

# RECENT ADVANCEMENT IN ELECTROCHEMICAL BIOSENSOR BASED ON REDOX ENZYME COMPOSITE MODIFIED ELECTRODE FOR THE DETECTION OF BIOMOLECULES

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## Abstract

Recent developments in electrochemical biosensors have resulted in new applications in a variety of disciplines, such as clinical diagnosis, food processing quality control, and environmental monitoring. The electrochemical biosensor is a widely used sensing device that transmits an electrical signal from biological activities. Electrochemical biosensors have led to significant advancements in detecting various biomolecules such as uric acid, glucose, cholesterol, lactate, and DNA, including cancer biomarkers, viruses, and antibodies. An electrode is a vital part of this kind of sensor since it serves as a strong support for immobilizing biomolecules (enzyme, antibody, and nucleic acid) and allowing electron flow. Redox enzymes are the foundation of enzymatic electrochemical biosensors, which use oxidation or reduction reactions to detect a substrate, which is then converted into an electrical signal. Immobilizing enzymes onto solid supports is a common method to enhance the performance of an enzymatic electrochemical biosensor. However, there are some challenges, such as potential loss of enzyme activity, difficulties in maintaining enzyme stability, and issues related to the reproducibility and consistency of enzyme immobilization. This review covers all challenges in enzymatic electrochemical biosensors. Numerous new biosensor platforms are announced each year as researchers look for various electrode materials. Additionally, the researchers should familiarize themselves with the practical methods used in electrode production in order to build a successful biosensor. This review highlights current developments in modifying enzyme-immobilized biosensors for detection of different biomolecules. The article further describes methods of enzyme immobilization and various enzymes with different nanomaterials for the development of novel enzymatic electrochemical biosensors.

**Keywords:** Biomolecules; Electrochemical biosensors; Enzyme; Immobilization; Nanocomposites

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## Introduction

The number of people suffering from various diseases and disorders is rising as a result of the expanding human population. The increasing demand for accurate and rapid detection of human diseases is driven by the need to enhance disease management and control strategies. Accurate and timely diagnosis significantly impacts patient outcomes and treatment decisions (Holmboe and Durning, 2014; Fredj *et al.*, 2023). Affected organisms usually experience changes in their bodies, most notably in the number of metabolites they contain. Certain molecules in the human body play a role in diseases like uric acid (UA), ascorbic acid (AA), glucose, etc. Biosensors now have a new option for the detection of biomolecules for disease analysis because of the quickly evolving fields of nanotechnology and semiconductor material production. In recent years, a variety of electrochemical biosensors have been presented for the detection of different diseases, stating advantages including low cost, sensitivity, selectivity, and quick response. A biological sensing system and an electrochemical transducer combine to form an electrochemical biosensor. (Bhalla *et al.*, 2016; Dai *et al.*, 2022). A biosensor is a device that generates signals commensurate with the concentration of an analyte it detects in a chemical or biological interaction. The working principle of electrochemical biosensors is based on the conversion of biochemical reactions into measurable electrical signals (Jesny *et al.*, 2016; Malecka *et al.*, 2020). These biosensors typically consist of a biorecognition molecule, such as an enzyme or an antibody, immobilized on an electrode surface. When the target analyte binds to the biorecognition molecule, it triggers a specific biochemical reaction that generates changes in electron transfer, leading to a measurable current or potential difference. These devices are mainly concerned with detecting current. The target analyte reacts specifically with the recognition factor (tissues, antibodies, DNA/RNA, enzymes, or other biomolecules), generating an electrical signal that the transducer transmits to the signal processor. Furthermore, the signal is amplified and the information is retrieved (Lazcka *et al.*, 2007; Menon *et al.*, 2020). The generation of electrochemical sensors has evolved over time, progressing from 1<sup>st</sup> to 4<sup>th</sup> generation sensors with increasing capabilities and advantages (Putzbach and Ronkainen, 2013). A comprehensive roadmap for electrochemical sensors involves the exploration of various materials and technologies to enhance sensing performance.

This includes research into graphene-based sensors, semiconductor metal-oxide-based sensors, MXene-based electrochemical sensors, and flexible sensors (Ashraf *et al.*, 2022), Dutta *et al.* (2023), and Sumitha and Xavier, (2023). An important factor influencing the electrochemical nano biosensor's performance is the surface science, surface engineering, and surface functionalization of the nanomaterials. Electrochemical biosensors, particularly those that are connected to enzymes, function by detecting an electric signal that is generated or drained by an electroactive species. Enzymes have outstanding catalytic activity and selectivity; however, they will gradually lose that activity, reducing the biosensor's lifespan (Choi and Yoon, 2023). Enzymes exhibit a high degree of specificity for their substrates, enabling the precise detection of target molecules. This specificity ensures that biosensors can accurately identify and quantify the presence of specific analytes, even in complex mixtures. Enzyme-based biosensors are effective in distinguishing target molecules from other substances that might interfere with the measurement. The enzyme's catalytic action transforms the target substrate into a detectable product, enhancing the signal-to-noise ratio and minimizing interference from other compounds.

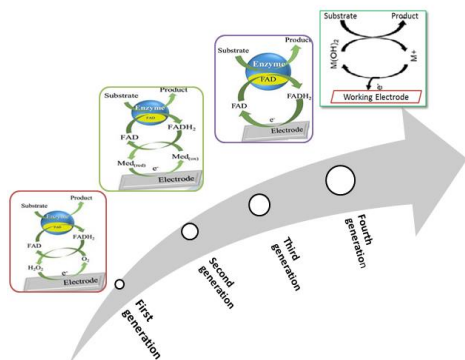
Enzymes typically function optimally under mild conditions (e.g., physiological pH and temperature), making them suitable for various applications, including biomedical diagnostics and environmental monitoring (Nguyen *et al.*, 2019).

Along with enzymatic electrochemical nanobiosensors supported by nanomaterials, research is also being done on novel enzymatic electrochemical nanobiosensors through the production of electrodes with improved conductivity employing nanotechnologies. To detect various biomolecules, a number of enzymes have recently been used, including glucose oxidase, cholesterol oxidase, lacase, horseradish peroxidase, etc. Electrochemical biosensor technology has made incredible strides recently in the detection of biomolecules.

The purpose of this review is to disseminate information regarding the advancement of electrochemical biosensors for the identification of biomolecules. This article explores the advancements in electrochemical biosensing based on several enzyme immobilization methods. For the purpose of detecting biologically significant compounds, this review will cover enzyme-immobilized nanomaterials.

## Generations of electrochemical Biosensor

The development of electrochemical biosensors is approached from many perspectives, as schematically represented in Figure 1.



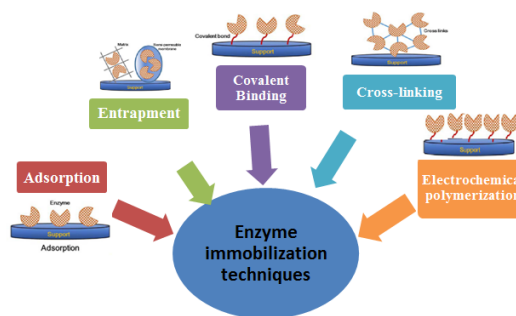
**Figure 1. Schematic representation of generational development of electrochemical biosensors**

In a first-generation biosensor, a transducer surface is used to immobilize the enzyme, and the analyte substrate is monitored by measuring the enzymatic product (such as  $\text{H}_2\text{O}_2$ , NADH, etc.) or by tracking the consumption of the co-factor (such as oxygen). Biosensors quantify the amounts of analytes and enzymatic reaction products that spread to the surface of the transducer and cause an electrical reaction. They are also called mediatorless biosensors. First-generation biosensors, which usually have reaction times of less than a second, have been shown to be extremely sensitive (Tseng *et al.*, 2009). Second-generation biosensors do not rely on oxygen for electron transport; instead, they use an electron acceptor or mediator to transfer electrons to the electrode. The mediator consists of small redox-active molecules that react with the electrode surface, and the enzyme-active site transfers electrons in the process to create a current signal proportional to the concentration of the analyte that was measured. According to this concept, electrons from the enzymatic reaction decrease the mediator, which is subsequently oxidized at the electrode to facilitate electron transport to the electrode. Due to redox mediator selectivity, the second-generation biosensor experiences mediator leaching and interference even though it is oxygen-independent (Mayorga *et al.*, 2011). Third-generation biosensors catalyze the conversion of the substrate to the product by direct electron transfer between the enzyme and electrode in the absence of mediators or co-substrates.

In order to help the electrons move from the electrode to the substrate molecule, the redox enzyme acts as an electrocatalyst. The selectivity of third-generation biosensors is higher due to their ability to function in a potential range that is nearer to the redox potential of the enzyme (Palmisano *et al.*, 2002). The final generation of biosensors, which uses a nanomaterial matrix with significant electrocatalytic activity, uses direct electron transfer via electro-oxidation of substrate to product. These biosensors integrate nanomaterials such as graphene, carbon nanotubes, and metal nanoparticles with biomolecules like enzymes or antibodies to detect analytes with high efficiency. This generation also focuses on miniaturization and integration for portable and point-of-care applications (Si *et al.*, 2013).

## Techniques for immobilization of enzymes on nanomaterials

Nanomaterials have emerged as versatile platforms for enzyme immobilization, enhancing stability and catalytic efficiency. Recent advances in nanotechnology and materials science have influenced enzyme immobilization technology, leading to novel approaches for enhanced enzymatic activity. Enzyme immobilization is typically accomplished using five techniques such as adsorption, physical entrapment, covalent binding, electrochemical polymerization, and cross-linkage, as shown in Figure 2, (Sun *et al.*, 2023).



**Figure 2. Schematic of enzyme immobilization techniques**

### 1. Non-covalent adsorption and deposition

Non-covalent adsorption and deposition are widely utilized methods in enzyme immobilization. This approach involves the binding of enzymes to surfaces through non-covalent interactions. Non-covalent adsorption and deposition play crucial roles in enzyme immobilization for biosensors due to their simplicity and efficiency. The process

involves adsorption, where enzymes adhere to surfaces through non-covalent interactions such as hydrophobic or electrostatic forces without forming permanent bonds. Second is physical adsorption, where enzymes are immobilized through physical forces like hydrophobic van der Waals or electrostatic interactions, ensuring reversible binding. Since these binds are weak, the enzyme's fundamental structure and activity remain unchanged, and it becomes immobilized in an arbitrary orientation on the surface of the electrode modified by the nanomaterial (Jesionowski *et al.*, 2014). This technique offers several benefits, such as minimal enzyme denaturation and reusability, as well as being simple to perform and cost-effective, requiring minimal equipment and reagents. This makes it accessible for various applications. However, non-covalent bonds are generally weaker than covalent bonds, leading to the potential desorption of the enzyme from the support, which can result in a loss of activity over time. In addition, this method can sometimes result in non-specific binding, leading to impurities or undesired interactions that can affect enzyme performance (Maghraby *et al.*, 2023; Mohidem *et al.*, 2023).

## 2. Physical entrapment

Physical entrapment is a technique used in enzyme immobilization where enzymes are enclosed within a matrix or membrane. This method involves creating a physical barrier often through non-covalent bonds or covalent to cage enzymes within gels, fibers, or capsules. The process helps maintain the native structure of enzymes, minimizing disruption, and offers stability for enzymatic activity. Physical entrapment can be achieved through various methods including mixing enzymes into a monomer solution followed by polymerization. It is a valuable approach in enzyme immobilization technologies providing a stable environment for the enzymes to function effectively (Datta *et al.*, 2013; Maghraby *et al.*, 2023). Therefore the technique may increase the stability of the enzyme and reduce denaturation and leaching of the enzyme. The technique also has the benefit of optimizing the enzyme's microenvironment by changing the encapsulating material to have the ideal pH, polarity, or amphiphilicity. Nevertheless a drawback of the technique is the mass transfer resistance that results from polymerization extension which tends to thicken the gel matrix and prevents the substrate from diffusing deeply enough to reach the active site of the enzyme.

Additionally, the process has a low capacity for loading enzymes and the polymerization process

may contaminate the support material (Nguyen and Kim, 2017; Maghraby *et al.*, 2023).

## 3. Covalent Binding

An established method of immobilizing enzymes is called covalent binding which is achieved by bonding the enzymes to support materials (such as agarose, porous glass, polyacrylamide, porous silica, etc.) via extremely strong and stable bonds (Wong *et al.*, 2008). The enzyme's functional group typically participates in binding through the glutamic acids, side chains of amino acids (lysine), thiol groups (cysteine), and aspartic. One important factor that regulates the stability of the covalent link between the enzyme and the support is the direction in which the enzyme binds. In certain cases, additional structural modifications to the enzyme are required to reveal certain capabilities that are necessary for covalent binding to take place. These changes to the reaction medium may cause the enzymes to become denaturated (Mohamad *et al.*, 2015). Covalent bonds offer a strong connection between the lipase and its carrier matrix enabling more frequent reuse than with alternative immobilization techniques and preventing the release of the enzyme into the reaction environment (Flickinger and Drew, 1999; Obzturk, 2001; Ovsejevi *et al.*, 2013). There is minimal chance of enzyme leakage from the support due to the strong bonds that covalent immobilization creates between the enzymes and the matrix. Covalent bonding necessitates a longer incubation period than adsorption because it takes several hours for the SAM to develop and the enzymes to link to it. To attain great homogeneity in the SAM a more sophisticated procedure necessitates careful attention to chemical purity (Hirsh *et al.*, 2010; Nguyen and Kim, 2017).

## 4. Cross-linking

Cross-linking is a technique extensively used in enzyme immobilization where enzymes are linked together via covalent bonds without the need for carriers (Siliang *et al.*, 2010). The process of cross-linking involves the use of multifunctional reagents to form intermolecular cross-linkages between the molecules of the enzyme. Glutaraldehyde is the most widely used cross-linking reagent because it is readily available and reasonably priced in large quantities. (Hanefield *et al.*, 2008; Gorecka and Jastrzebska, 2011). It's a unique active immobilized enzyme method that yields particles with controlled diameters (1-100  $\mu\text{m}$ ). It is extremely resistant to high temperatures and organic solvents. It is very attractive for industrial biotransformations because

of its excellent stability, volumetric efficiency, and optimal catalytic activity during recycling (Cao *et al.*, 2003). A straightforward technique for immobilization is crosslinking relies on the robust chemical bonding of biomolecules including enzymes hence minimizing enzyme leakage. The technique also has the benefit of allowing the microenvironment of the enzyme to be adjusted through the use of appropriate stabilizing chemicals through surface complementarity which contributes to increased stability (Honda *et al.*, 2005).

### 5. Electrochemical polymerization

Electrochemical polymerization techniques present an additional approach for immobilizing enzymes by producing polymers on the electrode surface. This technique involves initiating polymerization through electrochemical reactions typically oxidation of a monomer followed by the growth of a polymer film on the electrode's surface. It is a versatile method used to create polymer films with controlled properties for various applications. Conductive polymers produced via electrochemical polymerization are commonly used in the fabrication of electrochemical sensors. They exhibit sensitivity to specific analytes, making them valuable for sensing applications (Naveen *et al.*, 2017; Fomo *et al.*, 2019). Electrochemical polymerization provides a straightforward one-step process for producing homogeneous films with ease control over applied voltage. The disadvantages of this technique include the need for high monomer (0.05-0.5 M) and enzyme (0.2-3.5 mg ml<sup>-1</sup>) concentrations, as well as film deterioration from the overoxidation process which results from monomer depletion in the solution around the electrode (Fomo *et al.*, 2019; Goswami *et al.*, 2023).

### Nanomaterials for enzyme immobilization in electrochemical biosensors

Nanotechnology has gained significant attention due to its potential for developing novel electrochemical biosensors. Over the past few decades, nanomaterials with distinctive electrocatalytic properties such as significant adsorption capacity, extremely small size, vast surface area, and ease of surface modification have proven to be suitable for biosensing applications. Specifically in electrochemical sensors nanomaterials perform three multifunctional roles such as separation and preconcentration of the sample, immobilization of the bioreceptor and amplification of the signal (Lawal, 2023).

Nanomaterials are essential in electrode technology contributing to the development of robust electrode materials characterized by high electroactive surface area, enhanced porosity and superior electrical properties. Nanomaterials are currently popular for immobilizing certain enzymes. They improve the surface area to volume ratio which boosts enzymatic performance and the stability of the immobilized enzyme (Kuralay, 2019). A wide range of nanomaterials have been studied in relation to electrochemical biosensors, including nanoparticles, graphene, carbon nanotubes and nanostructured polymers.

### 1. Nanoparticle

Electrochemical biosensors make considerable use of nanoparticles to improve their sensitivity and efficiency. In order to improve the sensitivity of the detection metals with good electrical conductivity such as copper (Cu), platinum (Pt), gold (Au), and silver (Ag) are prepared onto the electrode surface. The free mobility of valence electrons in metals with designed nanostructures results in improved electrochemical characteristics. Metal-based electrochemical nanosensors have a number of disadvantages, including high cost and opaque appearance. Gold Nanoparticles (AuNPs) are known for their excellent conductivity. Since gold nanoparticles (AuNPs) have easy protein functioning and unusual biocompatibility they are the metal nanoparticles that are most frequently used in electrochemical biosensors. (Lipińska *et al.*, 2021) developed GOD/Fc/Au/SLG/GCE a biosensor based on glucose oxidase (GOD) deposited on gold nanoparticles embellished on a single layer graphene (Au/SLG) modified glassy carbon electrode detects glucose using 6-(ferrocenyl) hexanethiol (Fc-C6H12-SH) as an electron transfer medium. Figure 3 illustrates the electrochemical behavior of GCE, Au/SLG/GCE

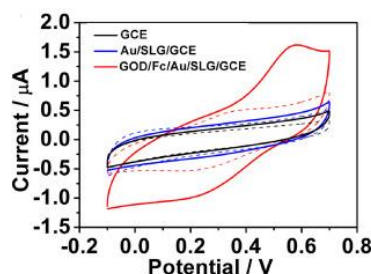


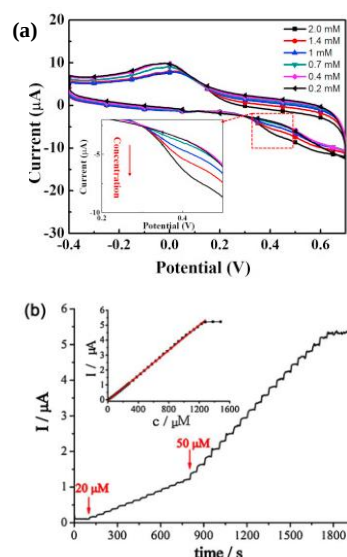
Figure 3. CV curves recorded for GCE, Au/SLG/GCE, and GOD/Fc/Au/SLG/GCE electrodes in PBS solution with and without 50  $\mu$ M glucose (solid line and dash line, respectively).

and GOD/Fc/Au/SLG/GCE toward glucose oxidation. The GCE and Au/SLG/GCE exhibit no redox peaks and poor capacitance in a 20 mL phosphate buffered solution without glucose. Anik *et al.* (2016) developed GSH-Px(immob)/Pt-NP/GCPE electrochemical biosensor for the detection of GSH. First, platinum (Pt) nanoparticles were placed on a glassy carbon paste electrode (GCPE). Next, gelatin was used to immobilize GSH-Px and last glutaraldehyde was crosslinked. The correlation coefficient  $R^2$  was determined to be 0.9968 with the linear range was between 10 and 250 mM.

## 2. Graphene

Graphene has recently garnered a lot of interest as a promising candidate for biosensors due to its exceptional electrochemical properties, adsorption capabilities, mechanical strength, and flexibility. Graphene, in particular, is one of the most often used carbon materials for electrode modification because of its long  $\pi$  bond and  $sp^2$  hybridized carbon atoms, which provide a wide range of usable potential. The three main constituents of graphene-based nanomaterials are reduced graphene oxide (rGO), graphene oxide (GO), and graphene. Graphene and its derivatives possessing high temperature resistance, a large specific surface area, and a high electron transport rate can be employed as biomolecule carriers or signaling devices to provide quantitative biomolecule detection (Kuila *et al.*, 2011; Justino *et al.*, 2017; Bai *et al.*, 2020). Graphene-based electrochemical biosensors have proven effective in the detection of many biomolecules, such as cholesterol, dopamine, glucose, and other analytes. Xuan *et al.* (2018) reported an electrochemical biosensor for measuring the concentration of glucose in actual human sweat that includes a working electrode (Au/rGO/AuPtNP/GOD/nafion). Figure 4(a), displays cyclic voltammetry data in a 0.05 mM PBS solution for a range of glucose concentrations (0.2, 0.4, 0.7, 1.0, 1.4, and 2.0 mM). The result revealed that with an increase in glucose concentration, the electrochemical oxidation peak current increases. The developed sensor exhibited good analytical activity with a linear range of 0.1 - 0.3 mM (1.8 mg/dl to 41.4 mg/dl) and a sensitivity of  $82 \mu\text{A}/\text{mMcm}^2$  towards glucose oxidation. Developed developed GC/RGO/PB/PTBO/CHIT-GOD biosensor for the detection of glucose. Figure 4(b) demonstrates the amperometric response of the GC/RGO/PB/PTBO/CHIT-GOD electrode when glucose is added one at a time and swirled in an air-saturated phosphate buffer solution (pH 7.4) with an applied potential of +0.2V. The current response increased as the glucose concentration increased and

reached steady state in 10 seconds. The inset figure shows the calibration curve for glucose detection at the GC/RGO/PB/PTBO/CHIT-GOD electrode. Its linear range was found to be 20 to 1090 M ( $R = 0.9995$ ,  $n = 34$ ), with a detection limit of 8.4 M at a signal-to-noise ratio of 3. The result also revealed an excellent response to glucose, with a sensitivity of  $59 \text{ mA M}^{-1} \text{ cm}^2$ .

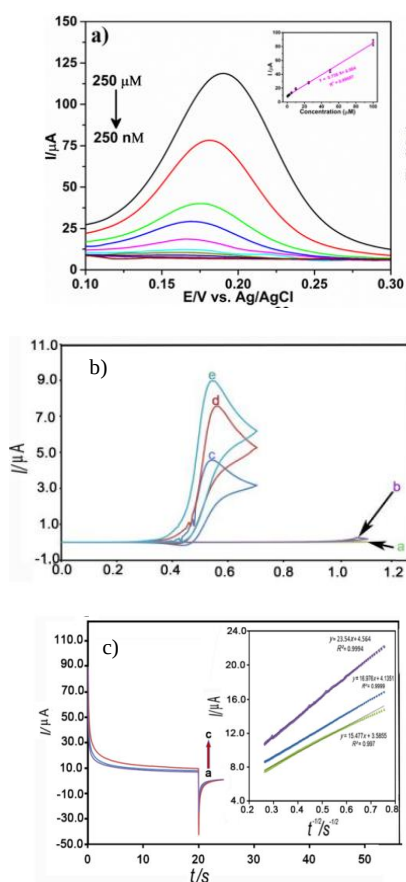


**Figure 4.** a) Cyclic voltammetry curves for Au/rGO/AuPtNP/GOD/nafion modified electrode for several glucose concentrations (0.2, 0.4, 0.7, 1.0, 1.4, and 2.0 mM) in 0.05 M PBS b) GC/RGO/PB/PTBO-GOD for successive addition of 500 M glucose

## 3. Carbon Nanotube

Carbon nanotube (CNT) has exceptional chemical and physical properties, such as a large surface area-to-volume ratio, high electrical conductivity, mechanical strength, and chemical consistency. However, CNT has drawbacks such as aggregation issues and challenging functionalization. Recently, viruses, viral pathogens, and vitamins have been identified with exceptionally high sensitivity and specificity by researchers worldwide utilizing CNT-based detection devices. Arumugasamy *et al.* (2020) reported GQDs@MWCNTs/GCE-modified electrodes for detection of dopamine. The steady and well-defined anodic oxidation peak curves of the electrode modified with GQDs@MWCNTs in the 0.1 M PBS solution (pH = 7.0) at different dopamine concentrations (250 nM-250 μM) are shown in Figure 5(a). The results revealed that the electrode modified with GQDs@MWCNTs has a strong electrocatalytic activity for dopamine sensing. The developed sensor had a linear range of 250 nM to 250 μM and a limit of detection of 95 nM.

Taei *et al.* (2015) reported a unique multi-walled carbon nanotube/carbon-paste modified electrode (ADAFCNPE) for the electrocatalytic measurement of glutathione (GSH) in hemolyzed erythrocytes. The electrochemical behavior of ADAFCNPE has been investigated using cyclic voltammetry and chronoamperometry in both the presence and absence of GSH, as shown in Figure 5(b). It was shown that the voltammetric detection of GSH was more sensitive when the electrode was modified using MWCNTs and ADAF.

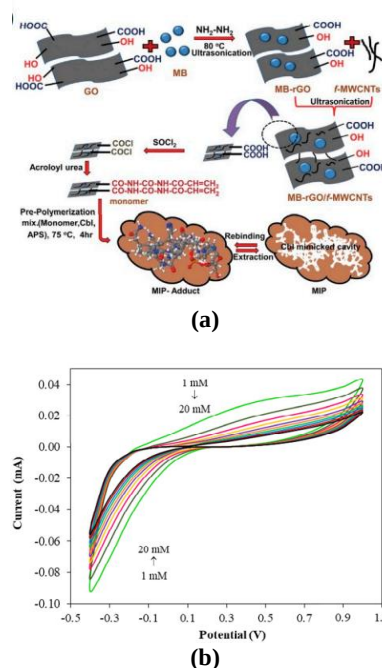


**Figure 5.** a) Differential Pulse Voltammograms at GQDs@MWCNTs nanocomposite modified GCE measures dopamine levels at different concentrations in pH = 7.0 PBS. b) Cyclic voltammograms for modified electrode in 0.05 M phosphate buffer (pH 6.0) at a scan rate of  $10 \text{ mV s}^{-1}$  in the absence (a) and presence (b) of  $400.0 \mu\text{M}$  GSH. c) The ADAFCNPE recorded chronoamperograms with and without GSH (a), as well as with and without 150 and  $500 \mu\text{M}$  of GSH.

#### 4. Nanostructured polymers

Nanostructured polymers, particularly conducting polymer nanostructures, are integral components of electrochemical biosensors used for

the detection of biomolecules. These advancements have applications in various fields, including healthcare and environmental monitoring. Singh *et al.* (2018) reported a three-dimensional biomimetic imprinted polymer with cyanocobalamin (Cbl) selectivity employing methyl blue-adsorbed functionalized multiwalled carbon nanotubes (f-MWCNTs) and reduced graphene oxide (rGO) composites appropriately functionalized with acryloylurea, as shown in Figure 6(a). This sensor was used for ultra-trace CBL detection using the differential-pulse voltammetry (DPV) technique. The sensor displayed a linear relationship with a detection limit of  $0.0056 \text{ ng/mL}$  and a concentration range of  $0.021$  to  $1.44 \text{ ng/mL}$  in real samples, with no interference observed. In particular, when used on actual samples, this sensor demonstrated excellent selectivity and sensitivity for CBL detection. An electrochemical glucose biosensor based on electrogenerated poly (pyrrole-3-carboxylic acid) into a composite film containing GO and immobilized GOD was developed by Thunyakontirakun *et al.* (2019). Figure 6(b) shows the CVs of the PP3C/GO/GOx modified electrode at different concentrations of glucose. The findings showed that the GOx-based biosensor that was built is appropriate for measuring glucose levels in the  $1\text{--}20 \text{ mM}$  range ( $R^2 = 0.9957$ ) with a  $0.05 \text{ mM}$  detection limit.



**Figure 6.** a) Schematic presentation of MB-rGO/f-MWCNT/AU-MIP Development b) CVs of PP3C/GO/GOx modified electrode for the detection of glucose at different concentrations at a  $50 \text{ mV/s}$  scan rate.

## Electrochemical Biosensor Development Using Enzymes

Enzyme-immobilized nanomaterials are extensively used in electrochemical biosensors for the detection of various biomolecules. These biosensors, commonly employed in the biomedical field, leverage enzymes to selectively detect vital biomolecules such as glucose, urea, and cholesterol. Enzymes such as laccase, horseradish peroxidase (HRP), glucose oxidase, cholesterol oxidase, and urease play crucial roles in various electrochemical biosensors, enabling the sensitive detection of specific analytes.

### 1. Horseradish peroxidase (HRP)

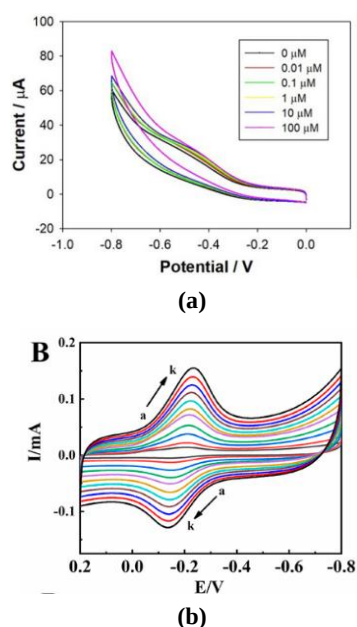
Horseradish peroxidase (HRP) is a plant-derived enzyme commonly used in various biochemical and biotechnological applications. In electrochemical biosensors, HRP is often immobilized on electrodes to facilitate the detection of specific analytes, such as hydrogen peroxide. Shin *et al.* (2020) developed horseradish peroxidase (HRP)-encapsulated protein nanoparticles (HEPNP/rGO/Au) as an electrochemical biosensor for the selective and sensitive detection of  $\text{H}_2\text{O}_2$ . As shown in Figure 7(a), CV was used to examine the electrochemical characteristics of the HEPNP/rGO/Au biosensor in the detection of  $\text{H}_2\text{O}_2$ . The result revealed that as  $\text{H}_2\text{O}_2$  concentrations

increased, reduction currents increased. Based on the responses from the CV, the HEPNP biosensor detection limit for  $\text{H}_2\text{O}_2$  was  $0.01 \mu\text{M}$ . A new and sensitive electrochemical biosensor, Nafion/HRP/ZnO @BP/CILE, has been developed by Feng *et al.* (2023). The direct electrochemistry of HRP on the Nafion/HRP/ZnO@BP/CILE was investigated using cyclic voltammetry, revealing the formation of two separate redox peaks. Moreover, CV was used to examine the impact of scan rate in the  $0.05\text{--}1.0 \text{ Vs}^{-1}$  range on the direct electrochemical behavior of HRP. The findings are displayed in Figure 7(b). It was observed that with an increase in scanning rate, the redox peak current increased gradually and the peak potential somewhat moved.

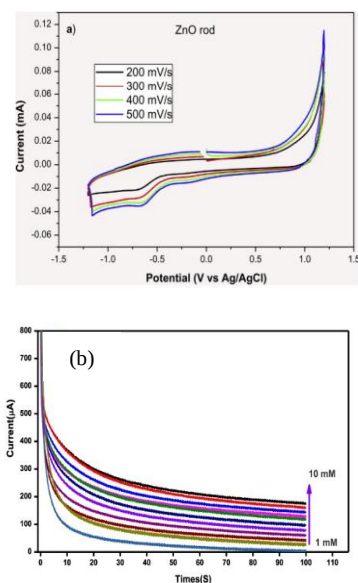
### 2. Glucose Oxidase

The enzyme glucose oxidase (GOD) is well-known for its ability to catalyze the oxidation of glucose to hydrogen peroxide and gluconic acid. This enzyme is commonly found in various organisms, including fungi and bacteria. It plays a crucial role in glucose metabolism. Glucose oxidase finds extensive use in clinical diagnostics, where it serves as a key component in glucose biosensors. These biosensors leverage the enzyme's ability to generate hydrogen peroxide in the presence of glucose, enabling quantification and the detection of glucose levels. Bagyalakshmi *et al.* (2020) developed ZnO rods, chitosan, and GCE electrodes for glucose detection. Cyclic voltage was used to examine the impact of the scan rate of the immobilized glucose oxidase (GOD) enzyme on the GCE-modified electrode, as shown in Figure 8(a). According to CV curves, an increase in the scan rate was seen following the assimilation of the glucose oxidase enzyme on the modified electrode, accompanied by an increase in the oxidation and reduction peak currents. According to these findings, the ZnO rods are essential for facilitating the direct electron transmission between the electrode surface and God. Maity *et al.* (2019) fabricated GOx/AuNP/PANI/rGO/NH<sub>2</sub>-MWCNTs modified SPCE for the detection of glucose. Figure 8(b) illustrates the amperometric detection of glucose using GOD/AuNP/PANI/rGO/NH<sub>2</sub>-MWCNTs developed as biosensors by varying glucose concentrations. The result showed the current increased linearly with a glucose concentration range of  $1 \text{ mM}$  to  $10 \text{ mM}$ . The developed glucose biosensor had a sensitivity of  $246 \mu\text{A mM}^{-1} \text{ cm}^{-2}$ , and its lowest detection limit was  $63 \mu\text{M}$ .

Wang and Chen, (2009) present the demonstration of DET using GOx electrodes without the use of nanomaterials. The enzyme



**Figure 7.** (a) CV of HEPNP/rGO/Au electrode for various  $\text{H}_2\text{O}_2$  concentrations (b) Cycle voltammetry of the Nafion/HRP/ZnO@BP/CILE at various scan rates (ranging from (a) to (k)).

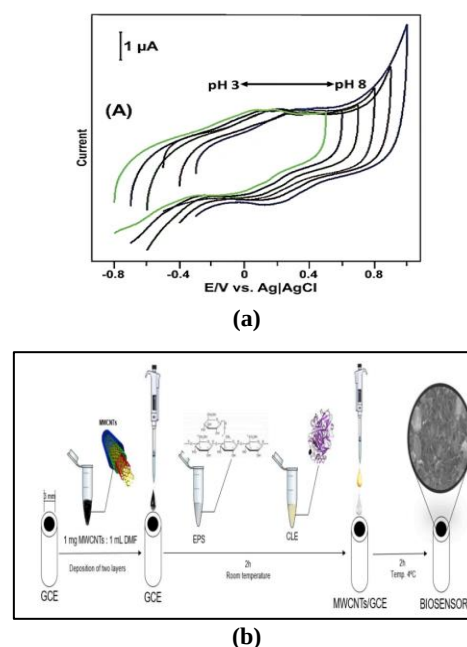


**Figure 8.** (a) CV of HEPNP/rGO/Au electrode for various  $\text{H}_2\text{O}_2$  concentrations (b) Cycle voltammetry of the Nafion/HRP/ZnO@BP/CILE at various scan rates (ranging from (a) to (k)).

efficiently oxidizes glucose after being immobilized with poly-L-lysine (PLL) and Nafion® while retaining its biocatalytic activity. The amperometric response of the Nafion-PLL-GOx modified electrode shows a sensitivity of  $0.75 \mu\text{A}/\text{mM}$  and a linear response with the concentration of glucose up to 10 mM. Yoon *et al.* (2019) developed a flexible electrochemical enzyme biosensor for immobilizing an enzyme on a flexible polymer electrode modified with a gold/MoS<sub>2</sub>/gold nanofilm. In this sensor, with the use of a chemical linker, the glucose-sensing biomolecule GOx was fixed to the gold/MoS<sub>2</sub>/gold nanofilm on the polymer electrode. This biosensor was also able to detect glucose dissolved in human serum and demonstrated improved amperometric glucose-sensing capability with a 10 nM detection limit, suggesting more sensitivity than that of previously reported flexible glucose sensors.

5.3 Laccase Laccase is an enzyme belonging to the family of oxidoreductases, and it catalyzes the oxidation of a variety of substrates through mono-electronic reactions. This enzyme is widely distributed in nature and is produced by various microorganisms, particularly fungi. Laccases play a crucial role in biological processes and have found applications in different industries due to their diverse properties. Multi-copper oxidase laccase (benzenediol: oxidoreductase, E.C. 1.10.3.2) is a green catalyst that combines the four-electron reduction of dioxygen to water with the one-electron oxidation of each of the four substrate molecules.

This laccase biosensor belongs to the third generation. A mediator is not required because of the direct electron transfer that occurs between the enzyme and the electrode. Laccases become immobile on the surface of the electrode, where they undergo oxidation by oxygen and subsequent reduction by the substrate, which serves as an electron donor for the oxidized version of the enzyme (Solomon *et al.*, 1996; Barrios-Estrada *et al.*, 2018; Koyappayil *et al.*, 2021). Palanisamy *et al.* (2017) reported a laccase-based biosensor using laccase immobilized on graphene-cellulose microfibers (GR-CMF) composite modified screen-printed carbon electrodes (SPCE) for the detection of catechol (CC). Laccase's redox electrochemical behavior is known to be pH-dependent. The direct bioelectrochemical behavior of the enzyme was investigated at a variety of pH values using cyclic voltammetry, as shown in Figure 9(a). It is evident that the laccase redox couple's anodic and cathodic peak potentials are significantly impacted by pH. An inventive sensing platform was presented by Li *et al.* (2012) that made use of laccase enzyme immobilized for the purpose of detecting dopamine on the surface of GCE coated with MWCNTs (laccase-EPS-MWCNTs/GCE). The steps needed to prepare the biosensor are schematically shown in Figure 9(b). Square-wave voltammetry, which has a detection limit of  $0.127 \mu\text{mol L}^{-1}$ , showed a linear



**Figure 9.** (a) Cyclic voltammograms of GR-CMF/laccase modified SPCE's at a scan rate of 100  $\text{mV/s}$  in various pH ranges (pH 3-8). (b) A schematic depicting the various stages involved in building the biosensor.

**Table 1. Enzyme immobilized electrochemical sensors for the detection of biomolecule**

| Modified Electrodes                                           | Biomolecule                   | Techniques | Linear Range ( $\mu\text{mol L}^{-1}$ ) | LOD ( $\mu\text{mol L}^{-1}$ ) | References                    |
|---------------------------------------------------------------|-------------------------------|------------|-----------------------------------------|--------------------------------|-------------------------------|
| laccase/Si/MWCNTs/SPE                                         | Dopamine.                     | DPV        | 1.30-85.5                               | 0.420                          | (Li <i>et al.</i> (2012)      |
| GOD/CoS-MWCNTs/NF/GCE                                         | Glucose                       | CV         | 0.008-1.5                               | 5                              | (Li <i>et al.</i> (2020)      |
| NTBC/LAC/GA                                                   | phenol                        | CV         | 0.5-150                                 | 0.5                            | (Romero <i>et al.</i> , 2017) |
| Co <sub>3</sub> O <sub>4</sub> /MWCNTs/gelatin/HRP/Nafion/GCE | H <sub>2</sub> O <sub>2</sub> | CV         | 0.74-19                                 | 0.74                           | (Kacar <i>et al.</i> , 2014)  |
| ChOx/Ag/GO/CS/ITO                                             | Cholesterol                   | CV         | 0.5-400                                 | 0.427                          | (Xu <i>et al.</i> , 2015)     |
| HRP-AuNCs/MWCNTs/CFUMEs                                       | H <sub>2</sub> O <sub>2</sub> | AMP        | 2-24                                    | 0.44                           | (Ren <i>et al.</i> , 2020)    |
| Laccase/MWCNT/GCE                                             | Dopamine.                     | SQW        | 0.1-10                                  | 3.63                           | (Wardak <i>et al.</i> , 2020) |
| GOD-CNT-PtNP-CS-MTOS-GCE                                      | Glucose                       | CV         | 1.2-6                                   | 3                              | (Kang <i>et al.</i> , 2008)   |
| rGO/Fe <sub>3</sub> O <sub>4</sub> /GOx/GCE                   | Glucose                       | CV         | 0.5-10                                  | 0.10                           | (Wang <i>et al.</i> , 2018)   |

CV: Cyclic Voltammetry; DPV: Differential Pulse voltametry; AMP:Amperometry; SQW: Square wave voltamtry

the connection between peak current and dopamine concentration. The range of this relationship was 2.99-38.5  $\mu\text{mol L}^{-1}$ . The biosensor demonstrated good selectivity even in the presence of uric acid, ascorbic acid, and other phenolic chemicals and was successfully used to determine the amount of dopamine in synthetic biological samples and pharmaceutical injections. Shown in Table 1.

## Challenges in the development of electrochemical biosensors

Electrochemical biosensors are essential for industrial, environmental, and biomedical applications. Numerous benefits of electrochemical sensors include their low cost, quick, real-time detection, and ease of use. However, enzyme-based electrochemical sensors face several significant challenges that need to be addressed for their further development and practical applications, such as first obtaining a low limit of detection (LOD). The reason that low-limit of detection (LOD) sensors are so important is that real samples frequently contain analytes in trace quantities. Secondly, another issue restricting the use of sensors in remote areas with variable temperatures has been their stability. Enzymes can denature and lose activity over time, affecting sensor stability and longevity. This issue is critical for applications requiring long-term monitoring (Ferrag and Kerman, 2020). Furthermore, achieving high selectivity and sensitivity is challenging due to potential interference from other substances in complex biological matrices. Improvements in electrode materials and bioreceptor configurations are necessary to enhance these attributes (Singh *et al.*, 2024). Next, integrating enzyme-based sensors with electronic systems for data acquisition and processing requires overcoming technical challenges related to signal transduction and noise

reduction (Kurbanoglu *et al.*, 2020). Finally, biocompatible sensors are necessary for in vivo applications in order to prevent immunological reactions and provide secure contact with biological tissues (Wijayanti *et al.*, 2023).

## Conclusion

The field of electrochemical sensors and biosensors has advanced with new discoveries in material science, nanotechnology, and custom manufacturing of biorecognition components. Enzyme-immobilized biosensors have drawn interest recently for both quantitative and qualitative investigations of a variety of target analytes in a number of domains, including agriculture, food quality assurance, the environment, biomedicine, and pharmaceuticals. This review discusses the many techniques for immobilizing enzymes, such as adsorption, covalent, cross-linking, entrapment, and electro-polymerization, in order to develop biosensors. In addition, this review provided various nanomaterials used for electrochemical biosensors, including nanoparticles, graphene, carbon nanotubes, and nanostructured polymers, as well as enzymes such as glucose oxidase, laccase, and horseradish peroxidase (HRP) for the detection of biomolecules. The integration of enzyme-functionalized nanomaterials into electrochemical biosensors represents a significant advancement in biomolecule detection, offering a balance between selectivity and sensitivity. However, ongoing research is essential to overcome challenges and enhance the sensitivity of these biosensors.

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