Improving accuracy and reliability of an electrochemical impedance spectroscopy aptamer-based biosensor

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Abstract

Impedance-based biosensors are effective tools for rapid and specific detection. However, they often rely solely on charge transfer resistance as an indicator of surface structural changes upon analyte binding and other valuable data are constantly ignored. In this work we explore different data obtained for a biosensor designed for water toxin (Saxitoxin) detection using electrochemical impedance spectroscopy. We established a safe potential range for ensuring sensor stability using cyclic voltammetry and determined the potential where the constant phase element values were more pronounced. The data showed that capacitance values could not reliably evaluate the system due to their high deviations. The impedance modulus (Z) revealed the highest number of acceptable frequencies and the values of the imaginary impedance (-Z") exhibited signal differences exceeding 30%, indicating their potential for signal enhancement during saxitoxin detection. The relative standard deviation can be reduced by increasing the concentration of the redox probe. The obtained results highlight the importance of carefully selecting parameters for characterizing the electrochemical behavior of the system to identify the most reliable and informative parameters for detection. This research contributes to broader electrochemistry sensing applications, opening new horizons for improving the accuracy and reliability of electrochemical analysis.

INTRODUCTION

Food safety and access to drinking water are global concerns highlighted by rapid population growth and globalization.\(^1,^2\) The contamination of food and water sources with harmful substances, such as bacteria, viruses, parasites and toxins pose significant social and economic challenges.\(^3,^4\) Saxitoxin (STX) is a natural toxin member of the so-called "paralytic shellfish toxins" (PST), which can reach dangerous levels during large proliferation episodes of certain aquatic species.\(^5,^6\) During those periods, contaminated water bodies used for recreation or drinking water supply represent health risk.\(^7,^8\)

Conventional detection and analytical methods for contaminants rely on labor-intensive techniques, which require qualified personnel, considerable finance support and time, hampering timely decision-making to prevent potential outbreaks.\(^9,^10\) In this sense, biosensors have emerged as a promising alternative for detecting contaminants. Biosensors employ a biological component coupled with a signal transducer to detect target analytes, offering high specificity, sensitivity, and rapid response times compared to traditional laboratory techniques such as mouse bioassays or high-pressure liquid chromatography\(^9–17\)

Aptamers (APTs) are DNA/RNA oligonucleotide sequences with great potential as biological recognition units in biosensor development. APTs do not require living organisms for production, exhibit high affinity and specificity for the target analyte, allowing for large-scale production, and easy functionalization.\(^18–20\) In a previous work\(^21\) we demonstrated the feasibility of an aptamer-based electrochemical impedance spectroscopy (EIS) biosensor for STX detection. Although many articles use EIS to access the charge
transfer resistance (R<sub>ct</sub>) to associate with changes in surface structure (analyte binding, for example)<sup>22,23</sup>, it represents just one parameter among a variety of information that is possible to obtain from such measurements.<sup>24-26</sup> This suggests a limited use of an outstanding technique.

In this work we analyze different parameters obtained from EIS measurements in response to STX using an electrochemical aptameric biosensor. We aim to improve the knowledge of the parameters impacting sensitivity, selectivity, and reliability of this type of biosensor. By testing the redox probe concentration, we could improve the sensitivity and accuracy of the measurements, ensuring reliable data interpretation.<sup>27,28</sup> We also evaluated the suitable potential range to be applied during electrochemical experiments. By defining the appropriate potential window, meaningful data were obtained, avoiding unwanted side reactions or electrode degradation.<sup>29</sup> The EIS response was investigated at various potentials within the established potential window. Finally, impedance data obtained from modified electrodes were evaluated to enhance the signal gain from the impedance measurements in order to improve the sensitivity and accuracy in detecting and characterizing electrochemical processes. By addressing these topics, we contribute to understanding and for the optimization of EIS measurements and data analysis for modified electrodes, which significantly improves biosensor technology development to obtain efficient and reliable detection methods.

**METHODS**

A commercially acquired oligonucleotide sequence (Aptamer - APT) was used, obtained from GenOne company. The structure was functionalized with a thiol group at the 3’ end of the following sequence: 5’-T TGA GGG TCG CAT CCC GTG GAA ACA GGT TCA TTG AAA AAA AAA AA-3’-C6-SH (K<sub>d</sub> = 133 nM in PBS-T), which was described by Zheng et al. as optimized for binding with STX.<sup>30</sup> Trizma®-HCl buffer (pH = 5), ethylenediaminetetraacetic acid (EDTA), 6-Mercapto-1-hexanol (MCH), SXT solution in HCl, potassium hydroxide, phosphate buffered saline solution (PBS, pH = 7.4) and potassium hexacyanoferrate II/III were acquired from Sigma-Aldrich.

For APT, the lyophilized solution was centrifuged (6000 rpm) for 10 minutes to ensure that all the content was available at the bottom of the tube. Then, it was reconstituted in 630µL of a 10 mmol.L<sup>-1</sup> Trizma®-HCl plus 1 mmol.L<sup>-1</sup> EDTA buffer solution (TE buffer), followed by agitation during approximately 5 minutes in a shaker at 200 rpm and stored in the freezer at -20ºC until use. Solutions containing APT, MCH, and STX were prepared by dissolving carefully measured quantities in PBS buffer containing a mixture of 10 mmol.L<sup>-1</sup> of K<sub>4</sub>[Fe(CN)₆] and 10 mmol.L<sup>-1</sup> of K₃[Fe(CN)₆].

The APT/MCH modified gold electrodes were produced at room temperature (25 ºC) by coating the electrode surface with 5.0 µL of a 1.0 µmol.L<sup>-1</sup> APT solution during 1h, followed by a second 1h coating using a 5.0 mmol.L<sup>-1</sup> MCH solution. The APT-STX complex formation on the modified APT/MCH electrodes was done with incubation in a 50 µg.L<sup>-1</sup> STX solution using a 5.0 µL drop for 1h. After each coating step, the surface was rinsed with the buffer solution to remove the non-bonded species.
A three-electrode system was used for the electrochemical characterization with an Ag/AgCl electrode as the reference and a platinum electrode as the counter electrode. The working electrode was made of gold deposited onto silicon with a surface area of 3.8 mm$^2$. The gold electrodes were cleaned prior to use following the methodology described by Serrano et al. (2021)\textsuperscript{[21]}. The EIS measurements were performed at room temperature (25 °C) using a volume of 10.0 mL with 10.0 mmol.L$^{-1}$ $K_4[Fe(CN)_6]$ and 10.0 mmol.L$^{-1}$ $K_3[Fe(CN)_6]$ in PBS (pH = 7.4) using a Metrohm Autolab Potentiostat/Galvanostat with an FRA2 frequency analyzer module. Frequency scan ranged from $10^{-1}$ to $10^5$ Hz with amplitude of 10.0 mV. The open circuit potential (OCP) was 0.22 V and used as the applied potential to the cell. Cyclic voltammetry (CV) measurements were performed in a range corresponding to ± 300 mV with respect to OCP, with a scan rate of 50 mV/s.

RESULTS AND DISCUSSION

Electrochemical measurements address the processes occurring at the electrode surface. It is known that higher electrolyte concentrations reduce the measured resistance\textsuperscript{[28]} Additionally, some studies describe how the theoretical fit of an electrochemical impedance spectrum can be improved by working with higher conductive solutions\textsuperscript{[25, 31, 32]}. So, increasing concentration could lead to better performance by making the system more accurate and more reliable.

The effect of the redox probe concentration on the electrochemical response was evaluated by varying the concentration of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ (1:1 proportion) between 2.5 and 15.0 mmol.L$^{-1}$ using bare gold electrodes, Fig. 1. As the concentration increases, the capacitive behavior becomes less dominant.

The data were fitted using a modified Randles equivalent circuit (inset of Fig. 1), using a constant phase element (CPE), instead of a capacitor to account for a non-ideal capacitance behavior. The impedance of a CPE is given by Eq. 1, where $Q$ is the parameter related to the electrode capacitance, and $\alpha$ is the dimensionless CPE exponent\textsuperscript{[33]}. The Warburg impedance component ($Z_W$), Eq. 2, accounts for the impedance associated with diffusion processes. $Y_0$ is the real part of admittance calculated at a specific angular frequency ($\omega$), when the phase angle ($\theta$) is zero. $Y_0$ describes the degree of diffusion or mass transport occurring in the system, indicating how easily ions or molecules can diffuse at the electrode interface\textsuperscript{[25, 33]}.

\begin{equation}
Z_{CPE} = \frac{1}{Q(j\omega)^{\alpha}}
\end{equation}

\begin{equation}
Y_0 = \frac{1}{Z_W} = (\frac{A}{\sqrt{j\omega}})^{-1}
\end{equation}
As expected, the solution resistance ($R_s$) and charge transfer resistance ($R_{ct}$) decreased inversely with redox probe concentration, while the $\alpha$ and $Y_0$ coefficients varied proportionally with increasing concentration (Fig. 2). All values vary linearly, except $R_{ct}$, which shows an exponential-like decrease, demonstrating a stronger dependence with the redox probe concentration.

The relative standard deviation (RSD) of the fitted parameters was calculated by dividing the standard deviation by the arithmetic mean value considering three distinct measurements on the same electrode (Table 1). For 10.0 mmol.L$^{-1}$ all deviations are already lower than 2.0%, which is below the acceptable limit of 5.0% according to the literature.[34] Therefore, this concentration was chosen as the working concentration for the next analyses. $R_{ct}$ displays the higher RSD values, which suggests that it could not be the best parameter for detection analysis.

![Table 1](image)

The potential window determines the limiting potential values to avoid undesired reactions, like electrode corrosion. CV was performed to evaluate the stability of the gold electrodes. Figure 3 shows the electrochemical response of the gold electrode in PBS solution and in the presence of $[\text{Fe(CN)}_6]^{3-/4-}$ redox couple (10.0 mmol.L$^{-1}$). Initially, for the PBS without the redox couple, the scan started at 0.22 V towards positive bias (oxidation) until 1.0 V, then reversed towards negative bias (reduction) until −0.6 V and back to 0.22 V to complete the cycle. An anodic peak starts at approximately 0.7 V, which indicates the onset of gold oxidation, followed by hydrogen evolution and consequent electrode degradation close to 1.0 V. When the scan changes direction to negative bias, an undesired cathodic peak of peroxide formation is also observed at around −0.25 V.[35, 36]

By repeating the measurement for a smaller potential window, between −0.1 V and 0.52 V (blue curve), no appreciable current peaks were observed, which indicates a safe potential window. With the addition of the $[\text{Fe(CN)}_6]^{3-/4-}$ redox couple the measurements were performed even for a smaller range, between −
0.1 V and 0.52 V. Significant current peaks at 0.276 V and 0.169 V were observed, corresponding to the oxidation of \([	ext{Fe(CN)}_6]^{3-}\) and reduction of \([	ext{Fe(CN)}_6]^{4-}\), respectively \(^{37,38}\).

EIS was performed at various positive potentials within the previously established safe potential window to evaluate the impact on the gain and reproducibility. Nyquist plots of Fig. 4 illustrate the impedance response for an APT/MCH modified electrode.

The values obtained from the equivalent circuit fit for each potential are presented in Fig. 5 for the electrode modified with APT/MCH (orange columns) and APT/MCH/STX (blue columns). \(R_{ct}\) is maximized around the OCP value (0.22 V) while CPE showed to be more pronounced at 0.07 V. The smallest value for Warburg impedance was obtained at the OCP.

The calculated RSD (for three distinct electrodes) after the APT/MCH electrode modification remained within the range of 5.0% for all \(\alpha\) values. For \(R_{ct}\), this range was obtained only for 0.22 V and 0.52 V, and 0.22 V only for \(Y_0\). After the additional modification with STX (APT/MCH/STX), less dispersive values were obtained for \(R_{ct}\), where only the −0.08 V potential resulted in RSD higher than 5.0%. \(\alpha\) and \(Y_0\) followed the same trend as prior to STX incorporation. Figures S1 and S2 in the supporting information highlight the RSD behavior.

Figure 6 shows the calculated signal gain \(\Delta S\% = \left| \left( S - S_0 \right) / S_0 \right| \times 100\), where \(S_0\) and \(S\) represent the values before and after the modification with STX, respectively. It is worth noting that \(\alpha\) shows a minimum change and large deviations, therefore it is not a suitable parameter to discriminate STX incorporation at any applied voltage. \(R_{ct}\) presented high gain (14.5%) and RSD below 5.0% for 0.22 V; for 0.07 V the gain is reasonable (13.5%) but unfortunately the RSD is close to 20%. Only one applied potential of -0.08 V for \(Y_0\) displayed acceptable RSD (1.0%) with incredible 40.5% signal gain. It suggests that \(Y_0\) at this specific potential may provide valuable information for STX detection.

IES data are often represented using Nyquist plots, where the real (\(Z'\)) and imaginary (-\(Z''\)) part of impedance are used. \(Z'\) provides information about the resistance component of the system and indicates how easily current can flow through it. -\(Z''\) reflects the capacitive component of the system and indicates the ability to store and release charge carriers. The modulus of \(Z\) is a measure of the overall impedance magnitude of the system. Another parameter of interest is the phase angle (\(\Phi\)), which is the phase difference between the applied voltage and the resulting current in the system. \(^{24,25}\) These four parameters, \(Z'\), -\(Z''\), \(Z\) and \(\Phi\) were analyzed as a function of the frequency for the five different potentials to assess the overall system response.

Figure 7 shows the selected data for RSD values less than 5.0%, all the others were omitted, where a total of 28 Voltage-Frequency combinations were selected for the investigated parameters. Eleven of those combinations (with gain around 18%) were obtained for the OCP potential of 0.22 V and frequencies in the range of 100–25 Hz. Within RSD values above 5.0%, most of the combinations with these parameters could be used to detect STX with a \(\Delta S\) higher than the obtained for \(R_{ct}\) at the OCP potential. It is worth
noting that 12 of the total 28 acceptable combinations arise from the impedance modulus $Z$. Figure S3 of the supporting information shows $\Delta S$ values of all the parameter combinations.

These analyses show the importance of carefully selecting the parameters for characterizing the electrochemical behavior of the system and the need of further investigation to identify the most reliable and informative parameters for STX detection.

**CONCLUSIONS**

A safe range of working potential, which guarantees the stability of the electrochemical system here studied, was found to be between −0.1 and +0.6 volts when compared with the Ag/AgCl reference electrode. Increasing the concentration of the redox probe improved the performance of the biosensor by diminishing relative standard deviations, mainly in respect to charge transfer resistance, which exhibited higher variability during the redox probe concentration study. It suggests that it may not be the best parameter for differentiating the signal depending on the probe concentration used. For this work, a 10.0 mmol.L$^{-1}$ concentration was found to be a good choice.

Comparison between the impedance spectra obtained before and after STX complexation revealed significant variations in the circuit elements and impedance parameters. Both the signal gain and relative deviation from the data were dependent on the potential applied during the measurement. This indicates that the data reading mode can be modulated to favor either the signal or the deviation.

The results indicated that capacitance is not a good parameter to evaluate STX complexation because of its negligible signal gain and high deviations. For conventional use of $R_{ct}$ at OCP, a signal gain of 14.5% was obtained. But an amazing 40.5% signal gain was found for $Y_0$ at −0.08V. We demonstrated that $Z'$, $Z''$, and $Z$ under specific Frequency-Voltage combinations allow optimization of sensing analysis.

By increasing the signal gain and reducing deviations between measurements, biosensors can be improved in accuracy and reliability. This work demonstrates the importance of exploring the different parameters available from impedance analysis to optimize biosensors analyses. It is important to highlight the possibility of faster analysis using single frequency measurements after identification of the parameters with suitable Voltage-Frequency combinations. Finally, this work contributes to a broader field of electrochemistry, opening new horizons for facilitating analysis and improving the accuracy and reliability of electrochemical sensors.

**DECLARATIONS**

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**Supplementary Figures**

Supplementary Figures S1 to S3 are not available with this version.

**Figures**
Figure 1

Electrochemical impedance spectra obtained for a bare gold electrode immersed in solutions containing different redox probe concentrations, [Fe(CN)$_6$]$^{4-/3-}$ (1:1 proportion). Inset, the equivalent circuit used for the fitting.
Figure 2

Data obtained from equivalent circuit fitting of Figure 1.
Figure 3

Cyclic voltammetry of bare gold electrode in a PBS solution only (red and blue) and PBS containing 10.0 mmol.L\(^{-1}\) of the [Fe(CN)\(\text{6}\)]\(^{3-/4-}\) redox couple (orange).
Figure 4

Nyquist plots from APT/MCH modified gold electrode for different applied potentials. Lines represents theoretical fit.
Figure 5

Values of $\alpha$, $R_{ct}$, and $Y_0$ obtained from the EIS curves fitting for APT/MCH (orange columns) and APT/MCH/STX (blue columns) electrode modifications. All standard deviations were calculated using three distinct electrodes.
Figure 6

Signal gain of the investigated parameters.
Figure 7

Signal gains of \( Z' \), \( Z'' \), \( Z \) and \( \Phi \) as a function of the frequency and different applied potentials. Only data with RSD values lower than 5.0 % are shown.