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Advances in Glucose Oxidase-Based Electrochemical Biosensors: Generational Development, Immobilization Strategies, and Nanostructure Applications

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ABSTRACT

Monitoring glucose levels is vital for the management of diabetes and other metabolic disorders, spurring extensive research into the development of reliable, sensitive and biocompatible glucose sensors. Glucose oxidase (GOx)-based electrochemical biosensors have evolved over time through three different generations, with each generation aiming to overcome the limitations of the previous one. First-generation sensors depend on oxygen as the electron carrier, while second-generation systems utilize artificial mediators that provide more stable and sensitive measurements at lower potentials. Third-generation sensors aim to enable direct electron transfer (DET) between the enzyme and the electrode, enabling the development of marker-free, biocompatible platforms suitable for continuous glucose monitoring. Enzyme immobilization methods such as physical adsorption, covalent binding, entrapment in polymer matrices, crosslinking and microencapsulation play a critical role in improving sensor performance, stability and reproducibility. Moreover, the integration of nanostructures such as graphene, carbon nanotubes, gold nanoparticles and hybrid composites on the electrode surface facilitates efficient electron transfer and improves sensor response by increasing surface area, conductivity and biocompatibility. This review provides a comprehensive overview of the basic principles, developmental processes and challenges of GOx-based glucose biosensors, with a particular focus on recent advances in enzyme immobilization strategies and nanostructured electrode materials. The integration of these approaches is anticipated to contribute significantly to the development of wearable, noninvasive, and continuous glucose monitoring systems, which are of great importance for personalized healthcare and diabetes management.

KEYWORDS

Electrochemical biosensor, glucose oxidase, glucose monitoring, enzyme immobilization, nanostructures, direct electron transfer, diabetes management

1. INTRODUCTION

1.1. Health importance of GOx measurement

Diabetes is a serious global public health problem characterized by hyperglycemia resulting from insufficiency or ineffectiveness of the hormone insulin. Irregular blood glucose levels are one of the main features of diabetes, which can lead to serious long-term complications such as cardiovascular disease, kidney failure, stroke, lower limb amputations and vision loss [1,2]. Therefore, accurate, fast and easy detection of blood glucose levels is of great importance for disease management.

Today, the rapidly developing socioeconomic structure has led to significant changes in the lifestyles and eating habits of individuals. In particular, diets high in carbohydrates have become more common, which is considered to be

one of the important factors that increase the incidence of diabetes. According to 2019 data from the International Diabetes Federation, approximately 463 million individuals worldwide live with diabetes. China has the highest patient population in terms of this disease with approximately 116.4 million individuals with diabetes and accounts for approximately 25% of the global burden. This increases the tendency for individuals to avoid high-carbohydrate foods, with a greater preference for products that are low in calories or contain sweeteners with low energy content. However, the compositionally diverse nature of food products increases the need for both selective and sensitive analysis methods for the detection of glucose [3-5].

Several analytical techniques have been developed to date for determining glucose, including fluorescence, optical, thermal, sonic, electrochemical and colorimetric methods. Although these methods usually have simple application steps, some of them do not provide the desired level of sensitivity and may be inadequate for glucose determination in complex sample matrices. To overcome these limitations, electrochemical approaches in particular have been intensively investigated in recent years and offer alternative solutions [6-8].

Sensor and biosensor technology for blood glucose measurement, especially electrochemical types, has had a decisive impact on patients' quality of life and diabetes treatment in recent years. Electrochemical glucose level measurement can be performed using enzymes (mostly glucose oxidase) or mimetic electrocatalyst materials (without enzymes) for the electrooxidation of glucose [9]. The main disadvantage of enzyme-based glucose sensors is the stability problem due to denaturation of the enzymes. Non-enzyme-based Glc sensors are stable but have lower selectivity and sensitivity. Various modified electrodes have been developed for enzymatic and non-enzymatic electrochemical Glc biosensors. Here we focus on glucose oxidase (GOx) based Glc biosensors [9].

The traditional approach to monitor blood glucose levels is by finger pricking, which causes pain and exposes patients to possible infections. With the recent advancement of wearable devices, there are opportunities to painlessly use alternative samples (sweat, saliva, urine) from patients for glucose testing [10]. The development of non-invasive biosensors as powerful medical devices continues to attract interest in the biosensor field [11].

1.2. Development process of GOx enzyme biosensors

Biosensors developed for the determination of glucose levels in blood are mainly based on glucose oxidase

(GOx) and less frequently on glucose dehydrogenase (GDH). GOx-based biosensors are highly selective to glucose molecules and are more stable than glucose dehydrogenase, but its activity depends on temperature, pH, and dissolved oxygen concentration in the sample. Since GDH-based sensors are not sensitive and selective for glucose determination due to their reactivity towards sugars other than glucose (such as maltose and xylose), research on biosensors based on glycooxidase has intensified [12,13].

The reaction catalyzed by the enzyme GOx in glucose biosensors is quite characteristic. The reactions that take place in glucose biosensors are given in Scheme 1. First, the flavin adenine dinucleotide (FAD) cofactor of the GOx enzyme is reduced by glucose and glucose is oxidized to the intermediate D-gluconolactone. Next, D-gluconolactone hydrolyzes with water to form gluconic acid, which is stable. Finally, oxygen re-oxidizes the reduced cofactor ($FADH_2$) in the glycooxidase enzyme and hydrogen peroxide (H_2O_2) is released in this reaction [14-16]. Finally, hydrogen peroxide is electrochemically oxidized against Ag/AgCl (Scheme 1) [17,18].

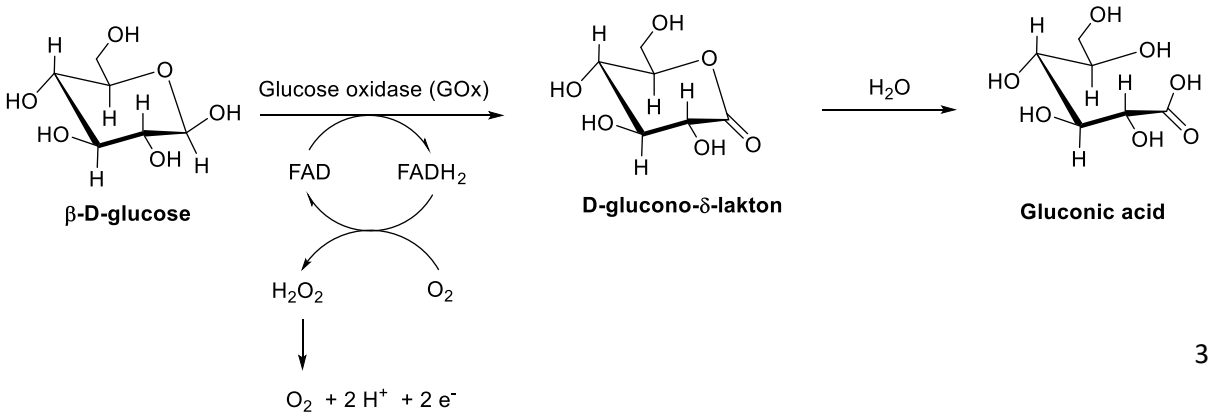
Proposed mechanisms for the oxidation half-reaction of β-D-glucose indicated in Scheme 1 have been reported in the literature[19].

The substrate being determined can be any of the products formed in the reaction. The fact that the products formed in the reaction have the same equivalents allows the quantification of other species by using any one of them for determination. Quantitative determination of glucose can be carried out by means of an oxygen electrode, since it consumes one oxygen molecule for each molecule of glucose oxidized, as indicated in Scheme 1[20].

Since the amount of peroxide produced on the electrode surface is equivalent to the amount of glucose, it is possible to determine the glucose level via H_2O_2 [21].

Some sensors measure the presence of H_2O_2 directly, while others measure indirectly through reactions such as color change with

a second enzyme (e.g. peroxidase)[19].



Scheme 1. Reaction of glucose with GOx enzyme

During the oxidation of hydrogen peroxide, other species sensitive to electro-oxidation (e.g. ascorbic acid (AA), uric acid (UA), etc.) can lead to signal interference and thus compromise enzymatic selectivity. On the other hand, glucose quantification based on oxygen consumption depends on the stability of dissolved O_2 in the analyzed sample, oxygen being a co-substrate of the enzymatic reaction; both of these phenomena cause errors in the measurement [22,23].

There are some challenges in developing specific biosensors to be applied to real complex samples. In systems operating at high potentials, the interference problem arises and reduces the reliability of the measurement since different reactions can be lysed in the catalyst. By incorporating structures that operate at lower potentials, the possibility of oxidation/reduction interferences at low operating potentials can be reduced and more reliable results can be obtained [24-27].

1.2.1. First generation GOx Biosensors

The first enzymatic glucose biosensor was introduced in 1962 by Clark and Lyons to determine the O_2 content of blood during an operation. [20,22,28]. GOx enzyme electrodes for glucose determination reported by Updike and Hick in 1967 were the first examples in this field [29].

The first generation of glucose sensors utilized oxygen as an electron mediator between GOx and the electrode surface [30]. The first generation of biosensors was based on the amperometric detection of H_2O_2 produced during the enzymatic oxidation of glucose. Amperometric measurement is performed with a Pt electrode at relatively high applied voltages [31]. Due to the relatively high voltage applied, electroactive substances in the sample medium such as acetaminophen, ascorbic acid and uric acid may be oxidized, which may interfere with the existing signal [12].

The presence of oxygen as electron mediator in first generation sensors offers some advantages (biocompatibility and low cost) but also certain limitations and disadvantages (the need for a certain level of dissolved oxygen, slow oxygen reduction, which leads to a longer sensor response time).

Oxygen has a low solubility in water and is present at different rates in different tissues, affecting the reaction rate, which in turn affects the response time and accuracy of the sensor. H_2O_2 formed by oxygen reduction can accumulate harmful to electrodes. H_2O_2 can also damage some

biomolecules, reducing sensor stability. The use of oxygen is practical but limiting [32]. Sensor activity can be severely affected, especially in low oxygen environments [33]. To overcome such problems, glucose sensors with artificial carriers (electron mediators) (e.g. ferrocene, ferrocyanide, p-benzoquinone) have been developed. Such sensors are characterized as second generation glucose sensors [34].

The first commercial glucose sensor in the first generation biosensor class was the YSI glucose sensor. YSI translated the working principle of Clark and Lyons (1962) into the first commercial glucose sensor in 1975 [19, 35].

1.2.2. Second generation GOx Biosensors

Second-generation biosensors have been developed by eliminating the disadvantages caused by oxygen by using different electron mediator molecules/materials that can replace oxygen in first-generation biosensors and by providing low voltage requirements. In sensor systems, electron mediators act as a bridge that rapidly transfers electrons between the enzyme and the electrode [34].

The advantages of these artificial electron carriers are that they provide faster and more stable electron transfer, lower operating potentials resulting in less interference and higher signal-to-noise ratio [33].

The fact that low voltage can be applied in second generation biosensors prevents interference with other redox active compounds in the sample, contributes to enzyme stability and provides advantages for the production of portable devices [36,37].

Second-generation glucose sensors facilitate electron transfer from the redox center of GOx to the electrode surface by introducing redox mediators, but in practical application, biocompatibility issues [38], stability issues [39], leaching into the sample environment [40,41], high cost and synthesis problems for some species (especially osmium complexes) [42], interactions of mediators with other active species in the sample limit their applications [43].

Second generation (mediator) biosensors started to be developed in the early 1980s and soon found their way into commercial applications. Especially in the field of glucose biosensors, systems using artificial mediators such as ferrocyanide, ferrocene, osmium complexes to eliminate oxygen dependence became widespread in this period [40].

The first example of artificial electron mediated glucose sensors was the ferrocene-based glucose sensor made by the Cass group in 1984.

The major advance with the use of redox mediators was the ability to detect glucose at lower applied potentials [22].

The first commercial examples of mediator glucose biosensors appeared in 1984, mainly used in portable blood glucose meters. These sensors offered more stable and faster measurements by providing electron transfer through the mediator instead of directly through oxygen. This was an important milestone in overcoming the limitations of the first generation (Clark-type) sensors [31,45].

1.2.3. Third generation GOx Biosensors

The disadvantages of redox mediators in second-generation sensors, such as toxicity, and the demonstration of direct electron transfer (DET) without a mediator have led researchers to design third-generation biosensors.

The DET occurs when electrons from the active site of the enzyme participating in the redox reaction are transferred directly to the electrode without the need for any mediator; this reduces one step in the electron transfer chain, eliminates mediator-induced toxicity, leakage and instability, and makes the biosensor more biocompatible and stable [45].

In DET-based biosensors, the enzyme should be immobilized on the electrode surface with the redox active site close to the electrode surface. Conductive nanomaterials such as carbon nanotubes, gold nanoparticles, graphene are often used to provide surface conductivity. Carbon nanotube modified electrodes increase DET efficiency by binding the enzyme in a directed manner [46].

Although GOx-based biosensors based on DET are not inherently well suited to DET because the FAD cofactor of the GOx enzyme is embedded in the protein structure, this has been made possible by some innovative strategies [45].

Third generation glucose sensors focus on achieving DET between GOx and the electrode without the need for redox mediators. However, third-generation glucose sensors are still in the research phase, they have not been commercialized [22,47]. Comparison of GOx Based Biosensors by Generations is given in Table 1 [19,22,31,33,48,49].

1.3. Types of binding in enzyme immobilization

The first enzyme immobilization studies were described in 1950 and have been further developed over time and methods for different immobilization have been established [19]. In all studies, the primary goal has always been to preserve the activity and natural conformation of the biomolecule [19].

Immobilization not only increases the durability of the enzyme, but also ensures that the enzyme can be easily removed from the reaction medium when desired.

For this reason, immobilization of enzymes has an important place in industry and health [50]. Immobilization techniques used in the literature for GOx enzyme: physical adsorption [51], covalent bonding [52], entrapment in polymer matrix [53], cross-linking [54] and microencapsulation [55].

Table 1. Comparative Overview of GOx-Based Glucose Biosensors by Generation

Generation	Fundamental Principle	Advantages	Disadvantages
1st Generation	Oxygen-dependent; Measures H_2O_2 from GOx-catalyzed rxn	-Simple design -Cost-effective -Enabled first commercial biosensors	- Relies on O_2 concentration -Requires high potential for H_2O_2 detection→ interference risk - Limited biocompatibility
2nd Generation	Mediated electron transfer (e.g., ferrocene, osmium complexes)	-Independent of oxygen -Lower detection potential -fewer interferences - Faster and more sensitive response -Low potential operation	- Mediator toxicity and leakage potential -Enzyme-mediator interactions need optimization - Stability may be limited
3rd Generation	Direct electron transfer (DET) ; no mediator used	-The electron chain is plain -Increased biocompatibility -Stable and low potential measurement -Biocompatible & simplified redox path -Low potential operation	- DET for GOx is inherently difficult (FAD embedded) -Requires nanostructured materials and engineering -Still limited in commercial use

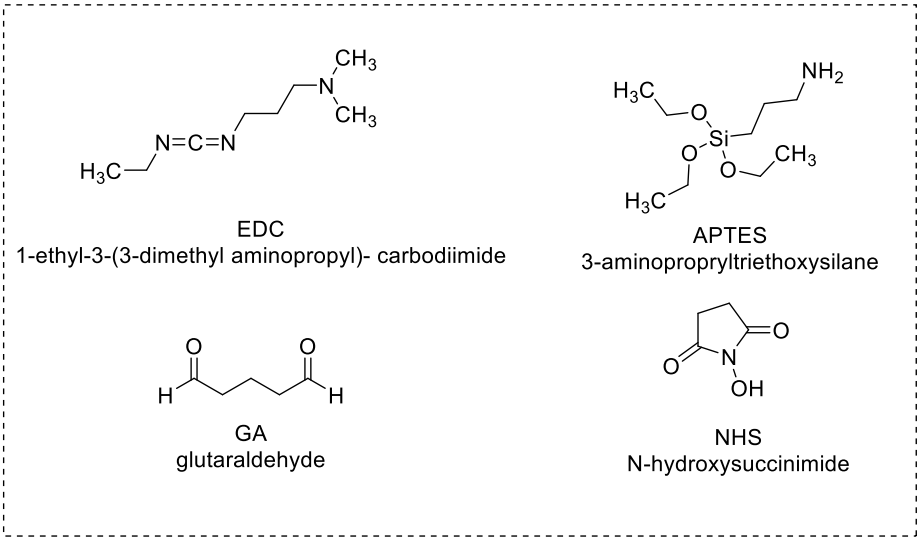
1.3. 1. Physical adsorption

The enzyme is attached to the surface by weak interactions (van der Waals, hydrogen bonds). This procedure involves the deposition of the enzyme on the surface of the solid support material or electrode. However, due to the weak binding forces, the immobilized enzyme is easily affected by changes in pH, temperature, solvent and ionic strength [56,57].

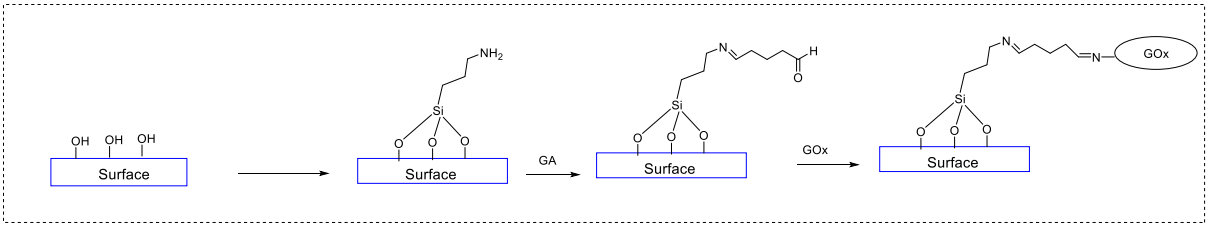
In physical adsorption, the enzyme is attached to the electrode surface by weak intermolecular interactions. It usually takes place on carbon electrodes or graphene surfaces [50, 58].

1.3.2. Covalent bonding

The enzyme is immobilized by covalently bonding to the surface through functional groups. In enzyme immobilization, if the enzyme is attached to the support material through a single chain, no rigidification is provided. If the enzyme is bound from more than one point, enzyme rididification is provided [54]. This method is more resistant to changes in pH, temperature, ionic strength. Glutaraldehyde, EDC/NHS (carbodiimide/succinimide), self-assembled monolayers or multilayers (SAM- self-assembly monolayers/ multilayers) and silane compounds (such as



Scheme 2. Organic structures used in enzyme immobilization



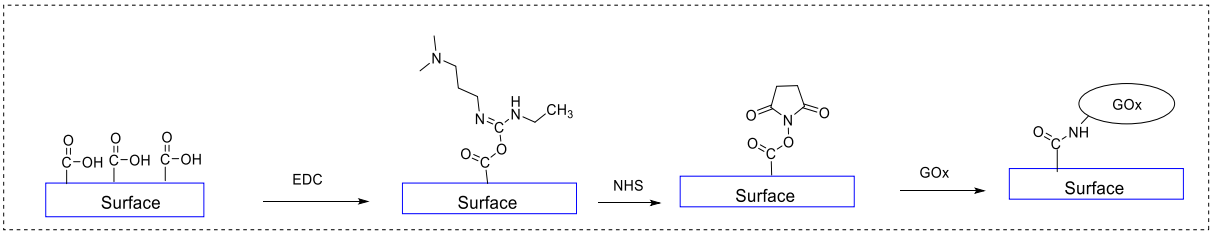
Scheme 3. The procedure for APTES-GA coating.

aminopropyltriethoxysilane) are frequently used for surface attachment of biomolecules [19, 54] (Scheme 2).

The Si atoms in APTES bind to the surface containing hydroxy group to form Si ether structures and the NH_2 group at the other end of the APTES molecule binds to GA (glutaraldehyde). The other aldehyde group of glutaraldehyde forms a glutaraldehyde imine bond with the free NH_2 group of the lysine amino acid in the GOx structure [59](Scheme 3).

EDC/NHS molecules are used to bind enzymes to electrode surfaces bearing carboxylic acid on the surface. The carbodimide group in the EDC molecule

reacts with carboxylic acid to form the O-acylurea structure. Subsequently, succinimide structures (2,5-dioxopyrrolidin -1-yl) are formed at the carbosilicic acid ends from the reaction with NHS. The free NH_2 groups in the GOx structure nucleophilically attack the carbonyl group in the R-CO-O-NR_2 structure and bind to the surface of the enzyme with an amide bond [60]. There are studies on the formation of SAMs and their use as a means of communication between redox active enzymes and the electrode surface [49] (Scheme 4).



Scheme 4. The procedure for EDC/NHS coating

1.3.3. Eembedding into polymer matrix (entrapment)

The enzyme is physically entrapped in a gel or polymer matrix (such as polyacrylamide)[29]. In entrapment in a polymer matrix, the enzyme is physically entrapped in a conducting or semiconducting polymer and controlled diffusion is achieved[38]. In the electrochemical polymerization method, the enzyme is embedded in the film formed by polymerization on the electrode surface. In this method, film thickness and porosity can be controlled depending on the desired property [61-64]. Nanostructure supported surfaces are used for immobilization. GOx is immobilized on surfaces such as carbon nanotubes, graphene, gold nanoparticles. Thanks to these nanostructures, high surface area and conductivity are provided, which provides an ideal environment for DET. Here, the electrode binding to the surface is by physical absorption and covalent bonding [65-69].

The enzyme immobilization technique in the design of GOx-based sensors has some disadvantages. Enzymes are easily inactivated during the immobilization process; therefore, it is difficult to achieve good stability and reproducibility and researchers are focusing their efforts on this step of biosensor fabrication[22]. Among many immobilization methods, the enzyme is fixed on the electrode surface by cross-linking using low-cost reagents, especially glutaraldehyde (GA) and bovine serum albumin (BSA)[22].

1.3.4. Cross-linking

Enzymes are bound to each other or to the carrier by cross-linking agents. The cross-linking method is based on covalent bonding between the carrier and the

biomolecule using one or more functional groups. Since there is covalent binding in this method, the possibility of enzyme desorption is very low. With this method, biomolecule immobilization can be done in different ways. Enzyme inhibition by cross-linking is based on electrochemical polymerization [70].

Altun et al. 2020 ferrocene-substituted 2,5-di(thienyl)pyrrole (SNS-Fc) was electrochemically polymerized in the presence and absence of 3,4-ethylenedioxythiophene (EDOT) and immobilized GOx by crosslinking and used for biosensing [70]. Altun et al. 2019 also prepared both homopolymer P(SNS-An) and copolymer P(SNS-An-co-EDOT) films and evaluated them for biosensing efficiency by incorporation of carbon nanoelements (carbon nanotubes and fullerene) and crosslinking of glucose oxidase. They reported that the copolymer showed superior performance as a biosensing interface compared to the homopolymer structure or previously reported P(SNS) biosensors [71].

Poly (2,5-dithienylpyrrole) (PSNS) and polypyrrole (PPy) derivatives are especially promising for electrochromic applications due to their low oxidation potential and easy synthesis by chemical and electrochemical methods. Despite all these positive properties, the polymer structure using PSNS derivatives as polymer matrix in enzyme biosensor applications, the effect of copolymerization, the use of carbon nanomaterial in the biosensor structure and the effect of the type of carbon nanomaterial were also systematically investigated [72].

1.3.5. Microencapsulation

The enzyme is surrounded by a semipermeable membrane, allowing controlled diffusion. One of the most ideal methods for enzyme immobilization is entrapment in an inert material (such as electropolymerized monomers, sol-gel matrices). The advantages of this method include milder conditions, a one-step process, low cost and high stability of the encapsulated enzyme. The limitations of entrapment are revealed in the diffusion barrier that the substrate and/or product must overcome. Long response time, difficulties in controlling pore size and possible enzyme release are the major disadvantages of the method [73-75]. Examples of materials used in encapsulation include photopolymerized polymers, chemically grown polymers (alginate, latex, etc.), electrochemically grown polymers (polypyrrole, polyaniline, polythiophene, etc.), and sol-gel matrices [57,76].

1.4. Use of nanostructures in electrode materials

The use of nanomaterials in biosensors has been increasing in recent years. Due to their structural properties and unusual properties, they can provide different properties according to the intended use in studies[77] . In order to emphasize the importance of nanostructures, it is evaluated under a separate heading.

The electrochemical performances of electrode materials depend on their unique properties, including but not limited to large specific surface area, high absorption capacity, superior redox properties, good conductivity, good biocompatibility, long-term cycle stability, good self-life and low cost [78-83]. The type of material used in electrode modification is very important. Modification of electrode surfaces is usually performed by various methods, including electrode deposition, drop casting and electrochemical polymerization [84]. Nanostructures can be used in electrode modification. The advantages of nanostructures such as large surface area, low background current, high potential range, low cost, chemical inertia, accelerating electrochemical reactions and increasing the electroactive surface area have led to increased studies with these structures [85,86]. Research on electrochemical biosensors has established a direct connection between the active center of the enzyme and the electrode through nanostructures, enabling DET between the electrode and the enzyme; this allows for measurement with higher sensitivity [87-89].

Graphene, a two-dimensional monolayer form of carbon, is a material of great interest in scientific and technological applications due to its excellent mechanical, optical, thermal and electrical properties [16,78]. Graphene oxide (GO) is a graphene derivative containing many functional groups (carboxyl, hydroxyl, epoxy, etc.) on its surface. At the same time, thanks to these functional groups on its surface, its dispersion in different solvents such as water increases and it allows materials with new properties to be obtained in the composite structure by interacting with other molecules [79]. Several biosensors based on graphene have been developed, which take advantage of its high specific surface area in addition to its optimal electrical conductivity to support enzyme immobilization thanks to its adsorption ability [22]. Graphene can be considered not only as an outstanding platform to support different biological molecules and nanomaterials such as metal or metal oxide nanoparticles (NPs), but also as an efficient interface between GOx or GDH and the underlying conductive support to develop third generation electrochemical biosensors [22].

Modifying the enzyme binding surface with metal nanoparticles, carbon-based

nanomaterials or biocompatible polymers are among the most common ways to create selectively advantageous surfaces for biomolecules. In 2019, Bük et al. reported that they modified gold electrode surfaces with a hybrid nanomaterial consisting of two different types of nanoparticles: gold nanoparticles (AuNPs) and carbon quantum dots (CQDs) [90]. ZnO nanostructures are used in glucose biosensors as their shape has a significant influence on their electrochemical properties [80]. In glucose sensor using Nanoflower/Ag nanoparticles (MoS_2 NF/AgNP); nanocomposites were synthesized by a simple chemical method and deposited directly onto a Pt electrode, then GOx was immobilized onto the modified Pt electrode by crosslinking [81].

4. CONCLUSION

The first generation of sensors was based on the most basic principles. However, their application was limited by the variable oxygen levels in biological samples. With the development of second-generation biosensors, this limitation was overcome with artificial mediators, enabling more precise and stable measurements. The third generation improves accuracy by establishing direct communication between the enzyme and the electrode, and the structure becomes simpler compared to Generation 2 biosensors, but studies are still ongoing to utilize this technology.

When looking at commercial applications, one of the key advantages of mediator structures is that they provide a balance in terms of both manufacturability and performance. While third-generation biosensors seem more ideal, the applicability of DET with enzymes such as GOx is still a technical challenge. To succeed in the third generation, research is ongoing on nanotechnology-based solutions such as carbon nanotubes, gold nanoparticles, directed immobilization techniques. If DET performance can be made stable and scalable, third-generation sensors could be label-free, biocompatible and very low-potential platforms for widespread use in the future. This could be particularly revolutionary in applications such as wearables and continuous glucose monitoring systems.

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6. Ethics approval and consent to participate

This study does not need any Ethics report.

7. Consent for publication

The Authors give consent for publication.

8. Authors' contributions

Abdullah BİÇER: Writing – original draft, Writing – review & editing,

Mesut IŞIK: Writing – original draft, Writing – review & editing

Disclosure statement

No potential conflict of interest was reported by the author(s).

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