

# Enzyme Based Biosensors

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Enzyme-based biosensors were the earliest biosensors. In 1962, Clark proposed the idea of enzyme electrodes for a glucose sensor. Subsequently, enzyme-based biosensors have been experimenting a massive growth in several applications.

biosensors

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nanozymes

laccase

glucose oxidase

horseradish peroxidase

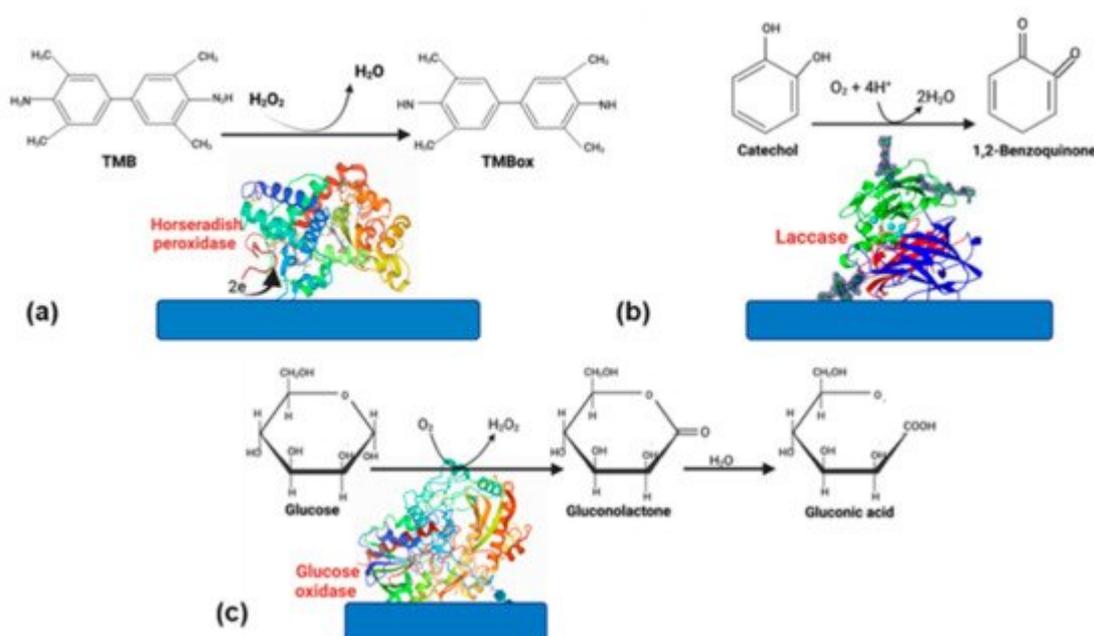
## 1. Introduction

The biological component used in enzyme-based biosensors is an enzyme. Enzymes are biological macromolecules with catalytic activity, high selectivity, and responsible for speeding up biochemical reactions under mild conditions [1]. These macromolecules can attach to one particular molecule or analyte, but not others to ensure the analyte selectivity. Due to their high specificity, simplicity, and scalability, enzyme-based biosensors represent a fast, precise, and continuous monitoring of analytes [2]. Additionally, the high specificity of enzymes enhances the ability to detect lower analyte concentration limits [3]. Additionally, the catalytic action can be influenced by the substrate concentration, temperature, pH, and inhibitor presence [3]. The enzyme functions could be the generation of electroactive species or an electroactive reactant's consumption, causing the direct measurement of the analyte [4], or for oxidation or reduction of a molecule, which can be monitored electrochemically [5].

The crucial factor in enzyme-based biosensors is the assembly or immobilization of the enzyme on the electrode surface [6]. If the immobilization is not correctly done, the accessibility of the active site, the stability through time, and the enzyme's reusability could be affected. The enzymes can be immobilized on the transducer surface to improve the stability and reproducibility of the detection. The choice of support material is essential for conferring stability, selectivity and even improve enzyme activity. Consequently, the support material must be inert, stable, and resistant [7]. The immobilization technique is highly significant; without immobilization, the enzyme cannot be stable and reusable. The immobilized enzymes can be used continuously and can maintain their catalytic activity. Adsorption, covalent bonding, crosslinking, encapsulation and entrapment are the main methods used for immobilization [8][9]. The simplest methods are adsorption, encapsulation, and entrapment. Adsorption is inexpensive and straightforward; however, the enzymes have weak associations with the support [7]. Entrapment gives the enzyme high stability; however, the matrix can interfere with substrates' diffusion to the enzyme's active site. Covalent bonding is the most used method because a stable complex between the enzyme and support is generated [6]. Nevertheless, the formation of the covalent bonding could affect the enzyme activity. Crosslinking immobilization improves the stability and efficiency due to the stable binding between enzymes, generally formed

with a reactant as glutaraldehyde. However, with reagents, conformational changes in the structure can affect the enzyme activity [10].

Even though the advantages of using enzymes, some disadvantages, such as the rapid loss of enzyme activity due to its interactions with the electrode surface, cause a biosensor's lifespan is only 2–4 weeks. However, if the enzyme is well stabilized, this can increase [11]. Therefore, choosing a suitable matrix and an excellent strategy to immobilize the enzyme [9]. Enzyme-based biosensors have tremendous applications in food, medicine, and environmental monitoring. Oxidoreductases and peroxidases are the most-reported enzymes in biosensors because they are very stable catalyzing oxide reduction reactions [12]. In this section, we will focus on glucose oxidase (GOx), horseradish peroxidase (HRP), and laccase (**Figure 1**); these enzymes have been successfully used for different applications in biosensors.



**Figure 1.** Enzymes commonly used in biosensors. (a) HRP, (b) laccase, and (c) glucose oxidase.

## 2. Horseradish Peroxidase

Horseradish peroxidase (HRP, EC 1.11.1.7) is an enzyme that belongs to the group of oxidoreductases. HRP is extensively distributed in nature, and its purification process is relatively simple. The principal source of extraction is the horseradish root. Some advantages reported are high activity and selectivity, resistance to inhibition by substances over a vast concentration, high operability, and reliability over a broad range of treatment conditions [13] [14]. In addition, it can catalyze organic and inorganic substrates' oxidation by reacting with  $H_2O_2$  and similar molecules [15]. Therefore, HRP has been widely used in biosensors for  $H_2O_2$  determination [11].

The detection mechanism of the  $H_2O_2$  biosensor depends on the electrode (modified or not) and whether the mediator is used or not. The mediator facilitates the electron transfer between enzymes and electrodes. In the

presence of a mediator, the  $\text{H}_2\text{O}_2$  in the solution is reduced by the HRP. On the other hand, the mediator is oxidized in the enzymatic reaction by itself. Moreover, the oxidized mediator is finally reduced on the electrode, with a change in the current. If the mediator is not present, the enzyme is converted to its oxidized form after being reduced at the electrode surface by direct electron transfer [16].

Hydrogen peroxide is an essential intermediate in enzymatic reactions. The detection of  $\text{H}_2\text{O}_2$  is essential in medicine, food, and environmental assays (Table 1). As a result, the development of biosensors for  $\text{H}_2\text{O}_2$  detection has been extensive [11]. Different materials have been reported to improve the response between the electrode and the HRP enzyme, for example, gold nanoparticles (AuNPs), cadmium sulfide, nanofibers, carbon nanomaterials. Carbon nanomaterials have unique properties, such as good biocompatibility, fast electron transfer, and excellent mechanical flexibility. Feizabadi et al. [11] immobilized HRP on a modified multi-walled carbon nanotube by  $\gamma$ -aminobutyric acid (GABA) on a glassy carbon electrode. The covalent bonding formed between the enzyme and GABA increased the stability and reproducibility of the biosensor. Due to its characteristics as low detection ( $0.13 \mu\text{M}$ ) and extensive linear range ( $0.2$  to  $281 \mu\text{M}$  for  $\text{H}_2\text{O}_2$ ), the biosensor could be used to quantify  $\text{H}_2\text{O}_2$  in human plasma.

Da Silva Freires et al. [17] developed a biosensor based on copper (I) sulfide ( $\text{Cu}_2\text{S}$ ) and HRP immobilized on a fluorine-doped tin oxide modified glass slide (FTO) for the determination of 1,4-dihydroxybenzene (DHB). The biosensor showed good selectivity for DHB, and good accuracy when DHB was determined in skin cream samples, presenting recovery percentages for the analyte in the samples between 99.89 and 100.70%, suggesting a good accuracy of the proposed method.

For cancer detection, exosomes can be an alternative as it is a non-invasive technique. Additionally, it is desirable for a cost-effective and instance detection in clinical diagnosis. Zeng et al. [18] developed a versatile biosensor to detect cancer-derived exomes (HepG2 cell-derived). HRP encapsulated DNA nanoflowers were the recognition elements and signal generation. A change of color was proportional to the concentration of exosomes. The system showed a satisfactory colorimetric response toward target exosomes within the working range from  $5.0 \times 10^3$  to  $5.0 \times 10^6$  particles/ $\mu\text{L}$  at a low detection limit of  $3.32 \times 10^3$  particles/ $\mu\text{L}$ . López-Marco et al. [19] used HRP in a 3D-printed graphene/polylactic electrode and compared the detection of  $\text{H}_2\text{O}_2$ . The AuNPs facilitate and enhance electron transfer. However, they found that biosensors without AuNPs displayed better stability over time. The response of biosensors was evaluated in human serum.

**Table 1.** Horseradish peroxidase biosensors.

Material	Transduction System	Application	Linear Range with a linear Correlation	Limit of Detection (LOD)	Ref.
Glass plate covered with fluorine-doped tin oxide (FTO)Copper (I) sulfide ( $\text{Cu}_2\text{S}$ ) and	Photoelectrochemical	Health	1,4-dihydroxybenzene (DHB)	10 nmol $\text{L}^{-1}$ up to 1 mmol $\text{L}^{-1}$ (R = 0.998)	4.0 nmol $\text{L}^{-1}$ [17]

Material	Transduction System	Application	Linear Range with a lineal Correlation	Limit of Detection (LOD)	Ref.
fluorine-doped tin oxide modified glass slide					
Encapsulated DNA nanoflowers of magnesium pyrophosphate crystals	Colorimetric	Health	Rapid screening of cancer-derived exosomes $5.0 \times 10^3$ to $5.0 \times 10^6$ particles/ $\mu\text{L}$ ( $R^2 = 0.9846$ )	$3.32 \times 10^3$ particles/ $\mu\text{L}$	[18]
Polydimethylsiloxane (PDMS) deposited into a polystyrene tube	Chemiluminescent	Health	Quantification of $\text{H}_2\text{O}_2$ as the oxidizing agent $0.06\text{--}10 \mu\text{M}$ ( $R^2 = 0.999$ )	$0.02 \mu\text{M}$	[20]
Modified multi walled carbon nanotube by $\gamma$ -aminobutyric acid	Electrochemical	Food, health, environmental	Detection of hydrogen peroxide $2.0 \times 10^{-7} \text{ M}$ to $2.81 \times 10^{-4} \text{ M}$ ( $R^2 = 0.998$ )	$0.13 \mu\text{M}$	[11]
3D-printed graphene/polylactic (PLA) electrode with gold nanoparticles	Electrochemical	Environmental and biomedical fields.	Hydrogen peroxide detection $25\text{--}100 \mu\text{M}$ ( $R = 0.996$ )	$11.1 \mu\text{M}$	[19]
HRP-encapsulated protein nanoparticles in an Au electrode surface	Electrochemical	Clinical applications	Hydrogen peroxide detection $0.01\text{--}100 \mu\text{M}$	$0.01 \mu\text{M}$	[21]
Modified platinum electrode covered with poly(4,7-bis(5-bromothiophen-2-yl) benzothiadiazole)	Electrochemical	Health	$17\beta$ - estradiol $0.1$ to $200 \text{ mM}$ ( $R^2 = 0.99$ )	$105 \text{ nM}$	[22]
Tungsten microwire modified with AuNPs and 3-mercaptopropionic acid	Electrochemical	Health	Determination of hydrogen peroxide $5 \text{ nM}$ to $5 \mu\text{M}$ ( $R = 0.999$ )	$800 \text{ pM}$	[23]
Modified acrylic microspheres	Electrochemical	Food	Chilli hotness determination $0.75\text{--}24.94 \mu\text{M}$ ( $R^2 = 0.992$ )	$0.39 \mu\text{M}$	[24] [25]

Glucose oxidase (GOx) is the principal enzyme used for the devices of glucose monitoring. It is a glycoprotein that possesses orthophosphates proteins. This enzyme has unique properties such as dispersibility in water, resistance to precipitation, and stability. All these advantages make GOx efficient for glucose monitoring in blood or saliva [25]. The continuous monitoring of glucose is vital in diabetes mellitus disease. Therefore, the production of a simple, cost-effective, accurate, and rapid sensor is essential. GOx has still been used for glucose detection due to its reliable stability and substrate specificity. The principle of its operation is the enzymatic oxidation of glucose and after the electrochemical oxidation of  $\text{H}_2\text{O}_2$  [26].

Electrochemical biosensors have been used to detect glucose using GOx (**Table 2**) [27]. The GOx enzyme converts the glucose to gluconic-d-lactone by reducing the flavin adenine dinucleotide (FAD) to FADH<sub>2</sub>. Then, H<sub>2</sub>O<sub>2</sub> is produced due to FADH<sub>2</sub> deoxidized by dissolved O<sub>2</sub>. Subsequently, the H<sub>2</sub>O<sub>2</sub> is oxidized to O<sub>2</sub> when a working potential is applied, and the electric current produced in the biosensor is proportionate to the glucose concentration [28]. The incorporation of nanomaterials as biosensor components has enhanced their performance. Bagyalakshmi et al. [27] prepared ZnO nanorods with chitosan. GOx was immobilized by the adsorption method. The ZnO nanorods were a successful platform for the immobilization of GOx due to their high surface area and displayed a good performance for displaying glucose. The use of carbon nanotubes in glucose biosensors have been improved the enzyme stability and specificity. Jayakumar et al. [29] reported an adsorbed osmium-based redox polymer crosslinked with GOx. It was possible to use less nanoconjugate due to the covalent bond between GOx and multiwalled carbon nanotubes (MWCNTs). Green approaches also have been made for the development of glucose biosensors. Yang et al. [30] developed an enzyme electrode based on AuNPs, PNE, and GOx for glucose detection by a green method. The biosensor presented high sensitivity to glucose and high response of fewer than 3 s. Redox mediator p-benzoquinone was added to enhance the linear detection range and sensitivity

**Table 2.** Glucose oxidase biosensors.

Material	Transduction System	Application	Linear Range with a Lineal Correlation	Limit of Detection (LOD)	Ref.
ZnO nanorods with chitosan	Electrochemical	Health	Glucose determination	10 $\mu$ M to 40 $\mu$ M (R = 0.9998)	[27]
Multi-walled carbon nanotubes and osmium redox polymer	Electrochemical	Health	Glucose determination		[29]
Au nanoparticles (AuNPs) and polynorepinephrine (PNE)	Electrochemical	Health	Glucose determination in human blood serum samples	0.003 mM to 3.43 mM (R <sup>2</sup> = 0.9987)	1.34 $\mu$ M [30]
Glassy carbon electrode with blend nanofibers of poly (vinyl alcohol) and poly(ethyleneimine)	Electrochemical	Health	Glucose in real samples	10 to 30 mmol L <sup>-1</sup> (R <sup>2</sup> = 0.971)	0.3 mmol L <sup>-1</sup> [6]
Screen-printed carbon electrode with platinum nanoparticles electrodeposited on Poly(Azure A)	Electrochemical	Food	Glucose quantification in real samples	20 $\mu$ M–2.3mM	7.6 $\mu$ M [31]

## 4. Laccase

Laccase (benzenediol: oxidoreductase, E.C. 1.10.3.2) is a multi-copper oxidase considered a green catalyst due to combines the four-electron reduction of dioxygen to water with the one-electron oxidation of four substrate molecules [32][33]. Laccase can be produced by insects, plants, bacteria, or fungi. It is considered a suitable enzyme due to its excellent catalytic properties [34][35]. The substrate range is vast, and they can oxidase different compounds, resulting in the application of numerous biotechnological applications such as environment, food, and biosensors (Table 3) [36].

The laccase biosensor is of the third-generation type. Due to direct electron transfer between the electrode and enzyme, there is no need for a mediator. Laccases are immobilized on the electrode's surface, and they are oxidized by oxygen and then are reduced by the substrate acting as electron donors for the oxidized form of the enzyme. A reduction current will be observed to reduce the products, which is proportional to their concentration. Unlike peroxidases, laccase-based biosensors only need oxygen and are already present in the solutions, so it is unnecessary to  $H_2O_2$  for its catalysis [37].

Laccase biosensors have been used widely for dopamine detection. However, even when some analytes' determination as dopamine has been studied extensively, novel techniques and approaches have been developed. Wardak et al. [38] constructed a laccase-based biosensor constructed by Soft Plasma Polymerization technique for dopamine detection. This technique enhances the sensitivity, and it was proved for pharmaceutical samples with satisfactory results. Furthermore, the use of polysaccharides has been explored. An exopolysaccharide (EPS) named botryosphaeran, and MWCNT were used to immobilize laccase on a glassy carbon electrode to detect dopamine. Even in the presence of other molecules as uric acid, the biosensor could determine the presence of dopamine [39]. Additionally, the fluorescence principle has been used for dopamine detection. Sangubotla and Kim [40] developed a fiber-optic biosensor with carbon dots and laccase. This material presents excellent features such as hydrophobicity, tunable photoluminescence, and biocompatibility.

**Table 3.** Laccase biosensors.

Material	Transduction System	Application	Linear Range with a Lineal Correlation	Limit of Detection	Ref.	
Laccase hybrid microflowers synthesized with $Cu_3(PO_4)_2 \cdot 3H_2O$	Optical	Health, clinical diagnosis application	Quantification of epinephrine	1–400 $\mu M$ ( $R^2 = 0.999$ )	0.6 $\mu M$	[41]
Carbon dots bio functionalized with 3-(aminopropyl)-triethoxysilane	Optical	Health. Clinical diagnosis application. Diagnosis of Alzheimer's and	Detection of dopamine	0–30 $\mu M$ ( $R^2 = 0.995$ )	41.2 nM	[40]

Material	Transduction System	Application	Linear Range with a Lineal Correlation	Limit of Detection	Ref.	
		Parkinson's diseases.				
Multi-walled Carbon Nanotubes modified glassy carbon electrode	Electrochemical	Diagnosis of Alzheimer's and Parkinson's diseases.	Dopamine detection	0.1 $\mu\text{mol}/\text{dm}^3$ to 10 $\mu\text{mol}/\text{dm}^3$ and from 10 $\mu\text{mol}/\text{dm}^3$ to 50 $\mu\text{mol}/\text{dm}^3$	3.63 $\mu\text{A}\cdot\text{dm}^3/\mu\text{mol}$ and 1.33 $\mu\text{A}\cdot\text{dm}^3/\mu\text{mol}$	[38]
$\text{Fe}_3\text{O}_4@/\text{SiO}_2$ microspheres stabilized onto glassy carbon electrode	Electrochemical	Health	Dopamine detection	1.5–75 $\mu\text{mol L}^{-1}$ (R = 0.9980)	0.177 $\mu\text{mol L}^{-1}$	[42]
Glassy carbon electrode layered with multi-walled carbon nanotubes using a film of botryosphaeran	Electrochemical	Health	Dopamine and spironolactone detection	2.99–38.5 $\mu\text{mol L}^{-1}$ (R <sup>2</sup> = 0.995)	0.127 $\mu\text{mol L}^{-1}$	[39]
Carbon paper electrodes with layered two-dimensional molybdenum disulfide ( $\text{MoS}_2$ ) in flowers ( $\text{MoS}_2\text{-F}$ ) and ribbons ( $\text{MoS}_2\text{-R}$ )	Electrochemical	Synthetic urine sample	Dopamine detection	0.1 to 0.5 $\mu\text{M}$ and from 1 to 5 $\mu\text{M}$ (R <sup>2</sup> = 0.993)	10 nM	[43]
6,9-bis(4-hexylthiophen-2-yl)-11H-indeno[2,1-b]quinoxalin-11-one (M1) polymerized on electrode surface.	Electrochemical	Environmental applications	Catechol in water	0.05–0.175 mM (R <sup>2</sup> = 0.994)	9.86 $\mu\text{M}$	[44]
Screen-printed carbon electrodes modified with carboxyl functionalized multi-walled carbon nanotubes	Electrochemical	Environmental application	Phenolics detection			[45]

biomolecules and nuclei acids. A higher level of ALP is related with tumors, biliary obstruction and diabetes [49]. Moreover, it can be used to detect organophosphate pesticide through catalyzes. Stéfanne e Silva et al. [50] immobilized ALP onto a modified non-commercial, low cost and nonrefundable pencil carbon graphite with three polymers derived from hydroxybenzoic acids for pesticide detection. Urease is an enzyme used for the urea detection, its levels are directly related to the protein intake and nitrogen metabolism in humans. Kim et al. [51]

developed a portable biosensor for real time monitoring of the flow of physiological fluids on a porous polytetrafluoroethylene.

**Table 4.** Other enzymes commonly used in biosensors.

Enzyme	Transduction System	Application	Linear Range with a Lineal Correlation	Limit of Detection (LOD)	Ref.	
Lipase	Electrochemical	Environmental application	Methyl parathion detection	0.1–38 $\mu\text{M}$	0.067 $\mu\text{M}$	[52]
	Optical	Health application	Triglycerides detection	100–400 mg/dL	15 mg/dL	[53]
Urease	Electrochemical	Health application	Urea detection	1.2–20 mM	1.1 mM	[51]
Tyrosinase	Electrochemical	Environmental application	Bisphenol detection	0.05–20 $\mu\text{M}$	0.011 $\mu\text{M}$	[54]
	Electrochemical	Food applications	Caffeic acid (reference polyphenol indices in beers and wines)	10–300 $\mu\text{M}$	4.33 $\mu\text{M}$	[47]
	Electrochemical	Environmental applications	Bisphenol detection	$5 \times 10^{-8}$ – $2 \times 10^{-6}$ mol L <sup>-1</sup>	12 nM L <sup>-1</sup>	[48]
	Electrochemical	Food applications	Benzoic acid detection		0.4 $\mu\text{mol L}^{-1}$	[55]
Lactate dehydrogenase	Electrochemical	Health applications	Pyruvate detection	$5 \times 10^3$ – $1.4 \times 10^5$ nM	8.69 nM	[56]
Alkaline phosphatase	Electrochemical	Environmental applications	Pesticide detection		20 $\mu\text{M}$	[50]

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