

# Inosine-Assisted Signal-On Electrochemical Biosensor for Selective Bacterial DNA Detection

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**Inosine-Assisted Signal-On Electrochemical Biosensor for Selective Bacterial DNA Detection**

**Seçici Bakteriyel DNA Tespiti Amacıyla Geliştirilmiş İnosin Tabanlı Sinyal-Artışlı Elektrokimyasal Biyosensör**

## SUMMARY

Accurate and rapid identification of *Shigella sonnei* and *Stenotrophomonas maltophilia* remains challenging due to their close genomic similarity to other Gram-negative bacteria and their limited coverage in routine molecular diagnostic panels. In this study, we report a label-free electrochemical DNA biosensing strategy for the sequence-selective detection of these two clinically relevant but under-recognized pathogens. The biosensor is based on a pencil graphite electrode modified with carboxyl-functionalized single-walled carbon nanotubes (SWCNT-COOH), providing a conductive and chemically active interface for probe immobilization. To minimize intrinsic guanine-related background signals, inosine-substituted DNA probes were employed, enabling a hybridization-induced "signal-on" response that arises exclusively from the target DNA. Stable and reproducible probe attachment was achieved through optimized  $-COOH-NH_2$  interactions between the SWCNT interface and amine-terminated probes. Under optimized conditions, the biosensor exhibited linear responses in the 20–80  $\mu g/mL$  range, with limits of detection of 6.1  $\mu g/mL$  for *S. sonnei* and 8.4  $\mu g/mL$  for *S. maltophilia*. The platform demonstrated effective discrimination against mismatched and non-complementary sequences and retained analytical performance in artificial saliva matrices, supporting its applicability in non-invasive sample environments. This work presents a simple and low-cost electrochemical approach for DNA-based detection of *S. sonnei* and *S. maltophilia*. The proposed sensing strategy highlights the potential of inosine-assisted, signal-on electrochemical readout as a modular platform for nucleic-acid detection, with relevance for decentralized and resource-limited diagnostic settings.

**Keywords:** Electrochemical sensing, carbon nanomaterials, label-free DNA detection, pathogen identification, bacterial diagnostic.

## ÖZ

*Shigella sonnei* ve *Stenotrophomonas maltophilia*'nın doğru ve hızlı tanımlanması, diğer Gram-negatif bakterilerle olan yakın genomik benzerlikleri ve rutin moleküler tanı panellerinde sınırlı düzeyde yer almaları nedeniyle halen zorluk teşkil etmektedir. Bu çalışmada, klinik açıdan önemli ancak yeterince tanınmayan bu iki patojenin dizi-seçici tespiti için etiketsiz bir elektrokimyasal DNA biyosensör stratejisi sunulmaktadır. Geliştirilen biyosensör, prob immobilizasyonu için iletken ve kimyasal olarak aktif bir yüzey sağlayan karboksil fonksiyonelleştirilmiş tek duvarlı karbon nanotüpler (SWCNT-COOH) ile modifiye edilmiş kurşun kalem grafit elektrot üzerine dayanmaktadır. İçsel guanin kaynaklı arka plan sinyallerini en aza indirmek amacıyla, inozin ile modifiye edilmiş DNA problemleri kullanılmış ve böylece yalnızca hedef DNA varlığında ortaya çıkan hibridizasyon kaynaklı "signal-on" yanıt elde edilmiştir. SWCNT yüzeyi ile amin uçlu problemler arasındaki  $-COOH-NH_2$  etkileşimleri optimize edilerek stabil ve tekrarlanabilir prob immobilizasyonu sağlanmıştır. Optimize edilmiş koşullar altında biyosensör, 20–80  $\mu g/mL$  aralığında doğrusal yanıt göstermiş; *S. sonnei* için 6,1  $\mu g/mL$  ve *S. maltophilia* için 8,4  $\mu g/mL$  tayin sınırları elde edilmiştir. Platform, uyumsuz ve tamamlayıcı olmayan dizilere karşı etkin ayırım yeteneği göstermiş ve yapay tükürük ortamında da analitik performansını koruyarak non-invaziv örneklerde uygulanabilirliğini ortaya koymuştur. Bu çalışma, *S. sonnei* ve *S. maltophilia*'nın DNA temelli tespiti için basit ve düşük maliyetli bir elektrokimyasal yaklaşım sunmaktadır. Önerilen algılama stratejisi, inozin destekli "signal-on" elektrokimyasal okumanın, nükleik asit tespiti için modüler bir platform olarak potansiyelini ortaya koymakta olup, merkezi olmayan ve kaynakların sınırlı olduğu tanı ortamları açısından önem taşımaktadır.

**Anahtar Kelimeler:** Elektrokimyasal algılama, karbon nanomalzemeler, etiketsiz DNA tespiti, patojen tanımlama, bakteriyel tanı.

Received: 12.05.2026

Revised: 17.07.2026

Accepted: 23.07.2026

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## INTRODUCTION

*Shigella* species are Gram-negative, diarrheagenic bacteria closely related to *Escherichia coli* and primarily transmitted through the fecal–oral route via contaminated food or water (Scott et al., 2025). By invading the colonic epithelium, *Shigella* species—comprising *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*—cause shigellosis, an intestinal infection ranging from mild diarrhea to severe bloody diarrhea and fever (Miles, Holt, & Mostowy, 2024). The disease predominantly affects young children in low- and middle-income countries with poor sanitation, while in high-income countries it is often linked to travel or close-contact transmission (Anderson et al., 2019). Moreover, the increasing antimicrobial resistance of *S. sonnei* has further accelerated its spread and limited treatment options (Mason et al., 2023).

*Shigella* species and *E. coli* are closely related Gram-negative *Enterobacterales* sharing extensive phenotypic and genotypic similarities, which complicates their differentiation despite distinct clinical characteristics (van den Beld et al., 2019). Conventional biochemical tests, serotyping, and molecular methods such as 16S rRNA sequencing and MALDI-TOF MS often fail to reliably distinguish *Shigella* spp. from *E. coli*, particularly due to the close similarity of enteroinvasive *E. coli* (EIEC) strains (Khot & Fisher, 2013). Moreover, increasing antimicrobial resistance in *Shigella* highlights the need for accurate bacterial identification (Mojica et al., 2022). *Stenotrophomonas maltophilia* is a multidrug-resistant opportunistic pathogen associated with severe nosocomial infections (Brooke, 2014), especially in immunocompromised or hospitalized patients, where it frequently forms biofilms on medical devices such as catheters and ventilators (Hafiz et al., 2022). Classical microbiological methods, including culture-based assays, immunological tests such as ELISA (Boonham et al., 2014), and DNA-based techniques like PCR (Wahab, 2026), are widely used for bacterial detection. Although PCR-based approaches can provide high

specificity, these methods often involve complex, time-consuming procedures requiring specialized equipment and expertise (Radomirović, Gligorijević, & Rajković, 2025).

Biosensors overcome many limitations of conventional analytical methods by providing rapid, cost-effective, sensitive, and selective detection with minimal sample requirements (Inês & Cosme, 2025). Electrochemical biosensors have emerged as promising tools for the rapid identification of clinically relevant bacterial pathogens through DNA hybridization, aptamer-based recognition, and nanomaterial-assisted sensing strategies. For instance, a gold nanostar-modified electrochemical genosensor provided highly sensitive and selective detection of *Escherichia coli* O157:H7 in river water, highlighting the effectiveness of nanomaterial-assisted DNA hybridization strategies for pathogen monitoring (Razmi, Hasanzadeh, Willander, & Nur, 2022).

Similarly, an electrochemical DNA biosensor for *Mycobacterium tuberculosis* achieved femtomolar sensitivity in sputum and urine samples, highlighting the potential of electrochemical platforms for rapid and affordable pathogen detection (Ramos-Sono et al., 2020). Beyond pathogen detection, electrochemical biosensing platforms have also been applied to therapeutic drug monitoring and cellular biomarker analysis. For instance, electrochemical systems have been developed for the sensitive monitoring of chemotherapeutic drugs such as 5-fluorouracil (Zouari, Barderas, Pingarrón, Raouafi, & Campuzano, 2024) and irinotecan (Alvau et al., 2018), as well as for evaluating potassium channel activity in cancer cells (Zouari et al., 2022), highlighting the versatility of electrochemical sensing technologies in biomedical and clinical applications.

Nanomaterials and polymers are widely incorporated into biosensors to improve sensitivity and selectivity by enhancing surface area, charge transfer, and mechanical stability (Jin & Mao, 2014; Tran,

Son, & Min, 2016). Among them, carbon nanotubes (CNTs) are particularly attractive due to their high electrical conductivity, chemical stability, and large surface area. Especially, single-walled carbon nanotubes (SWCNTs) exhibit excellent electrical mobility and chemical resilience, making them highly suitable for electrochemical biosensor applications (Saleh Mohammadnia, Roghani-Mamaqani, Ghalkhani, & Hemmati, 2023). In addition, surface functionalization of CNTs, commonly with carboxyl (-COOH) groups, improves interfacial reactivity and enhances selective target binding, thereby increasing sensitivity and lowering detection limits (Chen, Hou, Huo, Yang, & Fa, 2016). Accordingly, SWCNT-COOH was selected in the present study. While both SWCNTs and MWCNTs possess favorable properties for electrochemical biosensing, SWCNTs offer superior charge-transfer characteristics and higher aspect ratios, providing an efficient and accessible interface for DNA probe immobilization and hybridization (Som Sengupta, 2025).

This study introduces the first electrochemical DNA biosensors specifically designed for *S. sonnei* and *S. maltophilia*, two clinically relevant pathogens that remain challenging to identify using routine diagnostic methods. While *S. sonnei* is frequently misidentified because of its close genetic similarity to *E. coli*, *S. maltophilia* is often absent from standard diagnostic panels. The proposed platform enables the separate electrochemical detection of *S. sonnei* and *S. maltophilia* through species-specific probe architectures and successfully discriminates target sequences from mismatch and non-complementary oligonucleotides. The biosensor integrates a SWCNT-COOH-modified pencil graphite electrode (PGE) with amine-functionalized, inosine-substituted DNA probes to overcome guanine oxidation and background signal interference, a major limitation of CNT-based DNA sensors. PGEs were selected because of their low cost, disposable nature, rapid and practical operation, and suitability for nucleic acid sensing applications.

Although variations in electrochemical performance may occur among different manufacturers or production batches, all experiments in this study were conducted using electrodes from the same commercial brand and batch to minimize such effects. By suppressing nonspecific electrochemical responses and activating a signal-on mechanism only upon target hybridization, the platform differs from conventional label-free CNT-DNA architectures. The developed biosensors exhibited high analytical sensitivity, and excellent selectivity against mismatched and non-complementary sequences in both simple and complex matrices.

## MATERIALS AND METHODS

### Instrumentation

Electrochemical measurements were performed using an AUTOLAB 204.FRA32M system (Utrecht, Netherlands) with NOVA 2.1 software. A pencil graphite electrode (PGE), platinum wire, and Ag/AgCl electrode were used as the working, auxiliary, and reference electrodes, respectively. HB-grade graphite leads (Tombow, Tokyo, Japan) (0.5 mm × 60 mm) mounted in a Rotring T 0.5 mm mechanical pencil (Altenburg, Germany) ensured consistent handling. Sonication was carried out using an Elma ultrasonic bath.

### Chemicals

Ammonium chloride ( $\text{NH}_4\text{Cl}$ ), calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), glacial acetic acid, ( $\text{CH}_3\text{COOH}$ ) potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), potassium chloride (KCl), potassium ferricyanide ( $[\text{K}_3(\text{Fe}(\text{CN})_6)]$ ), potassium ferrocyanide ( $[\text{K}_4(\text{Fe}(\text{CN})_6)]$ ), sodium bicarbonate ( $\text{NaHCO}_3$ ), sodium hydroxide (NaOH), sodium chloride (NaCl), and urea ( $\text{NH}_2\text{CONH}_2$ ) were obtained from Merck (Darmstadt, Germany); dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ) and N,N-dimethyl formamide (DMF,  $\text{C}_3\text{H}_7\text{NO}$ ) from Carlo Erba (Val de Reuil, France); and EDTA disodium salt ( $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8$ ), sodium sulfate decahydrate ( $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ ), SWCNT-COOH (carbon purity >90%, 1.0–3.0 atom% carboxylic acid

functionalization, bundle dimensions of 4–5 nm in diameter and 0.5–1.5 µm in length), potassium thiocyanate (KSCN), and Trizma-HCl (NH<sub>2</sub>C(CH<sub>2</sub>OH)<sub>3</sub> · HCl) from Sigma-Aldrich (Munich, Germany). Buffers, including 0.5 M sodium acetate (ABS, pH 4.8), 0.5 M Tris (TBS, pH 7.0), and 0.5 M Tris-EDTA (TE, pH 8.0), each containing 20 mM NaCl, were prepared using ultrapure water (UPW).

Artificial saliva samples were prepared with UPW according to the following formula: 125.6 mg/L NaCl, 963.9 mg/L KCl, 189.2 mg/mL KSCN, 654.5 mg/mL KH<sub>2</sub>PO<sub>4</sub>, 200.0 mg/mL urea, 763.2 mg/L NaSO<sub>4</sub>·10H<sub>2</sub>O, 178.0 mg/L, 227.8 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O and 630.8 mg/mL NaHCO<sub>3</sub> (Gao, Gao, Xu, & Yu, 2015).

Oligonucleotides

DNA oligonucleotide stock solutions were prepared in TE buffer and stored at –20 °C until use. Two probe types were designed: guanine-containing

probes (PG-SON for *S. sonnei* and PG-MALT for *S. maltophilia*) for surface studies and inosine-based probes (PI-SON and PI-MALT) to eliminate background guanine signals prior to hybridization. For *S. sonnei*, probes targeted a methylase gene region (NC\_007384.1; positions 1,664,092–1,664,150) (Ranjbar, Behzadi, Najafi, & Roudi, 2017), while for *S. maltophilia*, a 16S rRNA gene region was selected (Hansen et al., 2014), ensuring high sequence specificity. Target oligonucleotides (TAR-SON and TAR-MALT) fully complementary to the probes were used for hybridization, while three-base mismatch (MM-SON, MM-MALT) and non-complementary (NC) sequences served as controls for selectivity evaluation.

To ensure rigorous selectivity assessment, the non-complementary (NC) sequence was verified via BLAST, confirming no significant homology with the probes or target sequences. Synthetic oligonucleotides are listed in Table 1.

Table 1. DNA oligonucleotides used in the study for the detection of *S. sonnei* and *S. maltophilia*

|                            | <i>S. sonnei</i>                                                                                                             | <i>S. maltophilia</i>                                                                                                    |
|----------------------------|------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| Amino-linked guanine-probe | <b>PG-SON: (GC:42%, T<sub>m</sub>:52 °C)</b><br>5'-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub> -GGCATTAGTACAGATTCTGA-3' | <b>PG-MALT: (GC:60%, T<sub>m</sub>:40 °C)</b><br>5'-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub> -CGCCATGGATGTTCC-3' |
| Amino-linked inosine-probe | <b>PI-SON: (GC:16%, T<sub>m</sub>:52 °C)</b><br>5'-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub> -IICATTATACAIATTCTCIA-3' | <b>PI-MALT: (GC:33%, T<sub>m</sub>:48 °C)</b><br>5'-(CH <sub>2</sub> ) <sub>6</sub> -NH <sub>2</sub> -CICCATIATITTTCC-3' |
| Targets                    | <b>TAR-SON: (GC:42%, T<sub>m</sub>: 52°C)</b><br>5'-TCGAATCTGTACTAATGCC-3'                                                   | <b>TAR-MALT: (GC:60%, T<sub>m</sub>: 56°C)</b><br>5'-GGAACATCCATGGCG-3'                                                  |
| Three-base mismatches      | <b>MM-SON:(GC:47%, T<sub>m</sub>: 55°C)</b><br>5'-TCAAATCTGGACTAACGCC-3'                                                     | <b>MM-MALT: (GC:47%, T<sub>m</sub>: 52°C)</b><br>5'-GTAACATCGATGGAG-3'                                                   |
| Non-complementaries        | <b>NC: (GC:40%, T<sub>m</sub>: 53°C)</b><br>5'-CCCGGGGTAATTTCTTTTAA-3'                                                       | <b>NC: (GC:40%, T<sub>m</sub>: 53°C)</b><br>5'-CCCGGGGTAATTTCTTTTAA-3'                                                   |

Electrode functionalization with SWCNT-COOH and bacteria-specific DNA probe immobilization

SWCNT-COOH (0.5–3 µg/mL) suspensions were prepared in DMF by sonication for 1 h at room temperature. Unmodified PGEs were modified by passive adsorption in which 110 µL of the SWCNT-COOH suspension was deposited onto the electrode surface and incubated for 1 h, followed by drying for 15 min at room temperature; DMF-treated electrodes served

as controls (Erdem, Karadeniz, & Caliskan, 2009). At each nanomaterial concentration, 100 µg/mL of probe was immobilized, and the resulting guanine oxidation signals were compared. The concentration yielding the highest guanine signal was selected as optimal for probe immobilization. Probe solutions were diluted with ABS. SWCNT-COOH-modified electrodes were immersed in 110 µL of probe solution for 1 h, then rinsed with ABS. Following nanomaterial coating, probe concentrations (5–80 µg/mL) were evaluated,

and 20 µg/mL was selected based on the highest and most reproducible guanine signal. Guanine-containing probes were used to assess surface saturation via guanine oxidation signals. Inosine-substituted probes showed no signal before hybridization but generated a guanine oxidation peak ( $\sim +1.0$  V) upon binding to target sequences (Subak & Ozkan-Ariksoysal, 2018).

### Probe–target hybridization

Target solutions were diluted in TBS (pH 7.0, containing 20 mM NaCl to maintain controlled ionic strength), and 110 µL aliquots of these target solutions were applied for hybridization by incubating with SWCNT-COOH and probes modified electrodes for 1 h at room temperature. Electrodes were then rinsed with TBS.

### Selectivity assessment

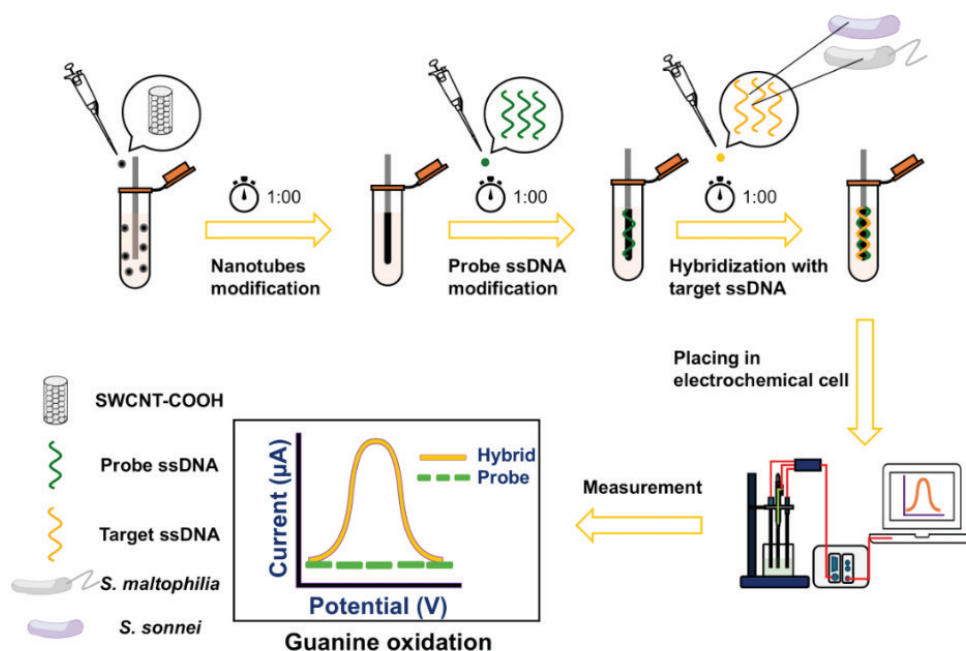
To demonstrate the biosensor selectivity biosensors, hybridization was performed not only with specific target oligonucleotides but also with MM, NC oligonucleotides, and mixtures of target oligonu-

cleotides, and NC oligonucleotide (MIX-SON (TAR-SON + NC) and MIX-MALT (TAR-MALT + NC)) using the same procedure.

### Electrochemical measurements

DPV measurements were conducted in ABS (pH 4.8, containing 20 mM NaCl to maintain controlled ionic strength) within the range of +0.4 V to +1.4 V, with a scan rate of 100 mV/s and a time interval of 0.5 s (step potential: 8 mV, modulation amplitude: 80 mV) (Kaya et al., 2024). CV measurements were performed in a 2.5 mM  $K_{3/4}[Fe(CN)_6]$  solution prepared in 0.1 M KCl, within the range of -0.05 V to +0.6 V, with a scan rate of 50 mV/s and a time interval of 0.05 s.

EIS measurements were conducted in a 2.5 mM  $K_{3/4}[Fe(CN)_6]$  solution, over a frequency range of  $10^5$  Hz to  $10^{-1}$  Hz, with an amplitude of 0.01 and decade frequency intervals (Isbilir et al., 2025). Figure 1. presents a schematic illustration of the working principle of the biosensor for the selective detection of *S. sonnei* and *S. maltophilia*.



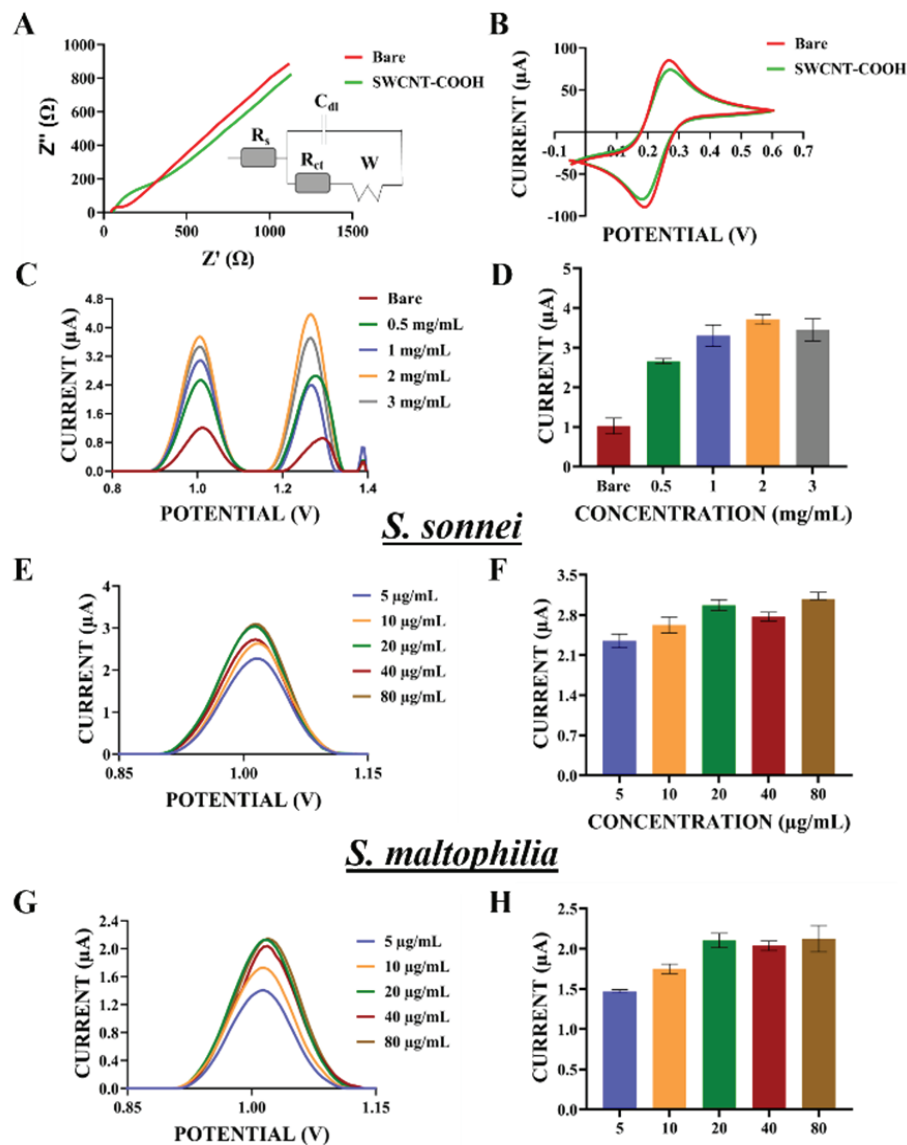
**Figure 1.** Schematic of the signal-on electrochemical DNA biosensor. SWCNT-COOH–modified electrodes were functionalized with a guanine-free ssDNA probe. Hybridization with complementary target ssDNA formed guanine-containing dsDNA, producing an increased guanine oxidation signal measured by DPV for selective detection

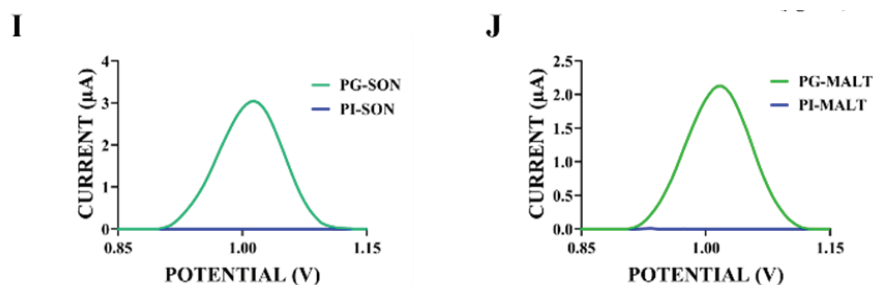
RESULTS AND DISCUSSION

Functionalization of PGEs with SWCNT-COOH and probe DNA

The performance of electrochemical DNA biosensors depends on the physicochemical properties of the electrode interface, which influence electron transfer and probe accessibility. Therefore, stable and site-specific immobilization of ssDNA probes is essential for efficient hybridization and high sensitivity. Chemical conjugation strategies, particularly carboxyl-amine coupling on nanostructured provides robustness and reproducibility (Rashid & Yusof, 2017).

In this study, SWCNT-COOH were used to increase the electrode surface area and provide reactive sites for ssDNA probe immobilization. Electrodes were first modified with SWCNT-COOH, followed by immobilization of sequence-specific oligonucleotides as described in the Materials and Methods section. Figure 2. shows the conductivity differences between unmodified and SWCNT-COOH-modified electrodes, along with voltammograms and histograms illustrating guanine oxidation currents after PG-SON and PG-MALT immobilization at different concentrations for the *S. sonnei* and *S. maltophilia* biosensors.





**Figure 2.** Electrochemical impedance spectra (Inset: Schematic of the Randles equivalent circuit used for electrochemical impedance analysis) (A) and cyclic voltammogram (B) of bare and 2 mg/mL SWCNT-COOH coated electrodes in 2.5 mM  $K_{3/4}[Fe(CN)_6]$  solution. Differential pulse voltammogram (C) and histogram (D) of guanine oxidation peak currents of bare electrodes and electrodes modified with 0.5, 1, 2, and 3 mg/mL SWCNT-COOH after coating with 100  $\mu$ g/mL of PG-SON. Differential pulse voltammogram (E) and corresponding histogram (F) of guanine oxidation currents for the electrodes modified with 2 mg/mL SWCNT-COOH and coated with 5, 10, 20, 40, and 80  $\mu$ g/mL PG-SON. Differential pulse voltammogram (G) and corresponding histogram (H) of guanine oxidation currents for the electrodes modified with 2 mg/mL SWCNT-COOH and coated with 5, 10, 20, 40, and 80  $\mu$ g/mL PG-MALT. Differential pulse voltammograms of (I) PG-SON- and PI-SON-modified electrodes and (J) PG-MALT- and PI-MALT-modified electrodes, demonstrating the elimination of probe-derived guanine oxidation signals following inosine substitution ( $n=5$ )

Figure 2A. shows a clear difference in impedance between bare and SWCNT-COOH-coated (2 mg/mL) electrodes. The impedance spectra of both electrodes were fitted using an RCRW equivalent circuit. The solution resistance ( $R_s$ ) was 47  $\Omega$  for bare and 55  $\Omega$  for SWCNT-COOH-modified electrodes. The capacitance ( $C$ ) decreased from  $1 \times 10^6$  to  $2 \times 10^5$  upon modification, while the Warburg impedance ( $W$ ) remained unchanged at 0.0009 for both surfaces.

The  $R_{ct}$  increased from  $67 \pm 26 \Omega$  for bare electrodes to  $139 \pm 6 \Omega$  after SWCNT-COOH modification, approximately a twofold rise. This increase is consistent with the negatively charged surface of SWCNT-COOH due to deprotonated carboxyl groups (Hassanpour & Petr, 2023). The higher  $R_{ct}$  of SWCNT-COOH-modified electrodes is attributed to electrostatic repulsion between the negatively charged ferri/ferrocyanide redox couple ( $Fe(CN)_6^{3-/4-}$ ) and deprotonated carboxyl groups on SWCNT-COOH, which hinders electron transfer at the electrode interface (Chiticaru, Pitan, & Ioniță, 2022). This behavior may also be influenced by pH-dependent surface charge, partial surface blocking, and nanomaterial film morphology, which collectively contribute to the increased charge transfer resis-

tance after surface modification. The SWCNT-COOH coating significantly modified the electrode interface and improved measurement reliability, reducing the RSD from 16% to 4%. This enhanced reproducibility reflects effective surface coverage and a homogeneous SWCNT-COOH layer, enabling more consistent electron transfer pathways for the redox probe.

CV analysis (Figure 2B.) showed that  $Fe(CN)_6^{3-/4-}$  redox currents decreased after SWCNT-COOH modification compared to bare electrodes. The mean peak current decreased from  $93.5 \pm 15.1 \mu A$  to  $80 \pm 3.2 \mu A$ , consistent with EIS results. This decrease confirms that the negatively charged SWCNT-COOH layer increases electron transfer resistance and forms a stable interface expected to influence DNA hybridization. After SWCNT-COOH functionalization of PGEs, concentrations (0.5–3 mg/mL) were optimized to achieve effective surface coverage and interface formation.

The SWCNT-COOH concentration range (0.5–3 mg/mL) was selected based on previous studies reporting effective electrode functionalization at similar loadings. Concentrations above 3 mg/mL caused nanotube agglomeration and non-uniform film for-

mation, potentially impairing electron transfer. Immobilization performance was evaluated using guanine oxidation signals following attachment of 100  $\mu\text{g/mL}$  amine-modified PG-SON probes (Erdem et al., 2009) (Figure 2C-D.). Lower SWCNT-COOH concentrations likely provided insufficient carboxyl sites for efficient probe immobilization, whereas higher concentrations may have produced thicker aggregated films with reduced conductivity and hindered electron transfer. As shown in Figure 2C-D., SWCNT-COOH modification significantly enhanced probe immobilization compared to bare PGEs through stable carboxyl-amine linkages, resulting in improved electron transfer and surface reactivity (Chen et al., 2016). Among the tested concentrations, 2 mg/mL SWCNT-COOH produced the highest and most reproducible guanine oxidation signal, with nearly fourfold enhancement relative to unmodified electrodes. A similar trend was observed for PG-MALT (data not shown). Therefore, 2 mg/mL was selected as the optimal SWCNT-COOH concentration for subsequent experiments.

A review of the literature indicates that the carboxylic acid groups on SWCNT-COOH are predominantly protonated at around pH 4.0, whereas they become fully deprotonated at alkaline conditions (approximately pH 10.0). Within the intermediate pH range (around pH 6.0), these groups have been reported to exist in a partially deprotonated state (Jones, Kim, Han, & Meyyappan, 2021). Examination of other studies indicates that, in the pH range 4–6, amino groups predominantly exist in their protonated form ( $-\text{NH}_3^+$ ) while carboxylic acid groups are deprotonated ( $-\text{COO}^-$ ), facilitating robust electrostatic interactions (Martínez, Godínez, & Robles, 2023). In the present study, the amino-modified ssDNA probes were immobilized onto SWCNT-COOH-functionalized electrodes at pH 4.8, where deprotonated surface carboxylates ( $-\text{COO}^-$ ) interact electrostatically with protonated amines on the probes ( $-\text{NH}_3^+$ ), thereby contributing to stable and specific surface attachment.

In addition to electrostatic interactions,  $\pi$ - $\pi$  stacking between DNA nucleobases and the graphitic SWCNT surface contributes to probe immobilization and stabilizes the attachment of ssDNA probes on SWCNT-COOH-modified electrodes (Lee et al., 2024; Roxbury, Jagota, & Mittal, 2013). Accordingly, EDC/NHS coupling chemistry was not employed in the present study, as the immobilization strategy was intentionally designed to take advantage of the intrinsic electrostatic and  $\pi$ - $\pi$  interactions between amino-modified ssDNA probes and SWCNT-COOH surfaces. This approach enabled a simpler and faster probe immobilization procedure while avoiding additional chemical activation and washing steps associated with carbodiimide coupling, thereby preserving the straightforward and cost-effective nature of the proposed biosensing platform.

Figure 2E. shows the differential pulse voltammograms recorded in ABS after immobilization of different probe concentrations (5–80  $\mu\text{g/mL}$ ) onto 2 mg/mL SWCNT-COOH-modified electrodes for electrochemical detection of *S. sonnei* (Li, Karadeniz, Canavar, & Erdem, 2012).

Figure 2F. presents the relationship between the average guanine oxidation current and PG-SON probe concentration. Among the tested concentrations, 20  $\mu\text{g/mL}$  produced the highest and most reproducible signal, showing a markedly higher response than 40  $\mu\text{g/mL}$  and a comparable response to 80  $\mu\text{g/mL}$ . Therefore, 20  $\mu\text{g/mL}$  was selected as the optimal probe concentration. This behavior likely reflects the balance between probe density and target accessibility at the electrode surface: low concentrations provide insufficient hybridization sites, whereas excessive probe loading may induce steric hindrance and reduce hybridization efficiency.

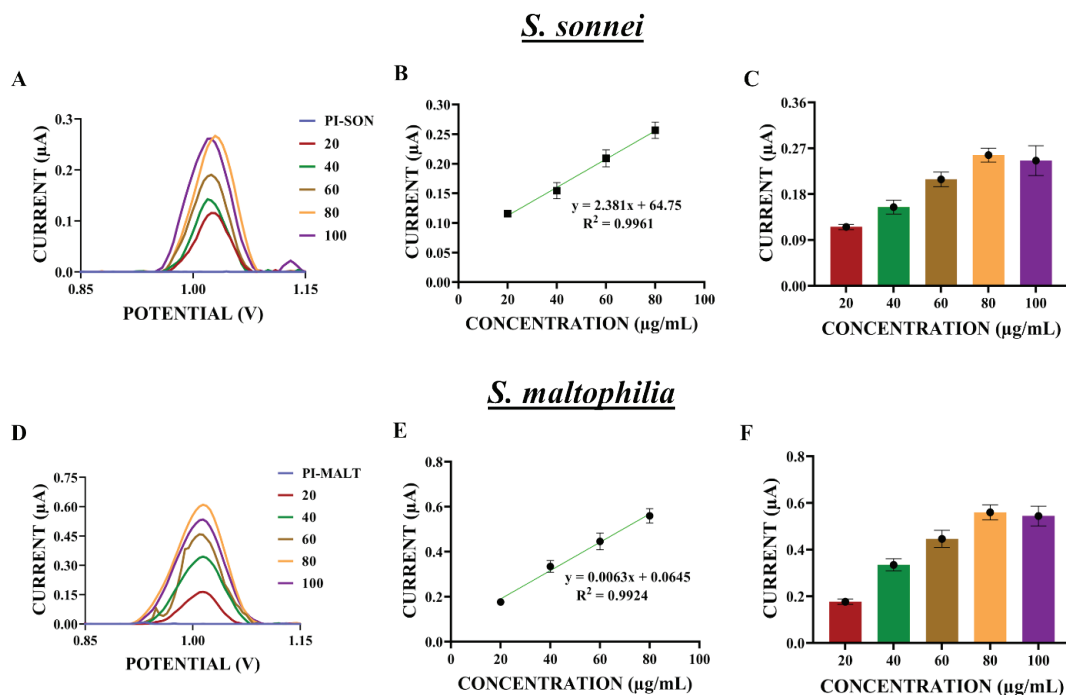
Figure 2G. shows the DPV responses recorded in ABS after immobilization of PG-MALT probes (5–80  $\mu\text{g/mL}$ ) onto 2 mg/mL SWCNT-COOH-modified electrodes for electrochemical sensing of *S. maltophilia*. As shown in Figure 2H., the guanine oxidation sig-

nal increased with probe concentration and reached a plateau at 20  $\mu\text{g/mL}$ , indicating optimal probe coverage and hybridization density. Like PG-SON, low probe concentrations resulted in limited surface coverage, whereas excessive loading may have caused steric hindrance and reduced hybridization efficiency. Therefore, 20  $\mu\text{g/mL}$  was selected as the optimal PG-MALT probe concentration. As shown in Figures 2I. and 2J., pronounced guanine oxidation peaks at approximately +1.00 V were observed for PG-SON and PG-MALT at the optimized concentration of 20  $\mu\text{g/mL}$ , whereas no such signals were detected for PI-SON and PI-MALT prepared and immobilized under identical conditions. This result confirms that inosine substitution effectively suppresses the background guanine signal before hybridization, allowing selective detection of target-derived guanine oxidation.

### Hybridization between probe DNA and target

Inosine-based probes were employed to eliminate background signals originating from guanine oxidation within the probe sequence. Since inosine is electrochemically inactive around +1.0 V, no signal is observed prior to hybridization. Upon hybridization with complementary targets containing guanine bases, a distinct oxidation peak appears near +1.0 V, enabling direct confirmation of target binding. Moreover, the peak current increases proportionally with the amount of hybridized target, allowing semi-quantitative evaluation of hybridization efficiency. This label-free strategy simplifies the assay while maintaining high sensitivity and specificity.

Figure 3. presents guanine oxidation currents obtained by DPV after hybridization of SWCNT-COOH/PI-SON and SWCNT-COOH/PI-MALT electrodes with their complementary targets (TAR-SON and TAR-MALT) over the concentration range of 20–100  $\mu\text{g/mL}$ .



**Figure 3.** Differential pulse voltammograms (A) obtained between +0.85 V and +1.15 V for hybridization with varying concentrations of TAR-SON (20–100  $\mu\text{g/mL}$ ) in the presence of 20  $\mu\text{g/mL}$  PI-SIN. Calibration curve (B) and histogram (C) showing the average guanine oxidation currents corresponding to TAR-SON concentrations. Differential pulse voltammogram (D) obtained between +0.85 V and +1.15 V for hybridization with varying concentrations of TAR-MALT (20–100  $\mu\text{g/mL}$ ) in the presence of 20  $\mu\text{g/mL}$  PI-MALT. Calibration curve (E) and histogram (F) showing the average guanine oxidation currents corresponding to TAR-MALT concentrations ( $n=5$ )

For *S. sonnei* detection, PGEs were modified with 2 mg/mL SWCNT-COOH and 20 µg/mL inosine-substituted PI-SON probes, followed by hybridization with TAR-SON targets (20–100 µg/mL) for 1 h. As shown in Figure 3A., no guanine oxidation peak was observed before hybridization due to the electrochemical inactivity of inosine, while a minor adenine peak appeared around +1.15 V; however, since the detection strategy is based on guanine oxidation, these minor adenine peaks were not analyzed. After hybridization with guanine-containing targets, a distinct oxidation signal emerged and increased with TAR-SON concentration.

The calibration plots in Figure 3B–C. show a linear response in the 20–80 µg/mL range, from which LOD and LOQ values were calculated using the  $3\sigma/s$  and  $10\sigma/s$  methods, where  $\sigma$  is the standard deviation of the intercept and  $s$  is the slope of the calibration curve. The LOD and LOQ for *S. sonnei* were 6.1 µg/mL (1.05 µM) and 20.30 µg/mL (3.51 µM), respectively. Compared with previous studies, reported detection limits include  $10^3$  cells/mL for a fluorescent aptasensor (Song et al., 2017), 37 CFU/mL (colorimetric) and 7 CFU/mL (SERS) for an aptasensor (He et al., 2024), and 1.7–24.7 CFU/50 mL for PCR-based detection in water samples (Hsu, Wang, Chen, Lu, & Chen, 2007). A direct comparison between DNA concentrations and CFU-based thresholds is not strictly appropriate due to differences in measurement units; however, these studies provide useful context for required analytical sensitivity in *S. sonnei* detection. In this study, the biosensor achieved a detection limit of 1.05 µM. Although this sensitivity is lower than that reported for amplification-based or signal-enhanced platforms, the proposed strategy enables direct, label-free detection of target DNA without enzymatic amplification or complex assay procedures. The incorporation of SWCNT-COOH improved the electrochemical response and facilitated reliable hybridization monitoring; however, the analytical sensitivity remains inherently constrained by the guanine oxidation-based detection mechanism. Therefore,

the developed platform may be particularly suitable as a simple post-amplification readout tool or for applications where rapid and straightforward nucleic acid analysis is prioritized over ultra-trace detection. It should be noted that PCR-based assays and other highly sensitive molecular diagnostic techniques benefit from target amplification mechanisms that are fundamentally different from the direct hybridization approach employed in this study. Consequently, the present biosensor is not intended to replace ultra-sensitive diagnostic platforms but rather to provide a rapid, straightforward, and cost-effective alternative for target DNA monitoring. Where lower detection limits are required, the proposed system may also be integrated as a post-amplification electrochemical readout following nucleic acid enrichment procedures. As shown in Figure 3C., the guanine oxidation current at 100 µg/mL TAR-SON decreased and showed lower reproducibility compared to 80 µg/mL, indicating surface saturation at 80 µg/mL. Therefore, 80 µg/mL TAR-SON was selected for subsequent experiments.

For *S. maltophilia* detection, optimized SWCNT-COOH (2 mg/mL) and PI-MALT probes (20 µg/mL) were hybridized with TAR-MALT (20–100 µg/mL) for 1 h. The voltammogram and calibration curve (Figure 3D–E.) show a linear increase in guanine oxidation current within 20–80 µg/mL, from which the LOD and LOQ were calculated as 8.40 µg/mL (1.82 µM) and 27.99 µg/mL (6.08 µM), respectively. Compared with previous approaches, fluorescent in situ hybridization reported an LOD of  $10^4$  CFU/mL (Hansen et al., 2014), while PCR-based detection for *S. maltophilia* yielded Ct values of 19.58 and 15.9 for different isolates (Gallo, Ramos, Ferreira, & Oliveira, 2013), where Ct reflects the cycle number at which fluorescence surpasses background levels, it does not provide precise bacterial quantification, limiting its utility for accurate infection staging and treatment planning.

These comparisons provide a qualitative view of analytical sensitivity rather than a direct equivalence

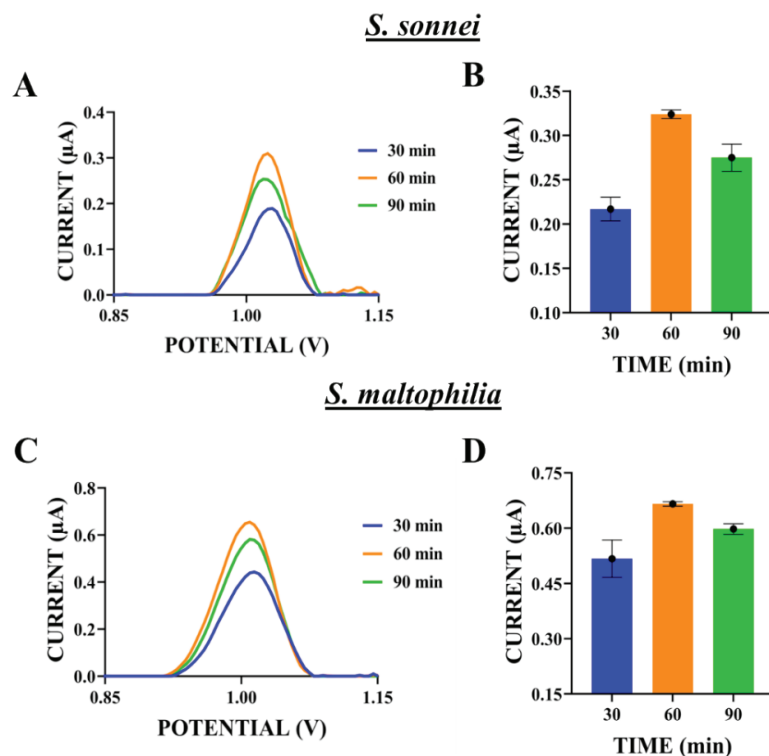
between DNA concentration and bacterial count, highlighting the capability of the developed biosensor for target DNA detection. Other electrochemical DNA biosensors have reported high performance, including a sandwich-type genosensor for *Vibrio cholerae* (LOD:  $1.25 \times 10^{-18}$  M) (Futra, Tan, Lee, Lertanantawong, & Heng, 2023), a CdS nanoparticle-based genosensor for *E. coli* O157:H7 (LOD:  $1.97 \times 10^{-14}$  M) (Abdalthai et al., 2015). The present SWCNT-COOH biosensor exhibited LODs in the low micromolar range for *S. sonnei* (1.05  $\mu$ M) and *S. maltophilia* (1.82  $\mu$ M) in buffer. Although these values are higher than those reported for amplification-based, nanoparticle-assisted, or multi-step electrochemical biosensing platforms, the present system relies on direct label-free guanine oxidation without enzymatic target amplification or additional redox indicators. The use of SWCNT-COOH as a nanostructured electrode modifier enhanced electron transfer and improved signal stability; nevertheless, the overall sensitivity remained in the low micromolar range. Consequently, the developed approach prioritizes simplicity, reproducibility, and operational convenience rather than ultra-sensitive detection at trace concentrations.

As shown in Figure 3F, guanine oxidation currents after TAR-MALT hybridization plateaued at 80  $\mu$ g/mL, consistent with TAR-SON saturation. Therefore, 80  $\mu$ g/mL TAR-MALT was used in subsequent assays to ensure optimal probe–target interaction.

The validation parameters of the developed biosensor include a linear detection range of 20–80  $\mu$ g/mL for both *S. sonnei* and *S. maltophilia*, with LOD/LOQ values of 1.05  $\mu$ M / 3.51  $\mu$ M and 1.82  $\mu$ M / 6.08  $\mu$ M, respectively. The system showed good precision, with repeatability (RSD%) values of 4.76% and 3.53%, and reproducibility (RSD%) values of 10.76% and 10.43% for *S. sonnei* and *S. maltophilia*, respectively.

The repeatability and reproducibility of the nano-material-modified electrodes were evaluated through intra-day and inter-day measurements. The intra-day RSD values were 4.76% and 3.53%, whereas the inter-day RSD values were 10.76% and 10.43% for the detection of *S. sonnei* and *S. maltophilia* target DNA, respectively, demonstrating good analytical precision of the developed platform. Long-term stability is particularly important for portable applications, where electrode robustness and shelf-life are critical. Therefore, future work will focus on disposable screen-printed electrodes to assess operational stability under real-world conditions.

After optimization, the effect of hybridization time was evaluated. Figure 4. shows DPV of guanine oxidation currents for SWCNT-COOH/PI-SON and SWCNT-COOH/PI-MALT electrodes hybridized with their respective targets at 30, 60, and 90 min under optimal conditions.

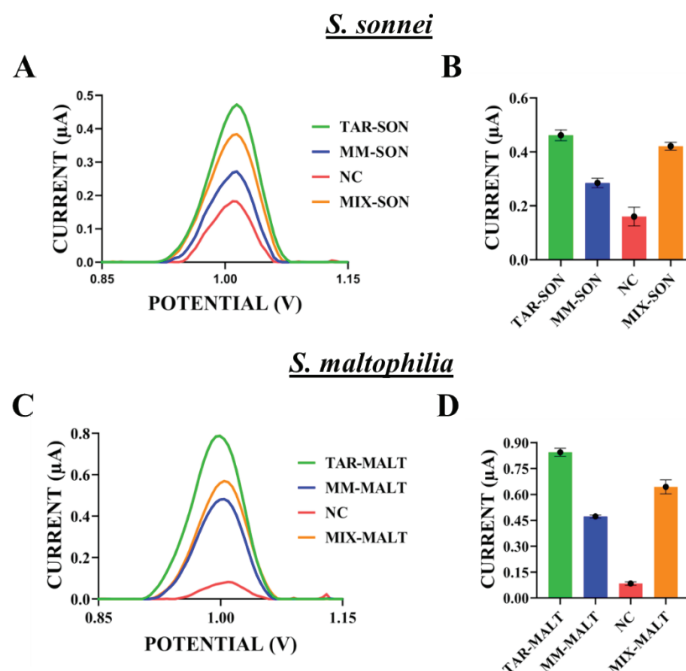


**Figure 4.** Differential pulse voltammograms (A) obtained between +0.85 V and +1.15 V after hybridization for 30, 60, and 90 min using 80  $\mu\text{g/mL}$  TAR-SON and 20  $\mu\text{g/mL}$  PI-SON. Histogram (B) shows the average guanine oxidation currents corresponding to each hybridization time. Differential pulse voltammogram (C) obtained between +0.85 V and +1.15 V after hybridization for 30, 60, and 90 min using 80  $\mu\text{g/mL}$  TAR-MALT and 20  $\mu\text{g/mL}$  PI-MALT. Histogram (D) shows the average guanine oxidation currents corresponding to each hybridization times ( $n=5$ )

For *S. sonnei*, SWCNT-COOH/PI-SON-modified electrodes were hybridized with 80  $\mu\text{g/mL}$  TAR-SON for 30, 60, and 90 min, and the highest guanine oxidation current was obtained at 60 min (Figure 4A–B.), which was selected as optimal. Similarly, electrodes for *S. maltophilia* (SWCNT-COOH/PI-MALT) showed the maximum response at 60 min under the same conditions (Figure 4C–D.), and this time was used for further experiments. Although 60 min provided the best signal, shorter hybridization times may be achievable by improving mass transport, using microfluidic systems, or optimizing probe surface density.

#### Selectivity of the developed DNA biosensors

Selectivity is a key validation parameter in electrochemical DNA biosensors, reflecting the sensor's ability to distinguish target sequences from closely related sequences with minor mismatches or from non-complementary strands, ensuring reliable analytical and diagnostic performance. Figure 5. shows DPV of guanine oxidation currents after hybridization of PI-SON and PI-MALT probes with target, 3-base mismatch, non-complementary, and target/non-complementary mixed (MIX-1, MIX-2) sequences for *S. sonnei* and *S. maltophilia* detection.



**Figure 5.** Differential pulse voltammograms (A) of hybridization with 20  $\mu\text{g/mL}$  PI-SON and different targets: 80  $\mu\text{g/mL}$  TAR-SON, MM-SON, NC, and MIX-SON (TAR-SON + NC). Histogram (B) shows the average guanine oxidation currents for each target ( $n=5$ ). Differential pulse voltammograms (C) of hybridization with 20  $\mu\text{g/mL}$  PI-MALT and different targets: 80  $\mu\text{g/mL}$  TAR-MALT, MM-MALT, NC and MIX-MALT (TAR-MALT + NC). Histogram (D) shows the average guanine oxidation currents for each target ( $n=5$ )

Selectivity for *S. sonnei* was evaluated by hybridizing SWCNT-COOH/PI-SON-modified PGEs with 80  $\mu\text{g/mL}$  TAR-SON, MM-SON, NC, and MIX-SON (TAR-SON + NC) for 1 h, followed by washing with TBS. As shown in Figure 5A–B., TAR-SON produced the highest guanine oxidation current, with a comparable response for MIX-SON, while MM-SON showed a moderate signal and NC the lowest current. The results demonstrate strong selectivity toward *S. sonnei* target DNA, with the 3-base mismatch showing a reduced signal compared to the fully complementary target but higher than the non-complementary sequence, indicating precise discrimination. These trends confirm sequence-specific selectivity and are consistent with hybridization-based DNA biosensors. The observed selectivity is attributed to the structural differences of the formed duplexes on the SWCNT-COOH interface.

As mentioned earlier, probe immobilization is stabilized by  $\pi$ – $\pi$  interactions between nucleobases and

SWCNT-COOH and electrostatic attraction between probe  $-\text{NH}_3^+$  groups and SWCNT-COOH  $-\text{COO}^-$  groups. This organized interface promotes hybridization with fully complementary strands, forming stable duplexes via Watson–Crick pairing and base stacking, resulting in high guanine signals. In contrast, 3-base mismatch strands disrupt hydrogen bonding and stacking, producing weaker hybrids, while non-complementary sequences do not form duplexes. These differences explain the clear separation between TAR, MM, and NC signals. Another indicator of selectivity is target discrimination in mixed samples. A MIX-SON solution (TAR-SON + NC) was tested (Figure 5A–B.), yielding a guanine oxidation current corresponding to 91% of TAR-SON alone, demonstrating strong selectivity even in the presence of non-complementary strands. This behavior is attributed to competitive binding, where TAR exhibits higher affinity due to complete Watson–Crick pairing and favorable base stacking, allowing it to outcompete NC at

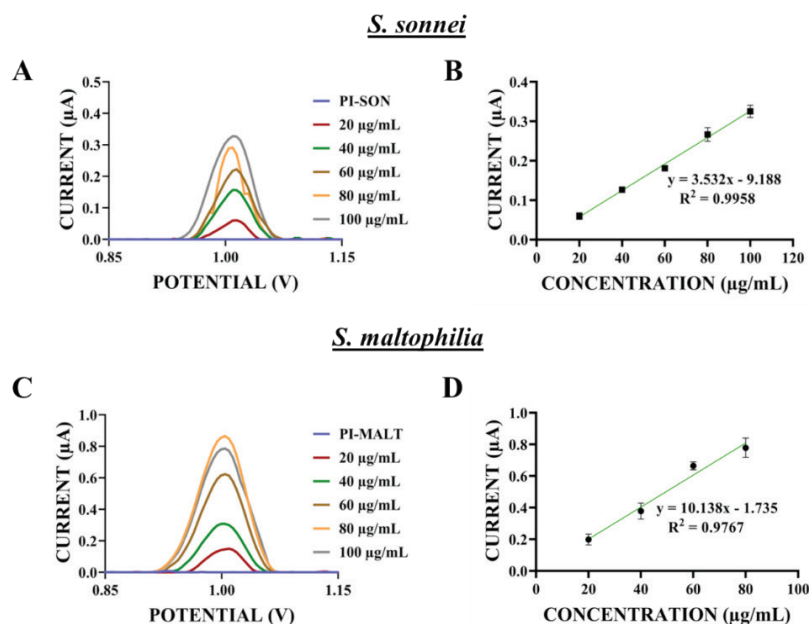
the probe interface. This strong selectivity toward *S. sonnei* is a key analytical advantage of the developed biosensor. The platform enables reliable discrimination between fully complementary, mismatched, and non-complementary sequences, overcoming a common limitation of nucleic-acid-based detection methods. This performance enhances the system's analytical robustness and enables precise sequence-level recognition. Selectivity for *S. maltophilia* was evaluated by hybridizing SWCNT-COOH/PI-MALT-modified PGEs with 80 µg/mL TAR-MALT, MM-MALT, NC, and MIX-MALT (TAR-MALT + NC) for 1 h, followed by TBS washing to remove non-specifically bound oligonucleotides.

As shown in Figure 5C–D, TAR-MALT produced the highest guanine oxidation current, while MIX-MALT generated a response corresponding to approximately 76% of the signal obtained for TAR-MALT alone. NC yielded minimal signal, whereas MM-MALT produced an intermediate response, consistent with partial hybridization. Although the relative decrease observed for the mixed sample was more pronounced for *S. maltophilia* than for *S. sonnei*, the developed biosensor still maintained clear discrimination between fully complementary, mismatched, and non-complementary sequences. This behavior may be attributed to the increased complexity of

the mixed environment, where limited interactions involving adenine–thymine, inosine–cytosine, and occasionally tolerated non-canonical base pairings (e.g., cytosine–thymine), as reported in the literature (Zhang, Tam, Wang, & Szostak, 2016), could contribute to a partial reduction in the effective hybridization efficiency of the target sequence. Nevertheless, the platform effectively distinguishes fully complementary targets from mismatched and non-complementary sequences, even in mixed samples, confirming its high sequence specificity. Future studies will evaluate genomic DNA from related Gram-negative bacteria to further assess diagnostic robustness under clinically relevant conditions.

#### **The use of developed biosensors in artificial samples**

*S. maltophilia* has been reported in the oropharynx and sputum, suggesting saliva as a potential reservoir. Likewise, *S. sonnei*, transmitted via the fecal–oral route and direct contact, may also be present in saliva, supporting non-invasive diagnostics. Figure 6 shows guanine oxidation currents for TAR-SON and TAR-MALT prepared in artificial saliva at different concentrations and hybridized with PI-SON and PI-MALT on SWCNT-COOH-modified electrodes, demonstrating effective performance in a biologically relevant matrix.



**Figure 6.** Differential pulse voltammograms (A) of hybridization using 20–100 µg/mL TAR-SON prepared in artificial saliva and 20 µg/mL PI-SON. Calibration curve (B) shows the average guanine oxidation currents corresponding TAR-SON concentrations. Differential pulse voltammogram (C) of hybridization using 20–100 µg/mL TAR-MALT prepared in artificial saliva and 20 µg/mL PI-MALT. Calibration curve (D) shows the average guanine oxidation currents corresponding TAR-MALT concentrations ( $n=5$ )

To demonstrate diagnostic applicability, *S. sonnei* and *S. maltophilia* target DNAs were analyzed in artificial saliva matrices. Target oligonucleotides (20–100 µg/mL) were prepared in artificial saliva diluted 1:1 with TE buffer to ensure suitable pH and ionic strength for hybridization while preserving matrix effects. As shown in Figure 6A., the guanine oxidation signal for *S. sonnei* increased in a clear concentration-dependent manner.

From the calibration plot in Figure 6B., the LOD and LOQ were determined as 8.52 µg/mL (1.48 µM) and 28.41 µg/mL (4.93 µM), respectively. While the LOD for *S. sonnei* in buffer was 1.05 µM, it increased slightly to 1.48 µM in artificial saliva, indicating that the nanomaterial-based biosensor maintains high analytical performance in complex matrices. The close agreement between buffer and saliva responses confirms good matrix compatibility and minimal interference from biological components, highlighting the platform's diagnostic reliability and translational

potential for pathogen detection. As shown in Figure 6C., the guanine oxidation signal increased with *S. maltophilia* target concentration up to 80 µg/mL. From the calibration curve (Figure 6D.), the LOD and LOQ in artificial saliva were 13.76 µg/mL (2.99 µM) and 45.86 µg/mL (9.97 µM), respectively, compared to a lower LOD of 1.82 µM in buffer. Consistent with *S. sonnei*, the slightly higher LOD in saliva confirms that the biosensor maintains sensitive and reliable detection in clinically relevant matrices, highlighting its diagnostic potential for real-world applications. These findings provide proof-of-concept for reliable performance in biological environments and support future validation with genomic DNA and clinical samples.

## CONCLUSION

This study reports the first electrochemical DNA biosensors specifically designed for the detection of *S. sonnei* and *S. maltophilia*, two clinically relevant pathogens. The incorporation of carboxyl-functionalized SWCNTs onto the electrode surface significantly im-

proved the DNA biosensors' reproducibility. Efficient immobilization of ssDNA probes onto the electrode surface was achieved in the DNA biosensor design through specific  $\text{-COOH/-NH}_2$  interactions between carboxyl-functionalized SWCNTs and amine-modified probes, ensuring stable and high-density probe attachment. Guanine-containing probes were utilized to fine-tune probe immobilization, with 20  $\mu\text{g/mL}$  established as the optimal concentration. In hybridization-based detection, the use of inosine-substituted probes enabled specific interaction with guanine-rich target sequences, producing a highly specific and readily discernible presence/absence signal, effectively captured through guanine oxidation by DPV, demonstrating the precision and reliability of the biosensing strategy. The biosensors exhibited a linear response over the 20–80  $\mu\text{g/mL}$  range, with LODs determined as 1.05  $\mu\text{M}$  for *S. sonnei* and 1.82  $\mu\text{M}$  for *S. maltophilia*. By enabling direct, label-free detection of target DNAs from *S. sonnei* and *S. maltophilia*, the proposed platform represents a proof-of-concept approach for rapid nucleic acid analysis. Although its sensitivity is lower than that of amplification-based methods, the assay eliminates enzymatic amplification steps, offering a simple, reproducible, and potentially more cost- and time-efficient alternative, with possible application as a post-amplification electrochemical readout when higher sensitivity is required.

Selectivity was also demonstrated, including successful differentiation of mismatched, non-complementary, and mixed sequences, underscoring the biosensor's ability to discriminate these pathogens with high specificity, thereby enabling precise and reliable pathogen identification in complex diagnostic settings. Detection was further validated in artificial saliva, with LODs of 1.48  $\mu\text{M}$  and 2.99  $\mu\text{M}$ , respectively; the close agreement with LODs obtained in buffer solution highlights that the developed biosensing platform preserves its analytical sensitivity and reliability even in complex biological matrices, underscoring its suitability for accurate pathogen detection under clinically relevant conditions. Future studies will focus on

extending this proof-of-concept platform to genomic DNA and clinical samples, as well as integrating the proposed strategy into disposable and portable electrochemical devices for point-of-care applications. In addition, the inosine-based signal-on approach may provide a versatile framework for the development of multiplex biosensing systems targeting multiple clinically relevant bacterial pathogens within a single analytical platform.

## ACKNOWLEDGEMENTS

This work was supported by the grant of Ege University Scientific Research Coordinatorship, Project Number of 24143.

## AUTHOR CONTRIBUTION STATEMENT

HOK: Methodology, Formal analysis, Investigation, Writing—Review & Editing. YT: Methodology, Formal analysis, Investigation. HK: Conceptualization, Formal analysis, Methodology, Validation, Writing—Review & Editing, Funding acquisition. SNT: Validation, Methodology, Formal analysis, Investigation, Writing—Review & Editing, Supervision.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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