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Original scientific paper

Electrochemical genosensor for detection of a mutation in the *adenomatous polyposis coli* gene (APC 1306)

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Abstract

Colorectal cancer is a type of disease that arises from alterations in the genome, making it challenging to diagnose at an early stage. The difficulty in early detection can complicate treatment and lower survival rates. These genomic alterations result in mutations in critical genes, including adenomatous polyposis coli (APC). Some of these mutations, including APC 1306, have been identified as potential biomarkers for early cancer diagnosis. Genosensors

offer a promising alternative to these detection methods; however, it is essential to optimize their characteristics, including sensitivity, stability, and reaction conditions, prior to use. In this study, an electrochemical genosensor was developed to detect a specific mutation using voltammetric techniques. The limit of detection was 8.56 pM for cyclic voltammetry and 29.76 pM for differential pulse voltammetry. The optimal incubation and hybridization temperature were determined to be 55 °C. The genosensor remained stable for 14 days, after which its performance gradually declined. The tested electrochemical detection methods demonstrate excellent performance for this type of genosensor, particularly in the presence of doxorubicin. This approach can also be utilized to identify various genetic alterations associated with other chronic degenerative diseases.

Keywords
DNA biosensor; DNA mutation; differential pulse voltammetry; cyclic voltammetry, colorectal cancer

Introduction

Cancer encompasses a group of diseases characterized by genetic, molecular, epigenetic, cellular, and histological alterations. These changes lead to uncontrolled cell growth, which can result in tumour formation and invasion of various tissues and organs, depending on specific conditions and disease stage [1-3]. Cancer is currently the second leading cause of death globally, with breast cancer, prostate cancer, and colorectal cancer being some of the most common types [4-7]. Colorectal cancer (CRC) is the third leading cause of death for both men and women worldwide, accounting for approximately 1.85 million cases and 850,000 deaths each year [8]. CRC development involves irreversible genetic damage that increases the susceptibility of intestinal mucosal epithelial cells to neoplastic transformation [9,10]. This process occurs gradually and involves the accumulation of point mutations in oncogenes and tumour suppressor genes, such as adenomatous polyposis coli (APC), p53, SMAD4 and KRAS, as well as other genes involved in DNA repair mechanisms. These genetic alterations happen at various stages of the disease [11,12]. The APC gene is a tumour suppressor gene, and alterations can occur in exon 15, which encodes 75 % of the protein [13]. The most frequently diagnosed mutations with potential as biomarkers include APC 1309, APC 1306, APC 1312, APC 1367, APC 1378, APC 1379, APC 1450, and APC 1465 [14]. Some variations manifest as deletions or point mutations. For example, APC 1306 has a specific alteration at nucleotide 126, changing from G to A, C or T [15]. The main issue with CRC is that diagnosis often occurs in advanced stages of the disease, typically based on the symptoms presented by the patient. Before reaching a definitive diagnosis, several screening methods can be used, including diagnostic imaging, faecal immunochemical test, faecal occult blood test, and multi-target faecal DNA tests. While these methods are noninvasive, colonoscopy is necessary to establish a definitive diagnosis. The colonoscopy enables tumour identification and biopsy. Unfortunately, biopsies are often conducted only after the disease has significantly progressed, which reduces the survival rate compared to cases diagnosed at an earlier stage [8,16-19]. Consequently, the search continues for noninvasive alternative methods to colonoscopy that can identify mutations before the development of polyps or cancer symptoms [20,21]. Emerging biosensor technologies offer a promising alternative to traditional colonoscopic examinations. These devices integrate a transducer with a biological component to transform interactions between biomolecules into measurable signals. The transducer can be optical, piezoelectric, or electrochemical. Additionally, biosensors are classified by the type of bioreceptor they employ, which can include enzymes, antibodies, nucleic acids, and other biorecognition elements. Devices specifically designed to detect genetic material are known as genosensors [22,23]. A genosensor typically comprises a functionalized DNA or RNA probe and a transducer that converts the interaction between

the probe and the target genetic material into a measurable signal. When the probe hybridizes to its complementary target, significant changes occur, enabling accurate identification and quantification of the target genetic material [24-26]. Genosensors can be designed to detect pathogens, genetic mutations, or biomarkers linked to specific diseases. These devices have demonstrated their usefulness across various fields, including medical diagnostics, infectious disease monitoring, and environmental and food safety applications. Their versatility and adaptability make genosensors essential tools in both genomic research and practical applications [27]. Some authors suggest that using mercaptoundecanoic acid (MUA) in the technique improves nucleic acid detection compared to other compounds. When combined with the probe, MUA prevents cross-linking of genetic material, thereby enhancing the detection limits of genosensors [28,29]. The limit of detection (LOD) for an analyte is the smallest amount of a substance that can be reliably identified as different from its absence, based on a specified confidence interval. This determination also considers the potential for false positives and false negatives [30]. Sensitivity and detection limits are crucial parameters; thus, genosensors are coupled to electrochemical transducers, enabling analysis with greater precision, reproducibility, and shorter analysis times than other complex and expensive methods [31-34]. Voltammetry is a prominent electrochemical technique that measures processes at the interface between electrodes and electrolytes. This method encompasses various voltammetric or controlled-potential techniques, in which the potential can be held constant or varied according to a predetermined schedule. The resulting current is then measured in response to the applied potential [35]. In summary, to develop an electrochemical genosensing platform for the quantification of the APC 1306 mutation. The proposed method is based on the fabrication of a biosensor by immobilizing specific DNA probes on gold electrodes, employing doxorubicin as an intercalating agent to amplify the electrochemical signal. The sensor's analytical performance will be rigorously evaluated to validate its applicability for clinical diagnostics.

Experimental

Reagents

Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, phosphate buffered saline pH 7.4 (PBS), 11-mercaptoundecanoic acid (MUA), ethylenediaminetetraacetic acid (EDTA), trizma® hydrochloride (Tris-Cl) and Doxorubicin (Doxo) were purchased from Sigma Chemicals (St Louis, MO); perchloric acid ($HClO_4$) was obtained from Merck (Mexico) and ultrapure sodium dodecylsulphate (SDS) was purchased from MP Biomedicals, Inc. (Solon, OH). The MUA solution was prepared in an ethanol solution (50 %), while the remaining solutions were prepared with Millipore Milli-Q ultrapure water (resistivity 18.2 MΩ cm) from Millipore Corporation (Milford, MA).

DNA oligonucleotides were purchased from T4 Oligo (Irapuato, Gto). Stock solutions of oligonucleotides were prepared in TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 8.0) and stored at -20 °C until use. Solutions diluted in 0.01 M PBS at pH 7.4 were prepared at concentrations ranging from 1 fM to 100 μM. The oligonucleotide sequences used in the experiments are shown in Table 1.

Table 1. *Oligonucleotide sequences*

	Sequence	Mismatch, %
Probe sequence (ssDNA)	5'- SH-$(CH_2)_6$-TCC AAT CTT TTC TTT TTT TAT TT-3'	-
Complementary sequence (dsDNA)	3'- AGG TTA GAA AAG AAA ATA AA-5'	0
Non-complementary sequence (ncDNA)	3'- AGG TT**C** GAA AAG **C**AA ATA AA-5'	10

Equipment and instruments

Screen-printed gold electrodes (SPGE) DS 250AT (Dropsens, Oviedo, Spain) were used as the working electrode, with a platinum (Pt) counter electrode and an Ag/AgCl reference electrode. The techniques were performed at room temperature using the Bioanalytical Systems BAS-100 workstation (West Lafayette, IN, USA). All potentials were measured relative to the Ag/AgCl reference electrode. A new SPGE was used for each assay. For scanning electron microscopy (SEM) characterization, a JEOL JSM-7800 (Japan) was used.

Preparation of genosensors

The working electrodes were cleaned using cyclic voltammetry (CV) under the following conditions: a scan rate of 100 mV s^{-1} and a potential range of -700 to 2000 mV, using 0.1 M $HClO_4$, before the preparation of the genosensor [36]. 10 µL of the working probe (ssDNA at 1 mM concentration from APC 1306) and 30 µL of MUA were incubated on the gold surface for 60 minutes. Afterward, the electrode surface was rinsed three times with Milli-Q water. This step is referred to as modification with MUCs (MUA + ssDNA).

Hybridization

The hybridization reaction was conducted by incubating 40 µL of 1 mM APC 1306 dsDNA on the surface of the MUCs-modified gold electrode for 60 minutes. This incubation resulted in the formation of the Au/MUCs/dsDNA complex. Following this, the electrode was rinsed three times with Milli-Q water to remove any excess complementary strands.

Intercalation

After forming the Au/MUCs/dsDNA complex, 10 µL of Doxo (20 µM) in PBS (0.01 M) was applied to the surface of the modified electrode. The solution was allowed to interact for 20 minutes in the dark room, after which the electrode was rinsed with Milli-Q water.

Experimental electrochemical conditions

The electrochemical experiments were performed using CV and differential pulse voltammetry (DPV). The CV settings were a scan rate of 50 mV s^{-1} and a potential range of -400 to 500 mV. For DPV, the experimental conditions included a scan rate of 50 mV/s, a sample width of 20 ms, a pulse amplitude of 50 mV, a pulse width of 50 ms, and a pulse period of 200 ms. Measurements were taken using 150 µL of a solution containing 2.5 mM $[Fe(CN)_6]^{3-/4-}$, diluted in 0.01 M phosphate--buffered saline (PBS) at pH 7.4.

Data processing

After obtaining the CV and DPV, the difference in the maximum anodic peak current for each electrode modification was calculated. Based on these values, the relative current change (I_R / %) associated with each modification step (Au, Au/MUCs, Au/MUCs/dsDNA, and Au/MUCs/dsDNA/Doxo) was determined (equation 1), following a modified procedure reported by García-Melo *et al*. [37].

$$I_R = \left(\frac{I_0 - I_M}{I_0} \right) 100 \quad (1)$$

where I_R is the relative current change, I_0 corresponds to the maximum anodic peak current of the Au/MUCs electrode, and I_M represents the maximum anodic peak current after modification with dsDNA or dsDNA/Doxo.

Genosensor sensitivity

SPGEs that were previously modified with Au/MUCs were exposed to 40 µL of various concentrations of dsDNA from the APC 1306 mutation. The concentrations used were 100 mM, 1 mM, 10 µM, 100 nM, 1 nM, 10 pM and 1 pM. After this step, Doxo was added, and I_R was calculated.

Temperature reaction

The effect of temperature on each stage of the genosensor construction process (Au/MUCs, Au/MUCs/dsDNA and Au/MUCs/dsDNA/Doxo) was evaluated at 25, 30, 37, 40, 45, 50 and 55 °C.

Genosensor stability

The stability experiment was conducted using a single SPGE for each assay. The working gold electrodes were modified with Au/MUC. Each electrode was stored at 4 °C for periods of 7, 14, 21 and 28 days. After the specified incubation periods, we proceeded with the following steps using Au/MUC/dsDNA and Au/MUC/dsDNA/Doxo to determine the genosensor lifetime.

Surface analysis of screen-printed gold electrodes by scanning electron microscopy

In this stage, the changes in the surfaces of the gold electrodes were evaluated after each modification during construction of the genosensor. To accomplish this, high-resolution scanning electron microscopy was performed using the JEOL JSM-F 7800, which offers magnifications ranging from 500 to 50,000×, an acceleration voltage of 1.5 kV, and a resolution of 15 kV. Each sample was analysed in duplicate.

Results and discussion

Figure 1 illustrates CV and DPV results from the construction process of the genosensor to identify the APC 1306 mutation. In CV (Figure 1A), the clean gold electrode exhibited a current of 107.5 µA for the $[Fe(CN)_6]^{3-}/^{4-}$ redox couple. After modifying the electrode with 1 µM of single-stranded DNA and MUCs, the maximum anodic current peak decreased to 64.25 µA. After the hybridization reaction with 1 µM of dsDNA, the maximum anodic current decreased to 57.25 µA. Following the addition of 20 µM Doxo, the current further decreased to 51.5 µA.

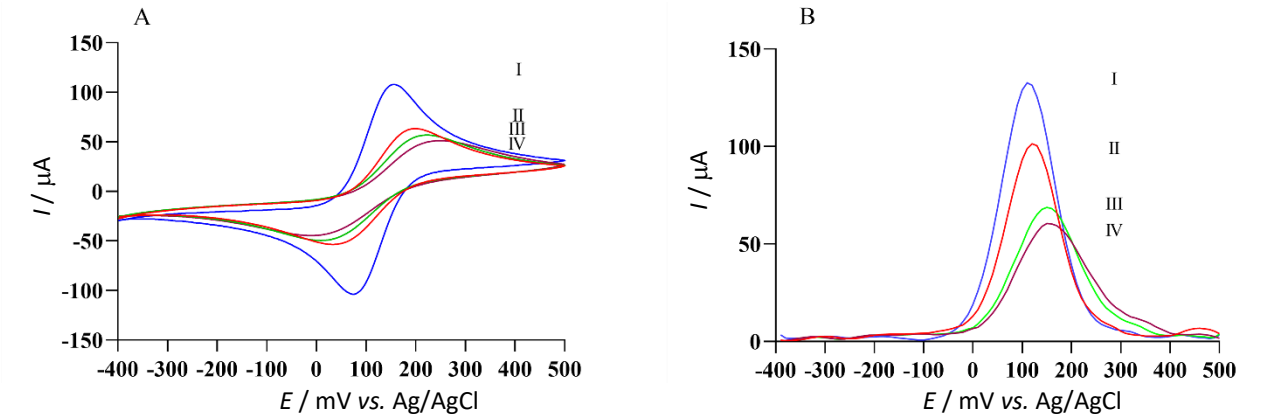

Figure 1. *Cyclic voltammogram (A) and differential pulse voltammogram (B) of 2.5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.01 M PBS (pH 7.4) for the detection of the APC 1306 gene. For both figures: Au (I), Au/MUCS (II), Au/MUCS/dsDNA (III), and Au/MUCS/dsDNA/Doxo (IV)*

A similar trend was observed in the DPV technique (Figure 1B), where the measured currents were 133, 101, 69.1 and 61.1 µA for the clean gold electrode (Au), Au/MUCs, Au/MUCs/ dsDNA and Au/MUCs/dsDNA/Doxo, respectively (I, II, III, and IV). The results obtained confirm the process of building the genosensor, as demonstrated by Rodrigues *et al.* [38]. They used electrochemical

techniques, such as CV, and observed a trend in the decrease of the electrochemical signal as the electrode modification increased, specifically when using different concentrations of DNA sequences for the detection of prostate cancer. This same trend was noted by Farzin *et al.,* who evaluated the detection of SARS-CoV-2 RdRP sequences at various concentrations through DPV and CV [39].

In both studies, the researchers monitored the electrochemical behaviour of the modified electrode and observed a gradual decline in the electrochemical signal until the target sequence was detected. They employed $[Fe(CN)_6]^{3-/4-}$ as a probe molecule and observed similar electrochemical response patterns after each modification of the working electrodes. This response is attributed to the interaction of $[Fe(CN)_6]^{3-/4-}$ with the working electrode. The rate of electron transfer is influenced by additional layers applied to the electrode surface, thereby facilitating sequence detection for diagnostic purposes in the biomedical field [40,41]

Genosensor detection limit

Figure 2 illustrates the genosensor's sensitivity in detecting the APC 1306 mutation. The device demonstrates a detection range from 1 pM to 1 μM. LOD was determined using the 3×*Sb* criterion, where *Sb* represents the standard deviation of the blank measurements, and matched to the corresponding equation of the linear portion of the graph. The LOD for CV was 8.56 pM, while for DPV it was 29.76 pM. The difference in electrochemical response between CV and DPV is due to the system's reaction involving surface or adsorption processes. Furthermore, charge-transfer kinetics are more efficient under continuous-sweep potential conditions in systems where the voltammetric response predominates [42,43]. In this analysis (CV), this technique has been observed to be effective in evaluating the electrode-electrolyte interface, adsorption dynamics, electrical double-layer organization, and charge-transfer processes associated with the formation of surface coatings on the working electrode, resulting in peaks of higher intensity than DPV, like this project [43-45]. On the other hand, in pulsed techniques such as DPV, it has been observed that if the capacitive relaxation time is long or the pulse time is too short, the capacitive current has not yet decayed sufficiently before the total current is measured. This results in a predominantly charge signal, which reduces the faradaic current fraction and limits the sensitivity and detection limit [46,47]. Furthermore, under our conditions, the electrode characteristics and the double-layer properties produce a faradaic current/charge current ratio in cyclic sweeps that is higher than in pulsed sweeps. Studies have shown that CV responses are highly sensitive in systems where oxide layers and microstructures form, reflecting the characteristics of the active surface and the electrode structure when charge transfer is coupled [48,49]. For this reason, the electrochemical response in CV is higher due to the continuous waveform and the contribution of the current associated with the functionalized biolayer, which leads to amplified surface processes, unlike those obtained with differential pulses [42,43,45]. In another study, Garcia-Melo *et al*. [37] reported a limit of detection of 43.92 pM for the CRC APC gene using CV. Although these are mutations in the same gene, the LOD values differ between their report and our study. According to Suback *et al*. [50], this is due to the sequences used and their base content, especially the presence of guanine, which can interfere with electron transfer and thus the electrochemical response. Regarding detection limits, different authors have reported detection limits of up to 8.75 fM for gastric [51] and 0.01 fmol for prostate [52] cancer using miRNAs as bioreceptors. For both studies, DPV shows higher sensitivity, as in this research for this type of analysis; however, the CV technique also has high potential in mutation identification, even for the efficient detection of nucleic acids of different origins, including those of bacterial type or molecules of interest such as vincristine, idarubicin, and epinephrine [53-57].

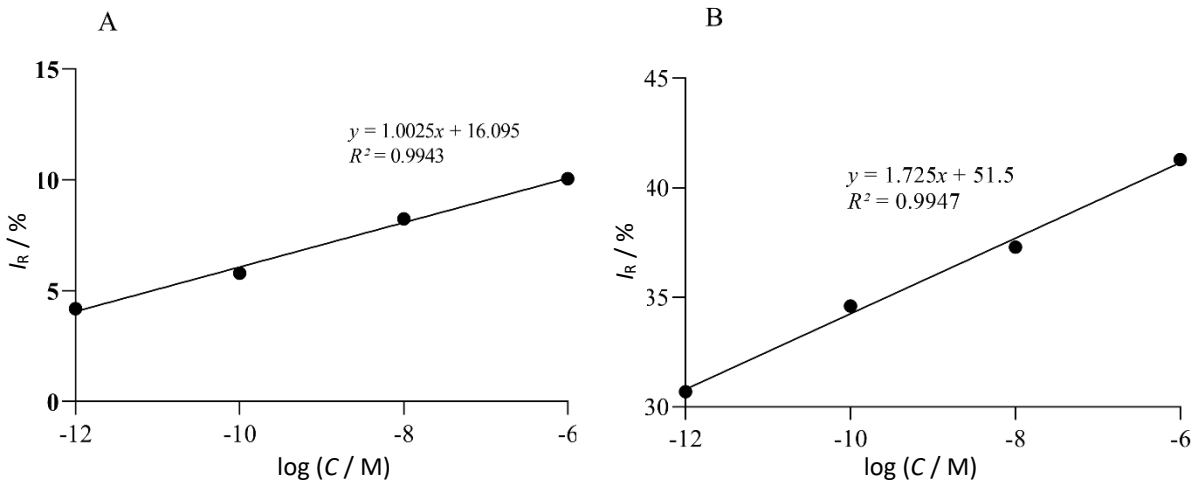

***Figure 2**. Linear relationship between the relative anodic peak current of $[Fe(CN)_6]^{3-/4-}$ (I_R) and the logarithm of the target DNA concentration: A) relationship represented by the CV technique; B) relationship represented by the DPV technique*

On the other hand, to increase the device's detection capacity, dsDNA interacted with Doxo, improving detection by up to I_R = 10 % compared to the previous hybridization process. This demonstrates the effect of the intercalating molecules in this type of genosensor (Figure 3).

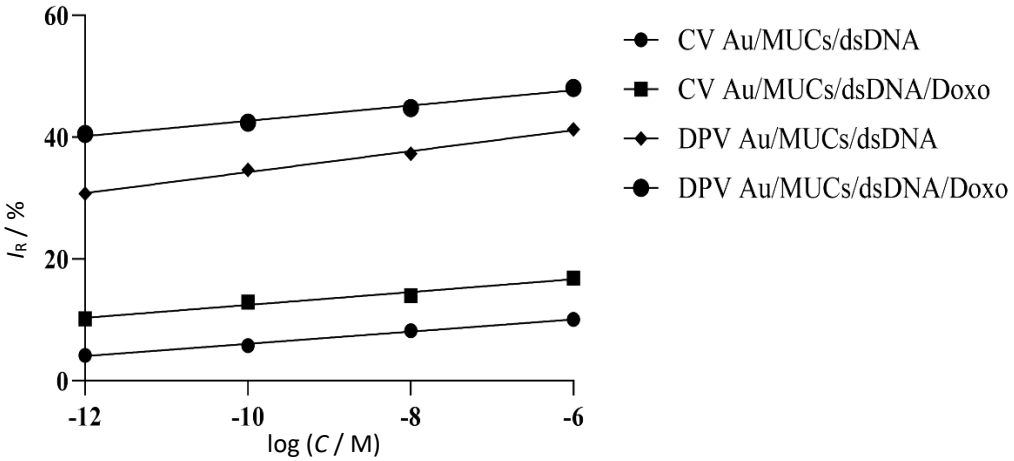

***Figure 3.** Linear relationship between the I_R of the anodic peak of $[Fe(CN)_6]^{3-/4-}$ and the logarithm of the target DNA concentration for the DOX/dsDNA/MUCS/Au and dsDNA/MUCS/Au electrodes, as determined by CV and DPV electrochemical techniques*

The results demonstrated superior electrochemical responses in both cases following intercalation with Doxo. DPV and CV exhibited greater detection sensitivity. Furthermore, our findings align with those of García-Melo *et al*. [37], who also reported enhanced detection sensitivity when using Doxo as a DNA intercalating molecule for this type of genosensor. A similar study by Hassani *et al*. [58] examined the effects of Doxo on dsDNA, applying DPV. The study revealed differences in signal intensity in the presence and absence of Doxo. In both studies, Doxo was effective in enhancing DNA detection signals. This enhancement occurs because Doxo tends to form hydrogen bonds with guanines in G-A base pairs, leading to a twist in the DNA strands. This twisting increases the surface area of the working electrode, thereby improving the detection of electrochemical signals [58-60].

Genosensor hybridization temperature

The hybridization temperatures evaluated for this assay ranged from 20 to 55 °C (Figures 4A and 4B). Each electrochemical response indicates successful hybridization of the DNA probe with its complementary strand, and these responses were unaffected by the temperature used during genosensor development. As noted in the previous section, the detection signals increased in the presence of Doxo. Optimal electrochemical responses occurred at 50 and 55 °C, whereas the weakest signal was detected at 25 °C. This suggests that the genosensor operates more efficiently at higher temperatures (as indicated by CV and DPV methods).

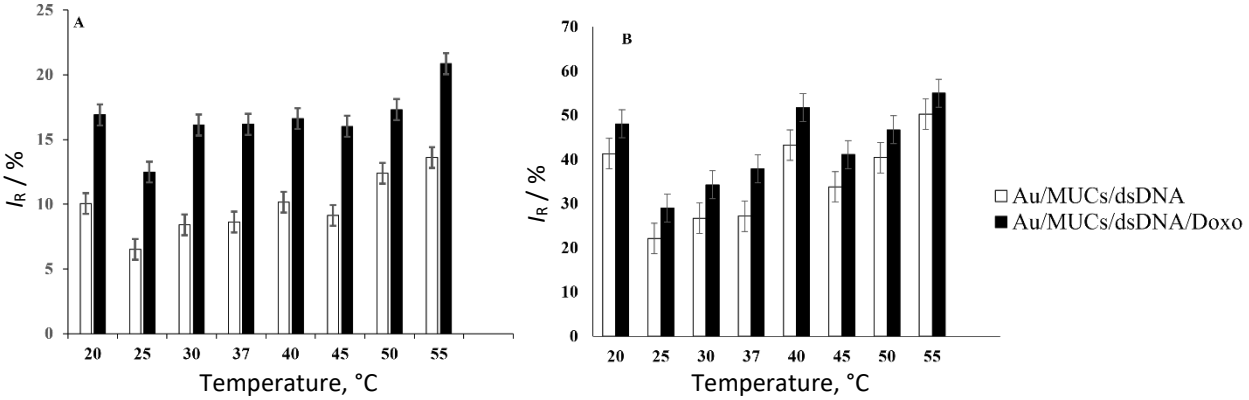

Figure 4. *Effect of incubation temperature on genosensor preparation, evaluated by CV (A) and DPV (B), respectively, where the IR% of the anodic peak of $[Fe(CN)_6]^{3-/4-}$ versus temperature was assessed for the Au/MUCs/dsDNA electrode without and with Doxo*

In this study, the genosensor exhibited an electrochemical response over the temperature range of 20-55 °C. A comparison of the present study's results with previous research reveals significant parallels. García-Melo *et al*. [37] also evidenced optimized hybridization detection at 55 °C. Concurrently, Ma *et al.* observed an increasing detection signal at temperatures above 25 °C in their study of thiolated DNA on gold electrodes. However, they reported a decline in CV readings and electrode deterioration at temperatures exceeding 90 °C. Consistently, this investigation showed an increase in the detection signal at temperatures above 20 °C, with a maximum response at 55 °C [61,62]. Likewise, Narang *et al*. designed a genosensor for detecting hepatitis B virus DNA and emphasized the determinant role of temperature in genosensor development, assessing the 5 to 45 °C range and identifying the highest response at 45 °C [63]. The results of this investigation, when compared with previous studies on MUC layer formation, indicate that the optimal electrochemical response occurs at temperatures above 45 °C. At these elevated temperatures, molecular resonance within the electrode structure increases, thereby enhancing molecular interactions and improving chemisorption [37,62,63]. For the hybridization assay, an optimal electrochemical response was observed specifically at 55 °C. This aligns with research by other authors who developed genosensors, which indicates that higher temperatures can decrease the rigidity of single-ssDNA, thereby promoting molecular interactions with its complementary strands. Nevertheless, the electrode's integrity may be compromised at temperatures exceeding 55 °C [37,62-64]. The current study found that increasing the temperature did not affect the electrochemical response after the interaction of DNA strands with Doxo. This observation contrasts with the findings of Du *et al*. [62], who reported that increased exposure temperatures impact the stability of the Doxo-DNA complex. Such a discrepancy might be attributed to variations in the DNA strands' characteristics, given that some bases, like adenine-thymine (A-T) pairs, demonstrate more favourable interactions with Doxo [62].

Genosensor stability

Figures 5A and 5B display the electrochemical responses of CV and DPV (respectively), of the genosensors over a 28-day storage period at 4 °C. Without Doxo, the genosensors maintained optimal performance for up to 7 days, at which point the I_R was 10.6 % for CV and 34.34 % for DPV. By day 14, in the absence of Doxo, the IR had diminished by 4.45 % (CV) and 21.82 % (DPV) compared with their respective initial (day 0) values. This represented an overall decrease of more than 50 % in the total electrochemical signal after 14 days of storage. When evaluated in the presence of Doxo, the genosensors exhibited an I_R of 16.9 % (CV) and 36.56 % (DPV) within the first 7 days. After 14 days under these conditions, the I_R values decreased to 7.75 % (CV) and 22.15 % (DPV), relative to the initial Doxo present values. A further reduction in the IR was observed on day 21, amounting to decreases of 81 % for CV and 62.85 % for DPV compared to the initial values recorded in the presence of Doxo. Finally, genosensors stored for 28 days exhibited negative I_R values.

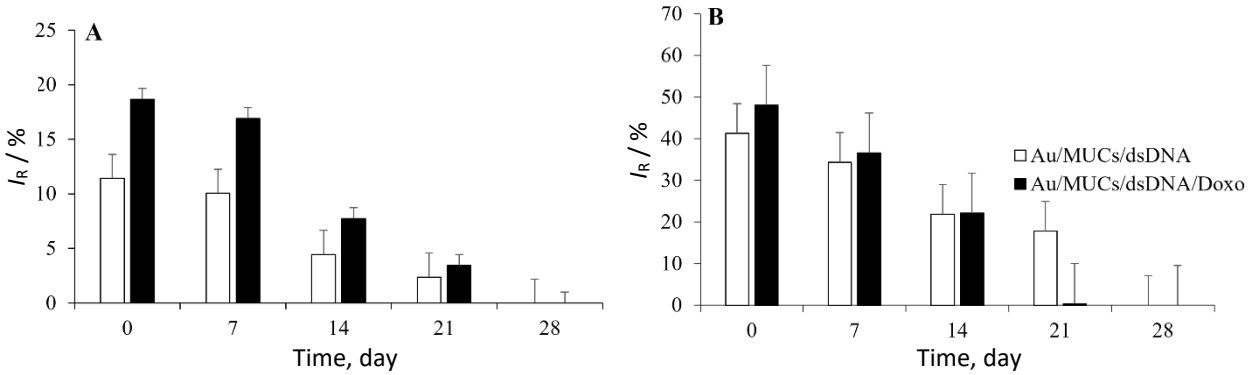

***Figure 5**. Electrochemical stability analysis of the genosensor over 28 days at 4°C. I_R values obtained through (A) CV and (B) DPV*

Genosensor selectivity

Figures 6a and 6b show that the system exhibits selectivity for the specific detection of dsDNA from the APC 1306 fragment relative to ncDNA containing 10 %-point mismatches (Table 1). In both electrochemical techniques (CV and DPV), a significant decrease in signal was observed, of 40 and 34 %, respectively, compared to the signal recorded for dsDNA in the presence of Doxo, which was 18.57 and 47.74 µA for CV and DPV, respectively. In this regard, several research groups have developed and optimized genosensor-based detection strategies to improve the measurement of this parameter. For example, Abad-Valle *et al*. [65] reported a highly selective, enzyme-based electrochemical genosensor for detecting thymine-rich 30-nucleotide sequences, with signal variations of 10-50 %. Similarly, Hybrid SWNT-DNA nanostructures [66] enable effective discrimination between complementary DNA sequences and those with mismatches or incorrect pairings. Likewise, Ngavouka *et al*. [67] employed nanolithography to enhance the selectivity of electrochemical genosensors. However, unlike previously reported approaches, this study employed a sequence corresponding to a clinically relevant colorectal cancer biomarker. This enabled effective discrimination between the target sequence and its identical complementary strand without the need for additives, nanomaterials, or additional amplification steps. Using our system, we observed a 50 % decrease in the electrochemical signal after hybridization of ssDNA with its dsDNA homolog. In contrast, ncDNA showed no significant electrochemical decrease, indicating low hybridization efficiency. Taken together, these results demonstrate that the proposed system constitutes an effective, highly selective, and specific strategy, maintains a simple design, and incurs no additional costs, representing a significant advantage for its potential clinical application.

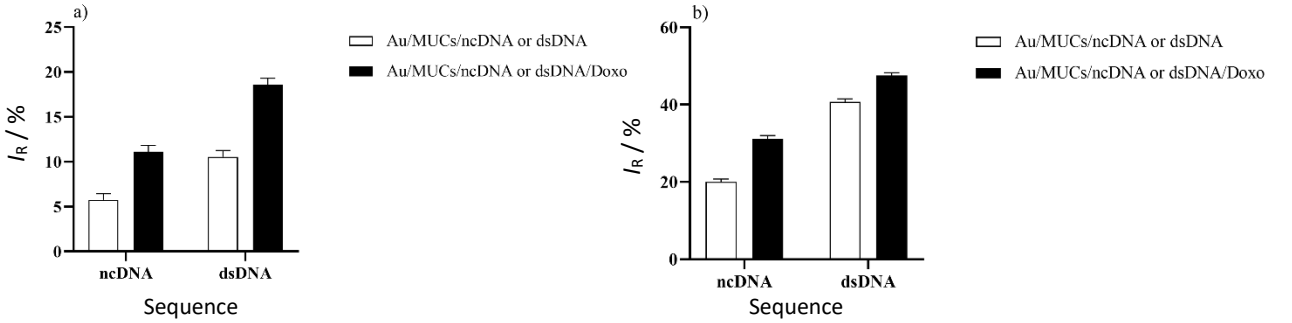

Figure 6. *Evaluation of the selectivity of the genosensor for the APC 1306 mutation by a) CV and b) DPV, where the* I_R *of the anodic peak of* $[Fe(CN)_6]^{3-/4-}$ *was evaluated with and without Doxo*

Scanning electron microscopy genosensor surface analysis

The results indicate surface changes on the SPGE working electrode after each stage of the genosensor construction process. Figure 7 shows the clean, unmodified SPGE, which presents a gold surface with rough, irregular topography, revealing areas of depression (dark) and elevation (bright). The most distinctive feature of the SPGE is its porosity, characterized by numerous pores of varying size and shape. The surface texture between the pores suggests a polycrystalline structure with visible “grains”.

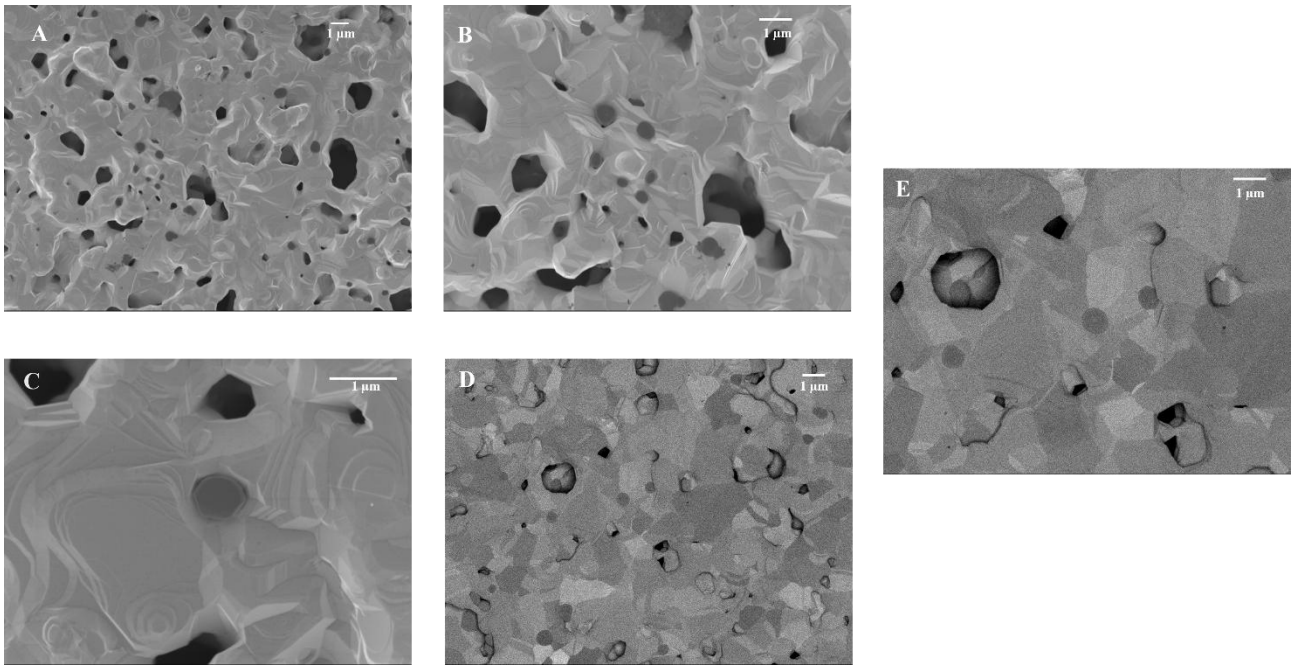

Figure 7*. High-resolution scanning electron micrographs of the SPGE gold surfaces. Images A, B and C were analysed using a top electron detector (UED) at magnifications of 5,500×, 10,000× and 20,000×, respectively. Micrographs D and E were analysed using a bac backscattered UED detector at magnifications of 5,000× and 10,000×, with a detection depth of 10 nm*

Figure 8 presents a micrograph of a surface characterized by a predominantly faceted and lamellar morphology, resembling overlapping 'leaves' with well-defined edges. Numerous dark, irregularly shaped pores and holes are visible, indicating deep depressions alongside folds or wrinkles. Additionally, the surface is covered with many tiny particles, averaging 9.37 nm in size.

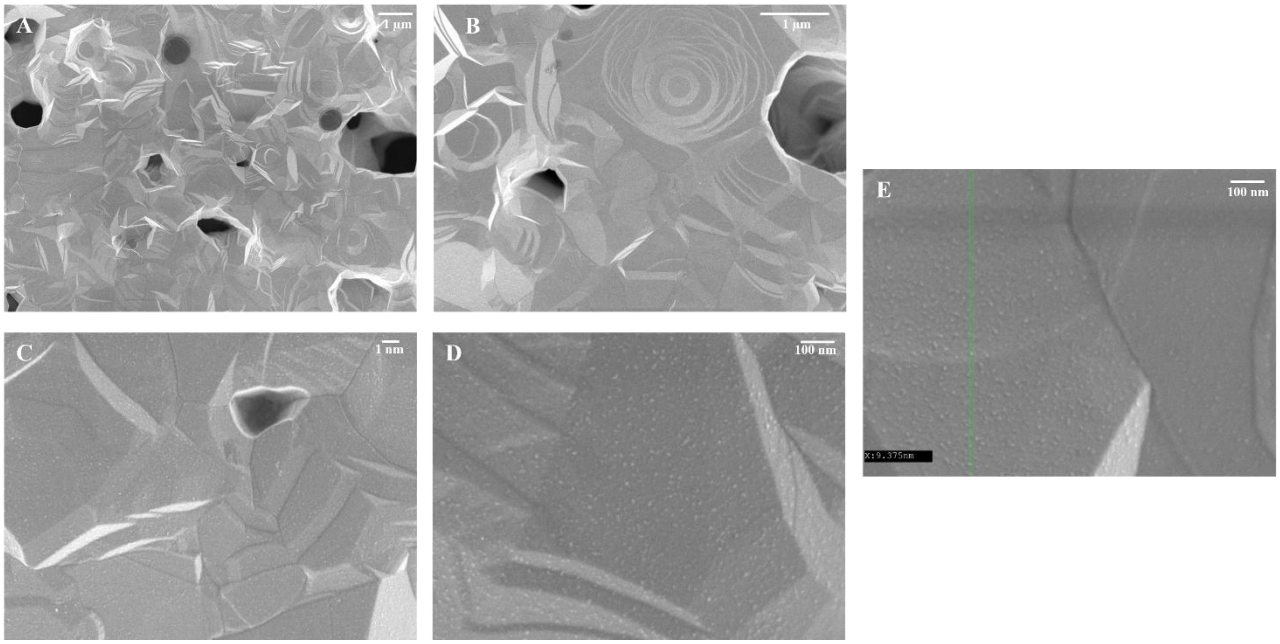

***Figure 8**. High-resolution scanning electron micrographs of the SPGE gold surfaces modified with the probe chain (APC 1306) and mercaptoundecanoic acid (MUA). Images (A, B and C) were obtained using a UED detector with magnifications of 10,000x, 20,000x and 50,00. Images (D and E) were obtained using a top electron detector (UED) at a magnification of 100,000×, highlighting the presence of particles with an average size of approximately 9.375 nm*

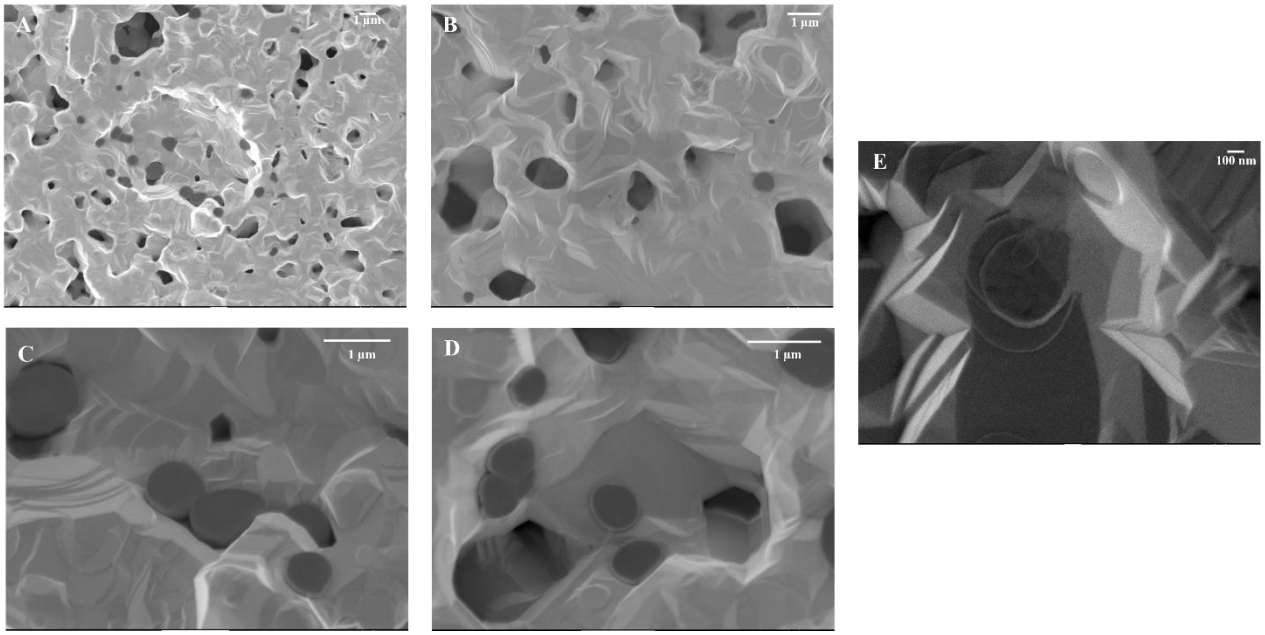

***Figure 9**. High-resolution scanning electron micrographs of SPGE gold surfaces modified with the complementary chain (APC 1306) and MUCs. Panels A and B were obtained using a UED detector with backscatter settings of 5,000× and 10,000×, respectively, with a penetration depth of 10 nm. Panels C, D and E were obtained using a top electron detector (UED) with magnifications of 20,000×, 22,000× and 50,000×*

These particles are consistent with the self-assembled monolayer system formed by mercaptoundecanoic acid and ssDNA, and their morphology aligns well with expectations for this type of system. In contrast, surface modification with dsDNA decreased pore size (Figure 9).

During the Doxo modification step, we observed a reduction in porosity and an increase in agglomeration. This may be linked to drug molecules that interact with the hybridized chains (Figure 10).

These findings confirm that the electrode surface undergoes significant changes at each stage of the genosensor design process. A notable change occurs in the final stage, when Doxo is intercalated into the hybridized chains, forming a thin film on the electrode surface. This phenomenon may explain the low electrochemical signal detected at this stage. While there is currently no direct evidence of Doxo intercalation via microscopy, the film likely formed due to the drug's ability to alter DNA structure during intercalation [68,69]. This aligns with the findings of Agudelo *et al.,* who characterized this process using fluorescence spectroscopy and molecular modelling. They demonstrated that Doxo induces structural twists in DNA upon intercalation [70]. Similarly, Zabost *et al*. [71] showed that this molecule can form noncovalent interactions with nucleic acids. This interaction enhances the electrochemical signal in CV because of the planar structure of Doxo, which favours such interactions. This response is further optimized in the presence of nitrogenous bases, such as guanine and adenine. In this context, the observed structural changes may be attributed to the high guanine and adenine content (85 %) of the probe used in this study. This composition likely promoted the formation of a surface film, which in turn blocked the electrode surface [62,63].

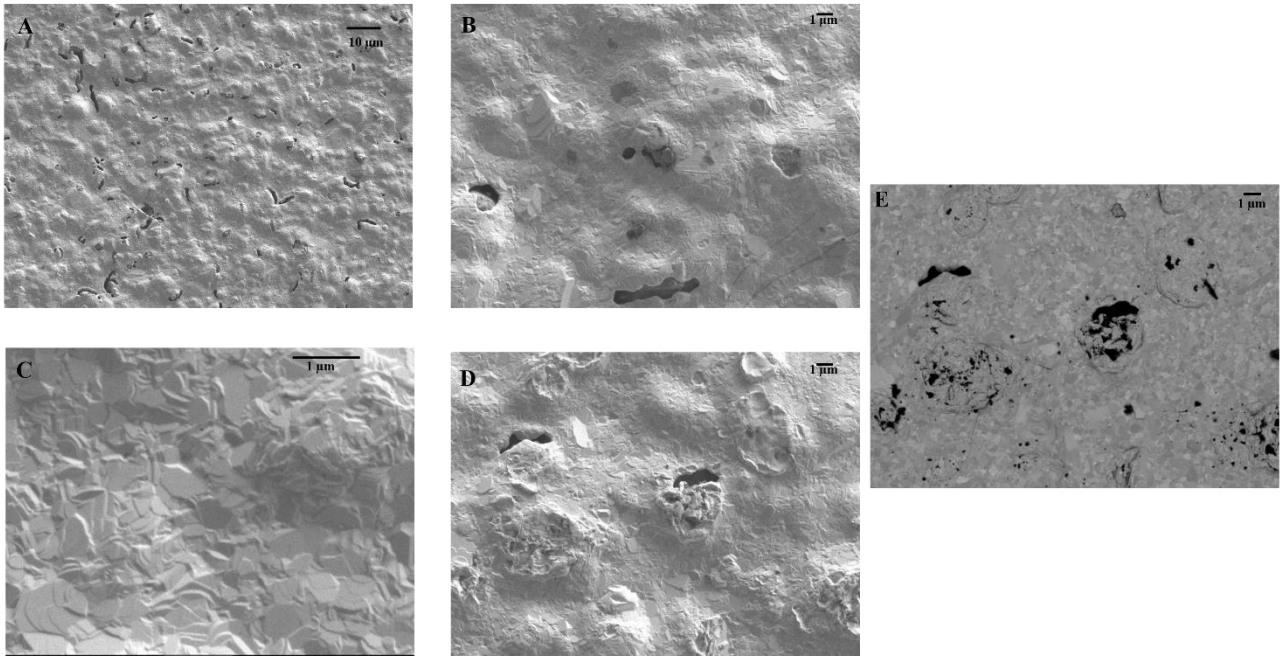

Figure 10*. High-resolution scanning electron micrographs of the gold surfaces of the doxorubicin-modified screen-printed gold electrodes (SPGEs) in the dsDNA/MUCs system (APC 1306). Panels (A, B, C and D) display images captured by an LED detector at magnifications of 1,000×, 5,000× and 20,000×, with a depth of 10 nm. Panel (E) presents micrographs analysed using a top electron detector (BED-C) at a magnification of 5,000×*

Conclusions

This study details the development of an effective genosensor for detecting the APC 1306 mutation. The immobilization of the probe on the gold electrode was confirmed by a decrease in current due to surface blocking, which was monitored using the $[Fe(CN)6]^{3-}/^{4-}$ complex. Interaction with Doxo significantly increased the anodic peak current, particularly during DPV, indicating higher sensitivity compared to CV. Doxo enhanced electrochemical detection by intercalating with DNA. The optimal temperature for the genosensors' efficiency was determined to be between 25 and 55 °C. The genosensor remained stable and quantifiable for up to seven days of storage. Scanning electron microscopy revealed morphological changes at various stages of genosensor processing, from the formation of the Doxo-induced thin film to the immobilization of MUCs. In summary, this study demonstrates the effectiveness of the developed genosensor for detecting the APC 1306

mutation. It also emphasizes the importance of factors such as temperature and stability, as well as the use of intercalators such as Doxo, in optimizing the electrochemical detection of genetic sequences. This positions the genosensor as a valuable tool for diagnostic applications in biomedical research, with potential uses in biotechnology and molecular medicine.

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