

Detection of Pathogenic *E. Coli* by Electrochemical Biosensors Based on Aptamers Designed by Bioinformatic Tools

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In this work, we present the in-silico design of a new aptamer (named Apt917) capable of interacting with *E. coli* and its evaluation as a recognition element in electrochemical biosensors. A search and bioinformatic analysis of various aptamers reported in the literature was carried out. Parameters such as low dissociation constant and short sequences were considered to design and model new hybrid sequences. The designed Apt917 has a minimum free energy and high percent to frequency, making it promising compared to the initial ones. Apt917 was synthesized and immobilized on gold nanoparticles-modified screen-printed electrodes. The evaluation of the obtained biosensor shows a promising detection of *E. coli* O157:H7 with limits of detection and quantification of 8 and 27 CFU/mL, respectively.

1. Introduction

Escherichia coli (*E. coli*) is one of the most widespread pathogenic bacteria in nature and it has been recognized that some special serotypes of this pathogen can produce enterotoxins, which cause abdominal pain, diarrhea, inflammation, ulcers, and even severe cases such as hemorrhagic enteritis (Zhao et al., 2018). Therefore, the quantitative detection of *E. coli* has always been an essential task in the environment, medicine, pharmaceutical industry, and food safety. Currently, there are several detection methods for *E. coli*, such as culture counts, immunological detection, and those based on molecular biology (Palomino-Camargo & González-Muñoz, 2014). However, these methods have drawbacks ranging from long times for the confirmation and identification of the type of microorganism, high costs or complexity in the tests (Zhao et al., 2018). Considering the above, there is a demand for fast, cost-effective, and sensitive methods to detect and identify bacteria or their components. In this sense, electrochemical biosensors have recently been considered attractive options to existing methods to detect pathogens (Albanese et al., 2019). These biosensors show excellent characteristics, including simplicity, specificity, low detection limit, simple operation, cheap, provide real-time measurement, portability, miniaturization, and rapid detection (Razmi et al., 2020). Electrochemical biosensors are defined as analytical devices that use biological/biochemical phenomena for the detection of target analytes, based on interaction with a bioelement attached to a transducer. This transducer converts the biochemical interaction into a quantifiable electrical signal (Justino et al., 2015).

In recent years, electrochemical biosensors with different types of transducers have been widely applied for the detection of pathogenic bacteria. Gold nanoparticles and carbon nanostructures are widely used in electrochemical transducers systems due to their excellent stability and electron transport properties (Shoai et al., 2018). On the other hand, it is crucial to select recognition elements with a high affinity for bacterial components or whole cells to ensure that detectable signals are generated, even at very low concentrations of the microorganism (Hoyos-Nogués et al., 2018). The development of new bioreceptors including aptamers offers higher specificity, which is a key advantage for detecting bacteria in complex matrices (Hianik, 2018). Aptamers are single short nucleic acids (NAs) sequences capable of binding specific molecules with high

affinity by folding into specific tertiary structures (Kinghorn et al., 2017). These molecules are commonly isolated by systematic evolution of ligands by exponential enrichment (SELEX), an evolutionary process in which successive rounds of selection and amplification are used to enrich a library of aptamers and create high-affinity aptamers (Wang et al., 2019). However, the selection of aptamers by this methodology is time-consuming, complex, and expensive. Hence, bioinformatics tools are being used to improve the affinity quality of these elements (Kurup et al., 2021).

A particular application to improve the efficiency of aptamer selections by computationally (in silico) methods is solving the three-dimensional structures of these NAs and their targets, and simulating the physical forces involved in the coupling to a target. Computational prediction of NAs secondary and tertiary structures and targets reveals the steric and energetic properties of each structure. These predictions allow modifying their selection groups to have a broader range of three-dimensional structures with more favorable free energy and provide essential information for molecular docking simulations. These simulations allow studying aptamer-target non-covalent interactions, including ionic interactions, hydrogen bonds, van der Waals forces, hydrophobic interactions, base stacking interactions, and shape complementarity.

In silico technologies allow to obtain aptamers in a short time and reduce the costs involved in the development through SELEX. For this reason, in this work we report the design and evaluation of a new aptamer for the detection of *E. coli* in an electrochemical biosensor. Various NAs sequences reported in the literature were used, and their three-dimensional structures were modeled. The new designed aptamer (named Apt917) was synthesized and immobilized on gold nanoparticles-modified screen-printed electrodes and their sensitivity and specificity towards *E. coli* O157:H7 were evaluated.

2. Experimental section

2.1 Bioinformatic design of aptamers

It was performed a search of scientific articles whose main objective was the detection of *Escherichia coli* by means of aptamer-based biosensors or the development of aptamers for the detection of *E. coli*. This bibliographic review was carried out in two databases: Scopus and Scielo (Scientific Electronic Library Online). The keywords used for the search were: aptamers, SELEX, biomarker, detection of *Escherichia coli*. The limit of detection (LOD), the dissociation constant (Kd), the strain of *E. coli*, the type of extracellular or intracellular target and type of measure used were considered. All the information was organized in a table to compare the results obtained from each of the articles reviewed. Aptamer sequences were used to obtain an alignment using the Clustal W algorithm and then distance trees were constructed to show common characteristics in the aptamer sequences. On the other hand, the sequences were analyzed using the free access program RNAfold Web server (v. 2.4.17) to identify the stability of the aptamers through the calculation of the free energy of each of the structures in two dimensions (2D) self-complementarity structure. The approach to the three-dimensional structure was developed using the free access program RNA composer (v. 1.0) (Antczak et al., 2017). Based on the sequences with the lowest dissociation constant and greater stability, a hybrid aptamer was designed considering the loops regions and performing base correction to increase stability.

2.2 Preparation and characterization of the biosensors

The biosensors were prepared on carbon-based screen-printed electrodes (SPEs, Italsens). The working electrodes of the SPEs were first modified by electrodeposition of gold nanoparticles (AuNPs) using chronoamperometry. For this, 100 μL of an aqueous solution of 1.0 mM of $\text{HAuCl}_4 \cdot \text{H}_2\text{O}$ ($\geq 99\%$, Sigma-Aldrich) in 0.5 M of H_2SO_4 were placed on the SPEs. Chronoamperometry was performed at -0.05 V vs. Ag/AgCl for 100 s. The AuNPs-modified SPEs were rinsed with Type-I water (Jose Luis Roperro-Vega et al., 2021). The aptamer designed in the present work was synthesized with a thiol group at the 5' end (Macrogen, South Korea). Immobilization of the aptamer on the SPEs was performed by self-assembly on the AuNPs surface via Au-S bond formation. Briefly, 20 μL of a 20-100 nM aqueous solution of the aptamer were placed on SPEs and incubated overnight (16 h). The biosensors obtained were rinsed with Type-I water. The effect of the concentration of the aptamer in the solution to be immobilized toward *E. coli* detection was evaluated. Changes in the electrochemical response of SPEs during their modification were evaluated by electrochemical impedance spectroscopy (EIS) at a scan frequency between 50,000 to 1 Hz, the amplitude of 10 mV RMS and 0 V vs. OCP (DC) by using an electrolytic solution of $\text{Fe}[(\text{CN})_6]^{3-/4-}$ couple (5 mM) and KCl (0.1 M). Surface characteristics of AuNPs-modified SPEs were studied by scanning electron microscopy (SEM) using a Quanta Field Emission Gun (Model 650) microscope operated at 15.0 kV. The images were obtained in secondary electron mode.

2.3 Evaluation of the biosensors on the detection of *Escherichia coli* O157:H7

E. coli O157:H7 (EC, ATCC 43895) was kept under cryopreservation at $-80\text{ }^{\circ}\text{C}$ in Luria Bertani broth (LBB, Merck) with 15% of glycerol. For the reactivation of the microorganism, 50 μL of cryopreserved material was added in 5 mL of LBB and incubated at $35\pm 2\text{ }^{\circ}\text{C}$ from 18 to 24 h before each assay, adjusting the concentration at $1\times 10^8\text{ CFU/mL}$ in 0.9 % w/v of NaCl. Then, 7 mL of NaCl (0.9 % w/v) with known concentrations of EC (0 to 1000 CFU/mL) were placed in an appropriate beaker, and the biosensor was immersed. The system was incubated at $25\text{ }^{\circ}\text{C}$ for 30 min and with constant stirring of 150 rpm. After that, the biosensor was rinsed with Type-I water prior to electrochemical measurements. The detection blank was 0.9 % w/v of NaCl solution without bacteria (0 CFU/mL). EIS measurements were performed using the same conditions used for the characterization of the biosensor. *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were used as model microorganisms to evaluate the selectivity of the biosensor. Electrochemical measurements and evaluation of the biosensor were performed on a potentiostat/galvanostat VersaSTAT 3 (Princeton Applied Research) controlled by Versastudio (v. 2.60.6.) software. The interaction of EC with the surface of WE limits the electron-charge transfer between the redox probe and the transducer. This is reflected in an increase in the impedance of the electrode. In the case of a Nyquist diagram, it will be reflected in an increase in the diameter of the semicircle, as long as the electrochemical system behaves in accordance with the Randles circuit. In fact, from this circuit it is possible to calculate the resistance to charge transfer (R_{ct}) on the electrode surface, whose values change proportionally with changes in the concentration of the microorganism. Thus, a normalized resistance change is calculated using equation 1 and whose numerical values are expected to be directly proportional to the concentration of the bacteria.

$$\Delta R_{Normalized} = \frac{R_{Apt917+EC} - R_{Apt}}{R_{Apt917}} \quad (1)$$

Where R_{Apt917} is the R_{ct} of the biosensor while $R_{Apt917+EC}$ is the R_{ct} of the biosensor in the presence of *E. coli* at a given concentration.

3. Results and discussions

3.1 Bioinformatic design of aptamers

Data from 19 articles were organized to consider the dissociation constant (K_d), limit of detection (LOD), type of biosensor, and signal measured. These results are published in Mendeley Data repository (J.L. Roperov-Vega et al., 2022). Aptamer sequences were between 32 and 88 nucleotides, one of them was reported as RNA sequence (Ec3), and seven recognized whole cell of *E. coli* O157:H7 strain. Alignment using whole aptamers sequences did not show a distribution pattern associated with *E. coli* strain, K_d , LOD, or molecular target. Despite the above, the S1 aptamer family (Table 1) was selected as a model for creating hybrid aptamer derivatives because they recognize the whole *E. coli* O157:H7 cells, have low K_d , a short length, and a high frequency of the structure with MFE.

Table 1: Main parameters of some aptamer that recognize *E. coli* O157:H7 cells and hybrid aptamer.

Aptamer	Sequence 5' – 3' Dot-Bracket representation	nt	ΔG° [Kcal/mol]	F** (%)	K_d nM
S1	TGGTCGTGGTGAGGTGCGTGT TGGGTGGT TGGATGAGTGTGTGGC*(((.....))).....	45	-3.51	22.75	10.33
S2	GCGGGAATAGGATGCGGCT TGGAAGGA GAGGTGTTGGTGGGTGGTG (((.....(((.....))).....)).....	45	-5.28	23.85	NR
S3	GTGCGGTGACG TGAGGGGG AGAGGCGTTGGTGTAGGCTGTTGGTG(((.....))).....	45	-8.86	55.93	NR
Apt517***	TTTTTACG TGGTGGT GCGGCT TGGAAGGAGAG GTTGA(((.....))).....	37	-3.98	17.35	---
Apt917***	TTTTTACG TGAGGGGG AGAGGCGTGC TGGAAGGAG GAGTTGA(((.....))).....	45	-5.40	61.95	---

*Bold letters indicate external loop nucleotides.

**Frequency of minimal free energy structure.

***This work.

Previous studies (Jayasena, 1999) have reported that larger aptamers are less selective and specific at the moment of detecting a target molecule. For this reason, hybrid aptamers were created without exceeding the number of nucleotides from S1. On the other hand, since the publication of SELEX technique (Tuerk & Gold, 1990) the structure of loop conformations has been gained attention due to its relation with the potential sites

to interact with a specific target. The selection of specific loops has been described to be helpful to detect whole cell of *E. coli* (Dua et al., 2016), and the presence of two external loops in the same aptamer could help to the target recognition as I-1 aptamer to *E. coli* O157:H7 (Lee et al., 2012).

Different combinations of two sequences from the external loop from S1 family were used to build hybrid aptamers. Also, five thymines were added at the 5' end, which provides flexibility to the aptamers and could improve the density of them on the surface of the electrode (Balamurugan et al., 2008). Finally, a thymine-guanine-adenine triplet was added at the 3' end to ensure base pairing and to improve the frequency of the MFE. With these considerations, two representative sequences were obtained and named Apt517 and Apt917 (Table 1). Spatial 2D conformation of Apt517 and Apt917 were elucidated using an RNA approximation. Unlike S1 family, the new hybrids have two external loops. However, the minor loop conformation of Apt517 does not have the same free nucleotides as S1 model. This change was observed too when the external loop from the S2 aptamer was used in Apt917. Only the external loop from S3 aptamer remains in its structure in the new hybrid aptamer (Figure 1). Finally, Aptamer Apt917 was chosen to attach to working electrode of the biosensor due to its minimum free energy (MFE) and high percent to the frequency of MFE.

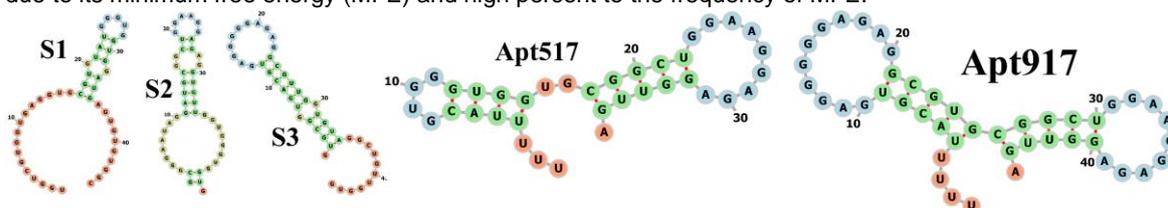


Figure 1: RNA 2D representation of family aptamer S1 and hybrid aptamer designed in this work (Apt517 and Apt917). Blue nucleotides represent external loops, green complementary and orange nucleotides with random disposition.

3.2 Electrochemical and structural characterization of the biosensors

The surface of the working electrode of SPEs was modified by electrodeposition of gold nanoparticles (AuNPs) by chronoamperometry, and the results are shown in Figure 2a. This method leads to obtaining well-distributed nanoparticles on the surface with sizes around 60-90 nm (Figure 2a, insert). Figures 2b-d show the effect that each of the modifications made has on the impedance of the electrode represented in the Nyquist diagrams. As expected, it is clearly observed that the electrode impedance decreases considerably when the AuNPs are deposited (red curves) compared to the signal from the new electrode (black curves). This is evidenced by the decrease in the diameter of the semicircle.

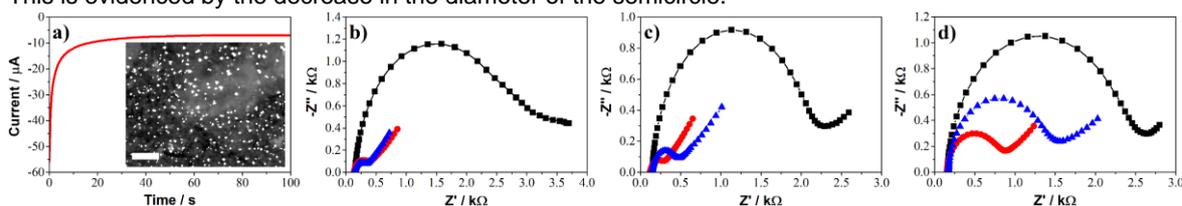


Figure 2: Current vs time response (a) in the chronoamperometric electrodeposition of AuNPs. Insert: SEM image of the AuNPs-modified surface of SPEs (scale bar 1 μm). EIS response of the biosensor by using 20 nM (b), 50 nM (c) and 100 nM (d) of aqueous solutions of Apt917. Curves black, red and blue correspond to SPE, SPE/AuNPs and SPE/AuNPs/Apt917, respectively.

For its part, the use of a concentration of aptamer of 20 nM does not clearly modify the impedance of the electrode (blue curve in Figure 2b). This can be attributed to the fact that this concentration is not sufficient to immobilize a considerable amount of the aptamer. On the other hand, the impedance of the electrode is increased when the concentrations of 50 and 100 nM are used (blue curves in Figure 2c-d), being notably higher at 100 nM. This effect is attributed to the immobilization of the aptamer on the gold nanoparticles.

3.3 Evaluation of the biosensors on the detection of *Escherichia coli* O157:H7

The prepared biosensors were evaluated in the detection of *E. coli* O157:H7 at concentrations between 0 and 1000 CFU/mL, and the results are shown in Figure 3. The biosensor prepared with a 20 nM solution of the aptamer does not show changes in the electrochemical impedance signal when exposed to different concentrations of the microorganism (Figure 3a). This is attributed to the possible low or null amount of the

aptamer on the surface of the electrode, which translates into a null sensitive response of the biosensor towards *E. coli*. Finally, this is evidenced by the fact that there is no correlation between the concentration of the microorganism and the quantified electrochemical signal (Figure 3b).

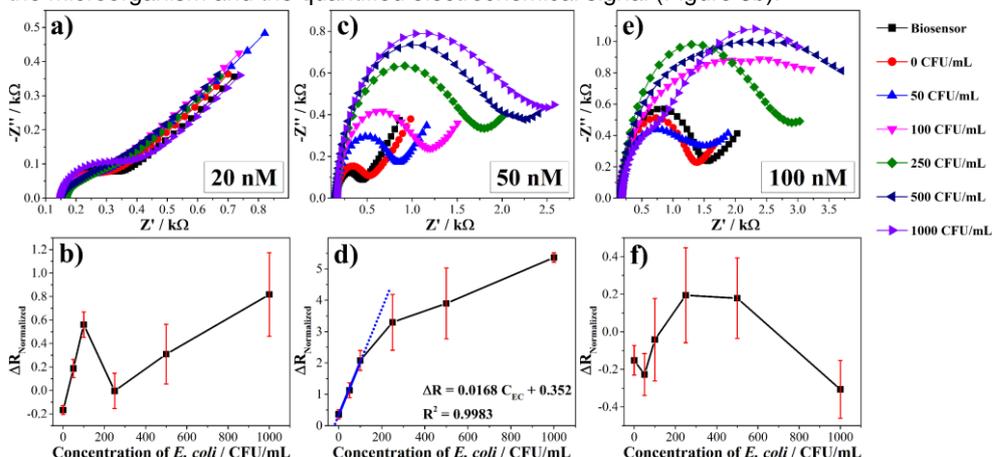


Figure 3: Results of the evaluation of the Apt917-based biosensor. The effect of the concentration of aptamer was evaluated. The linearization of response of each biosensor is presented on bottom.

The biosensor prepared with 50 nM of aptamer presented an adequate response since the electrochemical impedance signal increases as the concentration of *E. coli* increases (Figure 3c). It is important to mention that at low concentrations of *E. coli*, the biosensor presents an apparent linear response, as shown in the Figure 3d. Finally, the immobilization of aptamer is ensured when concentrations of 100 nM are used, but this results in a saturation of the surface of the electrode when interacting with the bacteria. This is reflected by the formation of two semicircles at concentrations above 100 CFU/mL (Figure 3d). Finally, this results that the electrode does not present a linear response between the measured signal and the concentration of *E. coli* in the matrix (Figure 3e).

The estimated values of the limits of detection and quantification calculated for the biosensor prepared with 50 nM of Apt917 are 8 and 27 CFU/mL, respectively. However, it is necessary to evaluate lower concentrations of the microorganism to carry out an adequate validation of the system.

Preliminary tests were carried out to evaluate the specificity of the biosensor prepared with 50 nM of Apt917 towards *E. coli* in the presence of other model microorganisms that may be present in water, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Concentrations of 50 and 100 CFU/mL of each microorganism were evaluated, and the results are presented in Figure 4.

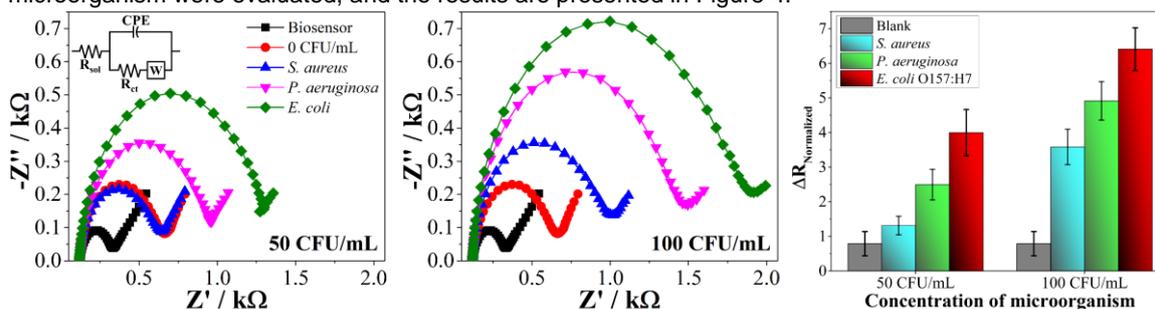


Figure 4: Results of the evaluation of the selectivity of biosensor towards *E. coli*.

The difference in the electrochemical response of the biosensor ($\Delta R_{\text{Normalized}}$) between the blank and *S. aureus* is not significant at a concentration of microorganisms of 50 CFU/mL. Considering that this microorganism is gram-positive, this indicates that the biosensor could differentiate this type of bacteria at low concentrations. In the case of *P. aeruginosa*, there is a significant difference with the *E. coli* response, but the biosensor is still capable of detecting the former, possibly related to this group of gram-negative bacteria. This is possible also considering that the aptamer has been designed to detect the whole *E. coli*, and not towards specific targets on the cell membrane.

4. Conclusions

An aptamer (named Apt917) was designed by bioinformatic tools based on sequences reported in the literature. Apt917 has two loops in its structure and can recognize whole cells of *E. coli* O157:H7. Apt917 was immobilized on gold nanoparticles-modified screen-printed electrodes. The electrochemical evaluation of the obtained biosensor shows that the device can be able to recognize *E. coli* at low concentrations. Also, the biosensor shows good specificity towards *E. coli* compared to a gram-positive bacterium such as *S. aureus*. However, at high concentrations of *P. aeruginosa* it is difficult to differentiate one or another microorganism. In any case, this two-loop aptamer-based system becomes an important basis for the subsequent design of new recognition molecules with increased specificity towards *E. coli* at low concentrations.

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