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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Desenvolvimento de Genossensores Eletroquímicos para a Detecção Simplificada do DNA do Vírus Zika

Aluno: Luiz Fernando Gabriel Luz

Orientador: Prof. Dr. João Marcos Madurro
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Aos vinte e quatro dias do mês de março de dois mil e vinte e cinco, às 14:00 horas, reuniu-se via web conferência pela *Plataforma Google Meet*, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica do Instituto de Biotecnologia da Universidade Federal de Uberlândia, assim composta: Drª. Cecilia de Carvalho Castro e Silva, Drª. Carla Eiras, Dr. Matheus de Souza Gomes, Dr. Gustavo Von Poelhsitz e Dr. João Marcos Madurro, como presidente da mesa e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o presidente da mesa, Dr. João Marcos Madurro apresentou a Comissão Examinadora e o candidato, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o senhor presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o candidato. Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato:

APROVADO.

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos

demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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Sumário

Lista de Figuras

Capítulo 1

Capítulo 2

Capítulo 3

Lista de Siglas

AFM: Atomic Force Microscopy

AuNPs: Gold Nanoparticles

CDC: Centers for Disease Control and Prevention

cDNA: Complementary Deoxyribonucleic Acid

CHIKV: Chikungunya Virus

CNTs: Carbon Nanotubes

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cys-GQD: Cysteamine and Graphene Quantum Dots

DENV: Dengue Virus

DFM: Dynamic Force Microscope

ELISA: Enzyme-linked Immunosorbent Assays

FTIR: Fourier Transform Infrared Spectroscopy

GQDs: Graphene Quantum Dots

gRNA: Genomic Ribonucleic Acid

IgG: Immunoglobulin G

IgM: Immunoglobulin M

LAMP: Loop-Mediated Isothermal Amplification

LOD: Limit of Detection

ML: Machine Learning

NS1: Non-structural Protein 1

PCB: Printed Circuit Board

PCR: Polymerase Chain Reaction

POC: Point-of-Care

QDs: Quantum Dots

rGO: Reduced Graphene Oxide

RNA: Ribonucleic Acid

RT-PCR: Real-time Reverse Transcription PCR

SEM: Scanning Electron Microscopy

SPCE: Screen-Printed Carbon Electrode

ssDNA: Single-stranded Deoxyribonucleic Acid

WHO: World Health Organization

ZIKV: Zika Virus

Resumo

Os biossensores são dispositivos analíticos inovadores que combinam um elemento de reconhecimento biológico com um transdutor, permitindo a detecção sensível e seletiva de diversos analitos. Entre suas aplicações, destaca-se a detecção de patógenos, como o vírus Zika, que representa um desafio significativo para a saúde pública. A busca por métodos rápidos, precisos e acessíveis impulsiona o desenvolvimento de novas abordagens baseadas em biossensores, especialmente os genossensores eletroquímicos.

Esta tese está estruturada em três capítulos principais. O primeiro capítulo apresenta uma revisão bibliográfica sobre os avanços na detecção do vírus Zika, abordando diferentes metodologias, com foco especial no uso de biossensores para essa finalidade. O segundo capítulo descreve o desenvolvimento de um genossensor eletroquímico para a detecção rápida e simplificada do DNA do vírus Zika, utilizando safranina como intercalador genômico sobre uma plataforma modificada com uma bicamada de cisteamina e pontos quânticos de carbono. Essa abordagem visa melhorar a sensibilidade e a especificidade da detecção, proporcionando um método eficiente e acessível. O biossensor demonstrou alta sensibilidade detectando 4,2 pg mL^{-1}, seletividade contra outros arbovírus (chikungunya e dengue) e boa estabilidade por pelo menos 45 dias.

Por fim, o terceiro capítulo apresenta o desenvolvimento de um novo genossensor eletroquímico baseado em óxido de grafeno e safranina para a detecção do vírus Zika. Essa estratégia busca explorar as propriedades únicas do óxido de grafeno para aprimorar a resposta eletroquímica. Este sensor obteve om um limite de detecção de 8,4 ng mL^{-1}. O genossensor manteve aproximadamente 80% da resposta após 60 dias, demonstrando boa estabilidade.

Palavras-chave: Vírus Zika, Nanomaterial, Safranina, Ninidrina, Biossensor Eletroquímico

Abstract

Biosensors are innovative analytical devices that combine a biological recognition element with a transducer, allowing the sensitive and selective detection of various analytes. Among their applications, the detection of pathogens, such as the Zika virus, which represents a significant challenge to public health, stands out. The search for fast, accurate and accessible methods drives the development of new approaches based on biosensors, especially electrochemical genosensors.

This thesis is structured in three main chapters. The first chapter presents a literature review on advances in the detection of the Zika virus, addressing different methodologies, with a special focus on the use of biosensors for this purpose. The second chapter describes the development of an electrochemical genosensor for the rapid and simplified detection of Zika virus DNA, using safranin as a genomic intercalator on a platform modified with a cysteamine bilayer and carbon quantum dots. This approach aims to improve the sensitivity and specificity of detection, providing an efficient and accessible method. The biosensor demonstrated high sensitivity, detecting 4.2 pg mL^{-1}, selectivity against other arboviruses (chikungunya and dengue), and good stability for at least 45 days.

Finally, the third chapter presents the development of a new electrochemical genosensor based on graphene oxide and safranin for the detection of the Zika virus. This strategy seeks to exploit the unique properties of graphene oxide to improve the electrochemical response. This sensor achieved a detection limit of 8.4 ng mL^{-1}. The genosensor maintained approximately 80% of the response after 60 days, demonstrating good stability.

Keywords: Zika virus, Nanomaterial, Safranin, Ninhydrin, Electrochemical biosensor

Capítulo 1: Electrochemical Biosensors for Zika Virus Detection: Bridging Diagnostic Challenges with Rapid, Portable Solutions and Future Innovation

O capítulo 1 corresponde a um manuscrito de artigo de revisão redigido nas normas do periódico Analytical Biochemistry (ISSN: 1096-0309).

Electrochemical Biosensors for Zika Virus Detection: Bridging Diagnostic Challenges with Rapid, Portable Solutions and Future Innovations

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Abstract: The ongoing threat posed by the Zika virus underscores the urgent need for rapid, sensitive, and accessible diagnostic tools. Traditional methods such as PCR and ELISA, while effective, are often impractical in resource-limited settings due to their complexity and cost. In contrast, electrochemical biosensors represent a transformative approach to ZIKV detection, combining rapid results with high sensitivity and the potential for point-of-care applications. The integration of advanced technologies, including CRISPR-based systems and nanomaterials, further enhances the capabilities of these biosensors, making them invaluable in the fight against ZIKV and similar viral infections. Future research should focus on optimizing these technologies for widespread use, ensuring that diagnostics keep pace with the evolving landscape of viral threats. This work reviews the development of electrochemical biosensors for detection of the Zika Virus, focusing on different biomarkers, electrode materials and detection platforms, in addition to discussing challenges and future perspectives.

Keywords: Zika Virus, Electrochemical Biosensors, Point-of-Care Diagnostics

Overview of Zika Virus

Zika virus (ZIKV), a member of the Flavivirus genus, was first identified in 1947 in Uganda and has since emerged as a significant global health concern, particularly following outbreaks in the Americas during 2015-2016[1], [2]. The primary vectors for ZIKV transmission are Aedes mosquitoes, specifically Aedes aegypti and Aedes albopictus, which facilitate mosquito-borne transmission[3], [4]. In addition to vector-borne transmission, ZIKV can also be transmitted vertically from mother to fetus during pregnancy and through sexual contact[4], [5]. The incubation period for the Zika virus is 3 to 14 days, and symptoms usually last 2 to 7 days[6]. The clinical manifestations of ZIKV infection are often mild, including symptoms such as fever, rash, conjunctivitis, and joint pain, with a significant proportion of infections being asymptomatic[6], [7]. However, severe outcomes such as Guillain-Barré

syndrome can occur during or shortly after acute Zika infection[7], [8] and congenital Zika syndrome can occur in babies of mothers infected during pregnancy[9], [10], characterized by microcephaly and other neurological defects, have raised alarms about the virus's impact on public health[8], [10]. Zika is contagious during the period of viremia, which usually lasts about a week after symptoms begin[6].

The global health implications of ZIKV are profound, particularly in regions where the virus is endemic. Figure 1 shows the list of Countries and territories with current or previous Zika virus transmission according to 2024 data available from the WHO.

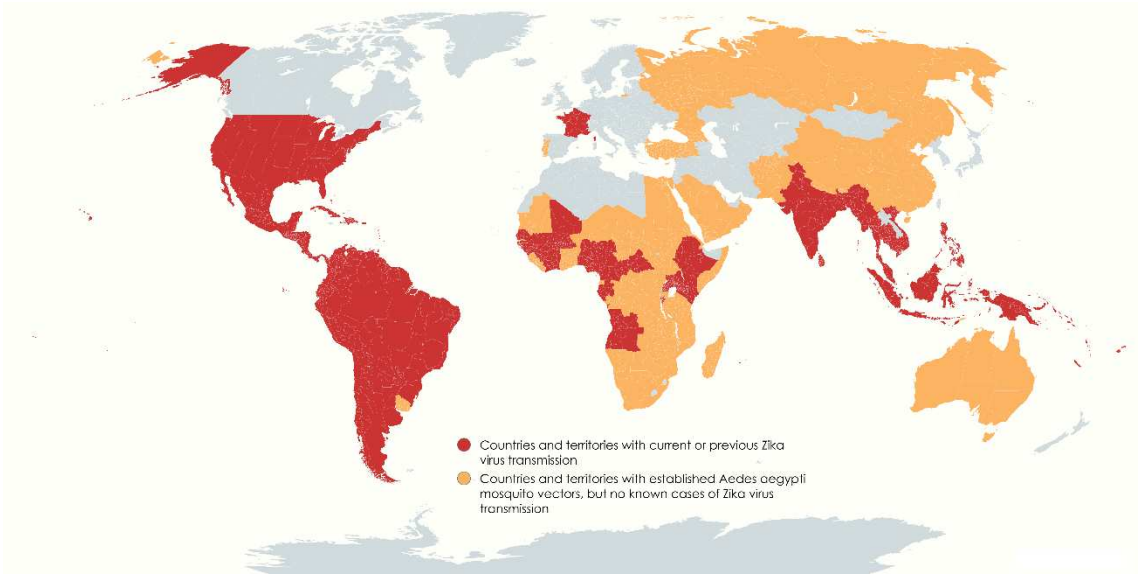

Figure 1. Countries and territories with current or previous Zika virus transmission according to 2024 data available from the WHO.

The World Health Organization declared ZIKV a Public Health Emergency of International Concern in 2016 due to its association with severe neurological complications and congenital anomalies[11], [12]. The emergence of ZIKV has highlighted the need for enhanced surveillance and research to understand its epidemiology and pathogenesis better[13], [14]. Approximately 1 million cases were documented in 2015, with a total of 7,126 deaths. More than 50 territories reported the occurrence of local transmission of the disease. However, from 2018 onwards, a reduction in the number of chikungunya cases was observed[15].

Challenges in Current Diagnostic Methods

Current diagnostic methods for ZIKV, including polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISA), face several challenges. These methods can

be time-consuming and costly, often requiring sophisticated laboratory infrastructure that may not be available in resource-limited settings[16], [17]. For instance, PCR, while highly sensitive, necessitates specialized equipment and trained personnel, which can be a barrier in endemic regions[16].

The diagnosis of ZIKV infections is primarily hindered by the need for effective biological markers that can be reliably detected across different bodily fluids. The principal biological markers associated with ZIKV include viral RNA[18], [19], [20], [21], Zika-specific immunoglobulin M (IgM) antibodies[6], [12], [19], [20], [22], [23], and non-structural protein 1 (NS1)[2], [6], [11], [20], [24]. Detecting these markers can significantly inform clinical decision-making and management of the infection. One of the most crucial biological markers detectable in ZIKV diagnostics is the viral RNA, which can be identified using techniques like polymerase chain reaction (PCR) across various bodily fluids including serum, urine, and saliva during the acute phase of infection[25]. PCR offers high specificity and sensitivity, particularly effective when performed early in the course of the disease. For instance, studies have shown that ZIKV RNA can persist longer in urine compared to serum, allowing for late-stage detection of the virus[17]. This persistence makes urine a valuable sample type for post-symptomatic diagnosis.

In addition to viral RNA, antibodies play a pivotal role in ZIKV detection. The presence of Zika-specific IgM can be analyzed through serological testing, which provides evidence of an immune response to the virus and is predominantly detectable after the first week of illness. ELISA tests are standard for this purpose, allowing the detection of IgM antibodies in serum samples[26]. Notably, due to cross-reactivity with other flaviviruses, such as dengue, care must be taken when interpreting serological results[17]. Furthermore, NS1 has gained attention for its utility as a marker in both early diagnosis and monitoring the severity of infection, and it can also be detected in serum through specific ELISA[26].

Emerging diagnostic technologies leveraging nanomaterials and biosensors also hold promise in enhancing the detection of biomarkers related to ZIKV. For instance, carbon nanotube-based biosensors can provide sensitive detection of ZIKV-specific proteins and nucleic acids, potentially offering rapid point-of-care diagnostics that might bypass traditional laboratory protocols[27]. These biosensors can also distinguish viral proteins such as NS1 with enhanced specificity[24].

Similarly, ELISA tests, although widely used, can suffer from cross-reactivity with other flaviviruses, complicating accurate diagnosis[17], [28]. Cross-reactivity among various arboviruses, such as Zika vírus, dengue virus, chikungunya virus, yellow fever virus, and West Nile virus, presents significant challenges in clinical diagnostics. These viruses share similar antigenic properties due to their phylogenetic relationships, which can lead to overlapping immune responses in infected individuals. For example, serological tests designed to detect ZIKV antibodies may misidentify antibodies generated in response to DENV infections, resulting in false-positive results and complicating differential diagnosis[2], [29]. The significance of this cross-reactivity is underscored during outbreaks when rapid identification of the specific virus is crucial to implement appropriate clinical management and public health interventions. Additionally, the continued evolution of these viruses may alter existing serological responses, highlighting the need for improved diagnostic assays that can differentiate among these closely related pathogens with greater specificity[30], [31].

Moreover, the overlapping symptoms of ZIKV with other arboviral infections such as dengue and chikungunya further complicate diagnosis[21], [32]. This diagnostic ambiguity can lead to underreporting and mismanagement of cases, emphasizing the need for rapid, portable, and sensitive detection tools that can be deployed in diverse settings[12], [33].

Need for Rapid, Portable, and Sensitive Detection Tools

The necessity for rapid and sensitive diagnostic tools is particularly acute in resource-limited settings, where traditional laboratory methods may not be feasible. Recent advancements in diagnostic technologies, such as loop-mediated isothermal amplification (LAMP) and point-of-care (POC) testing devices, offer promising alternatives for ZIKV detection[33], [34]. LAMP is a nucleic acid amplification technique that operates at a constant temperature, making it a more accessible option compared to traditional polymerase chain reaction (PCR), which requires multiple temperature cycles. This method can amplify specific DNA sequences with high specificity and sensitivity, thus providing rapid results within an hour even in field conditions[33]. It has been particularly useful in areas with limited laboratory infrastructure, allowing timely diagnostics and management of infections. Similarly, POC testing devices contribute to the immediate identification of viral pathogens, empowering healthcare workers to make informed clinical decisions without the delays associated with laboratory diagnostics. These portable tests often incorporate techniques such as electrochemical detection or molecular assays that enable quick turnaround times for

results, such as the detection of ZIKV proteins or antibodies in bodily fluids[19], [35] These methods are designed to be user-friendly and require minimal training, making them suitable for deployment in rural or underserved areas[36].

Furthermore, the integration of immunoassays and molecular techniques could enhance the specificity and sensitivity of ZIKV diagnostics, allowing for timely identification of infections and better management of outbreaks[28], [36]. The development of portable diagnostic devices that provide rapid results could significantly improve public health responses to ZIKV, particularly in the context of maternal and neonatal health, where early detection is crucial to prevent adverse outcomes[9], [26]. Furthermore, synergistic relationships between LAMP, POC devices and biosensors are critical to address the pressing need for effective diagnostics in the management of infectious diseases, especially in the face of outbreaks and co-circulating pathogens.

Biosensors - A Revolution in Diagnostics

Definition and Components of Biosensors

Biosensors are analytical devices that integrate a biological recognition element with a transducer to detect specific analytes, converting biological responses into measurable signals. The primary components of a biosensor include the bioreceptor, transducer, and signal processor. Figure 2 illustrates the components of a biossensor.

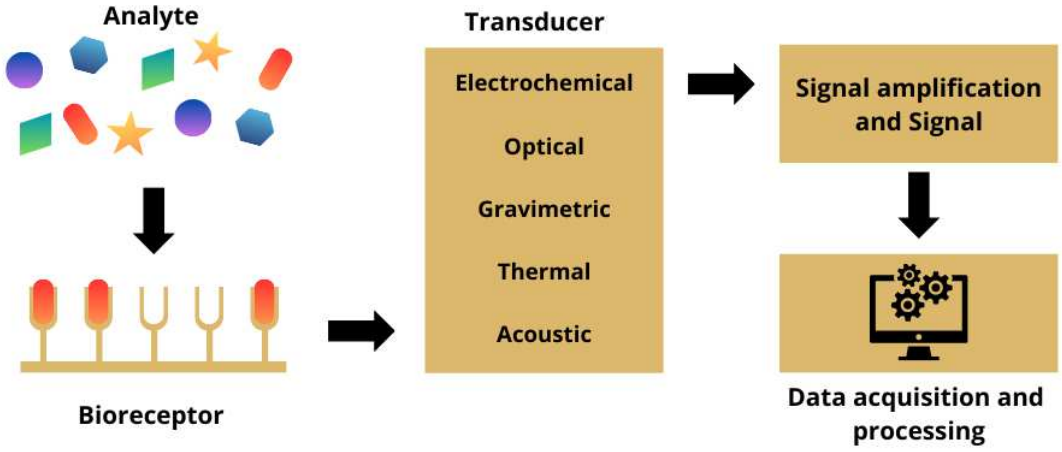

Figure 2. General operating diagram of a biosensor system.

A bioreceptor is a fundamental component of a biosensor, responsible for specifically recognizing a target substance (analyte)[37]. The transducer then converts this interaction into an electrical signal, which is processed and displayed by the signal processor[38]. This integration allows for real-time monitoring and analysis of various biological and chemical

substances, making biosensors invaluable in medical diagnostics and environmental monitoring[35], [39].

Types of Biosensors

Biosensors can be classified both according to the recognition elements, in the case of the bioreceptor, and by the transduction method. The bioreceptor can be of biological origin such as antibodies[9], [30], aptamers[40], [41], [42], [43], enzymes[24], [37], microorganisms[29] or even of synthetic origin such as nanostructured materials[30]. Cells, tissues, or entire organisms that can be used as bioreceptors to detect the presence of substances that affect their biological functions[37]. Biosensors can be classified into several types based on their transduction methods, including electrochemical[44], [45], optical[24], [46], thermal[37], electronic[47], and gravimetric transducers[48]. Among these, electrochemical biosensors are often regarded as the optimal choice due to their high sensitivity, rapid response times, and ease of miniaturization, ease of handling, low production cost, large-scale production[44], [49], [50], [51], [52], [53]. Electrochemical biosensors operate by measuring the current or voltage changes that occur during the biochemical reaction between the analyte and the bioreceptor[53]. Optical biosensor detect changes in optical properties (absorption, emission, refractive index) caused by the interaction of the analyte with the bioreceptor, while also effective, often require more complex setups and are generally less portable than their electrochemical counterparts[46]. Piezoelectric biosensors measure the change in the resonant frequency of a piezoelectric crystal when a mass attaches to its surface, although promising, are still in developmental stages and face challenges in terms of sensitivity and specificity[54]. Thermal Biosensors, these sensors monitor temperature changes that occur during chemical reactions. When an analyte interacts with a bioreceptor, there may be a variation in local temperature, which is measured and correlated to the concentration of the analyte[37]. Electronic biosensors detect changes in electrical conductivity or capacitance as an analyte binds to its bioreceptor. For example, graphene-based biosensors are used to detect electrical variations that occur with the adsorption of biomolecules to the transducer surface[37].

Advantages of Electrochemical Biosensors

Electrochemical biosensors offer several advantages that make them the ideal choice for various analytical applications. One of the main advantages is their high sensitivity, which enables the detection of extremely low concentrations of analytes, often in the femtomolar or

even attomolar range, depending on the sensor configuration and materials used[53]. Additionally, electrochemical biosensors have rapid response times, making them suitable for real-time analysis, with applications in infectious disease[44], [45], [50], [51], [52], [53] monitoring and food safety[55].

Another favorable characteristic is the ease of miniaturization and handling, which allows the development of portable devices. This promotes the use of electrochemical biosensors in POC settings, where tests can be performed quickly at the patient's bedside or in field environments, without the need for complex laboratories[17], [44], [53], [56], [57]. The low production cost can be a significant advantage, especially when using abundant nanomaterials such as graphene and carbon nanotubes, which not only enhance detection capabilities but also make the devices more accessible[25], [30], [38], [58].

Electrochemical biosensors also benefit from the possibility of large-scale production. The use of printing techniques and the integration of nanomaterials into electrodes make these sensors suitable for mass manufacturing, which can contribute to the availability and widespread implementation in the detection of pathogens, as demonstrated in applications related to foodborne pathogens[55].

Furthermore, electrochemical biosensors can be easily integrated with different transducers, such as thermal and gravimetric ones, further diversifying their applications in clinical diagnostics and environmental monitoring[59], [60]. This versatility, combined with their technical characteristics, makes electrochemical biosensors a powerful tool in various fields, from biomedicine to the food industry. Their portability and low cost also make them accessible for use in resource-limited settings, enhancing global health outcomes[54], [61].

Target Biomarkers for Zika Detection

Electrochemical biosensors designed for Zika virus detection typically focus on specific biomarkers such as viral RNA[17], NS1 protein[43], and IgM/IgG antibodies[26]. Immunoglobulin M (IgM) and Immunoglobulin G (IgG) antibodies provide significant specificity in identifying Zika virus antigens, making them reliable markers for infection detection [62]. Furthermore, the detection of IgM and IgG antibodies provides insights into the immune response and can indicate past infections[19]. For instance, serological assays that detect anti-Zika IgMtherm-class antibodies have received emergency use authorization due to their effectiveness in accurately identifying Zika cases, despite potential cross-reactivity with other flaviviruses like dengue virus[63]. The specificity of these antibodies

arises from their ability to bind uniquely to antigens associated with the Zika virus, such as the NS1 protein, which is noted for enhancing the sensitivity of assays specifically targeting Zika virus infections[64], [65].

Aptamer-based biosensors are noteworthy due to their inherent stability and ease of synthesis. Aptamers, which are short single-stranded DNA or RNA molecules, can be engineered to bind specific viral targets with high specificity, similar to antibodies, yet they offer notable advantages in terms of thermal stability and shelf-life[25], [66]. Unlike traditional antibodies, which may require complex and time-consuming production processes, aptamers can be synthesized through chemical means, allowing for rapid development and mass production. This flexibility in synthesis can be particularly beneficial in response to ongoing outbreaks, enabling quicker access to diagnostic tools, as highlighted by recent advancements in biosensor technology that leverage aptamer selection techniques such as SELEX (Systematic Evolution of Ligands by Exponential Enrichment)[66], [67]. Additionally, aptamers demonstrate lower susceptibility to degradation in challenging environmental conditions compared to antibodies, enhancing the utility of these biosensors in various settings[25], [66], while aptamers offer advantages such as stability and ease of synthesis [68].

Furthermore, the use of a system based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) which represents an adaptive immune system found in bacteria that allows the precise recognition and modification of DNA sequences, thus allowing the detection of specific nucleic acids[18], [41], [69]. The use of CRISPR-based systems has revolutionized biosensor design, allowing for highly sensitive detection of nucleic acids. For instance, CRISPR/Cas12a systems have been successfully integrated into electrochemical platforms to enhance sensitivity and specificity for viral RNA detection[70], [71]. This increase in sensitivity is due to the CRISPR system's ability to recognize specific nucleotide sequences and amplify them, minimizing false-negative results[18]. Additionally, aptamer-functionalized electrodes, which are sequences of DNA or RNA that bind to specific targets with high affinity, have been employed to selectively capture NS1 proteins, leveraging the high affinity of aptamers for their targets [62], [72].

The choice of target biomarkers is critical for the effective detection of the Zika virus. Viral RNA is a primary target due to its presence during active infection, while the NS1 protein, which is released in high quantities shortly after infection, serves as a reliable indicator of viral replication and early detection in the blood[62]. IgM antibodies are typically

detected during the acute phase of infection, indicating a recent immune response, while IgG indicates prior exposure and can be detected weeks after the initial infection[37] The integration of these biomarkers into electrochemical biosensors allows a comprehensive approach to Zika virus diagnostics, facilitating both early detection and serological studies. The combination of these advanced techniques enables the development of biosensors that can detect the Zika virus with high accuracy.

Case Studies of Innovative Biosensor Designs

Recent advancements in biosensor technology have led to innovative designs that enhance detection capabilities. For example, CRISPR-based electrochemical sensors have been developed for RNA detection, showcasing the potential of these systems for rapid diagnostics[18], [70]. Moço *et al*.[44] developed a groundbreaking electrochemical genosensor capable of detecting Zika virus genomic RNA in biological samples from infected patients. Their platform utilized graphite electrodes modified with electrochemically reduced graphene oxide and polytyramine, achieving exceptional sensitivity with a detection limit as low as 0.1 fg/mL of viral RNA. This technology was noted for its stability over a period of about 60 days and its rapid analysis time of approximately 20 minutes, making it a promising tool for point-of-care diagnostics in clinical settings. Lynch *et al*.[73] introduced a diagnosis platform that employs a universal DNA-hairpin probe for isothermal amplification of Zika virus RNA. This biosensor demonstrated a rapid detection capability, yielding results in less than an hour, which is essential during outbreaks where timely diagnosis can greatly impact public health responses.

Additionally, nanozyme-assisted amplification strategies have been employed to improve the sensitivity of CRISPR-based detection methods, enabling visual and electrochemical signal amplification[74]. The analysis of Zika virus detection methods using CRISPR technology, particularly Cas13 and Cas12-based methods, reveals several significant innovations. One of the main studies is developed by Gootenberg *et al*.[75], which presents a detection platform called SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing), enabling the specific detection of Zika virus RNA with increased sensitivity. This method has shown that detection could be performed in less than 1 hour, with a detection limit approaching 0.1 fM, highlighting its efficiency compared to traditional methods that often have longer response times and higher detection limits. Another relevant work, conducted by Meagher *et al*.[76], points to the use of paper-based sensors coupled with

CRISPR/Cas9 for rapid Zika RNA detection. This technique was developed in response to the emerging need for simple and sensitive diagnostic tests, with the total response time also around 1 hour, reflecting a significant advancement for resource-limited settings. Aptamer-functionalized electrodes have also shown promise in enhancing the binding efficiency and specificity for protein detection, further advancing the field of electrochemical biosensing[72], [77]. One significant advancement in this field is represented by Almeida *et al*.[43] in integrating DNA aptamers with graphene field-effect transistors (FETs) for the detection of Zika virus proteins further illustrate the synergy between nanotechnology and biochemistry in developing cutting-edge diagnostic tools. These aptamer-based FET devices show promise for direct virus detection and hint at the versatility of aptamer applications across various types of electrochemical biosensing platforms. Furthermore, the efforts by Jang *et al*.[77], who introduced a rapid biosensor utilizing truncated DNA aptamers to detect Zika virus envelope proteins in clinical serum samples. Their work showcases the effectiveness of using electrochemical pulse voltammetry for real-time detection, achieving highly sensitive measurements within a short turnaround time. This method highlights not only the rapidity of detection but also its applicability in clinical diagnostics, which is essential for mitigating outbreaks.

Comparison of Performance Metrics

When comparing the performance metrics of electrochemical biosensors with traditional methods, several factors must be considered, including the limit of detection (LOD), specificity, and time-to-result. Electrochemical biosensors have showcased significantly lower LODs, making them particularly advantageous for the detection of pathogens, including the Zika virus, at reduced concentrations[78], [79]. For example, Zika virus detection is pivotal for timely and effective public health responses, and studies have indicated that electrochemical biosensors can achieve detection limits in the nanomolar range, surpassing traditional methods like serum or plasma tests[80], [81]. Moreover, the specificity of these biosensors is enhanced through the use of tailored probes, such as DNA/RNA aptamers or antibodies, have been extensively used to target specific viral pathogens, greatly improving specificity and reducing cross-reactivity with non-target organisms[82], [83], and amplification strategies such as nanotechnology integration[84], [85] reduce cross-reactivity with other viral pathogens[71], [74]. The rapid response time of electrochemical biosensors, often within minutes, stands in stark contrast to the longer processing times associated with traditional laboratory methods, making them ideal for point-of-care applications[22].

Importance of Electrode Materials in Enhancing Sensitivity

The choice of electrode materials significantly influences the performance of electrochemical biosensors. Recent advancements have demonstrated that nanomaterials such as graphene, carbon nanotubes (CNTs), and gold nanoparticles (AuNPs) play pivotal roles in enhancing these biosensors due to their remarkably high electrical conductivity, substantial surface area, and excellent biocompatibility[58], [86], [87]. Figure 3 exemplifies materials that facilitate enhanced probe immobilization and conductive performance in biosensors. For example, CNTs and graphene are known for their large surface area and high electrical conductivity[88], [89], which are critical for increasing the rate of chemical reactions and enhancing signal transduction in electrochemical sensors. For instance, research has demonstrated that biosensors utilizing CNTs can achieve detection limits as low as 0.5 pg/mL in complex sample matrices, indicating a significant leap in sensitivity compared to traditional techniques[90].

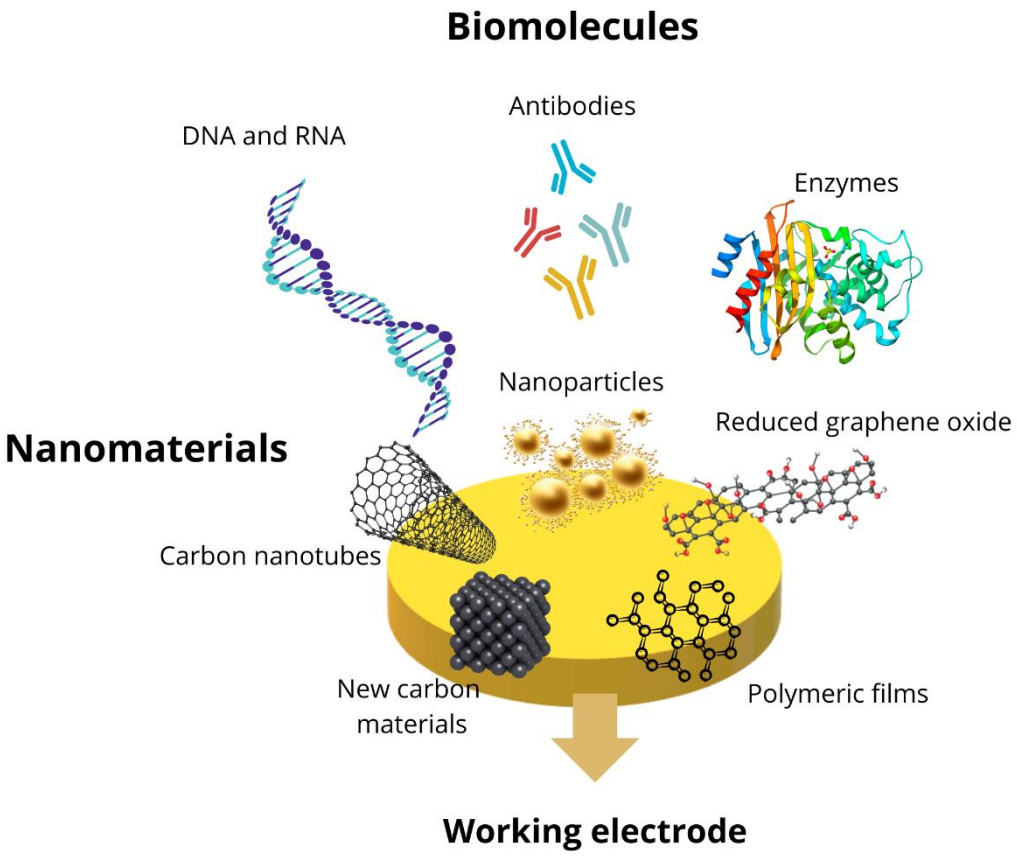

Figure 3. Examples of working electrode modifiers for biosensor construction

Furthermore, the integration of functional nanomaterials has been shown to improve LOD considerably in lab-on-chip microfluidic devices, allowing for rapid diagnostics in

healthcare settings. However, comprehensive evidence on the exact extent of these improvements varies across studies[91]. The strategic combination of nanomaterials with biomolecules further amplifies the signal response in biosensing applications. For instance, studies utilizing aptamer-functionalized CNTs have showcased rapid and sensitive detection of viral pathogens, achieving detection limits significantly lower than conventional methods[92]. The response mechanisms involving these materials help in achieving ultra-sensitive detections in the femtomolar range, attributable to high binding affinity and effective electron transfer facilitated by the nanomaterial interfaces[93]. Moreover, graphene-based materials excel not only due to their superior electronic properties but also from their adaptability in hybrid systems. Recent applications have showcased that graphene oxide incorporated with gold nanoparticles can lead to highly sensitive detection systems that provide LODs in the low femtomolar range[94]. This notable sensitivity is often tied to the plasmonic effects present in these nanomaterials, which enhance photonic interactions that amplify the detected signals[95]. Combined with flexible substrate technologies, these developments explore new frontiers for biosensing applications, particularly in point-of-care diagnostics, where rapid results and sensitivity are paramount[96]. The continual evolution of nanotechnology enables biosensors to capitalize on quality improvements, pushing detection limits further down. New materials and fabrication strategies have led to biosensing platforms capable of detecting biomarkers at attomolar concentrations across complex biological matrices[97]. The integration of these nanomaterials into biosensor designs can lead to significant improvements in sensitivity and detection limits for Zika virus diagnostics[98], [99].

Recent Advances in Electrochemical Detection of Zika Virus

Recent technological advancements in electrochemical detection methods have significantly improved the sensitivity and specificity of Zika virus diagnostics. Innovations such as lab-on-a-chip systems, which integrate multiple laboratory functions on a single chip, have been developed to facilitate rapid testing and analysis[100]. The detection of the Zika virus has become increasingly critical due to its association with severe congenital conditions. Various biosensing techniques have been developed to enhance the detection capabilities for the Zika virus, each with its unique methodologies, targets, limits of detection, and advantages. Table 1 summarizing detection devices for the Zika vírus. Ribeiro demonstrated the efficacy of a quantum dot-modified electrochemical immunosensing platform for the detection of Zika virus biomarkers, highlighting the benefits of quantum dots (QDs) in

enhancing sensitivity and detection limits. They noted that the inclusion of QDs in the biosensing assembly increases the electrode's functional area, facilitating the immobilization of biomolecules and improving detection performance[101]. Graphene oxide materials are frequently employed as enhancing substrates owing to their high surface area and electrical conductivity. The reduction of graphene oxide can increase the electroactive surface area, thus improving sensitivity. Furthermore, Park emphasized the advantages of using MXenes as electrochemical biosensors, which allow high electron conductivity and sensitivity in human serum analysis[47].

Table 1 - Comparative Analysis of Electrochemical Biosensing Technologies for Zika Virus

Principle	**Technique**	**Markers detected**	**Linear Range**	**Limit of detection (LOD)**	**Reference**
A platform based on graphite electrodes modified with electrochemically reduced graphene oxide and polytyramine-conducting polymer.	Differential pulse voltammetry	ZIKV gRNA	1.72 - 1.72×10^{10} copies mL^{-1}	1.72 copies mL^{-1} (0.1 fg mL^{-1})	Moço, *et al*.[44]
Rapid Electrical Pulse-Based Biosensor Consisting of Truncated DNA Aptamer for Zika Virus Envelope Protein	Pulse voltammetry	ZIKV E protein	10 pM-1 µM	90.1 pM	Jang, *et al*.[77]
Sensitive Zika biomarker detection assisted by quantum dot-modified electrochemical immunosensing platform	Cyclic voltammetry/ Electrochemcal impedance spectroscopy	anti-EP ZIKV	N/A	0.1 ng	Ribeiro, *et al*.[101]
Selection of DNA aptamer and its application as an electrical biosensor for Zika virus detection in human	Electrochemical capacitance Spectroscopy	ZIKV E protein	100 pM-10µM	38.14 pM,	Park, *et al*.[47]

serum					
Redox-Probe-Free Immunosensor Based on Electrocatalytic Prussian Blue Nanostructured Film One-Step-Prepared for Zika Virus Diagnosis	Cyclic voltammetry	ZIKV E protein	0.25-1.75 μg mL^{-1}	0.20 μg mL^{-1}	Santos, *et al*.[102]
Electrochemical immunosensor based on ZnO nanostructures immobilized with ZIKV-NS1 antibody on Printed Circuit Board (PCB)	Cyclic voltammetry	ZIKV NS1	0.1 ng mL^{-1} - 100 ng mL^{-1}	1.00 pg mL^{-1}	Faria, *et al*.[103]
A sensitive label-free impedimetric DNA biosensor based on silsesquioxanefunctionalized gold nanoparticles for Zika Virus	Electrochemical impedance spectroscopy	ZIKV ssDNA	$1.0x10^{-12}$ - $1.0x10^{-6}$ M	0.82 pM	Steinmetz, *et al*.[104]
Cyclic voltammetric PBG-based detection of the target DNA of Zika virus	Cyclic voltammetry	ZIKV target DNA	0.1-100 μM	0.1 μM	Bishoyi, *et al*.[105]
A single chip that distinguishes between Zika and Dengue infections using the non-structural protein 1 (NS1) as biomarkers	Electrochemical impedance spectroscopy	ZIKV NS1 protein	15.62-500 ng mL^{-1}	0.54 ng mL^{-1}	Sampaio, *et al*.[106]
Electrochemical magneto-immunoassay for detection of zika virus antibody in human serum	Electrochemical impedance spectroscopy	ZIKV NS1 protein	0.01-$9.8x10^{5}$ pg mL^{-1}	0.48 pg mL^{-1}	Castro, *et al*.[107]
Development and use of	Chronoamperometry	Anti-ZIKV	5-300	0.7 pmol L^{-1}	Alzate, *et*

genosensors specifically designed for the reliable detection of the Zika virus			pmol L^{-1}		*al*.[108]
A Sensitive and Specific Genomic RNA Sensor for Point-of-Care Screening of Zika Virus from Serum	Electrochemical capacitance Spectroscopy	ZIKV gRNA	N/A	78.8 copies μL^{-1}	Cheng, *et al*.[109]
Impedimetric electrochemical DNA biosensor for labelfree detection of zika virus	Electrochemical impedance spectroscopy	ZIKV cDNA	54-340 nM	25 nM	Faria, *et al*.[110]
Development of a new type of ZIKV electrochemical biosensor based on surface imprinted polymers and graphene oxide composites	Cyclic voltammetry	ZIKV strain	N/A	1 copy mL^{-1}	Tancharoen, *et al*.[111]

Additionally, electrocatalytic sensors utilizing Prussian Blue nanoparticles have been explored for amplifying the electrochemical signal associated with ZIKV detection. Santos *et al*.[102] work illustrates that the integration of redox-active materials enables the generation of a Faradaic current in the presence of ZIKV antibodies, thereby facilitating the measurement of specific antibody binding.

Furthermore, Cheng's research into genomic RNA sensors highlights the potential of integrating capacitive measurement techniques to detect ZIKV directly from serum samples[109]. This technology, along with portable electrochemical devices, facilitates immediate and accessible diagnostic capabilities, aligning with global health efforts to monitor and control ZIKV outbreaks effectively.

Challenges remain, particularly in addressing cross-reactivity among closely related viruses, which complicates differential diagnosis. Castro's exploration of electrochemical magneto-immunoassays identified that while antibody interactions enhance sensitivity, they are prone to interference from structurally similar proteins, requiring careful design and validation in new assays[107].

Point-of-Care Applicability

The integration of electrochemical sensors into point-of-care (POC) diagnostics is a significant advancement in the rapid detection of the Zika virus. The ability to deliver results quickly and accurately at the site of care is crucial in managing outbreaks and ensuring timely treatment. Portable electrochemical sensors can be coupled with smartphone-based readouts, enabling immediate results even in remote areas. Sharma *et al*.[112] discuss how the use of silver nanoparticle-enhanced biosensors can improve diagnostic efficiency and accessibility in outbreak-prone regions, highlighting the importance of such innovations in differentiating between co-circulating pathogens like Zika, dengue, and chikungunya, ultimately enhancing patient management and public health outcomes. Figure 3 represents a schematic of the application of a Point-of-care device.

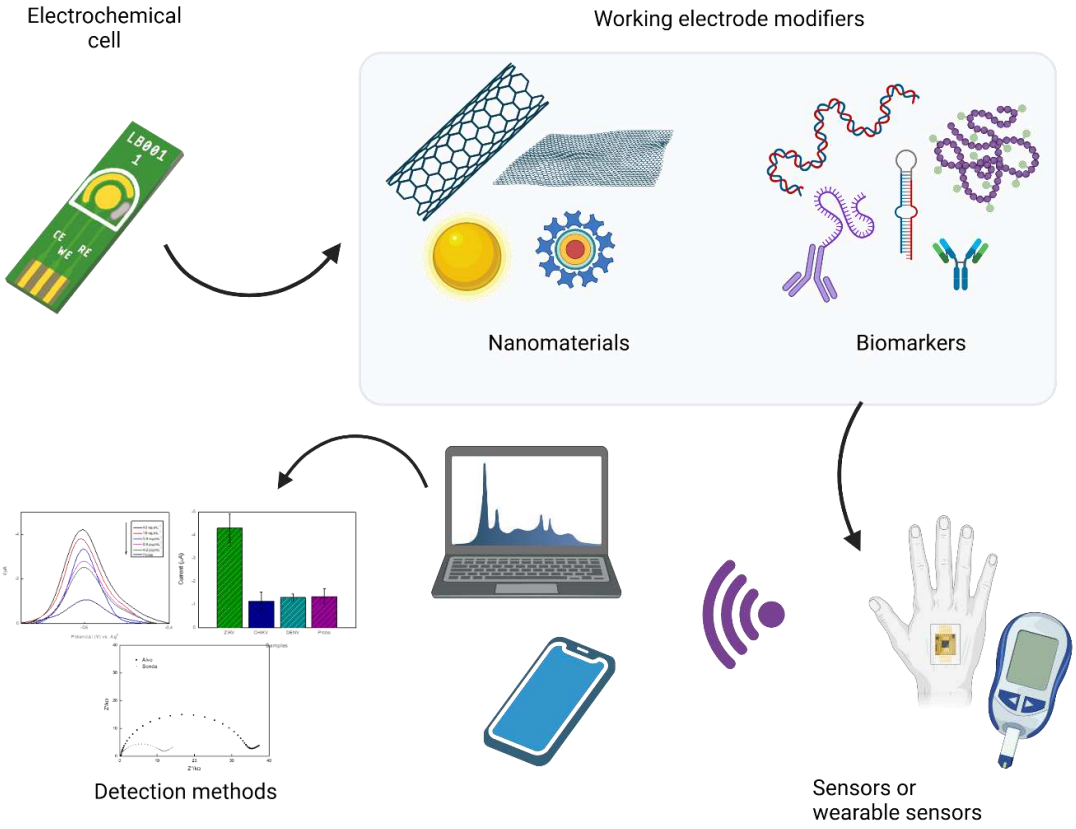

Figure 4. Schematic of the application of a point-of-care device.

Recent studies have highlighted the development of paper-based electrochemical immunosensors that allow for label-free detection of viral antigens, making them suitable for POC applications[113]. Dongen *et al*.[114] discuss the promising capabilities of CRISPR/Cas systems for nucleic acid detection, which are well-suited for on-site or portable testing. These technologies can yield rapid diagnostic results, further supporting the trend toward innovative

diagnostic tools that cater to real-world application demands. This trend is further supported by emerging research on wearable biosensors, such as those developed by Batra *et al*.[115], which demonstrate the versatility of biosensors in detecting viral pathogens across various formats, thereby contributing to rapid response capabilities in POC diagnostics.

Moreover, portable devices that utilize nucleic acid amplification techniques combined with electrochemical detection have shown promise in enhancing the accessibility and efficiency of Zika virus diagnostics[116].

Together, these studies highlight the transformative potential of portable diagnostic devices in addressing the urgent need for effective Zika virus detection, especially in resource-limited settings.

Multiplexing Capabilities

The simultaneous detection of multiple viruses, such as Zika, dengue, and chikungunya, is another critical innovation in electrochemical biosensing. The development of multiplexed biosensors allows for the detection of various pathogens in a single assay. These tools are of profound importance in regions where ZIKV, DENV, and CHIKV often co-circulate, complicating diagnosis and treatment[106], [117], [118], which is particularly beneficial in regions where these viruses co-circulate. Recent advancements in DNA aptamer-based sensors have demonstrated the capability to detect ZIKV NS1 protein alongside other flaviviruses, achieving high sensitivity and specificity[119]. For instance, research has demonstrated that integrated biosensing platforms can differentiate between these viruses with high sensitivity and specificity. Notably, a significant advancement was presented by Lee *et al.*[118], who developed a graphene oxide-based molecular diagnostic biosensor capable of simultaneously detecting ZIKV and DENV. This dual-target capability is crucial for managing outbreaks where co-infections are prevalent, thereby improving patient outcomes and public health responses[117], [118].

Furthermore, the application of DNA-hairpin probe for Zika virus RNA, as elucidated by Lynch *et al*.[120], illustrates another powerful methodology that enhances detection precision. Additionally, the use of microfluidic platforms has been explored to enhance the multiplexing capabilities of electrochemical sensors, enabling rapid and simultaneous analysis of multiple viral targets[116]

The integration of microfluidic platforms has further amplified the multiplexing capabilities of these electrochemical sensors. Microfluidics enables rapid, efficient handling of samples, thus streamlining the diagnostic workflow for multiple viral targets in a single assay. Studies indicate that the use of microfluidic systems can enhance diagnostic speed and accuracy for co-infected patients, facilitating better clinical decision-making and public health measures[117], [121]. Research by Sampaio *et al*.[106] demonstrates significant progress in developing microfluidic sensors for simultaneous detection of ZIKV and DENV directly from serum samples, improving the overall diagnostic process and comprehensively addressing co-infection scenarios

This approach not only streamlines the diagnostic process but also improves the understanding of co-infections, which is vital for effective public health responses.

Challenges in the electrochemical detection of the Zika virus

One of the primary challenges in the electrochemical detection of the Zika virus is cross-reactivity with related flaviviruses, such as dengue and chikungunya. This cross-reactivity can lead to false-positive results, complicating the diagnosis and management of infections. Studies have shown that various electrochemical sensors can exhibit significant interference from other viral proteins present in complex biological matrices[19], [105], [122]. Furthermore, sample matrix interference is a critical complication in electrochemical detection. Biological fluids contain numerous endogenous substances, including proteins, salts, and small molecules, which can impact analytical performance[109], [123]. For instance, the presence of high concentrations of uric acid can inhibit the electrochemical reactions necessary for the successful detection of viral components, leading to reduced sensitivity and specificity[56], [124]. Current research suggests that modifying the sensor surfaces with nanomaterials may mitigate some matrix effects by improving the selectivity and enhancing the interaction between the sensor and the target viral components[125], [126]. Nanomaterials such as gold nanoparticles and graphene oxides have been shown to significantly enhance the electrochemical signals, thereby compensating for the interference caused by complex biological matrices[102], [127]. The presence of these interfering compounds in serum or urine samples can lead to reduced sensitivity and specificity, necessitating the development of more selective detection methods[128]. Moreover, the methodology employed for sample preparation is crucial for minimizing matrix interferences. Techniques such as sample dilution, filtration, or enzymatic treatment are often utilized to

reduce the concentration of interfering substances before running electrochemical assays[53], [129]. However, these methods can introduce additional complexities and potential errors in quantifying the viruses[123]. It is therefore essential for researchers to innovate and develop electrochemical sensors that can selectively capture and detect ZIKV even in the presence of these challenging sample matrices, contributing to more reliable diagnostic capabilities.

The scalability and manufacturing of electrochemical sensors for Zika virus detection also present significant challenges. Issues related to the reproducibility of bioreceptors, cost of materials, and shelf-life of the sensors are critical factors that influence the widespread adoption of these technologies. For instance, the stability of bioreceptors in complex matrices can vary, leading to inconsistent performance across different batches of sensors[130], [131]. Furthermore, research directed toward developing cost-effective and reliable manufacturing methods is crucial to enhance the accessibility of these diagnostic tools in endemic regions[20]. Addressing these challenges is crucial for ensuring that electrochemical sensors can be produced at scale while maintaining their performance and affordability.

Regulatory and Commercialization Barriers

Regulatory hurdles represent another significant barrier to the commercialization of electrochemical sensors for Zika virus detection. The validation of these sensors in clinical settings is essential for gaining approval from regulatory bodies such as the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC)[132]. The process of obtaining these approvals can be lengthy and complex, often requiring extensive clinical trials to demonstrate the efficacy and safety of the sensors[124]. Additionally, the need for standardized testing protocols and quality control measures further complicates the pathway to market[133]. Overcoming these challenges is vital to the successful integration of electrochemical detection methods into public health strategies to manage disease outbreaks such as Zika Virus.

Future Directions in Electrochemical Detection of Zika Virus

Future research should focus on optimizing these biosensing platforms for real-world applications, including the integration of machine learning (ML) algorithms to improve detection accuracy and speed[134]. Research by Schackart and Yoon further emphasizes the role of machine learning in enhancing bioreceptor-free biosensors, which are traditionally limited by performance issues such as LOD and specificity[135]. The integration of ML

algorithms allows for the analytics of data generated by these biosensors, thus bridging the aforementioned gaps. This adaptation of ML is crucial, as it effectively replaces the specificity typically offered by bioreceptors through detailed data analysis. Additionally, Flynn and Chang discuss the opportunities presented by AI technologies in point-of-care biosensing, suggesting that AI can improve the accessibility and accuracy of health assessments performed at the patient level[136]. The review explores various computational advancements that promise to refine diagnostic methodologies, indicating a significant shift towards personalized health monitoring systems.

The integration of AI-driven technology and hybrid optimization algorithms holds significant promise for the future detection of the Zika virus. By employing advanced machine learning techniques, such as multilayer perceptron with a probabilistic optimization strategy, researchers can enhance the accuracy of predictions related to Zika outbreaks, ultimately facilitating better resource allocation for public health [137]. Moreover, this technology not only reduces forecast times but also improves the precision of geographical surveillance, thereby enabling rapid responses to potential outbreaks[137]. Additionally, the use of cloud computing for data processing ensures that collected health metrics are efficiently analyzed and securely stored, providing real-time insights into Zika virus dynamics[137]. As wearable health technologies become more integrated with AI, the potential for real-time monitoring of symptoms and risk factors associated with Zika virus infection will continue to expand, further contributing to what could be a more agile and responsive healthcare framework for managing outbreaks[138].

Conclusions

The ongoing threat posed by the Zika virus underscores the urgent need for rapid, sensitive, and accessible diagnostic tools. Traditional methods such as PCR and ELISA, while effective, are often impractical in resource-limited settings due to their complexity and cost. In contrast, electrochemical biosensors represent a transformative approach to ZIKV detection, combining rapid results with high sensitivity and the potential for point-of-care applications. The integration of advanced technologies, including CRISPR-based systems and nanomaterials, further enhances the capabilities of these biosensors, making them invaluable in the fight against ZIKV and similar viral infections. Future research should focus on optimizing these technologies for widespread use, ensuring that diagnostics keep pace with the evolving landscape of viral threats. Additionally, enhancing devices with the

use of AI, wearable technologies and effective data management frameworks is essential to promote early detection of viral infections, supporting a proactive approach to managing Zika virus outbreaks.

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Capítulo 2: Development of an electrochemical biosensor based on a cysteamine-CQD bilayer for detection of Zika Virus

O capítulo 2 corresponde a um manuscrito de artigo original redigido nas normas do periódico Electroanalysis (ISSN: 1521-4109).

Advanced Electrochemical Genosensor for Zika Virus Detection in Biological Samples Based on Cysteamine-Graphene Quantum Dots Bilayer

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Abstract
This paper describes a novel electrochemical genosensor designed for rapid and simplified detection of Zika virus DNA, using the biological dye safranin as biomolecular intercalator. The genosensor uses a gold-printed circuit board as electrode, modified with a bilayer formed by cysteamine and graphene quantum dots to immobilize oligonucleotides probes specifically designed for detection of Zika virus. The genosensor construction was monitored by scanning electron microscopy (SEM), dynamic force microscope (DFM) and Fourier transform infrared (FTIR). Electrochemical detection was carried out based on differential pulse voltammetry, monitoring the peak current of the DNA intercalator (safranin). The genosensor demonstrated high sensitivity detecting 4.2 pg mL^{-1}, selectivity against other arboviruses (chikungunya and dengue) and good stability for at least 45 days. These parameters indicate potential for use of this genosensor in medical diagnostic testing for Zika virus, aiming at early screening of patients, especially in epidemic situations.

Keywords: Electrode modification, safranin, PCB

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1. Introduction

According to the World Health Organization (WHO), the Zika virus (ZIKV) is considered a threat to global public health. This virus contains single-stranded RNA as its genetic material and is associated with cases of Guillain-Barré syndrome and microcephaly[1]. Given the tropical prevalence of the infection and the lack of effective treatments, it is essential to conduct accurate and timely diagnostics for disease control [2-4].

Molecular techniques, particularly the current diagnostic standard, reverse transcription polymerase chain reaction (RT-PCR), have emerged as the most sensitive and specific methods for detecting ZIKV, outperforming traditional serological assays[5,6]. However, this method faces challenges in endemic regions, primarily due to high costs, requirement for skilled personnel and sophisticated laboratory equipment, which can delay results and hinder timely access to diagnosis in underserved areas[2, 4, 7].

The need for rapid and precise diagnostics is further emphasized by the limitations of serological tests, which can suffer from cross-reactivity with other flaviviruses, complicating the interpretation of results[8, 9].

Biosensors are defined as analytical devices that incorporate a biological sensing element directly connected to a transducer, which converts a biological response into an electrical signal. This integration allows quantitative detection of analytes, such as biomolecules, pathogens, or toxins, making biosensors powerful tools across various fields, including medical diagnostics, environmental monitoring, and food safety assessment[10]. Biosensors have advantages over techniques like RT-PCR and enzyme linked immunonosorbent assay (ELISA) in terms of cost, accessibility and ease of use, requiring less complex equipment and highly trained personnel for operation[11].

Additionally, biosensors have low detection limits, allowing the detection of reduced amounts of analytes.

Among biosensors, electrochemical ones are among the most advanced, offering high sensitivity, selectivity, and speed, making them a superior option for clinical diagnostic applications[12].

Furthermore, public awareness and education regarding Zika virus transmission and prevention are crucial for controlling its spread. Studies have shown that informed populations are better equipped to engage in preventive measures, which can significantly reduce transmission rates[13,14]. The role of public health officials in disseminating accurate information and combating misinformation is vital, particularly in the context of emerging infectious diseases like ZIKV[15,16].

The integration of biosensors into public health strategies could facilitate community engagement by providing accessible testing options, thereby enhancing the overall response to Zika virus outbreaks[1, 3].

These advancements highlight the critical role of biosensors in enhancing the detection capabilities for ZIKV, ultimately contributing to better public health responses in affected regions. The potential for biosensors to provide timely and accurate results could significantly improve the management of ZIKV outbreaks, especially in areas where healthcare resources are limited[13, 16, 17].

The use of PCBs (Printed Circuit Boards) has been increasingly employed as electrodes in biosensors, among the different uses are detection of myeloperoxidase in blood plasma[18], monitoring of cancer cells[19] and monitoring in cryobiology applications[20]. Due to the technical and financial advantages offered by their ease of large-scale manufacturing, low cost, ability to be miniaturized, and high sensitivity, among other properties. Therefore, these characteristics would provide the opportunity to produce cheap and effective biosensors for a wide range of biomedical and environmental pollution applications[21].

Application of cysteamine for modifying gold electrodes in biosensor construction is justified by its capacity to form self-assembled monolayers, which notably enhance the electrochemical performance of the surfaces. When adsorbed onto the gold surface, cysteamine creates a layer that not only aids in the immobilization of biomolecules but also introduces new active sites that boost the sensitivity of the biosensor. Research demonstrates that cysteamine modification can increase the electrode surface coverage, leading to improved electron transfer and greater effectiveness in analyte detection[22, 23].

In addition, cysteamine has chemical properties that allow the formation of covalent bonds with functional groups in biomolecules, contributing to more specific and specific biosensors[24].

Graphene quantum dots (GQDs) are increasingly recognized for their significant role in the enhancement of electrochemical biosensors, mainly due to their intrinsic physicochemical properties, particularly their strong π interactions. The unique structure of GQDs, characterized by their zero-dimensional nanoscale and ample surface area, facilitates efficient electron transfer and advantageous π-π stacking interactions with biomolecules, thus amplifying the sensitivity and specificity of biosensing platforms[25,26]. These π interactions enhance the adsorption of target analytes, which is essential for the high performance of biosensors[25, 27].

This paper introduces a novel point-of-care genosensor for detection of Zika virus, using gold PCBs as electrodes, modified with a bilayer formed by cysteamine and graphene quantum dots functionalized with cysteamine and safranin, a fluorescent azo dye, used as biomolecular intercalator.

2. Material and methods

2.1. Chemicals and Apparatus

All the solutions were prepared with deionized and ultrapure water (resistivity of 18.2 MΩcm, Gehaka) and deoxygenated with ultrapure nitrogen. All the reagents were analytical grade and used without further purification. Sulfuric Acid (98%), sodium chloride (99%), dibasic sodium phosphate (99%) and monobasic sodium phosphate (99%) were obtained from Synth. Safranin ($C_{20}H_{19}N_4^+ \cdot Cl^-$, 95%) was obtained from Dinâmica. Citric acid and hydrogen peroxide (30%) purchased from Merck. Cysteamine and glycine were purchased from Sigma-Aldrich. Isopropyl alcohol (99%), acetone (99%) and ammonium hydroxide (28%) were purchased from Cromoline. Ethyl alcohol (95%) was purchased from Neon. The probes used (5'-CACTGAGTCAAAAAACCCCACGCGCTT-3') and complementary target (5'-AAGCGCGTGGGGTTTTTTGACTCAGTG-3') were obtained from Exxtend. All lyophilized oligonucleotides were prepared in buffered saline sodium citrate (0.03 mol L^{-1} sodium citrate, 0.3 mol L^{-1} NaCl, pH 7.4) to obtain the concentration of 100 μg μL^{-1}]. Zika (ZIKV), chikungunya (CHIKV), dengue (DENV) gRNAs were extracted using the QIAmp Viral RNA kit (Qiagen) and RNA was eluted with 75 μL of RNase-free water. Samples of gRNA extracted from serum of patients infected by arboviruses

were obtained from Adolfo Lutz Institute (Brazil, São Paulo, Ethics Commission number: 2.333.595).

The sensor utilized in this study is a three-electrode cell. The model was designed by the authors and produced by PCBWay, featuring working electrodes and counter electrodes made of gold. For the pseudo-reference electrode, silver ink was applied using screen printing techniques.

2.2. Instrumentation

Electrochemical measurements were performed using Differential Pulse Voltammetry (DPV) in a potentiostat CHI 760C (CH Instruments, USA). The surface morphology of the electrodes was assessed by scanning probe microscopy (SPM, Model 5100 N, Hitachi, Japan), performed in the dynamic force microscope (DFM) mode and by scanning electron microscope (SEM) using model Vega3 LMU (TESCAN, Czech Republic), operated at 10.00 kV. Fourier transform infrared (FT-IR) spectrum was recorded using a spectrophotometer FT-IR Frontier Single Range – MIR (Perkin Elmer, USA), in solid state with attenuated total reflectance accessory. The absorption spectra were obtained using a UV–Vis spectrophotometer (Shimadzu 1800, USA).

2.3. Board design and cleaning

Each printed circuit board (PCB) features a total of 27 electrodes, which were first cleaned with isopropyl alcohol and cotton to eliminate surface impurities. Subsequently, they were immersed in an ultrasonic bath for 15 minutes in solution composed of equal parts acetone, ethanol, and water. A second ultrasonic bath of 30 minutes was performed using solution composed of water, ammonium hydroxide (28%), and hydrogen peroxide (30%) in a 5:1:1 ratio. After this washing step, the electrodes were rinsed with deionized water, dried with nitrogen gas, and then placed in an oven at 40 °C for 15 minutes.

2.4. Quantum dots preparation

Graphene quantum dots (GQDs) were produced using a one-step pyrolysis method. 0.5 g of citric acid and 0.18 g of glycine were added to a 20 mL round-bottom flask and heated from room temperature to 200 °C. The solution changed color from colorless to yellow in about 3 min. The temperature was reduced to 150 °C and maintained for about 10 minutes, resulting in an orange color, when the heating was stopped. The GQDs were obtained by dispersing the solution obtained in 40 mL of distilled water. This GQD production methodology was described by Zhu[28].

2.5. Electrode modification

The pseudo-reference electrode was coated with a silver conductive ink using a pneumatic screen-printing machine. To make this print, screen printing was used with a mesh of 77 threads.

The surface of the working electrode was modified with cysteamine. A study was conducted using various concentrations of cysteamine, ranging from a minimum of 0.01 mol L^{-1} to a maximum of 0.5 mol L^{-1}.

These concentrations were allowed to interact with the surface of the working electrode for varying durations, with a minimum immobilization time of 1 hour and a maximum of 24 hours evaluated for each concentration.

The concentration defined for the subsequent experiments was 5 µL, 0.5 mol L^{-1}, maintained for 12 hours in a hermetically sealed system, in humid atmosphere and at room temperature. This time interval and this concentration of cysteamine were chosen because no significant changes in immobilization were observed after this period.

After this period, each electrode underwent a separate washing, being immersed for 10 seconds in deionized water in an agitated system. Subsequently, the electrodes were dried with ultrapure nitrogen gas and placed in a dry oven at a temperature of 40 °C for 15 minutes. After the formation of the cysteamine layer, 5 µL of a graphene quantum dots solution were applied onto the electrodes. To dry this solution, the electrodes were placed in the oven for 15 minutes at 50 °C. This process is performed to obtain a bilayer of cysteamine and graphene quantum dots (Cys-GQDs), which is used for immobilizing the oligonucleotides employed in this sensor.

2.6. Fabrication of genosensor and Zika Virus detection

Following the modification of the working electrode surface, 5 µL of a probe solution with a concentration of 8.18 mg mL^{-1} was applied onto the Cys-GQDs bilayer. This DNA sequence (5'-CACTGAGTCAAAAAACCCCACGCGCTT-3') was obtained using bioinformatics software and is specific to the Zika virus genome. The sensor with the deposited probe was stored in a sealed, humidified chamber for 40 minutes for immobilization by adsorption on the bilayer surface. This procedure was carried out at room temperature. Figure 1 shows the construction scheme of the genosensor.

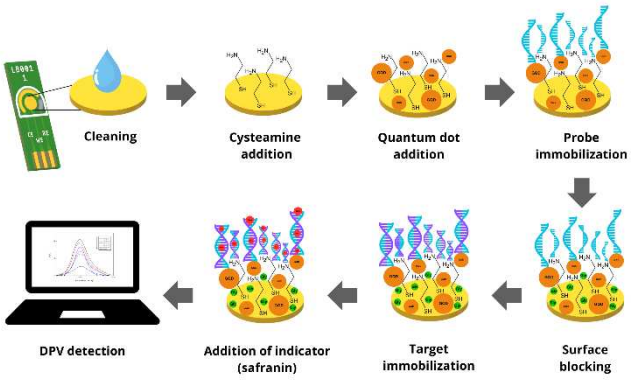


Fig 1. Scheme of the electrochemical genosensor construction.

After the immobilization of the probe, a surface blocking step was performed using glycine (5 µL, 10 mmol L^{-1}). During the blocking procedure, the bioelectrodes were stored in a closed and humidified system for 60 minutes at room temperature. A wash was performed to remove excess glycine, and then the electrode was dried. The purpose of the blocking procedure was to ensure that the interaction occurred only between the probe and the target during the hybridization step, avoiding non-specific interactions between the target and the working electrode surface. To form the double strand, 5 µL of the target was dripped onto the working electrode. The target is the complementary sequence of the Zika virus genome probe (5'-AAGCGCGTGGGGTTTTTTGACTCAGTG-3'. In the hybridization procedure, the genosensor platform used was humidified and sealed. In sequence, the platform was placed in an oven at 57 °C for 35 minutes. After this time, the washing and drying procedure were performed. The next step involved dripping the safranin solution (5 µL, 0.1 mmol L^{-1}) onto the working electrode, aiming at the safranin intercalation process in the double strand formed by the probe and target. This process was carried out in a closed and humidified system at room temperature for 1 hour. After this time, the washing and drying procedure was performed.

The final stage involved detecting the safranin intercalated in the double strand. For this, measurements by differential pulse voltammetry were performed. The scanning parameters were set to a scan rate of 50 mV s^{-1}, potential range of 0 to -1 V, equilibration time of 5 seconds, and supporting electrolyte of phosphate buffer (pH 7.4). In this procedure, the safranin reduction peak was monitored.

3. Results and discussion

3.1. Characterization of the electrode modified with cysteamine using SEM

Figure 2 shows the morphological analysis by SEM of the electrode modified with cysteamine.

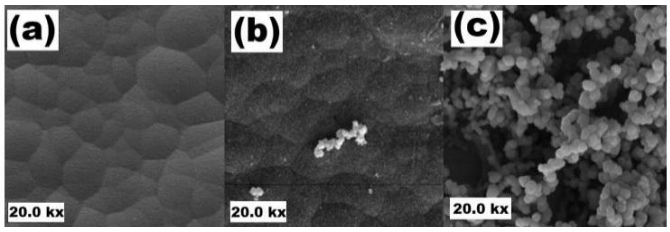


Fig 2. SEM images for the surface of (a) gold electrode without modification, (b) gold electrode modified with cysteamine (0.01 mol L^{-1}), (c) gold electrode modified with cysteamine (0.5 mol L^{-1}).

The results obtained reveal a remarkable change in the morphology of the electrode surface after treatment with cysteamine, compared to the unmodified electrode (Fig. 2a). Interconnected circular structures attached to the gold surface are observed onto the gold electrode modified with cysteamine (0.01 mol L^{-1}, Fig. 2b). With the increase in cysteamine concentration (0.5 mol L^{-1}), these structures are formed in significantly greater quantities (Fig. 2c). The images demonstrate significant differences between the electrodes, indicating that the cysteamine modification may lead to changes in the topography and physical properties of the gold electrode surface. Modification with cysteamine introduces positive sites for biomolecule interaction on the sensor. The thiol group (SH) forms a bond with the electrode surface, and the resulting exposed amino group (NH_2), which becomes protonated (NH_3^+) in solution, provides a positive charge that attracts negatively charged biomolecules.

3.2. Characterization of the genosensor by DFM

Dynamic force microscope (DFM) images reveal the development steps of the genosensor, from the initial modification of the electrode surface to the final interaction with the target. Figure 3 presents DFM images illustrating the modifications to the electrode surface at different stages of genosensor functionalization. Figure 3(a) shows the surface of the electrode modified with cysteamine (0.5 mol L^{-1}), representing the starting point for the functionalization of the device.

These images reveal a irregular topography, with an average roughness of 188.4 nm, indicating a surface that is already textured from the outset. In contrast, Figure 3(b) reveals the formation of the cysteamine and GQDs bilayer, a crucial step to anchor the detection probe. Notably, the surface at this stage becomes much

smoother and more uniform, with the average roughness decreasing dramatically to 2.6 nm, suggesting significant smoothing promoted by the

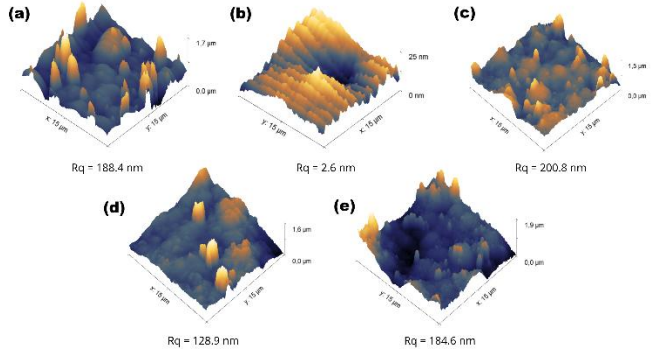


Fig 3. DFM images with Rq data of a) electrode with cysteamine (0.5 mol L^{-1}); b) cysteamine and GQDs bilayer; c) bilayer with immobilized probe; d) blocking with glycine of the surface of the modified electrode containing the probe; e) surface of the modified electrode containing probe and target.

cysteamine-GQD bilayer, creating a more controlled base. Figure 3(c) highlights the efficiency of probe immobilization, an essential component for genosensor selectivity. In this image, the surface returns to being more irregular and textured, with a significant increase in average roughness to 200.8 nm, evidencing the morphological impact of incorporating the biomolecular probe. The blocking step with glycine in Figure 3(d), demonstrates the strategy to avoid non-specific interactions. Compared to Figure 3(c), the surface in Figure 3(d) appears slightly smoother, with a reduction in roughness to 128.9 nm, although remaining rougher than the bilayer in Figure 3(b). This value suggests that glycine blocking partially fills spaces, but does not completely revert to the smoothness of the bilayer.

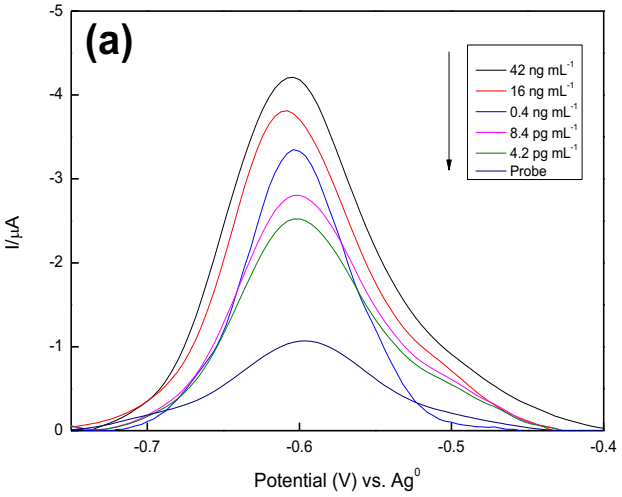
Finally, Figure 3(e) confirms the interaction of the target with the probe, demonstrating the genosensor's ability to recognize the analyte of interest. Visually, the surface of Figure 3(e) resembles that of Figures 3(a) and 3(c), remaining irregular and textured, with an average roughness of 184.6 nm. This value indicates that the probe-target interaction, while fundamental to the genosensor's function, does not induce drastic morphological changes on the surface in terms of roughness compared to previous steps, or that the change is not as prominent as probe immobilization. The comparison between these images offers a visual representation of the genosensor's operating principle, from the molecular recognition step to the generation of the detectable signal. Together, the sequential analysis of DFM images validates each step of the genosensor development, from the modification of the electrode surface to the specific interaction with the target. This rigorous methodological approach ensures the performance and reliability of the genosensor for accurate detection of the analyte of interest.

3.3. Characterization by FTIR of the modifications on the platform

Fourier Transform Infrared Spectroscopy (FTIR) was employed to compare the bilayer formed by cysteamine and graphene quantum dots with the individual components, cysteamine and GQDs. FTIR analysis revealed characteristic peaks in the spectra of the bilayer that were also present in the spectra of the separate cysteamine and GQDs components. This indicates the presence and interaction of both cysteamine and GQDs within the bilayer structure. The bands at 3200-3400 cm^{-1}, 1600-1700 cm^{-1}, and 1000-1100 cm^{-1} are maintained, indicating the presence of amino and thiol groups. However, a shift of the C-S band to a slightly lower frequency is observed, suggesting an interaction between the cysteamine and the GQDs. The complete analysis can be seen in the supplementary material.

3.4. Construction of the Calibration Curve

Different concentrations of the target were used to construct the calibration curve, ranging from 42 ng mL^{-1} to 4.2 pg mL^{-1}. As illustrated in Fig. 4, this curve was derived from measuring the electrochemical response of the intercalator within the double strand. It effectively establishes the relationship between the concentration of the target molecule and the electrochemical signal produced by the sensor. The lowest concentration detected was 4.2 pg mL^{-1}, demonstrating the high sensitivity of the genosensor, This remarkable ability to identify extremely low concentrations of the target molecule is a key factor in evaluating the genosensor's performance.



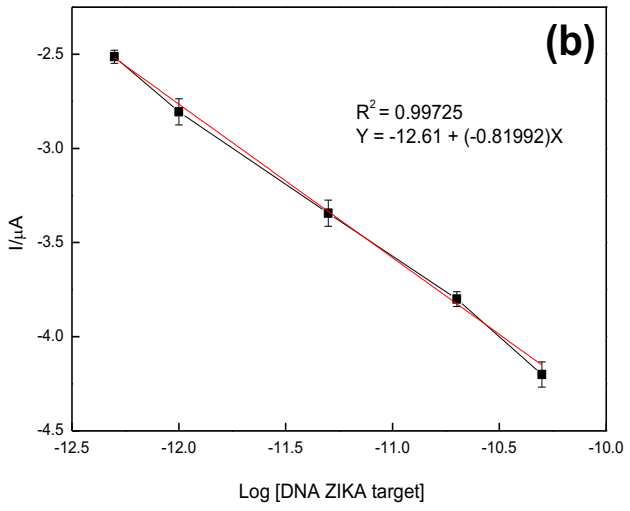


Fig 4. (a) Differential pulse voltammograms of the genosensor with potential ranging from 0 V to -1 V, modulation amplitude 60 mV, interval time 0.2s, scan rate 50 mV s^{-1}; in PBS solution (0.1 mol L^{-1}, pH 7.4) at different target concentrations, (b) calibration curve obtained from DPV.

3.5. Selectivity and specificity of the genosensor in ZIKV detection

To demonstrate the selectivity of the genosensor, a detection approach involving intercalation of safranin into double-stranded nucleic acids was used, based on the Differential Pulse Voltammetry technique. In this stage, human serum samples were quantified by spectrophotometry and enriched with genomic RNA from chikungunya (CHIKV), dengue (DENV), and Zika (ZIKV) viruses in a ratio of 10:1 (v/v), respectively, producing complex biological matrices. Detection was performed by monitoring the reduction peak of safranin (-0.6 V *vs.* Ag^0), where the current value observed was proportional to the concentration of the intercalator adsorbed on the surface following the specific hybridization of the probes with the viral targets. Selectivity and specificity were evidenced by the absence of significant signals in control samples (e.g., non-complementary gRNAs) and complex matrices, since the formation of double strands is essential for the intercalation of safranin and the generation of the response. The possibility of detecting ZIKV gRNA in physiological conditions (pH 7.4) using diluted serum without the need for prior sample treatment reinforces the applicability of the genosensor. Therefore, this genosensor combines the specificity of hybridization with the sensitivity of intercalator-mediated electrochemical detection, offering a robust platform for the differential diagnosis of arboviruses. This experiment can be seen in Fig 5 (a) and (b).

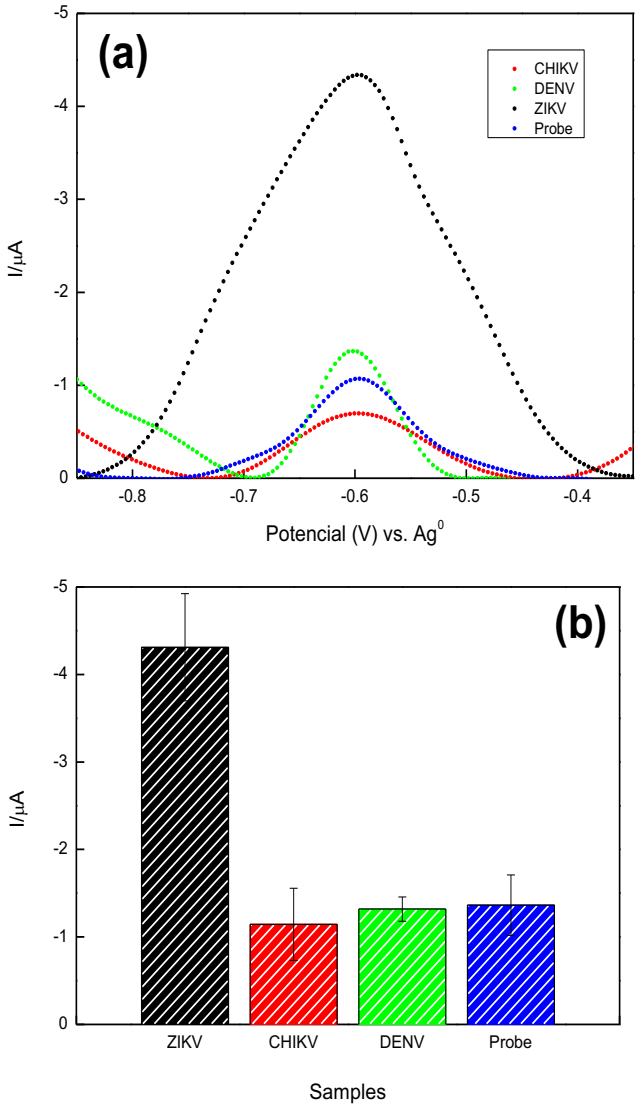


Fig. 5. a) Differential pulse voltammograms of the genosensor, 50 mV s^{-1}, potential range of 0 to -1 V, equilibration time of 5 seconds, in phosphate buffer (pH 7.4), using human serum enriched with genomic RNA of CHIKV, DENV and ZIKV, 10:1 ratio (human serum:gRNA), b) Histogram for current values for chikungunya virus (CHIKV), dengue virus (DENV) and Zika virus (ZIKV).

3.6. Storage stability analysis

The storage stability of the genosensor was assessed over a period of 45 days. For this assay, the bioelectrodes were positioned inside a sealed container and stored at room temperature and without light. The genosensor response retained 84% of its initial value over 45 days, implying a good degree of stability and functional integrity for the bilayer and immobilized DNA probe. The data from this experiment can be seen in Fig 6.

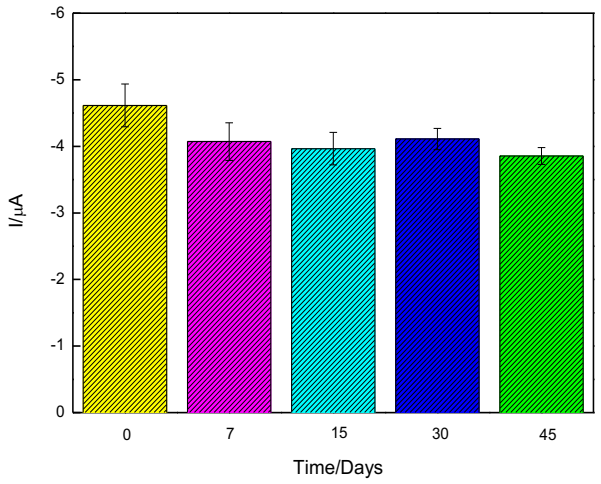


Fig 6. Genosensor response as a function of storage time.

4. Conclusions

In summary, an electrochemical genosensor was developed for Zika virus DNA detection, using a gold-printed circuit board electrode modified with a cysteamine/graphene quantum dot bilayer, and employing safranin as a DNA intercalator. The genosensor exhibited a low detection limit, high selectivity against chikungunya and dengue, and good stability, demonstrating a significant advancement in Zika diagnostics.

The combination of a three-electrode system, nanomaterial modification, and electrochemical principles provides a cost-effective, sensitive, and user-friendly platform for virus detection. This genosensor offers several advantages onto traditional diagnostic methods, such as PCR and RT-PCR, making it more accessible in resource-limited environments. The stepwise surface modification procedure ensures the proper immobilization of biomolecules, enhancing the genosensor's specificity and minimizing the risk of false-positive results.

The choice of safranin as a DNA intercalator, coupled with the utilization of differential pulse voltammetry for monitoring its electrochemical reduction, allows for both qualitative and quantitative analysis of Zika virus DNA. The genosensor's ability to operate at low analyte concentrations makes it well-suited for early detection, providing valuable information for timely therapeutic interventions.

Furthermore, the simplicity of the genosensor design and minimal training requirements for result interpretation enhance its applicability in various settings, including remote and underserved areas. Incorporation of GQDs improves sensor performance, ensuring high selectivity and sensitivity in target recognition. Although the genosensor specifically targets the Zika virus, its principles and methodologies pave the way for similar advances in the detection of other infectious diseases.

This genosensor overcomes limitations of current diagnostics, offering a valuable tool for public health initiatives against Zika and emerging infectious diseases.

5. Acknowledgements

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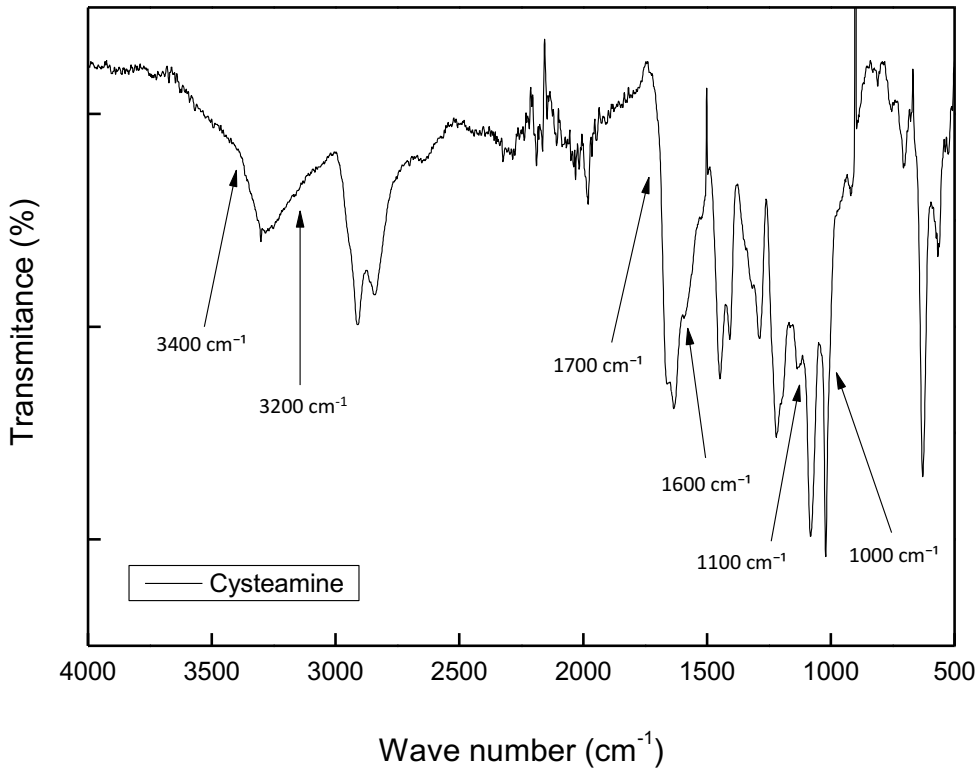
Comparative Analysis of FTIR Spectra

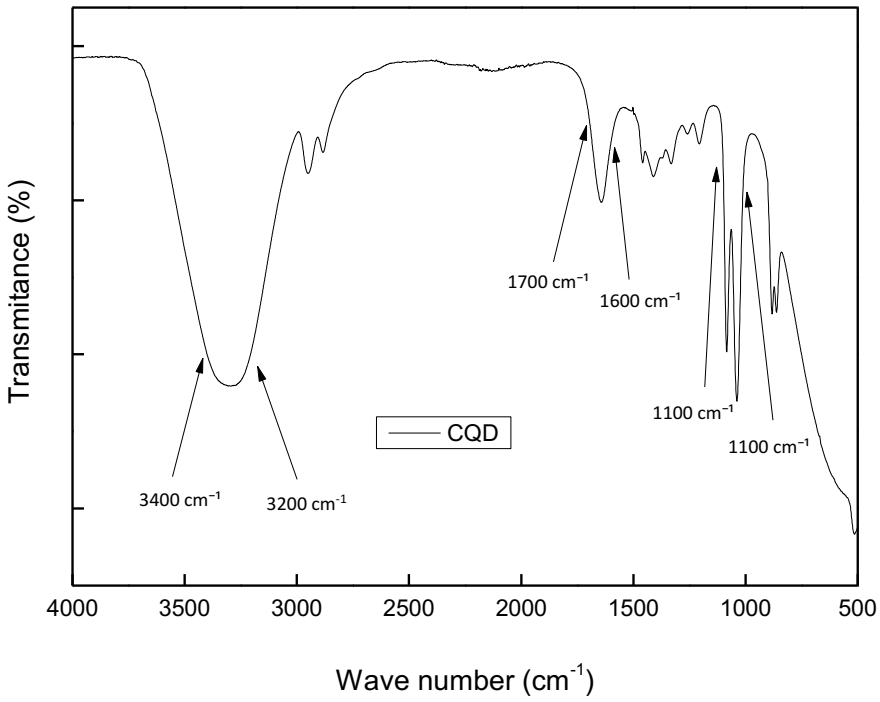
Cysteamine

The spectrum of cysteamine exhibits characteristic bands of amino and thiol groups. The region of 3200-3400 cm^{-1} displays broad and intense bands corresponding to N-H and O-H stretching, confirming the presence of amino and hydroxyl groups. At 1600-1700 cm^{-1}, N-H bending is observed, typical of primary amines. The band at 1000-1100 cm^{-1} corresponds to C-S stretching, characteristic of thiols.



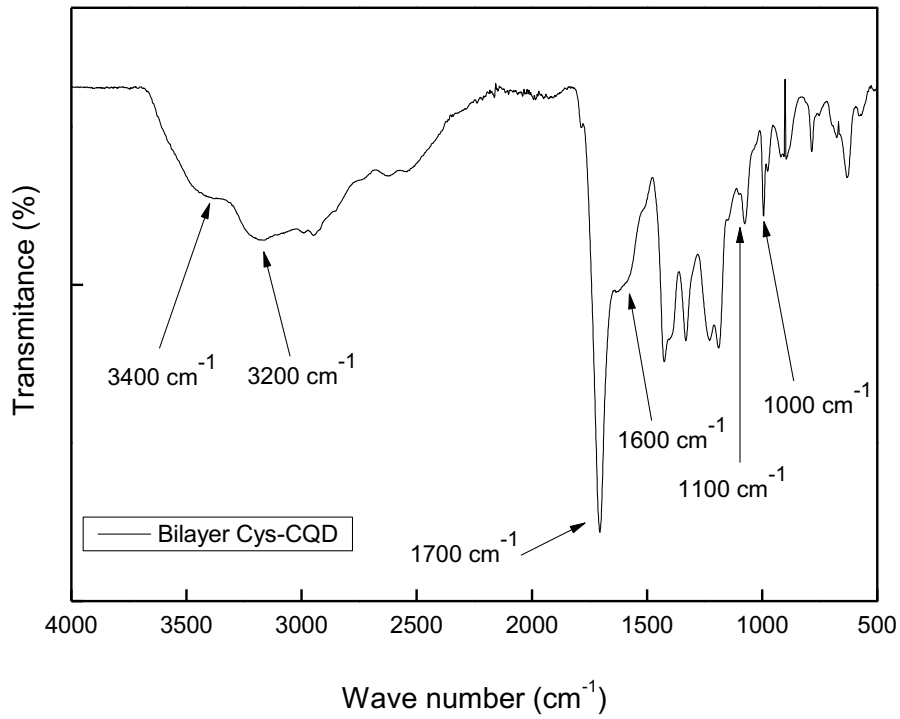
CQD

The CQD spectrum also shows bands similar to those of cysteamine, confirming the presence of amino, hydroxyl, and thiol groups. However, some differences can be observed, such as the lower intensity of the bands at 3200-3400 cm^{-1} and 1600-1700 cm^{-1}, suggesting a smaller amount of amino groups.



Bilayer Cys-CQD

The spectrum of the Bilayer Cys-CQD composite combines characteristics of the cysteamine and CQD spectra, revealing the presence of both components in the structure. The bands at 3200-3400 cm^{-1}, 1600-1700 cm^{-1}, and 1000-1100 cm^{-1} are maintained, indicating the presence of amino and thiol groups. However, a shift of the C-S band to a slightly lower frequency is observed, suggesting an interaction between cysteamine and CQD.



The comparison of the spectra reveals that the Bilayer Cys-CQD composite retains the main characteristics of cysteamine and CQD, indicating that both components are present in the structure. The shift of the C-S band suggests an interaction between the thiol group of cysteamine and CQD, possibly through the formation of a covalent bond. This interaction may be responsible for the unique properties of the composite, such as its ability to form thin films and its biocompatibility.

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2023 013776 8

Dados do Depositante (71)

Depositante 1 de 1

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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): GENOSSENSOR ELETROQUÍMICO PARA DETEÇÃO DE VÍRUS ZIKA

Resumo: A presente invenção consiste em um genossensor para detecção eletroquímica de uma maneira rápida e simplificada do DNA do vírus Zika utilizando-se o corante biológico safranina como intercalante, seja de maneira qualitativa ou quantitativa. O sensor é composto por uma Seq ID 01 e Seq ID 02 que são específicos um para o outro, e para a realização da detecção, foi utilizada técnica eletroquímica de voltametria de pulso diferencial. A superfície do eletrodo foi revestida com uma bicamada de cisteamina com quantum dot de grafeno dopado com glicina, que ajudou na eficiência da imobilização das moléculas de Seq ID 01 e Seq ID 02 respectivamente. A safranina foi utilizada como intercalante, pois é capaz de responder em sistemas eletroquímicos ao se ligar a bases de DNA.

Figura a publicar: 1

Capítulo 3: Development of a Novel Electrochemical Genosensor Based on Graphene Oxide and Safranin for Zika Virus Detection

O capítulo 3 corresponde a um manuscrito de artigo original redigido nas normas do periódico Biosensors and Bioelectronics (ISSN: 1873-4235).

Development of a Novel Electrochemical Genosensor Based on Graphene Oxide and Safranin for Zika Virus Detection

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Abstract: This study describes the development and characterization of a novel electrochemical genosensor for the rapid and sensitive detection of Zika virus (ZIKV). The genosensor is based on a nanocomposite film of reduced graphene oxide (rGO) and safranin, modified with a specific DNA probe derived from the ZIKV genome. The morphological characteristics of the film were investigated by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Calibration curves were constructed using different concentrations of complementary target DNA, with a limit of detection (LOD) 8.4 ng mL^{-1}. The genosensor retained approximately 80% of the response after 60 days, demonstrating good stability. Functionalization with the DNA probe allowed for the detection of ZIKV in samples, indicating the potential of the device for rapid and effective diagnosis. This genosensor represents a promising advance in ZIKV detection, with potential for clinical and field applications.

Keywords: Zika Virus, Graphene Oxide, Safranin, Ninhydrin

1. Introduction

The emergence of viral diseases, particularly those caused by RNA viruses such as the Zika virus, has necessitated the development of innovative diagnostic tools to facilitate rapid and accurate detection. Among the most promising advancements in this field are modified printed circuit boards (PCBs) integrated with nanomaterials, specifically carbon-based materials like graphene oxide and carbon paint, which have shown significant potential in biosensing applications. The utilization of these nanomaterials enhances the sensitivity and specificity of biosensors, making them ideal candidates for detecting viral RNA, including that of the Zika virus, which poses a substantial public health threat in many regions worldwide [1], [2], [3].

Graphene oxide, a derivative of graphene, is particularly noteworthy due to its unique physicochemical properties, including a high surface area, excellent electrical conductivity, and biocompatibility. These characteristics enable the effective immobilization of biomolecules, thereby facilitating the detection of viral genetic material through various biosensing techniques[1], [4]. Recent studies have demonstrated that graphene oxide can be functionalized to improve its interaction with target RNA sequences, which is crucial for the development of sensitive and selective biosensors[4], [5]. Furthermore, the incorporation of carbon paint into the design of PCBs allows for the creation of flexible and cost-effective biosensing platforms that can be easily integrated into diagnostic devices[6], [7].

The application of safranin, a fluorescent dye, in conjunction with graphene oxide and carbon paint, provides an additional layer of sensitivity in detecting Zika virus RNA. The properties of safranin can be used to amplify the signal generated during hybridization of the target RNA with its complementary strand, thus increasing the detection limit of the biosensor [8], [9]. This combination of materials not only improves the analytical performance of the biosensor but also allows for real-time monitoring of viral infections, which is essential for timely public health interventions [10], [11], [12].

Moreover, the integration of microfluidic technologies with these modified PCBs can further enhance the performance of the biosensing devices. Microfluidics allows for the precise control of fluid flow at the microscale, enabling the efficient mixing of reagents and samples, which is critical for achieving high sensitivity in viral detection [13], [14]. The combination of microfluidics with nanomaterials has been shown to facilitate rapid and

reliable diagnostics, making it a valuable approach in the fight against emerging viral threats such as Zika [15], [16].

In summary, the innovative use of modified printed circuit boards, carbon paint, and film using safranin and graphene oxide represents a significant advancement in the field of viral diagnostics. These technologies not only promise enhanced sensitivity and specificity in detecting Zika virus RNA but also pave the way for the development of portable, cost-effective, and user-friendly diagnostic tools. As research continues to evolve in this domain, the potential for these biosensing platforms to contribute to global health initiatives becomes increasingly evident [2], [3], [4], [5], [17].

2. Material and methods

2.1 Chemicals and Apparatus

All the solutions were prepared with deionized and ultrapure water (resistivity of 23.1 MΩcm, Gehaka) and deoxygenated with ultrapure nitrogen. All the reagents were analytical grade and used without further purification. Sodium chloride (99%), dibasic sodium phosphate (99%) and monobasic sodium phosphate (99%) were obtained from Synth. Safranin ($C_{20}H_{19}N_4^+ \cdot Cl^-$, 95%) was obtained from Dinâmica. The probes used (5'-CACTGAGTCAAAAAACCCCACGCGCTT-3') and complementary target (5'-AAGCGCGTGGGGTTTTTTGACTCAGTG-3') were obtained from Exxtend. All lyophilized oligonucleotides were prepared in buffered saline sodium citrate (0.03 mol L^{-1} sodium citrate, 0.3 mol L^{-1} NaCl, pH 7.4) to obtain the concentration of 100 μg μL^{-1}. Ninhydrin (ACS reagent) was purchased from Sigma Aldrich.

The sensor used in this study was developed as a three-electrode printed sensor from the company PCBWay. The working and counter electrodes were covered with a conductive carbon paint produced in the laboratory, and a conductive silver paint also developed in the laboratory was used for the reference electrode.

2.2 Instrumentation

Electrochemical measurements were performed using Differential Pulse Voltammetry (DPV) in a potentiostat CHI 760C (CH Instruments, USA). The surface morphology of the electrodes was assessed by scanning probe microscopy (Model 5100 N, Hitachi), performed

in the dynamic force microscope (DFM) mode, and by scanning electron microscopy (SEM) on an EVO MA10 Zeiss microscope.

2.3 SPCE construction and modification with Safranin and graphene acid

Each PCB board has a total of 27 electrodes that were cleaned with cotton soaked in isopropyl alcohol. These electrodes were then placed in a DEK 248 screen printing printer. The surface of the reference electrode was coated with a conductive silver ink made in the laboratory and with conductive carbon ink, both the working electrode and the auxiliary electrode were coated. After printing these electrodes, they were cured at 65 degrees Celsius for 40 minutes. Before use, the SPCEs were preconditioned by cyclic voltammetry in sulfuric acid solution (0.1 mol L^{-1}) between −1.5 and 1.8 V, 40 cycles, 100 mV s^{-1}, to ensure a surface free of impurities and to obtain a common voltammetric profile. They were then rinsed with deionized water and dried with N_2 gas. A solution was prepared in phosphate buffer (pH 6.4) containing 2.0 x 10^{-3} mol L^{-1} of safranin. In this solution, 1 mg of OG was added for each 1 mL of solution used. And simultaneously to the poly safranin film, the reduction of graphene oxide was performed. In the cyclic voltammetry, increases in each cycle can be observed in the anodic peaks observed at potentials -0.26 V and 0.35 V. This modification is shown in Figure 1.

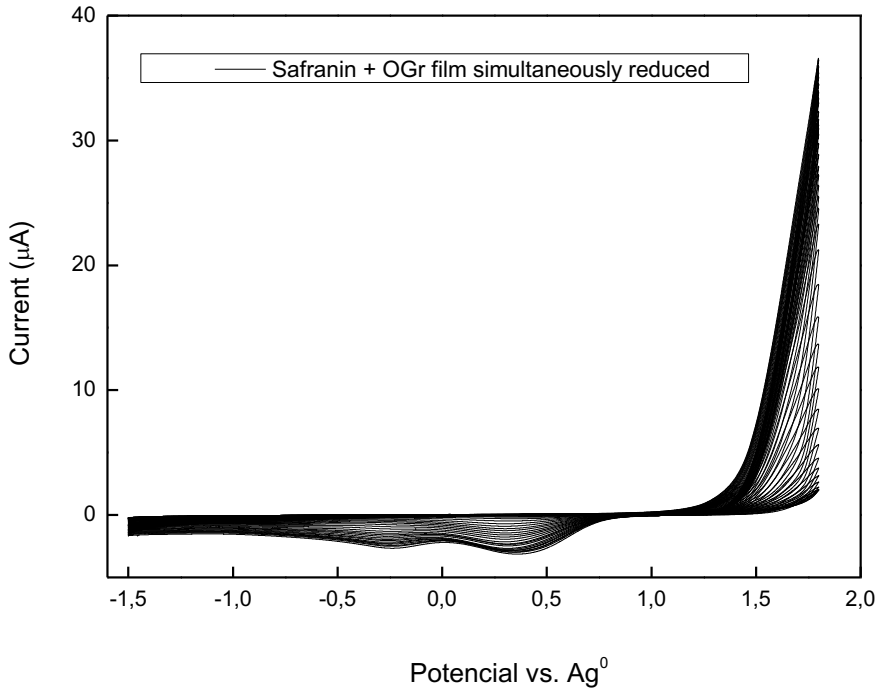

Figure 1. Cyclic voltammogram of the rGO-safranin nanocomposite film on a screen-printed carbon electrode (SPCE), demonstrating the electrochemical behavior of the modified electrode. Scan rate: 50 mV s^{-1}. Potential range: -1.5 V to +1.8 V vs. Ag^0

2.4 Genosensor construction

Figure 2 shows the construction scheme of the genosensor.

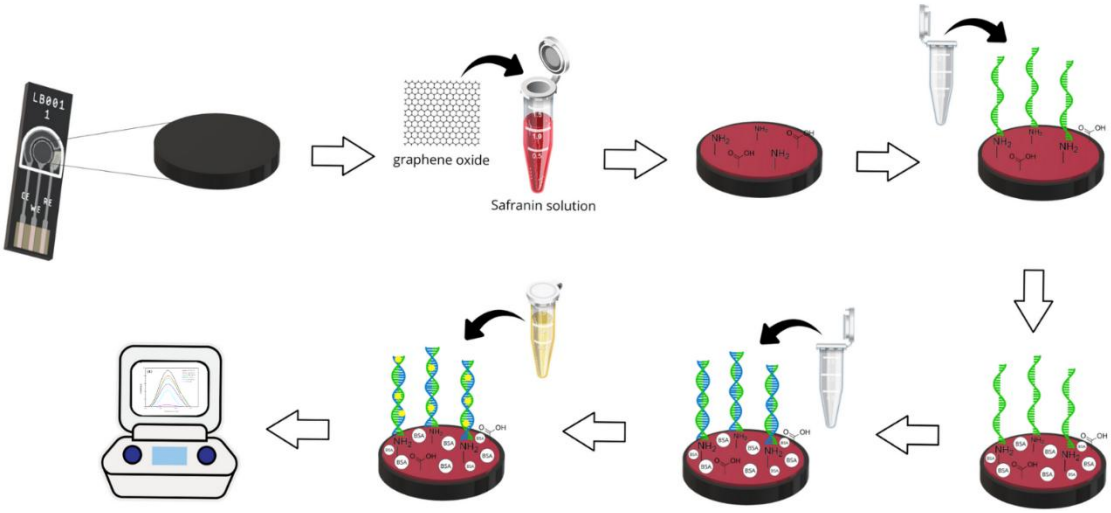

Figure 2. Schematic illustration of the electrochemical genosensor construction.

Oligonucleotide probe immobilization was performed after modification of the electrode containing reduced graphene oxide/polysafranin. The ZIKV-specific probe oligonucleotide was added to the modified electrode and kept sealed at room temperature for 35 minutes. After that, the electrodes were washed for 7 seconds in PBS and dried using N_2 gas.

After probe immobilization, a 3% BSA solution was added to block the surface. During this blocking procedure, the bioelectrodes were stored in a closed and humidified system for a period of 60 minutes at room temperature. The electrodes were washed for 7 seconds in PBS to remove excess BSA and dried using N_2 gas. To complete the immobilization, 5 µL of the target was added. During the hybridization process, the electrodes were placed in a humid chamber that was placed in an oven at 57 degrees Celsius for 35 minutes. After this time, the electrodes were washed in PBS and dried in N_2 gas.

After complete hybridization, a 5 µL ninhydrin solution was added to the SPCE/OGr:Poly safranine/dsZIKV, which was used as an intercalant in this study. After 20 minutes, washing in PBS and drying in N_2 were performed. DPV was recorded with potential ranging from − 0.7 V to +0.2 V, modulation amplitude 60 mV, interval time 0.2 s, scan rate 30 mV s^{-1}; interval time 0.2 s using an electrolyte PBS solution (0.1 mol L^{-1}, pH 7.4). The

final stage involved detecting the safranin that is present in the intercalated form in the double strand. For this, measurements by differential pulse voltammetry were performed. In this procedure, the safranin reduction peak was monitored.

The calibration curve was constructed by varying the concentration of the complementary ZIKAV target between 8,4 ng mL^{-1} to 420 µg mL^{-1}. Differential pulse voltammograms were recorded as analytical signal by indirect detection using ninhydrin as indicator in PBS solution (0.1 mol L^{-1}, pH 7.4).

3. Results and discussion

3.1 Morphological Characterization

The morphology of the rGO-safranin nanocomposite film deposited on the electrode surface was investigated using a combination of Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) as seen in Figure 3. SEM analysis provided insights into the overall microstructure and surface texture of the film, revealing the distribution of rGO and the formation of the safranin polymer. Figure 3 (a) - SPCE, the SEM image clearly displays an irregular and porous texture. Agglomerated particles are evident, forming a complex morphology that resembles flakes or aggregates of material, distributed in a heterogeneous manner. Moving to Figure 3 (b) - rGO-safranin nanocomposite film, a notable alteration in the surface texture is observed in the SEM image. Although the surface does not achieve complete smoothness, it exhibits a more continuous and coated appearance. This suggests that the nanocomposite film is deposited onto the electrode structure, resulting in a surface that appears less porous than the initial SPCE, with aggregates appearing flattened or integrated. Finally, Figure 3 (c) presents a morphology in the SEM image that appears to have reduced irregularity and porosity when compared to both Figures 3 (a) and (b). The texture observed still contains aggregates, but these appear to be smaller, more uniformly distributed, and less prominent, leading to a less porous surface overall. The images demonstrated a porous and rough surface, indicative of the successful deposition of the nanocomposite.

AFM, on the other hand, allowed for a higher resolution examination of the surface topography, quantifying the roughness and revealing details of the nanoscale features. Figure (d) supports the SEM findings by quantifying a root mean square roughness (Rq) of 127.2 nm. The representation of the surface topography in AFM reinforces the perception of a surface characterized by significant height variations, confirming the roughness and porosity

initially observed in SEM. Figure 3 (e) and the Rq value of 125.4 nm reflect the surface modification seen in SEM. This indicates a slight reduction in roughness compared to pure SPCE, although the roughness remains substantial. Visual analysis of the AFM image suggests a somewhat smoother surface, however the quantitative change in roughness is not drastic. Finally, Figure 3 (f) and an Rq value of 108 nm reveal the lowest roughness among the three samples analyzed. Despite this being the lowest Rq value, the roughness is still considerable. This indicates that the surface, although slightly flatter than Figures 3 (a) and (b), still exhibits significant height variations. This analysis confirmed the presence of nanostructures and provided information about the film's thickness and uniformity, crucial parameters for sensor performance.

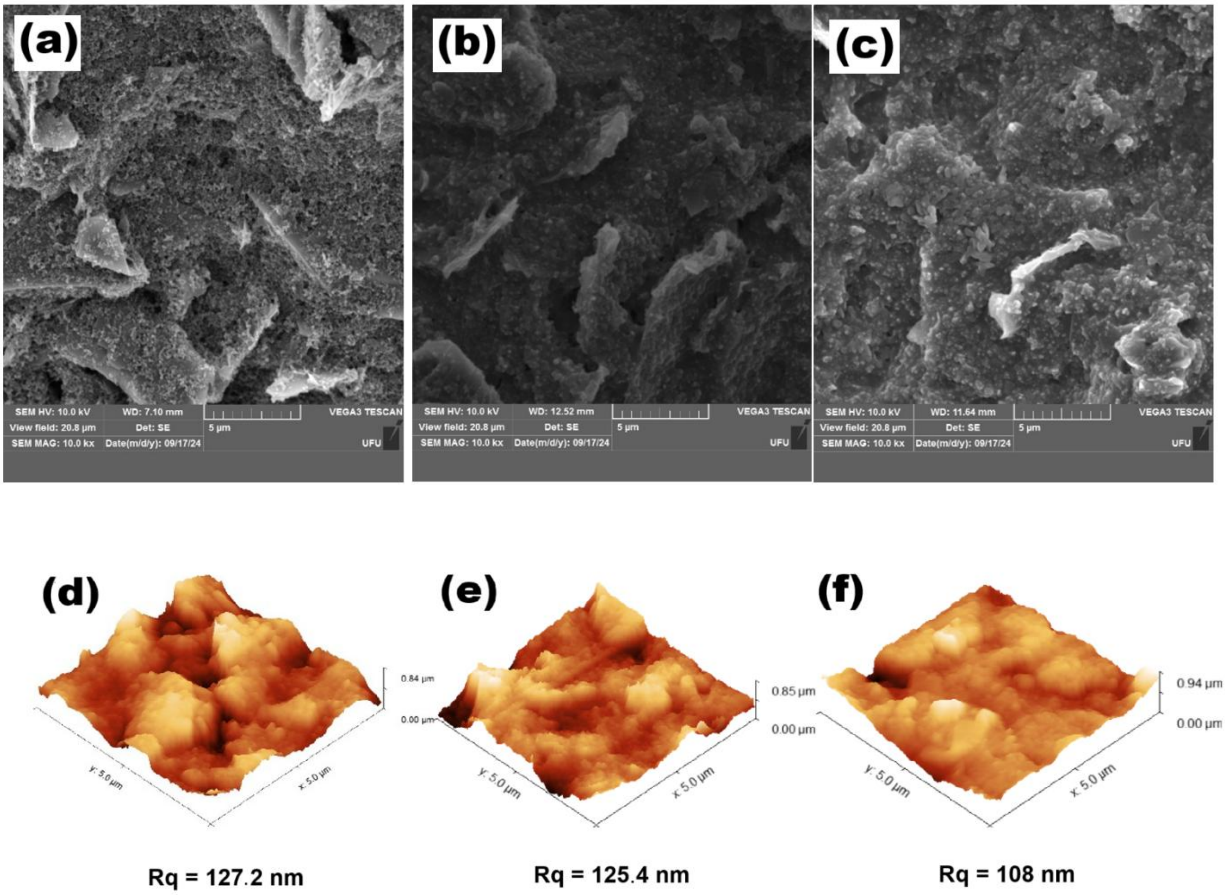

Figure 3. SEM images for the surface of (a) SPCE, (b) rGO-safranin nanocomposite film, (c) ZIKV target. (d), (e) and (f) AFM images to respective SEM images.

The infrared spectrum of the Safranin + rGO compound reveals the combination of the compounds through the presence and interaction of specific bands. The N-H band of Safranin at 3398 cm^{-1}, characteristic of amines, is observed in the compound, indicating the availability of amines on the surface. Simultaneously, the C=O band of rGO at 1724 cm^{-1}, typical of

carboxylic acids, also appears in the compound, suggesting a positively charged surface due to protonation at acidic pH. The shift or change in intensity of these bands in the Safranin + rGO compound compared to the individual spectra indicates the interaction between the amines of Safranin and the carboxylic groups of rGO, corroborating the combination of the compounds and the formation of a surface with available amines.

3.2 Functionalization, Detection of ZIKV and Selectivity

The genosensor was functionalized by immobilizing a specific DNA probe, designed from the ZIKV genome, onto the rGO-safranin film. The electrochemical signal was measured after addition of a sample containing the ZIKV target sequence. The interaction between the ZIKV target and the immobilized probe resulted in a detectable change in the electrochemical signal, confirming the successful detection of the virus. The utilization of a DNA probe specific to the ZIKV is critical for enhancing diagnostic accuracy and minimizing the risk of false positives associated with other related viruses, particularly within the flavivirus family. The specificity of DNA probes allows for the precise detection of ZIKV RNA, which is essential given the high cross-reactivity observed between ZIKV and other flaviviruses, such as DENV [18], [19].

Genomic RNA samples of CHIKV, DENV, and ZIKV were quantified using a Biodrop spectrophotometer and subsequently spiked into human serum at a 10:1 ratio (serum:RNA) to mimic complex biological matrices. Voltammograms with human serum revealed distinct electrochemical responses for each viral RNA target, with well-resolved oxidation peaks observed between −0.7 V and +0.2 V. The specificity of the genosensor was evident from absence of significant cross-reactivity signals, even when challenged with structurally related. This experiment can be seen in Figure 4 (a) and (b).

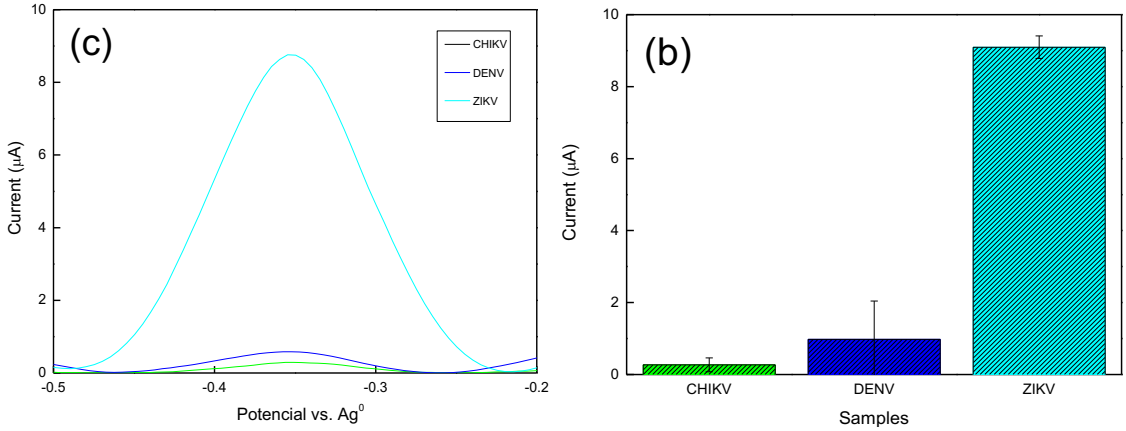

Figure 4. Figures of merit (a) Differential pulse voltammograms of the genosensor with potential ranging from − 0.7 V to +0.2 V, modulation amplitude 60 mV, interval time 0.2 s, scan rate 30 mV s^{-1}; using an electrolyte PBS solution (0.1 mol L^{-1}, pH 7.4) in human serum enriched with genomic RNA of CHIKV, DENV and ZIKV, 10:1 ratio (serum:RNA), (b) Histogram for current values for the CHIKV, DENV and ZIKV.

Figure 4 (a) presents the differential pulse voltammograms of the genosensor in human serum enriched with genomic RNA of CHIKV, DENV, and ZIKV. The voltammograms show distinct electrochemical responses for each viral RNA target, with well-resolved oxidation peaks observed between -0.7 V and +0.2 V. Figure 4 (b) displays a histogram summarizing the current values for each virus. The histogram highlights the clear differences in electrochemical signals between the three viruses, indicating the genosensor's ability to differentiate between them.

The absence of significant cross-reactivity signals, even when challenged with structurally related viruses, demonstrates the high selectivity of the genosensor. This selectivity is crucial for accurate ZIKV detection and minimizing false positives. The experimental results provide a robust proof of concept for the selectivity of the genosensor, demonstrated through differential pulse voltammetry (DPV) analysis under physiologically relevant conditions.

3.3 Calibration Curves and Genosensor Stability

To assess the quantitative performance of the genosensor, a calibration curve was constructed by measuring the electrochemical response by differential pulse voltammetry. Figure 5 (a) shows the differential pulse voltammograms of the genosensor at different target DNA concentrations. Figure 5 (b) presents the calibration curve constructed from these measurements, demonstrating a linear relationship between the electrochemical signal and the target DNA concentration. The genosensor exhibited a limit of detection (LOD) of 8.4 ng/mL and an upper limit of detection of 420 μg mL^{-1}, indicating high sensitivity and a broad dynamic range suitable for detecting ZIKV across various viral loads.

The stability of the genosensor was evaluated over a period of 60 days, as seen in the Figure 5 (c). The results showed that the sensor maintained about 80% of its initial response after this period, maintaining the functional integrity of the rGO/safranin nanocomposite, as

well as the immobilized DNA probe for a reasonable period. This indicates good stability and suggests the potential for practical application of the genosensor.

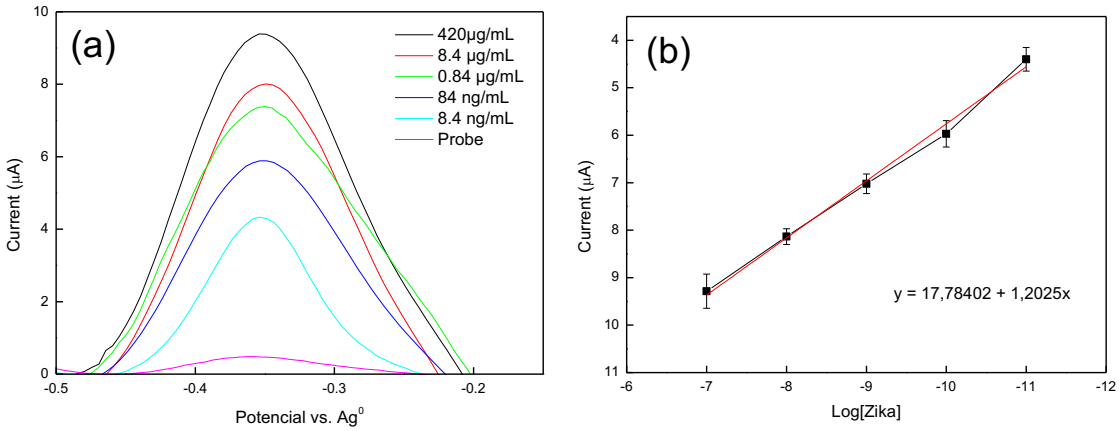

Figure 5. Figures of merit (a) Differential pulse voltammograms of the genosensor with potential ranging from -0.7 V to +0.2 V, modulation amplitude 60 mV, interval time 0.2s, scan rate 30 mV s^{-1}, using an electrolyte PBS solution (0.1 mol L^{-1}, pH 7.4) at different target concentrations, (b) Calibration curve.

Overall, the figures presented demonstrate the successful development of a highly selective, sensitive, and stable genosensor for ZIKV detection. The genosensor's ability to accurately detect ZIKV in complex biological matrices, such as human serum, highlights its potential for clinical application.

4. Storage Stability Analysis

As mentioned above, the storage stability of the genosensor was assessed over 60 days, showing an 80% retention of its initial response. For this assay, the bioelectrodes were positioned inside a sealed container and stored at room temperature and without light. This suggests that the rGO-safranin nanocomposite and the immobilized DNA probe maintain their functional integrity over a reasonable period. Further investigations into optimal storage conditions (e.g., temperature, humidity) could further enhance the shelf-life of the genosensor. The data from this experiment can be seen in Fig 6.

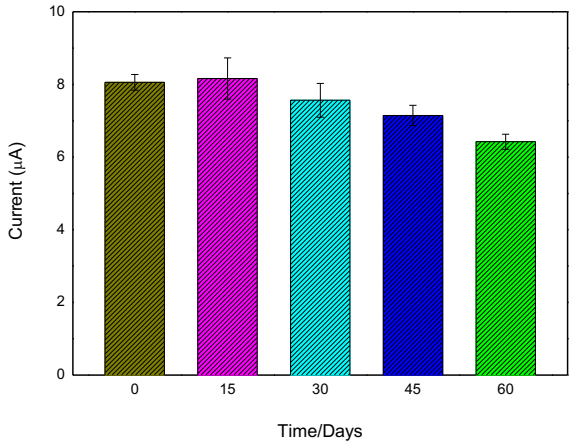

Figure 6. Relation between ip and storage time of the genosensor.

5. Conclusions

This study successfully demonstrated the development of a novel electrochemical genosensor for ZIKV detection based on a rGO-safranin nanocomposite modified with a specific DNA probe. The genosensor exhibited high sensitivity, a wide dynamic range, and good stability. The combination of rGO and safranin provides a biocompatible and conductive platform for DNA immobilization and enhanced electrochemical signal transduction. This genosensor holds great promise for rapid, cost-effective, and point-of-care diagnostics of ZIKV. Future work should focus on optimizing the sensor's performance, validating its use with clinical samples, and developing a portable device for field applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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