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# Bio-Based Nanofibers for Enzyme Immobilization: Advancing Voltammetric Biosensors Through Sustainable Materials

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**Abstract**

The replacement of conventional petrochemical polymers with bio-based alternatives has gained considerable attention due to their biodegradability, renewability, and lower environmental impact. The use of bio-based nanofibers as support matrices for enzyme immobilization addresses the common limitations associated with soluble enzymes, such as enzymatic instability under varying process conditions, including fluctuations in temperature and pH. Furthermore, immobilization allows enzyme reuse, leading to a significant reduction in process costs. This strategy combines the advantages of bio-based materials, such as biodegradability, renewability, and non-toxicity, allied with the intrinsic properties of nanofibers, such as nanoscale diameter, high surface area-to-volume ratio, and enhanced porosity. These features make bio-based nanofibers excellent platforms for biosensor development, where enzymatic biorecognition elements are employed to convert biochemical interactions into electrical signals. This is an emerging and multidisciplinary field with considerable room for improvement and innovation at every stage of development. A wide variety of bio-based materials can be optimized for nanofiber production via electrospinning, allowing the fabrication of support matrices with different functional groups and tailored characteristics for enzyme immobilization. Moreover, conductive nanomaterials can be incorporated into the electrospun solution, significantly increasing the electrode's active surface area and facilitating both mediated and direct electron transfer in the different voltammetric procedures. Advancements in this field will enable the development of improved biosensors capable of addressing various limitations across multiple application areas.

**Keywords:** Voltammetry; Nanotechnology; Electrochemical Biosensing

**Abbreviations:** SWV: Square Wave Voltammetry; DPV: Differential Pulse Voltammetry; CV: Cyclic Voltammetry; MET: Mediated Electron Transfer; DET: Direct Electron Transfer; FAD: Flavin Adenine Dinucleotide; EIS: Electrochemical Impedance Spectroscopy; ITO: Indium Tin Oxide; GLUOX: Glutamate Oxidase

## Introduction

Nowadays, there is a significant scientific effort focused on replacing synthetic chemical polymers with bio-based polymer materials. This substitution is driven by the desirable characteristics of these materials, such as environmental friendliness, biodegradability, renewability, and non-toxicity, which offer clear advantages over the environmental problems associated with conventional petrochemical polymers. Enzymes are biological macromolecules with catalytic activity, capable of increasing the rate of biochemical reactions by lowering the activation energy required for the process, without affecting the reaction equilibrium [1]. However, the use of soluble enzymes in industrial processes presents some limitations, such as high cost and enzymatic instability under different process conditions, including variations in temperature, pH, and the presence of surfactants or salts.

To overcome these challenges, enzyme immobilization has emerged as a promising solution, capable of increasing enzyme stability, reducing product inhibition, and enhancing functional properties. In addition to these advantages, enzyme immobilization allows for enzyme reuse, as it can be easily separated

from the reaction medium, resulting in a significant reduction in process costs [2]. There are several methodologies applicable to enzyme immobilization, which can be grouped into three main techniques: adsorption (physisorption or chemisorption), cross-linking, and encapsulation. Even among these well-established methods, there is still room for improvement to better understand the immobilization phenomena. Applying different studies, such as equilibrium, kinetic, and thermodynamic models for the adsorption process or different mathematical models for entrapment process, can help elucidate the interactions between the bio-based support matrix and the enzyme, thereby improving the desired applicability [3].

Bio-based polymers are being applied across various fields worldwide, with the development of bio-based nanofibers standing out among the different alternatives explored. Due to their intrinsic properties, such as nanoscale diameter, high surface area to volume ratio, and enhanced porosity, these fibers serve as excellent matrices for enzyme immobilization for biosensors development, offering greater accessibility and higher enzymatic activity. Furthermore, the use of different bio-based materials, including chitosan, gelatin, and cellulose, provides a wide variety of functional groups for this purpose, among others [3-5]. The electrospinning system is composed of four parts: a syringe containing the polymer solution, a metallic needle, a high-voltage power source, and a metallic collector. The process begins when the electric charges generated by the power source are transferred to the polymer solution through the metallic needle positioned at the tip of the syringe, inducing instability in the solution.

As the syringe is pressed, the polymer solution flows through the needle toward the collector. At this stage, as the solution exits the needle, repulsion between the solution's molecules occurs due to the electric charge, generating a force that opposes the surface tension and drives the solution in the direction of the electric field. An increase in the electric field causes the spherical droplet to deform into a conical shape. In this phase, nanofibers emerge from the droplet of the conical polymer solution, known as the Taylor cone, and are collected on the metallic collector, which is positioned at an optimized distance from the syringe. A stable charged jet can only be formed when the polymer solution has sufficient cohesive strength. During the process, the forces generated by the internal and external charges produce a whipping effect on the liquid jet as it moves toward the collector. This whipping effect allows the polymer chains within the solution to stretch, forming fibers with diameters small enough to be classified as nanofibers [6,7].

In addition to conventional electrospinning setup using a direct electrospinning setup, enzyme immobilization can be performed using different electrospinning configurations, which can influence enzyme stability and the functionalization of nanofibers. Coaxial electrospinning employs two concentrically aligned capillaries to produce nanofibers with a core–shell architecture. The outer capillary is loaded with a spinnable polymer solution that forms the protective shell, while the inner capillary contains the core solution, usually composed of the enzyme in a biocompatible medium. Triaxial electrospinning introduces a third, intermediate layer, allowing for additional spatial separation and functional complexity. This configuration enables the co-encapsulation of multiple enzymes or the integration of functional barriers (e.g., size- or charge-selective layers) to limit the diffusion of interfering compounds [3,8].

Moreover, the outermost layer can be functionalized with conductive nanomaterials, such as metallic nanoparticles, carbon nanotubes, or graphene/reduced graphene oxide [9,10]. The biopolymer matrix not only acts as a long-term stabilizing medium for these nanomaterials but also facilitates electron transfer between the enzyme in the core and the electrode surface, which is an important requirement for voltammetric biosensors. It is important to mention that the performance of voltammetric biosensors depends on the compatibility between the conductive nanomaterials and the biopolymer matrix. To achieve the desired functionality, a homogeneous distribution of conductive fillers must be ensured, as aggregation can impair conductivity and reduce sensor performance.

Therefore, future studies should focus on the development of new biopolymers that are soluble in aqueous or mildly acidic solvents is relevant, as these conditions can preserve the tertiary structure of enzymes during processing. Moreover, the incorporation of hydrophobic groups or emulsified phases can enhance the catalytic activity of enzymes, such as lipases by promoting lid opening, which exposes the active site and facilitates substrate access. Furthermore, the selection of an appropriate solvent–biopolymer–nanomaterial system is relevant not only for achieving the required properties for electrospinning, but also for ensuring the uniform dispersion of conductive nanomaterials. Indeed, interactions, such as hydrogen bonding, electrostatic forces, and π–π stacking between biopolymer and nanomaterials are essential for stabilizing the nanomaterials within the polymer matrix, preventing aggregation, and maintaining electrochemical functionality. For instance, these factors should be carefully considered and further investigated in the development of advanced voltammetric biosensors based on bio-based nanofiber materials.

Electrochemical devices have become essential in a world where the need for sensing, whether in environmental monitoring, forensic chemistry, or especially in disease diagnostics, has become indispensable [11]. When associated with enzymatic biorecognition elements, such devices rely heavily on detection mechanisms to convert biochemical interactions into electrical signals. Among the various electrochemical techniques, voltammetry, including square wave voltammetry (SWV), differential pulse voltammetry (DPV), and cyclic voltammetry (CV), has been widely recognized for its sensitivity and versatility, depending on the type of potential applied. In this context, SWV applies square


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wave pulses superimposed on a staircase potential, offering excellent signal-to-noise ratio and high efficiency in biosensing, particularly through enzymatic films and nanostructured electrodes. DPV, in turn, minimizes capacitive currents by applying potential pulses over a linear ramp, allowing also effective detection in complex biological matrices [12]. Finally, CV is extensively used to investigate redox properties and the reversibility of electron transfer processes in bioelectrochemical mechanisms [12,13].

The efficiency of these voltammetric techniques is closely tied to the electron transfer mechanism between the enzyme, nanofiber, and base electrode, with two main pathways observed: (i) mediated electron transfer (MET), in which redox mediators act as a bridge between the enzyme's redox centers and the base electrode, and (ii) direct electron transfer (DET), where a direct electrical connection is established between the enzyme's redox centers and the base electrode surface [11,14]. Although MET enables the detection of a broader range of enzymes, it typically requires higher operating potentials and may suffer from interference by electroactive species present in the environment. On the other hand, DET allows measurements at potentials close to the enzyme's native redox potential, thereby minimizing unwanted interferences [14]. However, achieving DET remains a challenge due to structural limitations, such as in the case of glucose oxidase, where the flavin adenine dinucleotide (FAD) cofactor is located approximately 17–22 Å from the protein surface, making DET both thermodynamically and spatially unfavorable [15].

Enhancing the performance of such biosensors depends on a set of integrated strategies aimed not only at overcoming thermodynamic and spatial constraints but also at improving sensitivity, selectivity, operational stability, and response time. These improvements primarily involve the use of supporting materials, optimized enzyme immobilization strategies, and tailored electrochemical architectures [11]. One effective approach is the incorporation of conductive nanomaterials [14], such as carbon nanotubes, graphene, and metallic nanoparticles (e.g., Au and Ag), which significantly increase the electrode's active surface area and facilitate both MET and DET processes [16]. Additionally, advanced enzyme immobilization strategies play a critical role in this optimization. Techniques such as entrapment, cross-linking, physical adsorption, and surface functionalization help preserve enzymatic catalytic activity and improve enzyme orientation relative to the electrode, thereby enhancing stability [17].

Therefore, the performance of voltammetric nanofiber enzyme-based biosensors can be substantially improved by integrating these approaches, enabling significant enhancements in key properties that are essential for their application in rapid and accurate diagnostics across various fields. As mentioned, in recent years, voltammetric biosensors have become established as efficient tools for sensing in various application fields, such as environmental monitoring, clinical diagnostics, pharmaceutical and food quality control, forensic and security contexts, among others. As these devices have their sensitivity, selectivity, and response time enhanced using nanofibers, such platforms enable efficient detection and quantification of relevant targets such as glucose, lactate, uric acid, $H_2O_2$, pesticides, nitrite, and many others.

In this context, Atilgan and collaborators [18] proposed the incorporation of a glassy carbon sensor modified with poly-ε-caprolactone nanofibers, chitosan decorated with montmorillonite modified with first-generation PAMAM dendrimer, and the enzyme glutamate oxidase (GluOx), for the detection of monosodium glutamate in food samples. The authors reported that the use of nanofibers enabled efficient enzyme immobilization due to the multiple covalent binding sites generated. Electrochemical characterization was performed using CV and DPV, showing good conductivity and electrocatalytic response when tested in the range of 0.0025 to 0.175 nmol $L^{-1}$. Additionally, a detection limit of 1.045 μmol $L^{-1}$ and a recovery rate of 103.152% demonstrated the sensor's efficiency.

In turn, Yildirim-Trigil and colleagues [19] developed an enzymatic biosensor for the detection of acetylcholine for clinical diagnostic applications. The biosensor was constructed using electrospun nanofibers composed of polypyrrole and chitosan, with the enzymes acetylcholinesterase and choline oxidase immobilized directly onto the nanofibers. Using CV and electrochemical impedance spectroscopy (EIS), a widely employed technique for sensor characterization, a detection limit of 5 μmol $L^{-1}$ was established, along with 30 days of operational stability. Another electrochemical sensor utilizing carbon bionanofibers impregnated with silver nanoparticles (AgNPs) for triglyceride detection was developed by Mondal and collaborators [20]. The mentioned material was electrophoretically deposited onto indium tin oxide (ITO) and treated with oxygen plasma to enhance enzyme immobilization capacity for lipase and glycerol dehydrogenase. Detection and characterization using EIS and CV resulted in a sensitivity four times higher than that of the non-modified enzymatic sensor (1.232 μA $mg^{-1}$ $dL^{-1}$ $cm^{-2}$ vs. 0.33 μA $mg^{-1}$ $dL^{-1}$ $cm^{-2}$).

Finally, a biosensor for the detection of organophosphate pesticides using acetylcholinesterase was developed by Tun and collaborators [21]. The biosensor, based on the modification of a screen-printed carbon electrode with cellulose nanofibers, graphene oxide, chitosan, and the target enzyme, enabled the detection of chlorpyrifos in a linear range of 25 to 1000 nmol $L^{-1}$, with detection and quantification limits of 2.2 nmol $L^{-1}$ and 73 nmol $L^{-1}$, respectively. Thus, as observed, nanofiber-based biosensors associated with enzymes demonstrate excellent performance in sensitivity, selectivity, and response time, proving to be versatile and effective platforms especially when coupled with strategies that enhance these properties.

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