

Article

Gold Nanoparticle-Enhanced Graphene Immunosensor for Rapid Detection of *Gardnerella vaginalis* in Clinical Diagnostics

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Abstract: The global market increasingly demands alternative rapid diagnostic tools, such as disposable biosensors, to meet the growing need for point-of-care clinical testing of infectious diseases. Bacterial vaginosis (BV), a common infection caused by *Gardnerella vaginalis*, requires efficient and accurate detection methods to improve patient outcomes and prevent complications. However, existing diagnostic approaches often lack sensitivity, specificity, or rapid response times, highlighting the need for innovative biosensing solutions. In response to this challenge, we developed a peptide-based electrochemical biosensor for the specific detection of *Gardnerella vaginalis*. The sensor was designed to achieve high sensitivity, selectivity, and stability, with detection performed through electrochemical techniques. Cyclic voltammetry (CV) was employed to monitor electron transfer kinetics at the electrode surface, while electrochemical impedance spectroscopy (EIS) provided insights into changes in resistance and capacitance during peptide binding. The sensor fabrication involved covalently bonding anti-*Gardnerella vaginalis* peptides to a gold nanoparticle (AuNP)-modified graphene electrode, significantly enhancing bioreceptor immobilization stability and increasing the surface area for target binding interactions. The incorporation of AuNPs improved signal amplification due to their high surface-to-volume ratio and excellent conductivity, leading to enhanced sensor performance. The biosensor demonstrated a low detection limit (LOD) of 0.02305 µg/mL, with a rapid response time of 5 min across various concentrations of the target *Gardnerella vaginalis* antigen. The results confirmed specific and selective binding to the pathogen marker, with minimal interference from non-target species, ensuring high accuracy. The combination of graphene, AuNPs, and peptide bioreceptors resulted in robust signal enhancement, making this biosensor a promising tool for fast and reliable point-of-care diagnostics in clinical settings.

Keywords: bacterial vaginosis; BV diagnostics; electrochemical immunosensors; *Gardnerella vaginalis* detection; rapid test



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1. Introduction

Gardnerella vaginalis is a Gram-variable bacterium primarily associated with bacterial vaginosis (BV), a common yet often asymptomatic vaginal infection in women. BV has been linked to numerous health issues, including increased susceptibility to sexually transmitted infections (STIs), preterm labor, and pelvic inflammatory disease. The accurate and timely detection of *G. vaginalis* is crucial for effective management and treatment, especially because untreated cases can lead to adverse reproductive and general health outcomes [1,2].

Traditional diagnostic techniques for *G. vaginalis*, including culture methods and PCR assays, while reliable, are often time-consuming and require laboratory resources. Hence, there is a strong clinical need for a rapid, sensitive, and cost-effective diagnostic tool to enable early detection of *G. vaginalis* in point-of-care settings [3–6].

In recent years, there has been growing interest in the development of rapid and sensitive diagnostic tools that can provide timely information for both patients and healthcare providers. Advances in electrochemical sensor technology present a promising avenue for the development of effective point-of-care diagnostic devices. Electrochemical sensors offer several advantages, including ease of use, real-time response times, low cost, and the potential for miniaturization [1,6].

The electrochemical rapid sensor for the detection of *Gardnerella vaginalis* aims to utilize specific biochemical interactions to detect the presence of this bacterium in vaginal samples. By employing various electrochemical techniques, including impedance spectroscopy and voltammetry, the sensor can achieve high sensitivity and specificity, allowing for the detection of *Gardnerella vaginalis* even at low concentrations. Electrochemical sensors are analytical devices that detect specific biological or chemical analytes by measuring electrical signals resulting from redox reactions or changes in charge transfer at the electrode surface. These measurements, which include techniques such as voltammetry, amperometry, and impedance spectroscopy, provide valuable insights into analyte concentration, binding interactions, and reaction kinetics [7–9]. These sensors are particularly advantageous for pathogen detection due to their high sensitivity, simplicity, and potential for miniaturization. Electrochemical sensors operate based on the following primary mechanisms [2,9–11]:

- (a) Amperometry: Measures the current resulting from redox reactions of the analyte at the sensor's electrode [12].
- (b) Potentiometry: Measures voltage changes corresponding to the concentration of the analyte.
- (c) Electrochemical Impedance Spectroscopy: Tracks the resistance changes as the analyte binds to the sensor surface. For *G. vaginalis* detection, the sensor is designed to recognize specific biomolecular markers (such as DNA sequences, proteins, or metabolites) associated with the bacterium, translating the biochemical interactions into quantifiable electrical signals [13].

Electrochemical sensors for pathogens are typically developed using functionalized electrodes that have been modified to capture the target organism or its components specifically. The detection mechanism is based on biological recognition elements, such as antibodies, aptamers, or DNA probes, that selectively bind to *G. vaginalis* [14].

For *G. vaginalis*, DNA probes targeting specific sequences within the bacterium's genome, antibodies against *G. vaginalis* surface proteins, or aptamers that specifically bind to bacterial metabolites are commonly used [15]. The specificity of these elements ensures high accuracy and low false positives.

This work not only addresses the clinical need for rapid and reliable detection methods for bacterial vaginosis but also contributes to the broader field of biosensor development. The proposed peptide-based electrochemical sensor, functionalized with gold nanoparticles on a graphene electrode, enhances sensitivity and specificity through optimized biorecognition interactions. This study evaluates key performance parameters, including detection limit (LOD), response time, and stability under different storage conditions. Furthermore, by facilitating timely and accurate diagnosis, the developed sensor has the potential to improve patient management and outcomes by enabling healthcare providers to offer targeted treatment options, reducing complications and recurrence rates. In this study, we present the design, fabrication, and performance evaluation of the electrochemical sensor, as well as its potential applications in clinical settings. Through this innovation, we aim to enhance

the diagnostic capabilities for *Gardnerella vaginalis* and contribute to better reproductive health outcomes. The combined use of gold nanoparticles (AuNPs) and graphene offers several advantages over using either material alone, particularly in applications such as electrochemical sensing, catalysis, and biomedical devices (Table 1). The synergy between these materials enhances their individual properties, leading to superior performance. Here are the key advantages:

(a) Enhanced Electrical Conductivity

- Graphene is a highly conductive material with excellent electron mobility.
- Gold nanoparticles provide additional electron transfer pathways, improving charge transfer kinetics.
- Combined effect: The integration of AuNPs with graphene enhances conductivity further, making them ideal for electrochemical sensors and electronic devices.

(b) Increased Surface Area and Active Sites

- Graphene has a large surface area, which supports high molecular adsorption.
- Gold nanoparticles provide additional active sites for chemical interactions.
- Combined effect: The hybrid structure increases the density of active sites, improving catalytic and sensing capabilities.

(c) Improved Biocompatibility and Functionalization

- Gold nanoparticles are highly biocompatible and can be functionalized with biomolecules (e.g., antibodies, enzymes, DNA).
- Graphene supports functionalization but may require modifications.
- Combined effect: The hybrid material facilitates biomolecular interactions, making it useful in biosensors, drug delivery, and medical diagnostics.

Table 1. A comparison of the analytical characteristics of the immunosensors developed in this work with relevant immunosensors for *G. vaginalis* detection based on the literature.

Type of Sensor	Detection Mechanism	Target Biomarker	Advantages	Challenges	References
Electrochemical Sensor	Amperometric, voltametric, or impedimetric detection of electrochemical changes at an electrode surface upon target binding	<i>G. vaginalis</i> DNA, proteins, or sialidase enzyme	High sensitivity and specificity; rapid real-time results	Requires functionalized electrodes; sensitive to biofouling	[12]
Nucleic Acid-Based (PCR/NAAT)	Amplification of specific <i>G. vaginalis</i> DNA sequences (e.g., PCR)	<i>G. vaginalis</i> DNA	High accuracy and specificity; robust quantitative analysis	Requires specialized equipment; longer processing times	[16]
Immunochromatographic (ICT) Tests	Electrochemical or optical detection based on sialidase activity	Proteins associated with <i>G. vaginalis</i>	Rapid, easy-to-use, and can be used as point-of-care test	Limited sensitivity; prone to cross-reactivity	[17]
Biosensor with Sialidase Detection	Electrochemical or optical detection based on sialidase activity	Sialidase enzyme (marker of BV)	Specific to <i>G. vaginalis</i> ; reliable BV marker	May miss BV cases as sialidase levels can vary	[4]
pH-Based Sensor	Measures vaginal pH changes indicating bacterial vaginosis	pH > 4.5 (indicative of BV conditions)	Simple, quick; minimal equipment required	Low specificity for <i>G. vaginalis</i> alone; pH can be influenced by various factors	[1]

The combination of gold nanoparticles and graphene results in a highly conductive, stable, and multifunctional material with superior properties compared to using either

component alone. This hybrid structure is particularly valuable in biosensing, catalysis, energy storage, and medical applications, where enhanced performance is crucial.

2. Materials and Methods

2.1. Chemicals

Potassium hexacyanoferrate (III), methanol, sodium nitrite, and hydrochloric acid were purchased from Chempur (Piekary Śląskie, Poland). Phosphate-buffered saline was obtained from Sigma-Aldrich (Poznan, Poland). Alumina slurries, 0.3 m, were purchased from Buehler (Lake Bluff, IL, USA). Sulphuric acid, potassium hydroxide, hydrogen peroxide, ethanol, and methanol were supplied by Pol-Aura (Dywity, Poland). Graphen and gold nanoparticles were purchased from Sigma Aldrich (Poznań, Poland).

Fmoc-Rink Amide AM Resin (0.7 mmol/g) and DL-Dithiothreitol (DTT) were purchased from Iris Biotech GmbH. Fmoc (Fluorenylmethyloxycarbonyl)-protected amino acids, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OBzl)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, and Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Met-OH and Fmoc-Phe-OH were purchased from CSBio (Shanghai) Ltd. (Shanghai, China), Fmoc-His(Boc)-OH from CEM Corporation, 11-mercaptoundecanoic acid (11-MUA), Oxyma pure (Ethyl cyano(hydroxyimino)acetate), N,N'-Diisopropylcarbodiimide (DIC), Piperidine, 99%, extra pure, Triisopropylsilane (TIS) from Sigma-Aldrich, N,N-Dimethylformamide, 99.8% (DMF) and Trifluoroacetic Acid (TFA) for synthesis, were gained from VWR International, LLC (Radnor, PA, USA). Ethyl dieter was collected from POCH S.A (Gliwice, Poland). Aqueous solutions were made using double-distilled sterile water (ddH₂O).

2.2. Instrumentation

Potentiostat-galvanostat system (PalmSens4, Palmsens, Houten, The Netherlands) was used. Screen printed electrode (Palmsens, The Netherlands) was used as a working electrode.

3. Results

3.1. Production and Purification of Vaginolysine (VLY) Protein from *Gardnerella vaginalis*

The gene encoding the recombinant protein was chemically synthesized with codon optimization for expression in an *Escherichia coli* host (GenScript, Piscataway, NJ, USA). This synthetic gene was inserted into the pET-51b(+) vector using the Sall and HindIII restriction sites, resulting in an N-terminal Strep-Tag II and a C-terminal 10 × His-Tag fusion. The obtained plasmid was then transformed into competent *Escherichia coli* BL21(DE3) cells (#C2527, New England Biolabs, UK). Additional recombinant proteins, including Upa3, UreaD, Oppa, and Sip, served as negative controls. All recombinant proteins were induced in 1000 mL of LB broth (#2020, A&A Biotechnology, Gdansk, Poland) using 0.1 mM isopropyl β-D-thiogalactoside at an OD₆₀₀ of 0.5, at 37 °C for 3 h with shaking at 180 rpm. After induction, cell cultures were centrifuged, and the resulting pellets were lysed in 50 mL of buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10% Triton X-100, 70,000 U/mL lysozyme (#62971, Merck, Darmstadt, Germany), and 15 µg/mL DNase I (#10104159001, Merck, Germany), adjusted to pH 8.0. The recombinant proteins were subsequently isolated from the lysate using IMAC chromatography with His-Select Nickel Affinity Gel (#P6611, Merck, Germany), employing a column gravity approach with 1 mL of resin (Table 2).

Table 2. Recombinant protein IMAC chromatography conditions.

Recombinant Protein IMAC Chromatography Conditions					
Chromatography Step	Upa3 Genebank: WP_006688445.1	UreD Genebank: AAF30840.1	Oppa GenBank: CAX37285.1	VLY Genebank: ACD39459.1	Sip Genebank: AAG18474.1
Column Wash	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 50 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 10 mM imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 20 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 25 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 5 mM Imidazole, pH = 8.0 20 CV
Elution	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 150 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 250 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 100 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 250 mM Imidazole, pH = 8.0 20 CV	50 mM NaH ₂ PO ₄ , 300 mM NaCl, 250 mM Imidazole, pH = 8.0 20 CV

3.2. Peptide Synthesis and Modification

The peptide with a sequence of 11MUA-KKK-AHYADFYLTNVM-NH₂ was synthesized on Rink Amide resin (0.08 mmol) by microwave-assisted Fmoc solid-phase peptide synthesis (SPPS). An automated microwave peptide synthesizer, Initiator+ Alstra™ (Biotage, Sweden), was utilized for peptide chain elongation. Fmoc-amino acid derivative (0.5 M, 5 equiv.), DIC (0.5 M, 5 equiv.), and Oxyma (0.5 M, 5 equiv.) in dimethylformamide (DMF) were used in two 5 min couplings at 75 °C. The fluorenylmethoxycarbonyl (Fmoc) protecting group was removed using a 20% piperidine solution in DMF at room temperature (1 × 3 min, 1 × 10 min). 11-MUA (11-mercaptoundecanoic acid) (0.5 M, 5 equiv.) was introduced at the N-terminal end of the peptide under standard coupling conditions during synthesis (1 × 7 min, 75 °C). The 11-MUA-labeled peptide was cleaved from Rink Amide resin for 2 h using the cleavage cocktail consisting of TFA/H₂O/DTT/TIS (88/5/5/2) before being precipitated with anhydrous, cold diethyl ether and lyophilized. The modified peptide was analyzed using an analytical reverse-phase high-performance liquid chromatography (RP-HPLC) method to assess its purity and identity. A Prominence-i LC-2030C Plus system (Shimadzu, Kyoto, Japan) with a UV detector (λ = 224 nm) equipped with a Jupiter Proteo column (90 Å, 4.6 × 250 mm, particle size 4 µm) was used. The method was 60 min long with a linear gradient method starting from 5 to 95% solvent B at a flow rate of 1 mL/min, where solvent A was water and B was acetonitrile as eluents containing 0.1% TFA. Following mass spectrometry measurement, the expected molecular weight was confirmed, indicating that the peptide was successfully synthesized and modified.

Afterward, a semi-preparative reverse phase HPLC system (Shimadzu/Prominence Modular HPLC, column: Luna C8(2), 5 µm 100 Å, 21.2 × 250 mm, with UV detection at λ = 224 nm, using the gradient method from 30 to 70% solvent B for 60 min at a flow rate of 15 mL/min, where solvent A was water and B was 80% acetonitrile as eluents containing 0.1% TFA) was utilized to purify the crude product.

The effective synthesis and purification were validated by an ESI mass spectrometer. A single quadrupole mass spectrometer LCMS 2020 Shimadzu (Kyoto, Japan) operated in positive ion mode (+) was used to perform analysis in the following conditions. The ionization source was set to a temperature of 250 °C, with a nebulizing gas flow rate of 1.5 L/min. The samples were injected into the ESI source at a flow rate of 0.4 mL/min, using a mobile phase composed of 60% acetonitrile in 0.1% formic acid (FA).

Characterization of the 11-MUA-labeled peptide: white solid; synthetic yield: 89%; HPLC purity > 97%; Rt: 23.88 min. ESI MS of peptide estimated value: 2026.9 (g/mol); observed value (m/z): 508 [M+4H]⁴⁺, 677 [M+3H]³⁺, 1015 [M+2H]²⁺.

3.3. Biomaterials Preparation and Identification by Reference Method

The Biotechnology Laboratory isolated recombinant proteins at the Institute of Biotechnology and Molecular Medicine following standard procedures. For verifying the efficacy of the produced antibodies, a control experiment was conducted. The possibility of detection of *Gardnerella vaginalis* protein using anti-protein *Gardnerella vaginalis* antibodies was confirmed in the ELISA test (A qPCR reaction detected the presence of the bacteria and their concentrations (CFU).

3.4. Procedure of Electrode Modification

Prior to electrochemical measurements, the commercial carbon electrodes were modified with graphene and Au nanoparticles in order to achieve a novel sensor with improved sensitivity to *Gardnerella vaginalis*. Graphene dispersion was prepared by mixing 5 mg GPH (0.2% in acetic acid, pH 5). The gold nanoparticles (AuNPs) were prepared through the reduction of HAuCl₄ with trisodium citrate solution in aqueous solution. Gold nanoparticles (AuNPs) can be synthesized by various methods, but in this work the Turkevich method (citrate reduction) was used. The size of the nanoparticles depends on the synthesis conditions, such as the ratio of reagents, temperature, and reaction time [18]. The synthesis reaction can be summarized as follows:



Using the drop-and-dry method, chemically modified screen-printed carbon electrodes (SPCEs) were developed. A micropipette was used to deposit 10 μL of the nanomaterials' composite dispersion onto the SPCEs, which were then dried at room temperature in a desiccator. The sensitive elements of the modified electrodes contained either GPH (GPH sensor) or GPH combined with AuNPs (GPH-AuNP sensor). After preparation, the electrodes were stored at 4 °C (Figure 1).

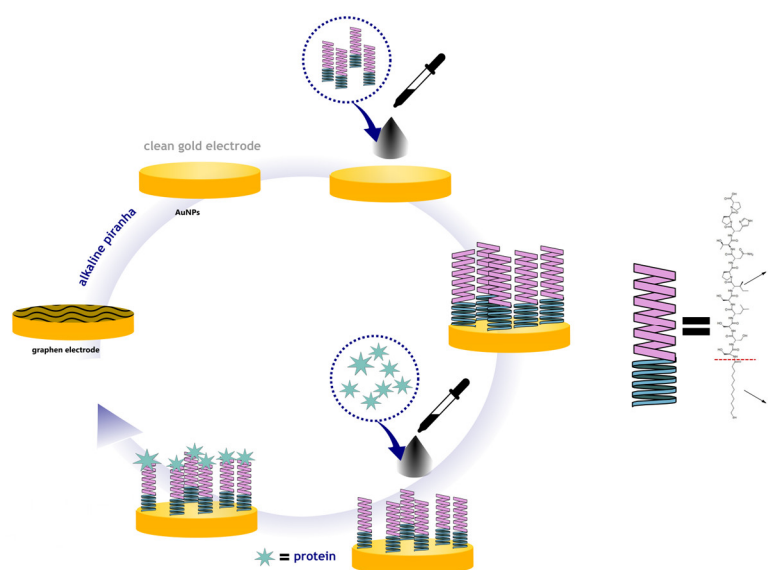


Figure 1. Mechanism of graphene electrode with gold nanoparticle film modification using the peptide.

To prepare for detection, 10.0 μL of a Pept solution ($1000 \times$ diluted in PBS) was applied to the working electrode. The device was then oven-dried at 37°C until the solvent had fully evaporated. Following this step, 20.0 μL of protein solution was added for detection. Between each construction and detection step, the electrode was gently rinsed with PBS solution (0.1 mol L^{-1} , pH 7.4). To prepare the device for detection, 10.0 μL of a Pept solution ($1000 \times$ diluted in PBS) was applied to the working electrode and oven-dried at 37°C until the solvent had completely evaporated. Next, 20.0 μL of the protein solution was added for the detection step. Between each construction and detection step, the electrode was gently washed with PBS solution (0.1 mol L^{-1} , pH 7.4).

3.5. Electrochemical Measurements

All the electrochemical tests: the cyclic voltammetry and electrochemical impedance spectroscopy (EIS) were conducted using a Palmsens 4 potentiostat/galvanostat system (Methrom, Autolab, Utrecht, The Netherlands) in the standard three-electrode configuration. Carbon electrode was used as a working electrode modified with a film from the dispersion of graphene and AuNPs, $\text{Ag}|\text{AgCl}$ (3.0 mol L^{-1} KCl) as a reference electrode, and wire of platinum as a counter electrode (Pt).

All the electrochemical tests were carried out in 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ in 0.01 M PBS that was previously deaerated. In cases of the electrochemical impedance spectroscopy measurements (EIS), the frequency ranged from 10 kHz to 1 Hz with 40 points. The amplitude of the AC signal was 10 mV. Each potential was held constant for 60 s before each measurement to obtain steady-state conditions. Obtained data were subjected to the analysis using EIS Spectrum Analyzer according to the proposed electric equivalent circuit (EEQC). The procedure described above is a standard measurement procedure used in biosensing.

3.6. Immunosensor Fabrication

A large set of GCE and GCE with graphene film samples was tested and studied during the optimization of process parameters to obtain an efficient modification procedure. Each surface was washed in an ultrasonic cleaner for 5 min in methanol and ddH₂O and dried in an argon stream before the modification procedure.

The electroactive area of the GCE and GCE with graphene film was determined using various scan rates (from 10 to 500 mV/s) in 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ prepared in 0.01 M PBS with an equimolar mixture of redox probe, as shown in Figure 2. This estimation was made by applying the following Randles–Ševčík equation:

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} \nu^{1/2} C$$

where I_p is the peak current (anodic or cathodic), n represents the number of electrons transferred ($n = 1$), A is the electroactive surface area, D is the diffusion coefficient ($D = 7.6 \times 10^{-6}\text{ cm}^2/\text{s}$ for the redox probe in 0.01 M PBS), and C is the concentration of the redox probe.

Figure 2 compares cyclic voltammograms between the graphene electrode and the graphene electrode modified with gold nanoparticles, measured in a 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ solution in 0.01 M PBS at scan rates from 10 to 500 mV/s. The difference in peak potentials (ΔE_p) for the GPH electrode with AuNPs was 200 mV, while it was 243 mV for the GPH alone, indicating enhanced reversibility for the redox pair with the AuNP-modified GPH electrode. These findings demonstrate that GPH with AuNPs provides significantly better electrochemical performance than GPH alone.

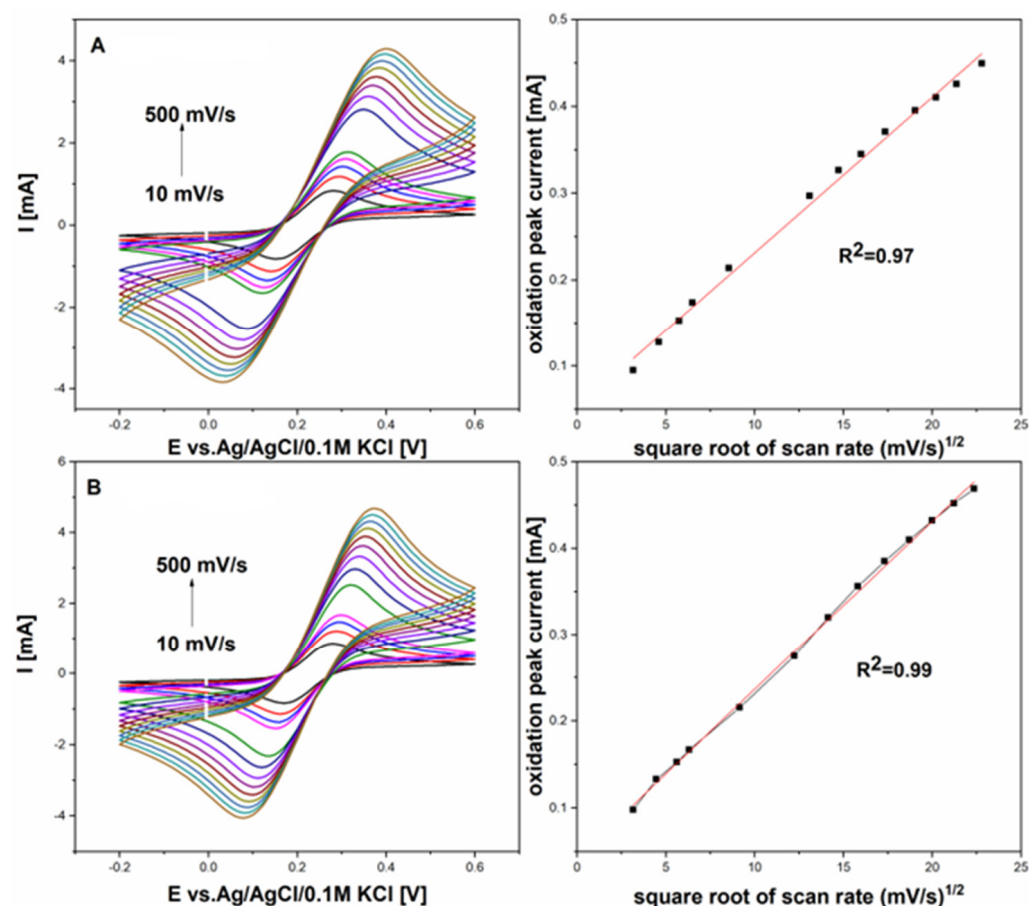


Figure 2. Cyclic voltammograms recorded at different scan rates (10–500 mV/s) using (A) bare GC electrode and (B) graphene with AuNPs.

In Figure 3A, the changes recorded during the successive steps of sensor modification are easily observable through the CV. Following the previously discussed alterations after incubation in AuNPs, a noticeable decrease of 0.5 μ A in the current peak heights from the oxidation and reduction of $[\text{Fe}(\text{CN})_6]^{3-}/4^-$, along with an increase in their separation by 0.04 mV, is evident after peptide addition. For the EIS data analysis (Figure 3B), an electric equivalent circuit was built (Figure 3C), a derivative of Randles circuit with parallel capacitance replaced by the constant phase element (CPE).

To enhance data presentation, the impedance spectra are displayed over a frequency range from 10 kHz to 8 Hz. For improved analysis of the EIS data, the spectra were fitted to an equivalent electrical circuit (EEC), as depicted in Figure 3C, with the fitting results summarized in Table 3. The chi-square parameter was used to assess the goodness of fit.

Table 3. Electric equivalent circuit (EEC) utilized for fitting and data analysis.

Stage	R_{ct}/Ω	$Q/\mu\text{F} \times \text{s}^{(1-n)}$	n/-	Chi-Square
Bare GPH electrode	308.93	59.40	0.38	2×10^{-3}
AuNPs	78.06	7.30	0.64	1×10^{-4}
Peptide	905.80	0.76	0.98	3×10^{-4}

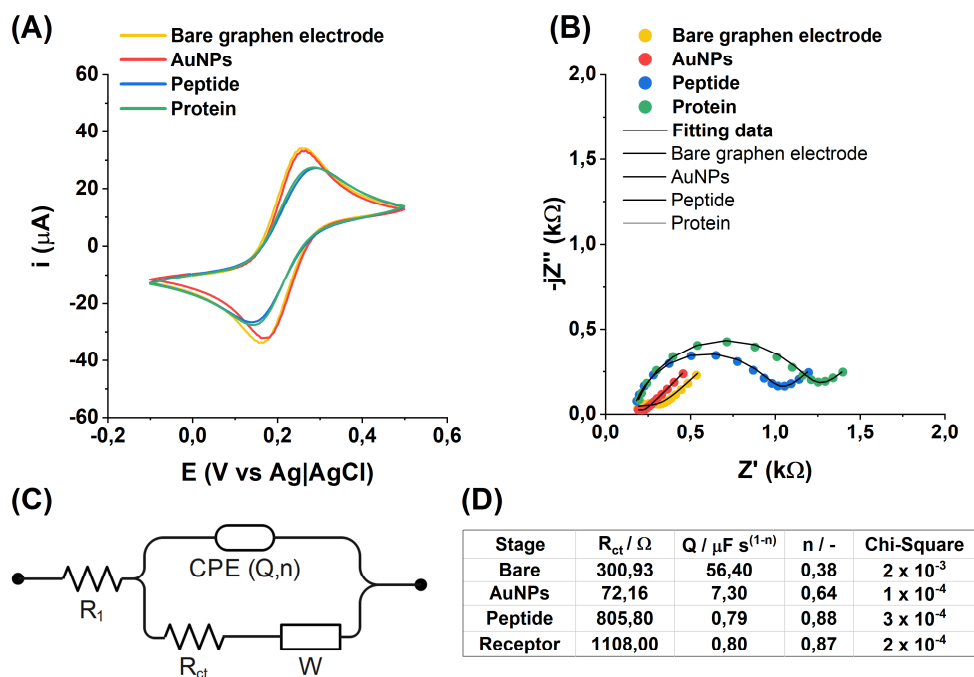


Figure 3. Results obtained from electrochemical tests: (A) CV study for each stage of sensor modification, (B) impedance spectra for each stage of the sensor modification, (C) electric equivalent circuit (EEQC) utilized for fitting and data analysis. (D) values obtained during the fitting of experimental data.

3.7. Electrochemical Characterization of the Immunosensor

The correctness of successive modifications of the electrode was verified using the EIS technique. Electrochemical measurements were conducted in a buffer solution prepared in PBS with a pH of 7.4, containing 5 mM $K_3[Fe(CN)_6]$ and 5 mM $K_4[Fe(CN)_6]$. This redox system was specifically chosen because it facilitates the analysis of changes in electron transfer kinetics on the electrode surface. Such changes are indicative of modifications occurring at each stage. By comparing the results obtained after each modification step, it is possible to detect even subtle variations and assess whether each step was performed correctly. This approach ensures the reliability and accuracy of the modification process, providing a robust method for step-by-step validation. EIS measurements inform about changes in resistance that occur on the electrode surface.

3.8. Biosensor Selectivity, Repeatability and Stability Studies

One of the key requirements for developing any immunosensor is ensuring its stability under normal conditions [19]. However, it is equally important to have the ability to store the immunosensor before use while maintaining its functionality. Unfortunately, electrochemical biosensors containing biological components often exhibit limited stability under standard conditions due to irreversible structural changes that may occur over time.

To evaluate the stability of the prepared biosensor, impedance spectra of the fully prepared electrode (ready for target protein detection) were recorded. Following this, the electrode was rinsed with deionized water and dried using an argon stream. Next, the prepared samples were stored under three different conditions:

- At 5 °C, without immersion in any buffer;
- At 20 °C, without immersion in any buffer;
- At 5 °C, immersed in TBS buffer for 1 h.

After the designated storage period, the electrodes were once again rinsed with deionized water, dried with argon, and their impedance spectra were recorded for a second

time. To ensure reliability, all tests were conducted on multiple samples, and the results are presented in Figure 4.

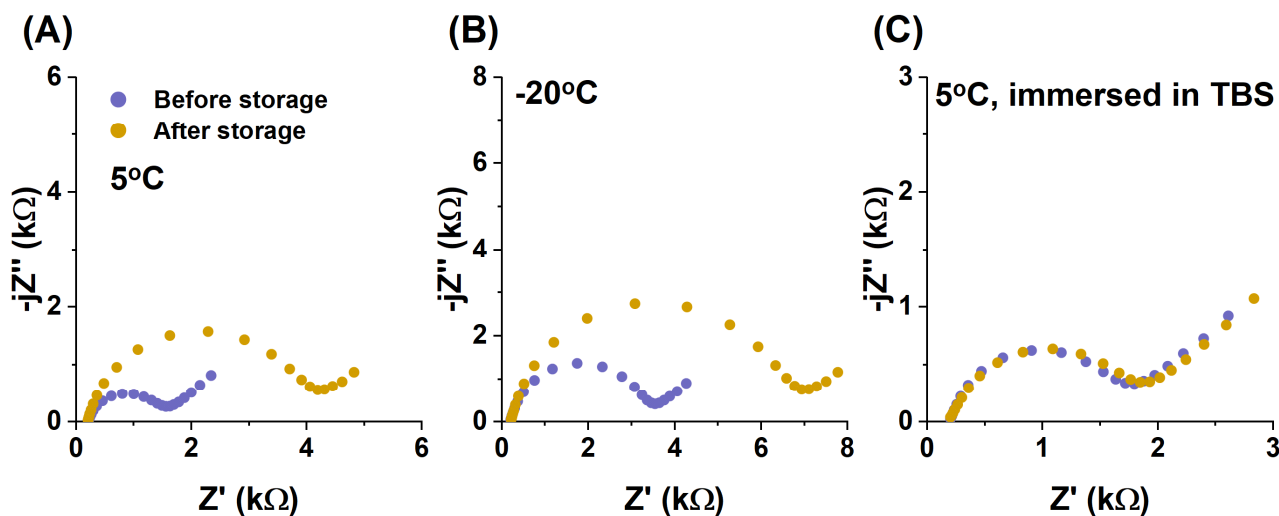


Figure 4. Impedance spectra of the peptide-based sensor after incubating in solutions with protein in different storage.

This study provides insights into the effects of different storage conditions on biosensor stability, helping to optimize conditions for long-term usability.

To examine the selectivity of the presented peptide-based sensor, potentially interfering samples included *Mycoplasma hominis*, *Ureaplasma*, and *Streptococcus agalactiae*, along with PBS buffer. The concentration of bacteria was maintained within the same order of magnitude to ensure comparable results. EIS spectra were recorded after a 2 min incubation period.

As shown in Figure 5, none of the negative controls exhibited a significant increase in impedance; for individual samples, the percentage change in Rct remained below 10%. This establishes 10% as the threshold for differentiating between positive and negative samples.

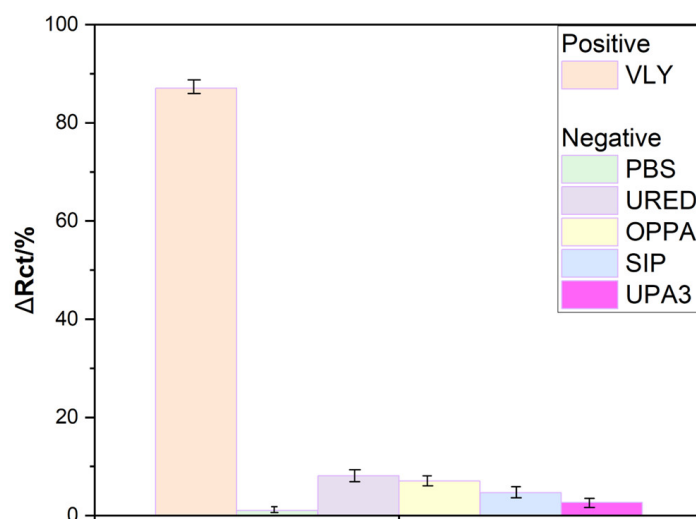


Figure 5. The selectivity of the peptide-based sensor tested versus positive *G. vaginalis* sample and other marker proteins: Ured (*Ureaplasma parvum*), Oppa (*Mycoplasma hominis*), Upa3 (*Ureaplasma urealyticum*), Sip (*Streptococcus agalactiae*). PBS bare buffer solution is shown as blank and reference sample.

Figure 6 shows the impedance spectrum recorded during additions of the positive protein (*Gardnerella vaginalis*) solution with increasing concentrations. All R_{ct} change values were calculated from the following equation:

$$\text{Sensor response} = \frac{R_{ct}^{\text{Test}} - R_{ct}^{\text{Basic}}}{R_{ct}^{\text{Test}}} \times 100(\%)$$

where R_{ct} Test is for the sample, and R_{ct} Basic is for the fully prepared peptide-based sensor.

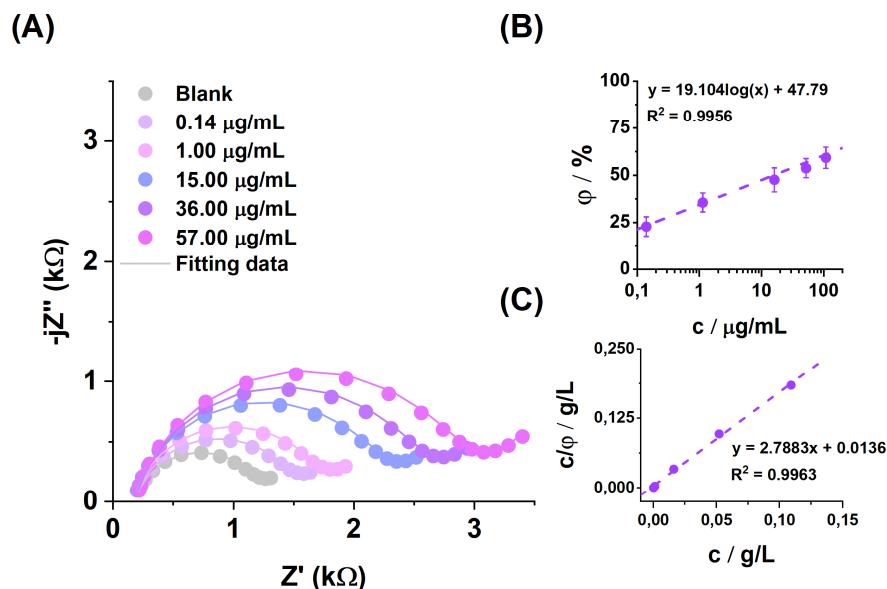


Figure 6. (A) The impedance spectra of the peptide-based sensor, after incubation in solutions with different protein concentrations, were recorded in 5 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4[\text{Fe}(\text{CN})_6]/0.01$ M PBS. (B) The relationship between the sensor response, expressed as the change in R_{ct} (ΔR_{ct}), and the protein concentration was also registered in 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]/0.01$ M PBS. Error bars denote the confidence interval ($\alpha = 0.05$, $n = 3$). (C) Langmuir adsorption isotherm.

The EIS was used to investigate the metrological performance of the biosensor detecting the *Gardnerella vaginalis* bacteria by spotting the solutions with different concentrations on the surface of electrodes and incubating them for an optimal time (Figure 6).

The limit of detection was calculated from the relation $\text{LOD} = 3 \times \text{SD}/\text{slope}$, where SD is the standard deviation in the low concentration range.

The calculated LOD value is 0.02305 $\mu\text{g/mL}$. To gain further insight into the process by which the VLY protein attaches to the surface of the modified electrode, the Langmuir adsorption isotherm, as illustrated in Figure 6, can be constructed. This approach provides a deeper understanding of the adsorption mechanism and enables the determination of binding parameters essential for characterizing the interaction.

4. Conclusions

The developed electrochemical peptide-based sensor demonstrated high sensitivity, selectivity, and stability in detecting vaginolysine as a positive marker protein for *Gardnerella vaginalis*, making it a viable candidate for rapid and reliable diagnostic applications. The strategic use of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) provided crucial insights into the electrode modifications and confirmed effective antibody binding and electron transfer properties. The EDC/NHS functionalization protocol enabled stable and precise immobilization of anti-*Gardnerella vaginalis* peptide on the gold nanoparticle-modified graphene surface, significantly enhancing the sensor's sensitivity.

The integration of gold nanoparticles on the graphene electrode surface played a pivotal role in amplifying signal response due to their excellent conductivity and large surface-to-volume ratio, allowing for the detection of low pathogen concentrations. The peptide-based sensor exhibited rapid and specific binding to *Gardnerella vaginalis* antigens, with minimal cross-reactivity, underscoring its high selectivity. These properties position the sensor as a promising tool for point-of-care diagnostics in clinical environments, potentially advancing the early detection and management of *Gardnerella vaginalis* infections.

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References

1. Koumans, E.H.; Sternberg, M.; Bruce, C.; McQuillan, G.; Kendrick, J.; Sutton, M.; Markowitz, L.E. The Prevalence of Bacterial Vaginosis in the United States, 2001–2004; Associations with Symptoms, Sexual Behaviors, and Reproductive Health. *Sex. Transm. Dis.* **2007**, *34*, 864–869. [[CrossRef](#)] [[PubMed](#)]
2. Wang, J. Electrochemical Biosensors: Towards Point-of-Care Cancer Diagnostics. *Biosens. Bioelectron.* **2006**, *21*, 1887–1892. [[CrossRef](#)] [[PubMed](#)]
3. Campos, C.A.; Lima, B.S.; Trindade, G.G.G.; Souza, E.P.B.S.S.; Mota, D.S.A.; Heimfarth, L.; Quintans, J.S.S.; Quintans-Júnior, L.J.; Sussuchi, E.M.; Sarmiento, V.H.V.; et al. Anti-Hyperalgesic and Anti-Inflammatory Effects of Citral with β -Cyclodextrin and Hydroxypropyl- β -Cyclodextrin Inclusion Complexes in Animal Models. *Life Sci.* **2019**, *229*, 139–148. [[CrossRef](#)] [[PubMed](#)]
4. Heller, A.A.; Spence, D.M. A Rapid Method for Post-Antibiotic Bacterial Susceptibility Testing. *PLoS ONE* **2019**, *14*, e0210534. [[CrossRef](#)] [[PubMed](#)]
5. Roselletti, E.; Sabbatini, S.; Perito, S.; Mencacci, A.; Vecchiarelli, A.; Monari, C. Apoptosis of Vaginal Epithelial Cells in Clinical Samples from Women with Diagnosed Bacterial Vaginosis. *Sci. Rep.* **2020**, *10*, 1978. [[CrossRef](#)] [[PubMed](#)]
6. Tomás, M.; Palmeira-de-Oliveira, A.; Simões, S.; Martinez-de-Oliveira, J.; Palmeira-de-Oliveira, R. Bacterial Vaginosis: Standard Treatments and Alternative Strategies. *Int. J. Pharm.* **2020**, *587*, 119659. [[CrossRef](#)] [[PubMed](#)]
7. Bu, S.; Liu, X.; Wang, Z.; Wei, H.; Yu, S.; Li, Z.; Hao, Z.; Liu, W.; Wan, J. Ultrasensitive Detection of Pathogenic Bacteria by CRISPR/Cas12a Coupling with a Primer Exchange Reaction. *Sens. Actuators B Chem.* **2021**, *347*, 130630. [[CrossRef](#)]
8. Muzny, C.A.; Schwebke, J.R. Biofilms: An Underappreciated Mechanism of Treatment Failure and Recurrence in Vaginal Infections. *Clin. Infect. Dis.* **2015**, *61*, 601–606. [[CrossRef](#)] [[PubMed](#)]
9. Wang, B.; Wang, H.; Lu, X.; Zheng, X.; Yang, Z. Recent Advances in Electrochemical Biosensors for the Detection of Foodborne Pathogens: Current Perspective and Challenges. *Foods* **2023**, *12*, 2795. [[CrossRef](#)] [[PubMed](#)]
10. Justino, C.I.L.; Rocha-Santos, T.A.; Duarte, A.C.; Rocha-Santos, T.A. Review of Analytical Figures of Merit of Sensors and Biosensors in Clinical Applications. *TrAC Trends Anal. Chem.* **2010**, *29*, 1172–1183. [[CrossRef](#)]
11. Cesewski, E.; Johnson, B.N. Electrochemical Biosensors for Pathogen Detection. *Biosens. Bioelectron.* **2020**, *159*, 112214. [[CrossRef](#)] [[PubMed](#)]
12. Labib, M.; Sargent, E.H.; Kelley, S.O. Electrochemical Methods for the Analysis of Clinically Relevant Biomolecules. *Chem. Rev.* **2016**, *116*, 9001–9090. [[CrossRef](#)] [[PubMed](#)]

13. Vidic, J.; Manzano, M. Electrochemical Biosensors for Rapid Pathogen Detection. *Curr. Opin. Electrochem.* **2021**, *29*, 100750. [[CrossRef](#)]
14. Zhang, R.; Wang, Y.; Qu, X.; Li, S.; Zhao, Y.; Zhang, F.; Liu, S.; Huang, J.; Yu, J. A Label-Free Electrochemical Platform for the Detection of Antibiotics Based on Cascade Enzymatic Amplification Coupled with a Split G-Quadruplex DNAzyme. *Analyst* **2019**, *144*, 4995–5002. [[CrossRef](#)] [[PubMed](#)]
15. Hilbert, D.W.; Smith, W.L.; Chadwick, S.G.; Toner, G.; Mordechai, E.; Adelson, M.E.; Aguin, T.J.; Sobel, J.D.; Gygax, S.E. Development and Validation of a Highly Accurate Quantitative Real-Time PCR Assay for Diagnosis of Bacterial Vaginosis. *J. Clin. Microbiol.* **2016**, *54*, 1017. [[CrossRef](#)] [[PubMed](#)]
16. Cartwright, C.P.; Lembke, B.D.; Ramachandran, K.; Body, B.A.; Nye, M.B.; Rivers, C.A.; Schwebke, J.R. Development and Validation of a Semiquantitative, Multitarget PCR Assay for Diagnosis of Bacterial Vaginosis. *J. Clin. Microbiol.* **2012**, *50*, 2321–2329. [[CrossRef](#)] [[PubMed](#)]
17. Muzny, C.A.; Schwebke, J.R. Pathogenesis of Bacterial Vaginosis: Discussion of Current Hypotheses. *J. Infect. Dis.* **2016**, *214* (Suppl. 1), S1–S5. [[CrossRef](#)] [[PubMed](#)]
18. Kimling, J.; Maier, M.; Okenve, B.; Kotaidis, V.; Ballot, H.; Plech, A. Turkevich Method for Gold Nanoparticle Synthesis Revisited. *J. Phys. Chem. B* **2006**, *110*, 15700–15707. [[CrossRef](#)] [[PubMed](#)]
19. Grieshaber, D.; MacKenzie, R.; Vörös, J.; Reimhult, E. Electrochemical Biosensors—Sensor Principles and Architectures. *Sensors* **2008**, *8*, 1400–1458. [[CrossRef](#)] [[PubMed](#)]

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