

Incubation of ciprofloxacin antibiotic on activated electrode

Lastly, an antibiotic standard solution (mother solution) was prepared, with a concentration of 5.18 M. It was done by dissolving the pharmaceutical reference standard of ciprofloxacin in PBS buffer solution.

The electrode was incubated in a range of 5 different analyte concentrations, starting with the smallest concentration. These different ciprofloxacin antibiotic solutions' concentrations are 0, 0.005, 0.05, 5 and 50 µM. Once prepared, the different solutions were placed in different Eppendorf tubes in which the SPCE's working electrode portion is immersed. So, no agitation was performed in this step. Each electrode incubation took the optimal timing of 1.5 h and was performed at room temperature (22 °C).

Electrochemical conditions

A portable potentiosat (*Palmsens Sensit Smart*™) characterized the electrochemical signal by using techniques of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). They measured the modifications performed on working microelectrodes. All measurements were quantified in 2.5 mM ferro-ferricyanide ($[Fe(CN)_6]^{3-/4-}$) and a solution of phosphate buffer saline (PBS). The pH of the PBS solution is pH 7.4, with a concentration of 10 mM phosphate buffer and 3 mM KCl.

Regarding cyclic voltammetry measurements, the potential was scanned between − 0.2 V and 0.6 V at a

scan rate of 0.05 V/s. Electrochemical impedance spectroscopy data was recorded for an initial potential Edc of 0.158 V (potential applied during measurement), a potential amplitude (RMS) Eac of 0.01 V, and a frequency range varying from 200 000 Hz to 2 kHz.

Results

Preparation of electrodes and electrochemical and morphological characterization

This section includes scans and spectra obtained as a result of each step applied in the methods Sect. "Methods". First, the SPCE were pretreated by applying $E_{dc} = 1.0$ V, for 90 s in 0.1 M KCl, to activate the pseudo-reference microelectrode.

Then, bare electrodes are subjected to CV and EIS for electrochemical characterization of the SPCEs after pretreatment (Fig. 2, corresponding to Sect. "Preparation of electrodes"). The charge transfer of the bare SPCE electrode is controlled through a 2.5 mM solution of ferrocyanide/ferricyanide $[Fe(CN)_6]^{3-/4-}$ redox probe. The CV scan is represented in Fig. 2A. ΔE is close to 200 mV, showing a limited charge transfer rate. The EIS spectra is represented in Fig. 2B, the charge transfer resistance being around 1500 Ω.

The CMA deposition was carried out by cyclic voltammetry. The scans obtained are presented in Fig. 3, corresponding to the method detailed in Sect. "Incubation of aptamer on cma activated electrode". The current decreases strongly after the first cycle, showing that the electropolymerized film limits the charge transfer. 15 cycles were carried out and the charge transfer of the CMA-modified SPCE electrode is presented in Fig. 4. There is nearly no charge transfer in the presence of 2.5 mM $[Fe(CN)_6]^{3-/4-}$ redox solution.

Figures. 3 and 4A show expected changes to the electrode's electrochemical structure, during (Fig. 3) and following (Fig. 4A) CMA deposition. They confirm that the diazonium salt was effectively fixed on the SPCE electrode (corresponding to methods Sect. "Incubation of aptamer on CMA activated electrode").

Fig. 4B shows the change in electrochemical spectrum (EIS spectra) after the SPCE is incubated in the prepared ciprofloxacin aptamer (method detailed in Sect. "Incubation of aptamer on CMA activated electrode").

Morphological characterization of the screen-printed carbon electrode

The surface morphologies' investigations of bare screen-printed carbon electrode (SPCE), SPCE/CMA, CMA/aptamer and CMA/aptamer/ciprofloxacin were carried out by scanning electron microscopy (SEM) (Fig. 5). A different morphology of the surfaces of the bare electrode (Fig. 5A) and of the CMA modified validates the CMA deposition step (Fig. 5B). After the chemical deposition of the aptamer onto CMA layer, no considerably visible morphological modifications can be reported on the surface of the working electrode (Fig. 5C). SEM is not able to differentiate between the morphology of activated surface

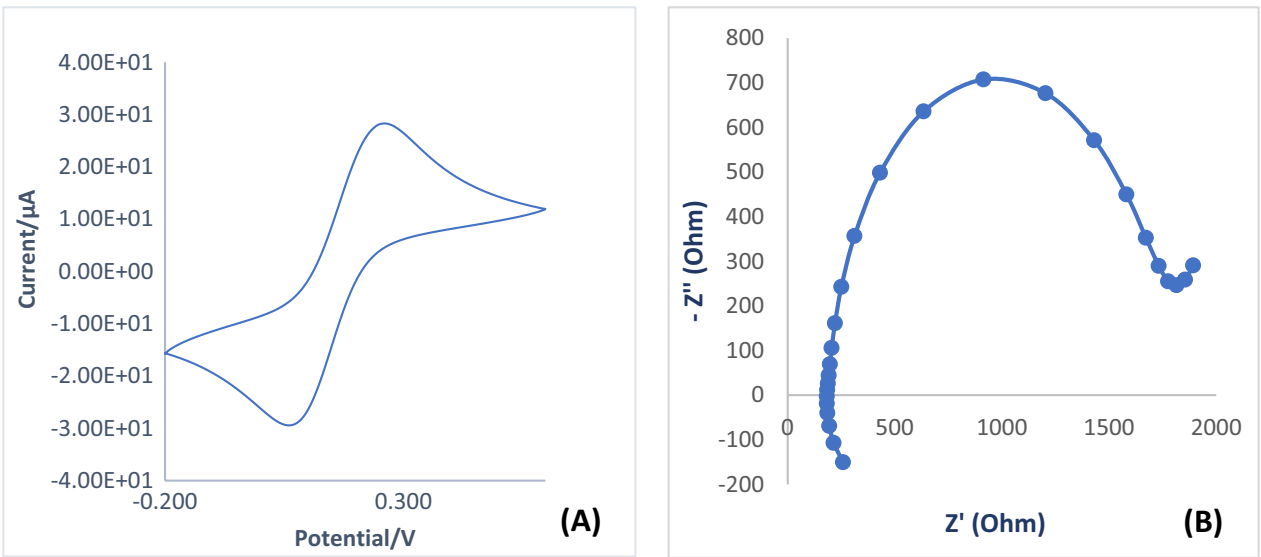

Fig. 2 **A** Cyclic voltammetry (CV) after SPCE pretreatment (bare electrode measurements) The oxidation peak's potential value for this CV scan is 0.259 V ± 0.005. **B** Electrochemical Impedance Spectroscopy (EIS) spectra after SPCE pretreatment (bare electrode measurements).

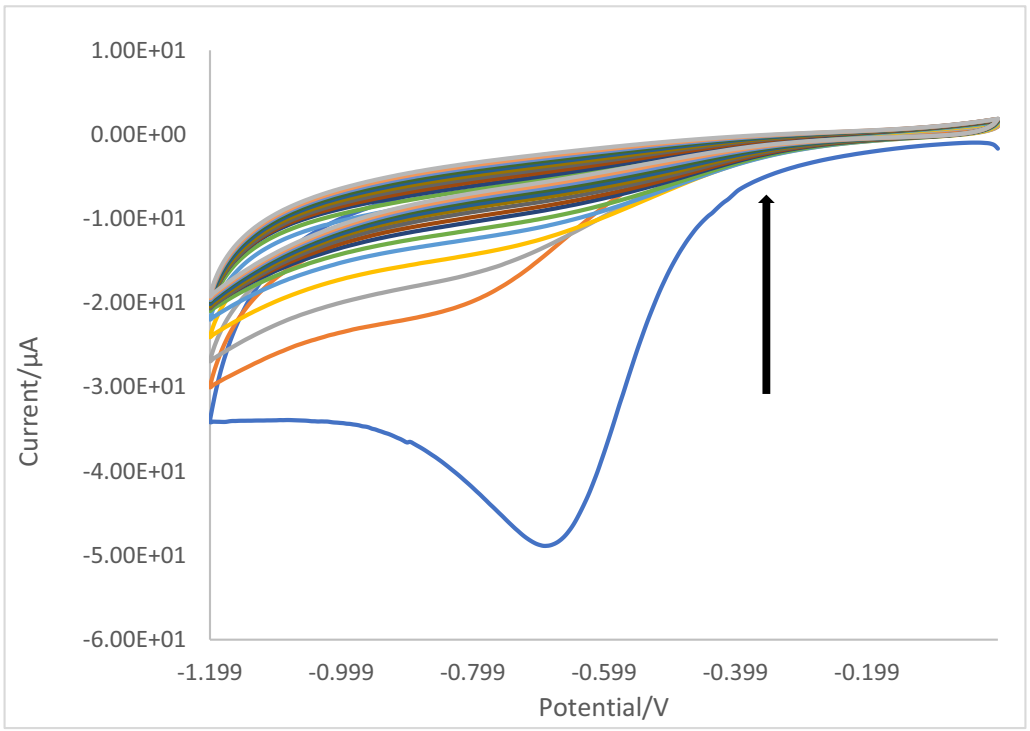

Fig. 3 Scans using cyclic voltammetry (CV) for CMA deposition. This CV scan shows 15 CV cycles, used to deposit diazonium salt on the surface of screen-printed carbon electrodes

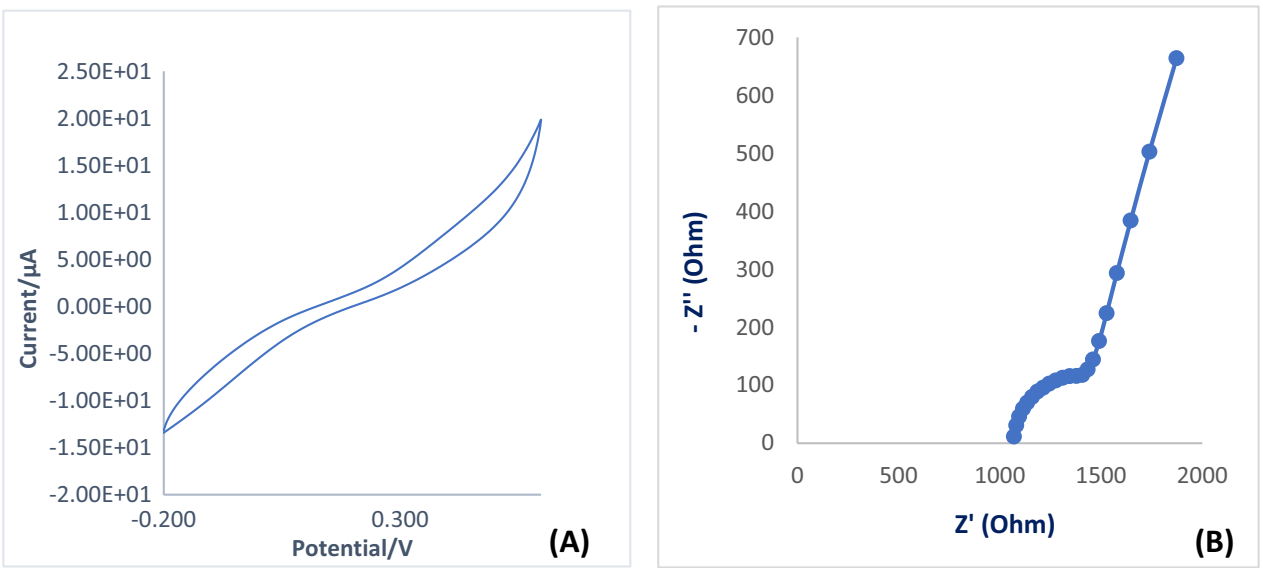

Fig. 4 **A** Cyclic Voltammetry (CV) scan after CMA deposition. This figure represents a CV scan, following the previous diazonium salt deposition. **B** Electrochemical Impedance Spectroscopy (EIS) spectrum after aptamer incubation. This figure represents an EIS spectrum, following the incubation of the CMA-modified electrode with the ciprofloxacin aptamer

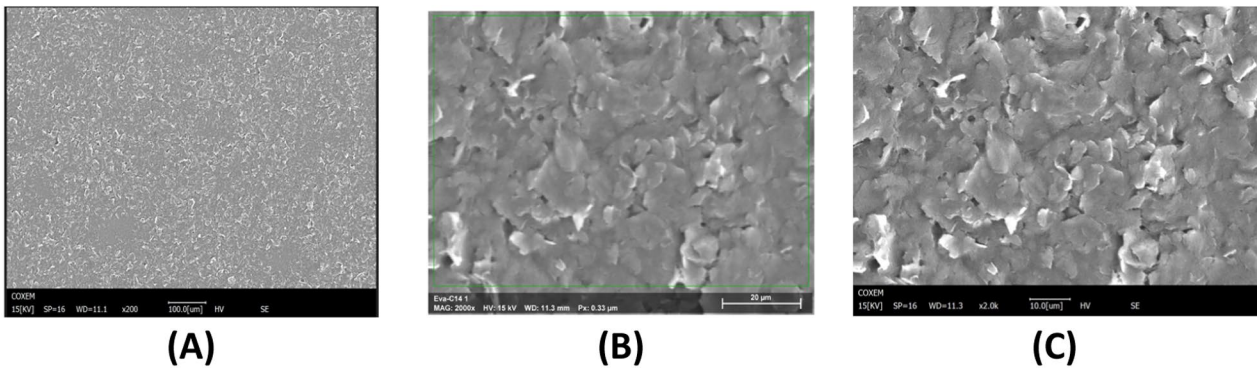

Fig. 5 **A** Scanning Electron Microscope image of bare screen-printed carbon electrode (working electrode) surface. **B** Scanning Electron Microscope image of CMA-modified screen-printed carbon electrode (working electrode) surface. **C** Scanning Electron Microscope image of screen-printed carbon electrode surface, after incubation of the working electrode with the ciprofloxacin aptamer

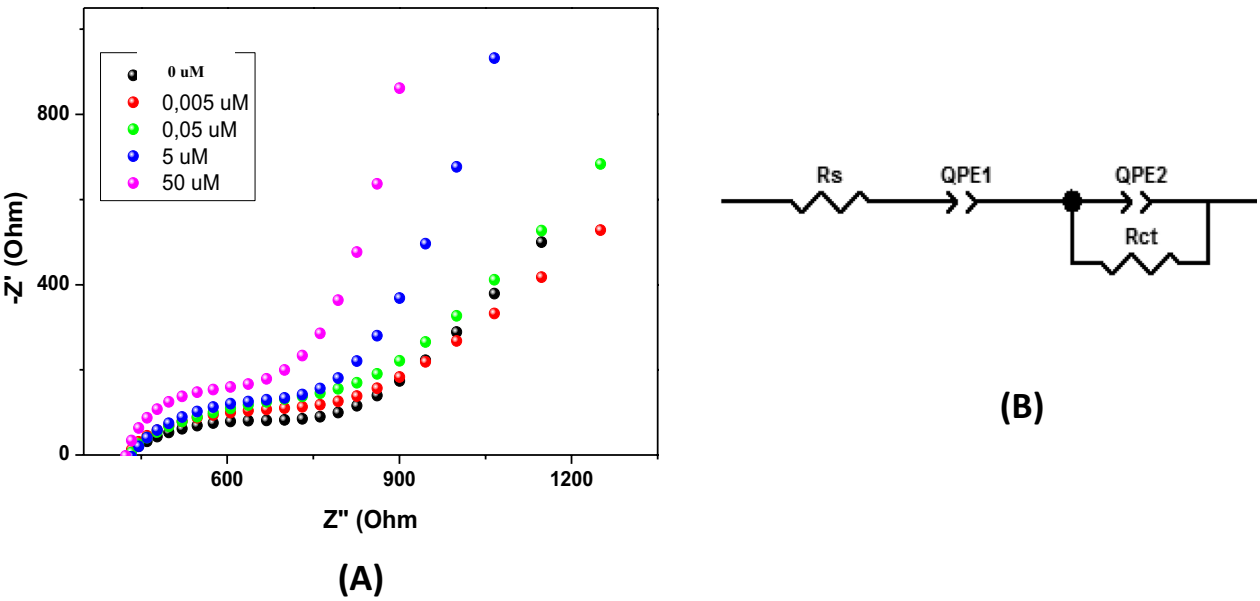

Fig. 6 **A** Nyquist plot showing the EIS response of aptasensor after incubation in different concentrations of ciprofloxacin. **B** Equivalent circuit used for the mathematical fitting of the EIS responses of the aptasensor

and aptamer-incubated electrode, because the order of magnitude of the aptamer is smaller than the range detected by the SEM.

Impedimetric responses to ciprofloxacin

Ciprofloxacin incubation on the CMA/aptamer-activated SPCE is detailed in methods' Sect. "Incubation of ciprofloxacin antibiotic on activated electrode". From the EIS spectra (Fig. 6A), we can see a very remarkable variation in response amplitudes with increasing ciprofloxacin standard concentration, which is an indicator of the relationship between the latter and the impedance of the ciprofloxacin sensor.

The raw spectra were normalized to allow their comparison (Fig. 6). EI data were fitted using Z-View software (software from Scribner Associates) with the equivalent circuit shown in Fig. 6B. This equivalent circuit is applicable in the high frequency range; the Warburg element being omitted.

The first two elements of the circuit arise from polarization of the surface/electrolyte interface (SEI) [31]. The immobilization of ciprofloxacin was evaluated through the resistance element of the second part of the circuit. The pseudo-capacitance can be calculated from the constant phase element QPE2 [32].

Results from the fitting can be found in Table 1.

Table 1 Parameters of the electrical equivalent circuit for the aptasensor in the presence of different concentrations of ciprofloxacin

Cipro conc (µM)	Rs	QPE1	n1	QPE2	n2	Rct	ΔRct
0	500 ± 10	$1.28 \pm 0.02^{E-07}$	0.53 ± 0.01	$6.60 \pm 0.1^{E-09}$	0.64 ± 0.01	253 ± 5	Initial
0.005	430.8 ± 8	$1.22 \pm 0.1^{E-07}$	0.54 ± 0.01	$6.62 \pm 0.1^{E-09}$	0.64 ± 0.01	303.5 ± 6	19.96 ± 0.4
0.05	418.7 ± 8	$5.25 \pm 0.04^{E-08}$	0.50 ± 0.01	$4.04 \pm 0.1^{E-09}$	0.61 ± 0.01	323.5 ± 6	27.86 ± 0.5
0.5	426.6 ± 8	$2.01 \pm 0.04^{E-06}$	0.79 ± 0.02	$5.78 \pm 0.1^{E-09}$	0.63 ± 0.01	378.4 ± 8	49.56 ± 1
50	376.4 ± 8	$2.09 \pm 0.04^{E-06}$	0.93 ± 0.02	$4.09 \pm 0.1^{E-10}$	0.52 ± 0.01	502.3 ± 10	98.54 ± 2

The ΔRct% values shown in Fig. 7 were calculated according to: ΔRct% = ((ΔRct(Cipro) - ΔRct(initial))/ ΔRct(initial))*100

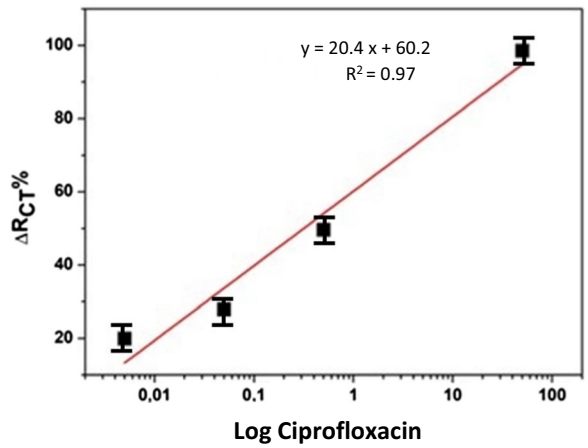

Fig. 7 Calibration curve representing the EIS response of aptasensor, after incubation in different concentrations of ciprofloxacin

The calibration is presented in Fig. 7. A linear relationship is obtained between ΔRct% and log of ciprofloxacin concentration with a satisfying correlation coefficient of 0.97, judged satisfying at these low ranges of concentration. The limit of detection LOD was calculated as LOD = 3σ/S where σ is the standard deviation of three different blanks and S is the slope of the curve (S = 8.84); therefore LOD = 3 nM (1 ng/mL). The dynamic range is from 10 nM to 100 µM.

The reproducibility between two aptasensors is 10%. In total, 10 fully functional aptasensors were retained for their data. Remaining aptasensors were discarded due to errors during electrochemical characterization. This is probably due to manufacturing errors of the screen-printed carbon electrodes since the protocols and conditions were the same for all SPCEs. In order to investigate the specificity of the impedimetric sensor for the ciprofloxacin detection, the effect of the interfering compounds tobramycin, ceftriaxone, ofloxacin and norfloxacin on the aptasensor response was investigated. These compounds belong to the class of antibacterial agents, some of them (ofloxacin and norfloxacin) displaying a similar chemical structure to ciprofloxacin. No significant changes in the impedimetric responses were observed for these antibacterial agents, at a concentration of 10 µM. Moreover, the sensor response for up to 100 µM of ciprofloxacin was tested in the presence of the 1/10 diluted pre-immune serum. Pre-immune serum is the serum extracted prior to immunization, which means that this type of serum acts as a control. It can be purchased through available commercial kits for research purposes. These tests done on it show no change in the impedimetric response, corroborating the high specificity of the serum-based final device and its ability to operate in real blood samples.

Results obtained from graphene electrodes.

This part shows graphs obtained from the same electrochemical process followed for the screen-printed carbon electrodes. It reports the results computed using graphene electrodes (Figs. 8 to 10), which do not require activation of the pseudo-electrode (Fig. 8A).

Except for this step, the protocol followed is the same for both types of electrodes. In the protocol, CMA is deposited on G1 graphene electrodes - just like on SPCE electrodes - through 15 cycles of cyclic voltammetry (Fig. 9). Reported here are the scans and spectra obtained for each step (Figs. 8B, 9, 10), only for comparison purposes with those obtained from the screen-printed carbon electrodes.

Results obtained from electrode preparation and aptamer incubation on screen-printed graphene electrodes show a relatively good response of these electrodes to the protocol.

Figures 8 to 10 highlight the electrochemical changes following each method step applied on the graphene electrode G1. These results can be compared to those obtained for the carbon electrodes (Figs. 2 to 4). They show that graphene electrodes are also capable of withstanding aptamer incubation (Fig. 10B). These results are for comparison purposes only, no quantification or impedimetric analysis was performed for G1. When comparing the ΔE of the graphene electrode to that obtained with the carbon electrode, it is divided by 2, and the peak

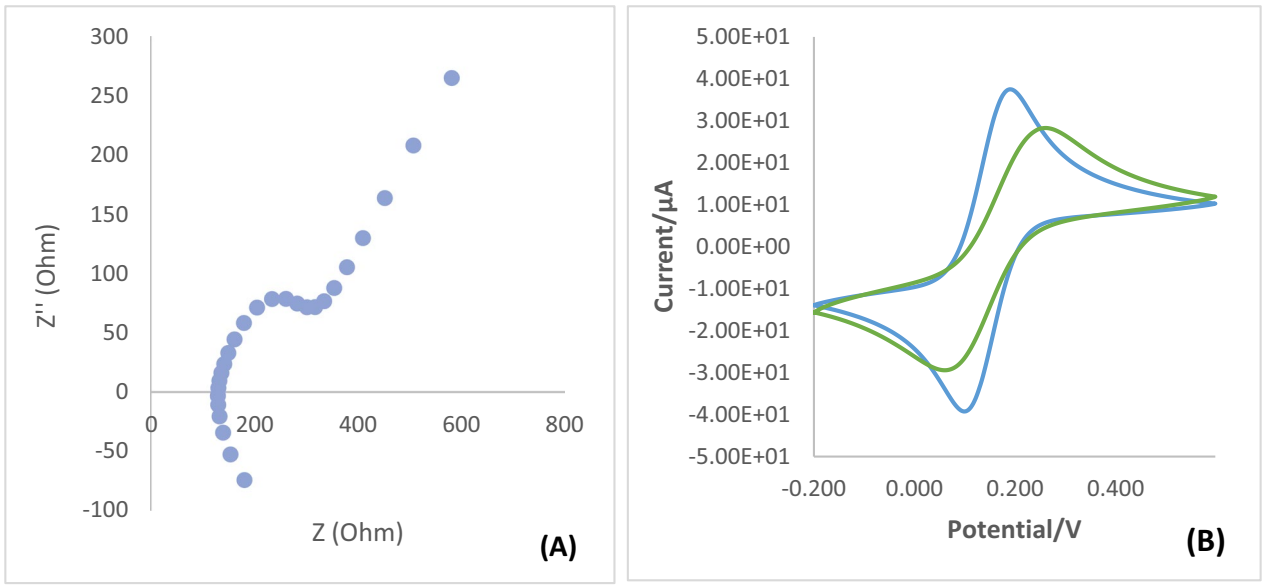

Fig. 8 **A** Graphene electrode (G1) response spectra after EIS application on the bare electrode. **B** Overlaying of the CV scans for both graphene electrode (blue) and carbon electrode (green). Graphene electrode (G1) response scan after CV application on the bare electrode corresponds to the scan in blue color, whereas Cyclic voltammetry (CV) after SPCE pretreatment (bare electrode measurements) is in green color (Fig. 2A)

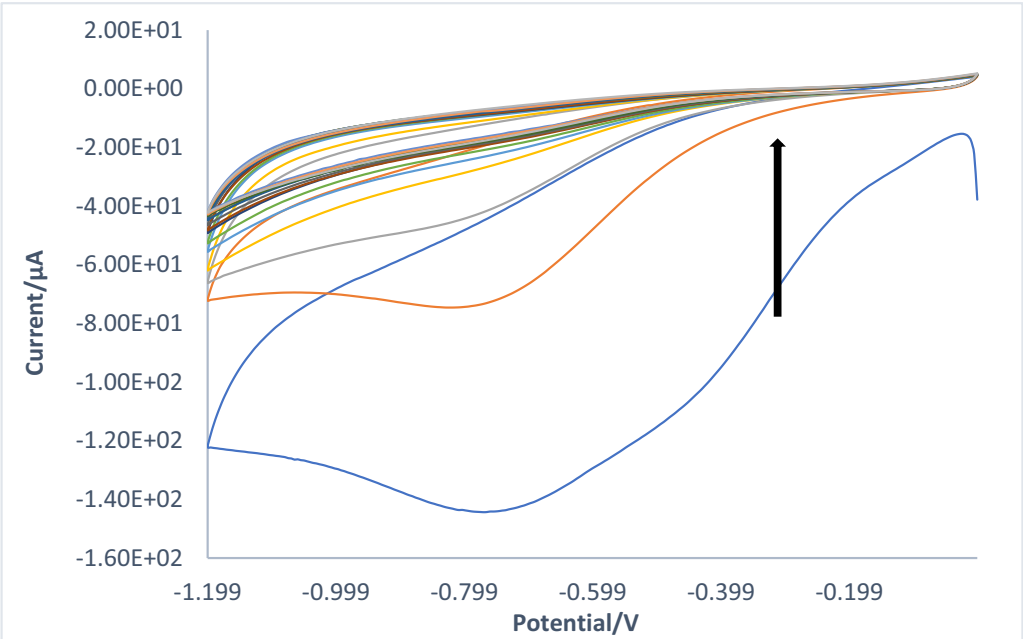

Fig. 9 Graphene electrode (G1) cyclic voltammetry (CV) scan for CMA deposition. This scan shows 15 CV cycles, used to deposit diazonium salt on the surface of graphene electrodes

intensities are increased by 30%, showing a higher electron transfer rate on graphene electrodes (Fig. 8B).

Discussion

Microbiological testing sheds the light on decreased susceptibility and increased resistance of bacteria for ciprofloxacin. Testing of antibiotics, whether for quality control purposes related to medications or environmental

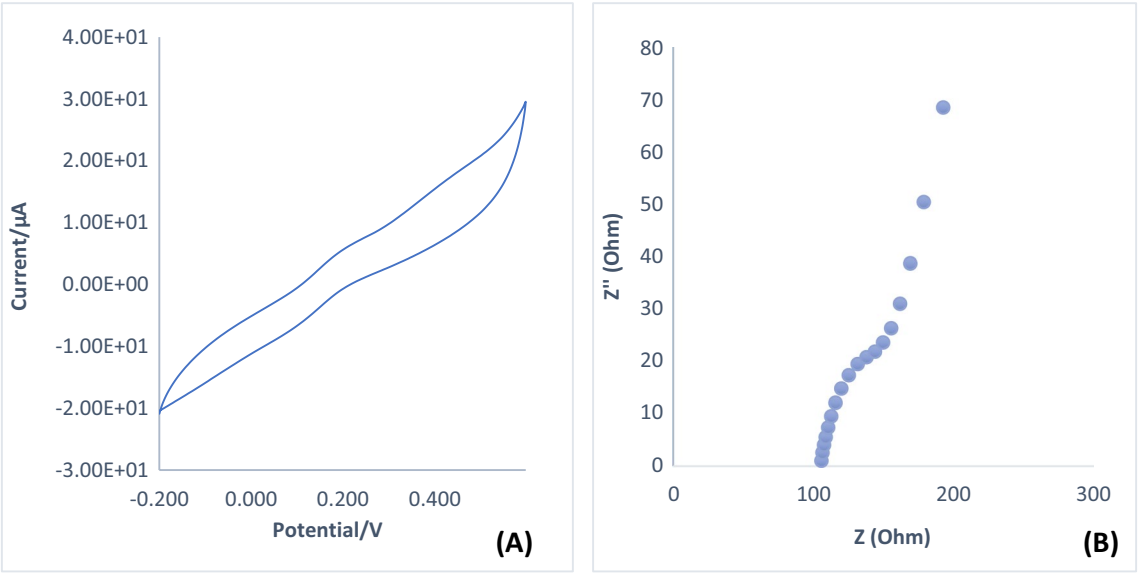

Fig. 10 **A** Graphene electrode (G1) response scan after CV application, following CMA deposition. **B** Graphene electrode (G1) response spectra after EIS application, following incubation with the aptamer

residues from factories or sewage, has several impacts. In addition, offering counterfeit antibiotics or antibiotics that do not meet guidelines and norms has an impact on patient safety and blooming of bacterial resistance to further treatments. Hence it is important to offer time-critical ways to detect the quality of antibiotics.

The current study sheds the light on antibiotics in all their plausible applications. This portable device allows for control on drug substances, control from sewage systems and environmental samples, as well as real-time quantification of antibiotics levels in human fluids. Noting that, albeit this study does not offer validation nor proof to the biosensor's applicability to the mentioned various scenarios; these practical applications will be conducted in our future work.

This technique offers less time-consuming analyses, less solvents used (better environmental impact), and less overall expenses. Better sensitivity and specificity of results are also achievable through this method, since less concentrations can be detected as compared to conventional methods. Microgram and nanogram amounts of antibiotics are easily detected through this portable potentiostat technique, as compared to milligram amounts using the well-known chromatography and spectroscopy instruments. Optimization of this technology can further allow the detection of picogram amounts of antibiotics, important for toxicological studies and control checks in the food and beverage industry.

This current work—albeit not providing fundamental originality in the concept since numerous publications are already available on the topic of ciprofloxacin aptasensor—does offer some originality. The originality of this work lies in the protocol used to attach the aptamer on the screen-printed carbon and graphene electrodes. Recording impedimetric responses which vary as a function of the tested ciprofloxacin concentrations, shows that this protocol can be applied for both carbon and graphene materials. Electrochemically applying diazonium salt (CMA) and activating it using EDC/NHS combination provided a synergistic effect on the screen-printed electrodes. Hence immobilizing the aptamer on the SPE, blocking it with ethanolamine and incubating it with the ciprofloxacin molecule. This process yielded a 3 nM detection limit, which is the innovative part of our research. Reaching a nanomolar detection without the use of nanoparticles or nanomaterials to enhance selectivity. Making this process time-efficient and budget-friendly for mass productions. As well as applicable for field transfer since it doesn't contain nanoparticles that can be unstable outside controlled laboratory conditions. Moreover, throughout available literature on ciprofloxacin detection, a scarce number of aptasensor-based studies used graphene electrodes as means to detect and quantify antibiotic molecules.

Results obtained for graphene electrodes (Figs. 8 to 10) can be compared to those obtained for the carbon electrodes (Figs. 2 to 4). This study resulted in computing electrochemical results of graphene electrodes. Upon comparison with the established carbon/graphite screen-printed electrodes, shapes and electrochemical characterization showed that graphene electrodes are also capable of withstanding aptamer incubation. Yet, a main

limitation from these results is that they are for comparison purposes only. Since this study did not quantify nor perform impedimetric analysis for G1.

The use of aptamers as a detection probe leads to a highly sensitive portable device. Compared to other classical approaches such as antibody-analyte system, antibodies sandwich method, enzyme-linked immunosorbent assay (ELISA) and other biological means, aptamers are a less expensive tool and result in repeatable detection rates. The other methods often depend on the provider and can be affected by several factors such as storage conditions, shelf-life and specific dilution or usage solutions.

The following part provides a detailed comparison of the analytical performance of the fabricated ciprofloxacin sensor. Comparing analytical parameters like limit of quantification, limit of detection and linear ranges as reported by recently published sensors.

A study published in 2021 [33], evaluated the detection of ciprofloxacin in milk samples using an aptamer platform of detection. This electroanalysis of antibiotics was performed via reduced graphene oxide and a functionalized nanogold dendrimer. The limit of quantification was 1 nM, and linear range of 1 nM to 1 µM. Their results are close to the ones obtained in our study, also being in the micromolar range. However, they used different voltammetry techniques, such as square wave voltammetry, differential pulse voltammetry and chronoamperometry techniques, more appropriate to the scaffold used.

Our study focused on the use of impedimetry, instead of voltammetry, due to its technical advantage when quantifying biomolecules. Impedimetry can be used for non-electroactive molecules, being classified as a more general technique. Hence, biological molecules that are not electroactive can still be quantified through electrochemistry, by applying impedimetric protocols. Confirmed through the literature, impedimetric biosensors are reported to show superior performance [34] when compared to voltametric immunosensors (DNA-based sensors) which are within limits that are acceptable [35, 36].

Another study in 2022 [37] tested ciprofloxacin detection via a photochemical aptasensor. Under the optimized concentration and incubation time of the aptamer, a photoelectrochemical detection platform with high efficiency and low cost for ciprofloxacin was prepared. Study showed that the trend of photocurrent of PEC sensor changed with the increase of ciprofloxacin concentration. Increase of ciprofloxacin concentration resulted in the rise in photocurrent signal. The corresponding detection range included 1 nM to 1500 nM and 0.3 nM of detection limit (S/N = 3). Compared to our results, this electrochemical method offers lower detection limits, due to the photocurrent property of their aptasensor. Optimization of our method is essential to reach this nanogram level. Since this study [37] concluded that their results are consistent with results of the national standard method for detection of ciprofloxacin in food samples.

A ciprofloxacin assay deemed ultrasensitive, used fluorescently labeled ciprofloxacin aptamer and carbon nanotubes in the form of nanocomposite [38] to detect the antibiotic in food samples. When optimized, the limit of detection is 0.63 ng/mL with a linear range of 0.63 to 80 ng/mL. Their study also proved a nanomolar range of detection using an aptasensor, not very far from the attempted concentrations (50 nM as the lowest concentration) used in this study. The use of nanoparticles [38] enhanced detection and allowed for lower concentrations.

Another category of sensors commonly used is immunosensors. A piezoelectric immunosensor method was used to detect ciprofloxacin, using magnetic carbon nanocomposites [39]. The limit of detection obtained was 2 ng/mL (6 nM), whereby the linear range of concentrations was set at 5 to 400 ng/mL.

An impedimetric immunosensor [40] is reported in the literature, used to detect ciprofloxacin in wastewater environmental samples. An anti-ciprofloxacin antibody was used and immobilized on a printed carbon electrode. The observed Rct changes resulted in a linear ciprofloxacin concentration range of 10^{-5} (3 pM) to 1.0 µg/mL.

Comparison of the analytical performance of our aptasensor, using a simple portable instrumentation, with data already published in the literature that a nanomolar limit of detection is reached, is essential for the control of food and environmental samples. An overview of the literature also reports that electrochemical sensors using nanocomposites or other additions can increase the sensitivity of their sensors. This can be considered too for further work on the methodology.

However, the advantage of the protocol we provided lies in the fact that it is low-cost and does not require expensive preparations or instrumentations. The materials used can be safely prepared and do not degrade rapidly, allowing safe transport for field analyses. Combined with the fact that we are using SPCE and a portable potentiostat, our protocol offers a diverse range of detection whether in food samples, rivers, lakes, or environmental samples, or in pharmaceutical manufacturing or storing locations.

Conclusion

This study provided a quantification method of ciprofloxacin antibiotic in samples. The method relied on incubating a ciprofloxacin aptamer on screen-printed carbon electrodes. With electrochemical response analyzed via a

portable potentiostat. This portable potentiostat allowed detection at low levels, like reported literature that used a similar ciprofloxacin quantification process, albeit with a regular potentiostat hooked to a laboratory computer. So, this reported protocol bypasses the logistical walls of a laboratory, by adapting antibiotic bioanalysis for in-field applications.

Noting that this approach is novel in terms of the experimental protocol followed to immobilize the aptamer on the electrodes, with diazonium salt being the key to synergistic effect between the different materials. Moreover, the aptamer is immobilized on either a screen-printed carbon electrode or graphene electrode. Then, it binds the target molecule, allowing the detection and quantification of antibiotics in an active pharmaceutical ingredient or in consumable substances. Although results reported in this study offer values reaching nanomolar detection limit, additional work can decrease detection to femtomolar. This will provide better analytical use of the nanotechnological aspect of this biosensor. A point-of-care testing device can monitor patients in a timely basis at hospitals. It can also be taken to pharmaceutical storage facilities or manufacturers, where tests on the pharmaceutical dosage forms are done on-the-spot. This process can facilitate market withdrawal of substandard medications. Thus, saving both time and resources between testing time and effective market withdrawal, causing less concern on the Public Health front.

These current results have opened the door towards perspective studies, pertaining to the application of this same protocol and its optimization on screen-printed graphene electrodes. These types of electrodes can further enhance the selectivity of the aptasensor to ciprofloxacin found in complex matrices, since graphene is an electrochemically favorable material with conductive properties more specific than carbon. A valuable aspect when it comes to environmental or physiological matrices, containing numerous interfering sources.

Abbreviations

DNA	Deoxyribonucleic acid
SPCE	Screen-printed carbon electrode
CMA	Carboxymethylaniline
LOD	Limit of detection
CV	Cyclic voltammetry
G1	Graphene electrode
EIS	Electrochemical impedance spectroscopy
EDC	Ethylcarbodiimide hydrochloride
NHS	N-hydroxysuccinimide

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Author contributions
EH carried out the research, methodology development and drafted the manuscript. JS revised the article for intellectual content and managed the work's resources. SH was responsible for this project's conception. IAMF developed the methodology. NJR revised and edited the manuscript. AE revised the article for intellectual content and managed the work's resources. All authors read, reviewed and approved the final manuscript.

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Availability of data and materials
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Declarations

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Not applicable.

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Competing interests
The authors declare no competing interests.

Author details
[1]Department of Chemistry and Biochemistry, Faculty of Arts and Sciences, Holy Spirit University of Kaslik, P.O. Box 446, Jounieh, Lebanon. [2]School of Medicine and Medical Sciences, Holy Spirit University of Kaslik, P.O. Box 446, Jounieh, Lebanon. [3]Université Claude Bernard Lyon 1, ISA, UMR 5280, CNRS, 5 Rue de la Doua, 69100 Villeurbanne, France. [4]Department of Psychology, College of Humanities, Effat University, 21478 Jeddah, Saudi Arabia. [5]Applied Science Research Center, Applied Science Private University, Amman, Jordan.

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