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The Principles and Recent Applications of Bioelectrocatalysis

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Bioelectrocatalysis is a phenomenon concerned with biological catalysts, which accelerates the electrochemical reactions. The bioelectrocatalysis has been widely explored by the research community in various aspects. Enzymes can catalyze different chemical reactions in living organisms by enzymes as the most important biological catalysts. These enzymatic biocatalysts are commercially available and commonly called enzyme electrodes. Electron transfer between the active center of the enzyme and the electrode can be performed either by direct electron transfer (DET) or by means of mediators (*i.e.* mediated electron transfer (MET)), which are discussed in details in the review presented. Therefore, different strategies have been used to increase the efficiency and stability of bioelectrocatalysis. In this review, different strategies of the bioelectrode designs are discussed and the application of the common bioelectrodes used in the biosensors are presented in the various fields. Moreover, a summary of the research status in the applications of bioelectrocatalysis in biosensors and biofuel cells was provided.

Keywords: Electrochemistry, Bioelectrocatalysis, Enzyme, Immobilization, Biosensors, Fuel cell

INTRODUCTION

Bioelectrocatalysts are known as biological materials which accelerate the redox reactions. The function of biological catalysts is similar to that of the conventional catalysts used in the traditional chemical industries. The difference between biological catalysts and their conventional counterparts is that biological catalysts cannot usually act as a conductor to transfer the electrons, while conventional catalysts have high conductivity. Also, bioelectrolytes act in milder conditions and lower temperatures than traditional electrocatalysis, due to their high level of stability and activity at low temperatures and neutral pH.

The electrocatalysts have extensive practical applications, including energy conversion, as storage agent in the gas systems [1-10], biofuel cells (BFCs) for miniaturized biocompatible power supplies in the implantable medical diagnostics devices, drug delivery systems [11-15], as well as their wide use in analytical applications [16-19]. Potter, for the first time in 1912, introduced whole cells as biological catalysts [20]. Subsequently, microbial cells were used as bioelectrocatalysts in different fields such as microbial fuel cells, biosensors and bioelectrochemical systems [21]. Whole cells have been applied as a new generation of catalysts due to their advantages such as high stability, persistent growth and good efficiency. Enzymes as biological catalysts have unique properties which accelerate the chemical processes occurring both inside and outside the living cell [22]. Although the properties of bioelectrocatalysis have been known for long time, the first application of enzymes as biocatalysts was carried out in 1970.

These enzymatic biocatalysts are commercially available and commonly called 'enzyme electrodes', which

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are applied in biosensors and also under development for use in the BFCs and biobatteries. The main enzymes include cytochromes, ferredoxins, copper proteins, flavoproteins, and the coenzymes nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) [23]. Leland Clark, in 1954, for the first time introduced an enzyme-based biosensor for the detection of oxygen reduced at a platinum cathode and called it the Clark electrode [24]. In this system, the first glucose sensor was introduced to determine glucose [25]. Glucose oxidase (GOx) is a typical flavin enzyme with flavin adenine that oxidizes β-$_D$-glucose to $_D$-glucono-1,5-lactone, while this enzyme itself is reduced from GOx (FAD) to GOx ($FADH_2$). GOx ($FADH_2$) reacts with oxygen dissolved in the solution, producing hydrogen peroxide. The decrease in the oxygen concentration, due to its reaction by GOx, causes a decrease in the current. This decrease is related to the concentration of reacted glucose. Bio-electrodes can be divided as first-, second-, and third-generation bioelectrocatalytic systems.

The first one was not based on direct electron transfer. In these biosensors, the measured response of hydrogen peroxide oxidation at a Pt electrode has a linear relation with the amount of the target analyte, as shown in Fig. 1. Therefore, they have some drawbacks including high potential required for peroxide oxidation, and the relation between the biosensor response and the amount of dissolved oxygen in the sample, which means that the concentration of oxygen should not change during the determination.

In the second generation of biosensors, oxygen replaced with the redox mediators, which have electron transfer ability from the biocatalyst active site to the electrode surface, as shown in the Fig. 1. The presence of mediators in the reaction leads to less potential required for biosensors based on GOx [26]. Compounds such as ferricyanide, ferrocene compounds, azine dyes, quinones, *etc.* are used as redox mediators in the second generation of biosensors [21].

The third generation of biosensors transmits electrons directly from the active biocatalyst to the electrode surface without mediator or oxygen [27] (Fig. 1). The activation potential of these biosensors is close to the redox potential of the enzyme and, thus, these kinds of biosensors have high selectivity. Only a few numbers of biological catalysts are capable of direct electron transfer. Extensive studies have been performed to develop immobilization strategies orienting the active site of the catalyst close to the electrode. Therefore, many researchers investigated the development of the systems based on DET in the analytical device by protein modification with genetic or chemical engineering techniques [28-30], and or new interfacial technologies [31, 32]. To ensure the electron transfer between enzyme and substrate/co-substrate, the presence of cofactor is essential. The cofactor can be firmly bound to the enzyme structure and release from it during the reaction. Cofactors that are commonly used for glucose-oxidizing enzymes are FAD, NAD and pyrroloquinoline quinone (PQQ).

This chapter describes the electron transfer mechanisms of bio-electrolysis by dividing it into two main categories: mediated bioelectrocatalysis and direct bioelectrocatalysis. Also, the techniques to enhance the efficiency of bioelectrocatalysis and their applications in biosensors, biofuel cells, and bioelectrosynthesis are briefly described.

ELECTRON TRANSFER MECHANISMS

Direct Electron Transfer

In the direct bioelectrocatalysis, the reactions occur through a DET mechanism and electron transfer between the electrode surface and enzyme occurs directly in the absence of any mediator. In the 1960s, the first direct electrochemical of redox proteins and enzymes like peroxidase [33] and cytochrome [34,35] were reported. The discovery of direct bioelectrocatalysis by enzyme was reported in the 20th century, and also, the first enzymatic oxygen electrode was made through immobilization of the enzyme laccase on carbon black electrode [36]. Hill and his colleagues in 1981 described the successful coupling of electron transport between a modified gold electrode with adsorbed 1,2-bis(4-pyridyl)ethene, and the soluble terminal oxidase/nitrite reductase of Pseudomonas aeruginosa [37]. They found out that enzyme and mediator cannot be in solution and then applied the mechanism of direct bioelectrocatalysis. Furthermore, in 1984, Yaropolov *et al.* discovered the direct bioelectrocatalysis by hydrogenase as enzymes which catalyze the oxidation reaction of hydrogen on a carbon-black electrode [38]. In this work, the hydrogen atmosphere on the electrode was determined by

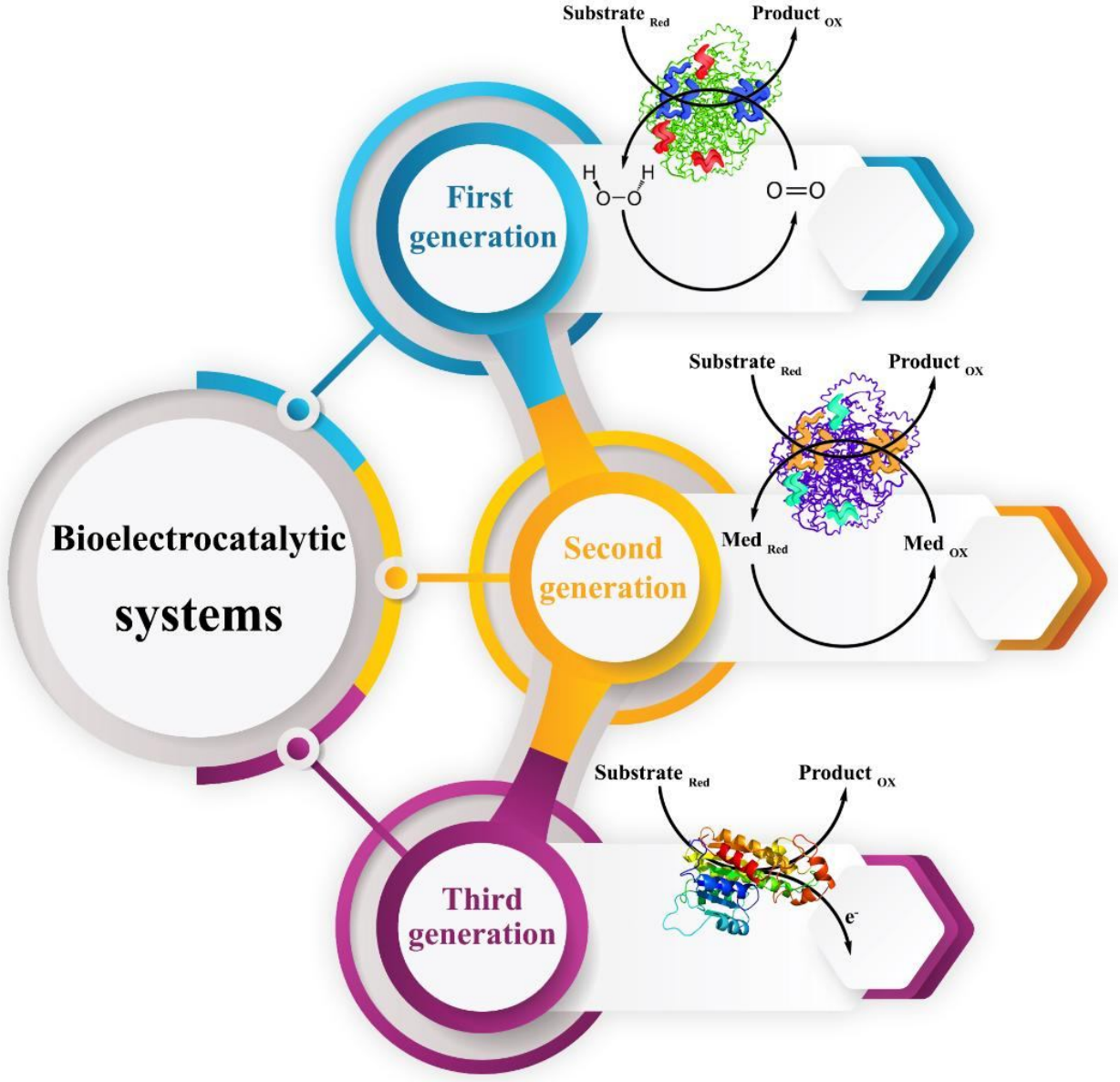

Fig. 1. Schematic description of the (A) first, (B) second and (C) third generation of bioelectrocatalytic systems.

immobilized hydrogenase and the hydrogen equilibrium potential equal to 0.0 V was established. Once the hydrogen molecule was absent, the zero current potential was shifted to positive values and cathodic current at positive overvoltages (*vs.* $E_{reduction}$) was seen. The anodic current generated by the enzyme electrode was related to the hydrogen oxidation reaction [39].

DET requires the provision of specific conditions including: 1**)** the distance between the electron donor (cofactor) and acceptor should be minimal in order to allow fast electron transfer and improve bioelectrocatalysis performance [40], 2) orientation of enzyme-containing a

cofactor onto the electrode surface is critical to keep the electron tunneling distance below 2 nm [12]. Armstrong research group showed that the presence of carboxylate groups at the surface of pyrolytic graphite edge (PGE) electrodes accelerated the reduction of O_2 to H_2O by bilirubin oxidase (BOx) due to improved enzymatic orientation [41]. However, only a successful orientation and a small enough distance between the redox cofactor and the electrode surface cannot guarantee the direct transmission of electrons because orientation may limit the access to the enzyme, and consequently bioelectrocatalysis may not be successful [12].

Enzymes that have been investigated in direct electron transport between a multicopper oxidase (MCO) (laccase) and a carbon electrode for enzymatic O_2 reduction include laccase and BOx, cellobiose dehydrogenase, pyrroloquinoline quinone-dependent glucose dehydrogenase and GOx [36,42-44]. For example, the electrons are received directly by the enzyme laccase from an electrode and then applied to reduction of oxygen to water.

Mediated Electron Transfer

The amperometric detection of blood glucose in diabetic patients initiated the scientific studies on the mediated enzymatic electron. Scientists wanted to convert glucose to gluconolactone stoichiometrically without the interference of oxygen and the consequent H_2O_2 production.

The presence of nonphysiological molecules with multiple redox states in the environment can play the role of oxygen as the electron receptor, which eliminates the need of O_2 or H_2O_2 detection at an electrode surface. These nonphysiological molecules called mediator are immobilized on the surface of the electrode, and carry out the electron transfer from the enzyme to the electrode surface. The compounds used as a mediator should have some special features [22], including:

1) The interaction between the mediator and the active center of the enzyme must be fast (the mediator must be a "specific" substrate of the enzyme);

2) The reduction/oxidation potential of a mediator should be close to that of the desired reaction.

3) The mediator must be subject to electrochemical oxidation (or reduction) on the electrode fabricated from a given material under conditions close to reversible ones.

All known intermediates do not necessarily have all of the above characteristics, and Table 1 presents the features of some specific intermediates used in bioelectrocatalysis in the last five years [45-58].

The electrochemical potential of a redox mediator (E^0_m) must be more positive than the reduction potential of the redox cofactor for an enzymatic oxidation reaction, and also, in order to reduce the cofactor and following reduction in the substrate, the reduction potential of the mediator should be more negative than that of the redox cofactor.

Most experimental evidence indicated that the magnitude of the potential difference between the mediator and redox potential of the enzyme active site is 50 mV to 170 mV which will provide the minimum overpotential needed to reach the maximum enzyme-mediator electron transfer rate. The reaction quotient defines the cell potential as per the Nernst equation, Eq. (1) and should be considered.

$$E = E^{\circ} + \frac{RT}{nf}\ln\frac{Q}{R} \quad (1)$$

Considering the importance of reaction quotient, MET can represent the pseudo-reversible enzymatic reaction and also, in the case of some hydrogenases, both the catalytic reductive and oxidative reactions can be observed in the same experiment.

For the first time, Adam Heller in the late 1980s developed organometallic osmium-containing hydrogel polymers as a bioelectrocatalytic redox mediator to mediate electron transfer for GOx and later for laccase and Box [59-61]. When a mediator is chosen from a series of electron mediators with suitable electronic characteristics, they should be able to interact with the enzyme as a substrate. Recently, Milton group indicated that naphthoquinone derivatives have high ability to undergo MET for one type of glucose-oxidizing enzyme [62]. However, no activity was observed for other types of this enzyme even though the enzymes having the same redox cofactor. However, their potentials are expected to have a negligible effect on the required overpotential of MET, reemphasizing the importance of cofactor accessibility by the electron mediator [62].

Table 1. Electrochemical Properties of Certain Mediators

Name	Enzyme	Electrode	Ref.
9,10-Phenanthrenequinone (PQ)	Formate dehydrogenase (FoDH1)	GCE	[45]
Toluidine blue O/DNA	Peroxidase	Toray carbon paper electrode	[46]
Viologen-functionalized polymer	Bilirubin oxidase	GCE	[47]
1,1′-Trimethylene-2,2′-bipyridinium dibromide	FoDH1	Ketjen Black	[48]
Naphthoquinone	Flavin adenine dinucleotide	MWCNT/FDH electrode	[49]
Cytochrome c	NADH	Gold electrode	[50]
Bis-pyrene-ABTS	Bilirubin oxidase	GCE	[51]
Cobaltocene/cobaltocenium	MoFe protein	Planar glassy carbon disk electrode	[52]
N-hydroxyphthalimide	Histone acetyltransferases	GCE	[53]
$Ru(NH_3)_6^{3+}$	DNA	GCE	[54]
Iodine	nBu4NI	Platinum plates	[55]
Terminal deoxynucletidyl transferase	NADH	GCE	[56]
Flavins	Shewanella oneidensis MR-1	Carbon cloth anode	[57]
Riboflavin and flavin adenine dinucleotide	Pseudomonas aeruginosa	C1018 carbon steel	[58]

STRATEGIES FOR INCREASING THE EFFICIENCY AND STABILITY OF BIOELECTROCATALYSIS

High Surface Area Electrodes

The advantage of utilizing electrodes with high surface area is increasing the current densities in bioelectrocatalytic. Porous electrodes such as carbon fiber paper [63], carbon felt [64], carbon cloth [65] and graphene [66] with metallic nanoparticles, nanorods, and carbon nanotubes (CNT) provide a high accessible surface area, thermal and chemical stability, and high current density. These electrodes have a minimal size with a high active surface area that can generate a large current and be used for the miniaturization of biocatalytic devices such as biosensors and BFCs [61]. Due to their three-dimensional (3D) porous network, high surface area porous carbon materials can enhance the number of loaded biocatalysts, which increases the substrate diffusion rates than other electrodes. Carbon felt or carbon cloth with high electric conductivity, stability, and vast surface area are widely used in microbial cultures. Although most studies have focused on porous carbon felt applied in microbial biocatalytic systems because of the decrease in electrode fouling and large volume, which can permeate into cells [67], similar electrode materials with high current can be used for enzymatic systems.

Immobilization Strategies

One of the important roles of enzymes as natural biocatalysts is their capacity to increase the rate of virtually all chemical reactions within a cell. Enzymes increase the rate of reactions without altering the equilibrium between the reactants and the substrates [68]. Enzyme immobilization is a strategy for placing of enzyme at the

surface of a phase (matrix/support) different from the one for substrates and products. Inert polymers and inorganic materials that are usually used as substances for the immobilization of enzymes are known as carrier matrices.

Immobilized biocatalysts (enzymes and whole cells) have been known for about 100 years while Nelson and Griffin published the first paper in this field in which yeast invertase was adsorbed onto the charcoal and catalyzed the hydrolysis of sucrose [69]. The immobilization of the biocatalysts has several advantages such as improved stability, activity, selectivity, and they may be reused several times, reducing the costs of a biocatalyst, and thus, of the entire process. The characterization of the immobilized enzyme can be attributed to the properties of both the enzyme and the carrier materials. Various strategies were used for immobilization of enzymes which are categorized as follows:

Adsorption immobilization. One of the most common and straightforward immobilization techniques is physical adsorption. In this technique, firstly, the electrode is incubated in an enzyme solution, and then enzymes are physically adsorbed on the support using various mechanisms such as hydrogen bonds, multiple salt linkages and Van der Waal's forces. Although this method has advantages such as high speed and simplicity, physical adsorption is weak and interactions between support and enzyme result in the lack of immobilization long term stability [70]. Some of the carriers generally used to immobilize enzymes by physical adsorption include activated carbon, bentonite, kaoline, collagen, alumina, Amicon-AP10, diatomaceous earth, silanized alumina, silica gel, calcium carbonate, titanium, propyl agarose, nitrocellulose fiber, and cheesecloth [71].

Covalent immobilization. The chemical immobilization methods generally involve covalent attachment of enzymes to a water-insoluble matrix; cross-linking with the use of a multifunctional and low molecular weight reagent; and co-cross-linking with other neutral substances, for examples, proteins [72]. One of the important purposes of the covalent immobilization is the long-term stability of the immobilized enzyme. Covalent immobilization usually takes place between the enzyme and the electrode surface with the electrodes being usually gold and carbon electrodes. Liu *et al.* introduced the self-assembled monolayers (SAMs) technique, as a simple and effective immobilization method, in which an organic monolayer film was formed by the spontaneous assembly of -SH of thiol or sulfur compound onto the gold electrode [73]. This novel immobilization approach exhibited better performances in terms of response rate, sensitivity, operational stability, and fabrication simplicity, which is utilized to immobilize tyrosinase for the determination of phenolic compounds. In the case of carbon electrodes, carbon surfaces are typically functionalized with groups like nitro ($-NO_2$), bromo (-Br) or hydroxyl (-OH) that can interact with homo- or heterobifunctional cross-linkers, binding to the protein at the electrode surface. In the covalent binding method, enzymes are strongly immobilized on the surface and leaching rarely occurs.

Entrapment immobilization. The entrapment method of immobilization is based on the localization of an enzyme within the membrane. The basis of the method is to polymerize the hydrophilic matrix in an aqueous solution of the enzyme and the polymer mass decomposes to specific particles because there is no connection between the enzyme and the polymer matrix [74]. Parameters such as the amount of enzyme loaded, its distribution, activity through the carrier and its stability are essential to developing these systems. The gelation of the biocatalyst on the membrane surface is due to concentration polarization and, as a result, the mass transport limit reduces the efficiency of the catalyst [75]. The process of entrapment restricts rotation and unfolding movements but permits substrate recognition and binding as well as catalysis. Entrapment of the enzyme into membrane can take place in two ways: one is entrapment after the membrane has been made and or the other is entrapment as the membrane is being made. The entrapment technique is used for solid and liquid membranes [76].

Cross-Linking immobilization. Immobilization of enzymes can be obtained by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble carrier material [77]. The cross-linking of the enzyme is an expensive and inefficient method, so some proteins act as a support and reduce the activity of the enzyme [78]. Since the enzymes covalently bind to a support matrix, desorption does not occur easily. This method has attractive advantages such as simplicity

and strong chemical bonding achieved between biomolecules [78]. It is the main disadvantage of this immobilization strategy that activity of the enzyme is possibility lowered due to the distortion of the enzyme conformation and the chemical alterations of the active site during crosslinking.

In general, the immobilization of the enzyme can be used to determine the stability of the bioelectrodes from a few minutes to several hours for adsorption and to months for entrapment and encapsulation. It is important to remember that many environments contain protein, and therefore, essential to choose an immobilization strategy able to protect the enzyme by protecting proteases.

Enzyme Cascades

Enzyme cascades based on the combination of different enzymes in delicate scaffolds open new opportunities in biosynthesis [79], biocatalysis [80] and bioassays [81]. An important strategy for increasing the output signals is the use of multi-enzyme cascades as catalysts that increase sensitivity to the identification of biomolecules. The use of DNA as a structural scaffold is a robust method for assembly of enzyme cascades. Due to the programmability of DNA hybridization and the predictability of its secondary structure, DNA provides versatile and addressable platforms to precisely organize enzyme components at the nanometer scale [82,83]. Whitesides group in 1998 reported the use of an enzyme cascade to regenerate nicotinamide NAD at low overpotentials [83]. Generally, enzymatic cascades act by couplings with reactions as the product of one enzyme is the substrate of the other one. However, there are a limited number of enzymes that can form the cascades, because a rigorous match of the substrate/product between the coupled enzymes is needed. As a result, making other kinds of enzymatic cascades using new designs is still attractive, for which there is a competition between scientific and industrial research. The use of enzymatic cascades as bio-electrolyte catalysts has been shown in numerous studies to increase the current density. However, the efficiency of these systems is often confined by the mass transport of intermediate substrates among individual enzymes. Therefore, the locating enzymes of cascade in the vicinity of the electrode surface will improve the flux, which in turn leads to a further increase in the current density. For this purpose, shorter crosslinkers such as dimethyl suberimidate, glutaraldehyde or bismaleimides have been used to conjugate enzymes in the cascades [84]. Applying these conjugates, instead of free enzymes in the anode, improves the detection sensitivity of biosensors and efficiency of the biofuel cell [85].

Nanostructured Electrodes

About 30 years ago, the first evidence of the direct bioelectrocatalysis for laccase absorbed on black carbon was reported [36,86]. The Kano group made a carbon aerogel (22 nm average pore size) of polyvinylidene fluoride film on glassy carbon with adsorbed laccase or bilirubin oxidase**.** The use of conductive nanomaterials such as CNT or different nanoparticles for biocathode modification causes an increase in the output signal. Due to the size of the flexible enzyme and rigid nanoparticles (or other nanoparticles), the transmission distance of the electron is reduced without decreasing the enzyme activity [87,88]. Also, these modified nanostructured electrodes increase the catalytic activity of the transducer and the enzymatic reaction improved on the electrode surface. In addition to using carbon nanoparticles and CNT for modified electrodes, films containing graphene, metal and metal oxide nanoparticles have been widely used successfully for direct dioxygen bioelectrocatalysis. The electroanalytical characterizations of nanomaterials are very important in biosensing applications and also to realize the mechanism of electron transfer. The semiconductor nanomaterials like conducting-polymer nanomaterials [89-91], organic-inorganic nanocomposites [92], metal [41,93-95], metal oxides [96], CNTs [97-104] and semiconductor quantum dots [105-107] are very attractive for designing high-density protein arrays. Some nano-scale materials, depending on their nanocrystalline structure, exhibited excellent electron transport properties.

Typically, CNT is one of the most popular nanomaterials in the fabrication of biosensors because it accelerates DET rate and compatibility to biocatalysts. CNTs can be attached to enzymes with various methods such as direct physical adsorption [41], covalent binding [108], mechanical compression [109], or connection with carbon walls through aromatic molecules [110]. The improvements of DET enzymes such as PQQ-dependent

dehydrogenases [111,112], laccase [113] and Box [114] with the above methods have been proved. Rubianes *et al.* [115] reported a modified carbon paste electrode with more ability in oxidation of H_2O_2 in comparison to the bare carbon paste electrode. In this paper, the overvoltage reduction of the hydrogen peroxide decreases with the incorporation of GOx into the composite material, and therefore, the selectivity and sensitivity of glucose biosensor increased in the presence of CNTs.

The CNT attachment to the redox center of protein enzymes enhanced the performance of direct bioelectrocatalysis by attaching enzyme redox cofactors to the end of CNTs or modifying CNTs with molecules that can be inserted into enzymes. For example, the current density of bioelectrocatalytic reduction of oxygen can be increased using π-electron-rich compounds functionalized CNTs, such as anthracene or naphthoquinones [116,117]. These molecules accelerate DET with proper orientation in direct bioelectrocatalysis. Direct conjugations of GOx on multi-wall carbon nanotubes (MWCNTs) grown on a platinum substrate have been used to create biosensing arrays where the CNTs both immobilize the enzymes and act as mediators [118].

Also, biosensors modified with CNT have been indirectly used to measure a wide range of biomolecules. Galactose biosensor was designed by the chitosan film containing single-wall carbon nanotube (CHIT-SWCNT) which was chemically crosslinked with glutaraldehyde and free aldehyde groups and produced a substrate for the covalent immobilization of galactose oxidase [119].

Many other nanomaterials, such as graphene, metal, metal oxide, conducting polymers, etc., are used to fabricate nano-structure bioelectrodes to improve the efficiency of bioelectrocatalysis of oxidoreductase enzymes. Metallic nanoparticles increase the conductivity of enzyme-modified electrodes and are widely employed in biofilm cells. Because of their similar size to some of the oxidoreductase enzymes used in biofilm cells with high active surfaces area, they can decrease the electron transfer distance and then facilitate this process at the bioelectrodes surface [88].

In 2018, Simchi *et al.* fabricated an enzyme GOx with 3D-networks of graphene nanosheets immobilized on vertically aligned gold nanorods. In this work, the enhancement of electron transfer from the enzyme to the gold nanostructure was confirmed by covalent conjugation with 3D networks of reduced graphene oxide nanosheets [120]. The combination of Au nanoparticles with CNTs was employed to catalyze the NADH oxidation at low overpotentials, which is used in hybrid biofuel cells. The hybrid bioelectrode containing the Au nanoparticles supported on NH_2-functionalized MWCNTs shows a high ability to regenerate NAD^+ species [121].

Metal oxide nanomaterials have properties suitable for immobilization of biocatalysts and improve the rate of bioelectrocatalysis. Two galactose biosensors have been developed based on MWCNTs/Co_3O_4/chitosan and graphene/Co_3O_4/chitosan nanocomposites, respectively. The analytical performance of these biosensors was compared in the detection of galactose [122]. Zanini *et al.* developed an L-lactate amperometric biosensor made with a layer-by-layer (LbL) film composed of poly(diallyldimethylammonium) (PDDA)/AuNPs/lactate oxidase supported onto a thiol-modified polycrystalline gold electrode (PGE) surface. The biosensor was used for the evaluation of L-lactate in standard solutions and also in commercial dairy products [123]. Finally, it can be concluded that a combination of different nanomaterials into bio-electrodes is an essential strategy for enhancing the performance of bioelectrocatalytic systems.

APPLICATIONS OF BIOELECTRO-CATALYSIS

Biosensors

Electrochemical sensors consist of an electrical transducer that monitors the electrochemical signals generated during reactions by using potentiometric, impedimetric, amperometric, or voltammetric systems. In biosensors, active functionalized sensing biomaterials present on the electrode act as a catalyst, and it must efficiently catalyze the biochemical reaction of the compounds to give the desired signals [124,125]. Generally, electrochemical biosensing devices are constructed using a two- or three-electrode configuration, one of which is functionalized by a biorecognition element as the working electrode.

-Potentiometric biosensors: based on ion-selective

electrodes or ion-sensitive field-effect transistors. The output signal is generated by the accumulation of ions at an ion-selective membrane.

-Impedimetric biosensors: based on changes in impedance (Z), resistance (Ω), or capacitance at the electrode surface.

- Voltammetric/amperometric biosensors: based on changes in current at the surface of the electrode. In voltammetry, a variable potential is applied while in amperometry the applied potential remains constant.

Biosensors, depending on the kind of target analytes, have widespread applications in numerous fields such as clinical/medical, food/agriculture, and environmental science. Enzymes are highly specific biological catalysts that are capable of detecting low concentrations of target agents without interference. The advantages of these systems are their low cost, fast response and application in portable devices without the need for preparing the samples, but they also have drawbacks such as less stability and, in some cases, slow electron transfer.

Clinical/medical sensors. Biomedical sensors are a particular type of biosensors that provide the necessary interface between the biological material and electronics systems and finally detect medically relevant parameters. GOx was the first enzyme used in medical biosensors for glucose monitoring in diabetic patients [25]. Domestic application of glucose biosensors accounts for 85% of the giant global market [126]. Nowadays, glucose biosensor is one of the most common enzymes employed in electrochemical biosensor. The group of Fusco in 2018 developed a biosensor for glucose detection with high current density [127]. In this work, polymer films of polythiophene were electrosynthesized in aqueous solution onto CNTs modified gold electrodes. Pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) is one of the most widely employed enzymes for glucose conversion, which is immobilized on the surface of the modified electrode. Polythiophene deposition significantly improves the bioelectrocatalysis of PQQ-GDH so that the process of glucose detection happens at 0 V *vs.* Ag/AgCl instead of -200 mV *vs.* Ag/AgCl.

Cancer is defined as the uncontrollable growth of cells that invade and cause damage to surrounding tissue. It is the second leading cause of death worldwide after cardiovascular diseases. Therefore, there is an urgent need for developing a rapid, highly accurate, and comprehensive method that can be applied for the detection of cancer. Biosensors have several potential advantages over other methods of cancer diagnosis, especially due to their reduced assay time, portability, high sensitivity and selectivity, simplicity, miniaturization and flexibility. Oral cancer appears as a growth or sore in the mouth and is currently the sixth most common cancer [128]. Malhotra *et al*. developed a non-invasive, label-free biosensor based on nanostructured hafnium oxide (hafinia) deposited onto indium tin oxide (ITO) coated glass for oral cancer biomarker (CYFRA-21-1) detection in the human saliva [129]. Studies have been performed on the effect of orientation arising due to biomolecules (*e.g*., protein A or G, NHS-LC-biotin, cysteine, histidine, lysine) on the sensing properties of this biosensor and to use the $nHfO_2$ based smart platform for detection of other cancer biomarkers.

Environmental sensors. Due to the increasing growth of environmental pollution, the development of an innovative and sensitive technique is urgently required for legislative actions on environmental pollution control and early warning. In recent decades, biosensors have been developed as portable and warning systems due to properties such as fast, specific, reusable, and uninterrupted operations. Among various pollutants, determination of heavy metals, phenolic compounds, organophosphorus, and carbamate pesticides is a major concern, considering their great contribution to increasing pollutant levels. Pesticides are considered as a major cause of environmental pollution, which are divided into three categories of insecticides, herbicides, and fungicides. The enzyme of acetylcholine esterase (AChE) has been typically used in constructing biosensors for the detection of organophosphorus and carbamate pesticides because these compounds inhibit the activity of acetylcholine esterase. The Cui research group developed an electrochemical AChE biosensor for detection of organophosphorus pesticides (OPs) by adsorption of AChE on chitosan (CS), TiO_2 sol-gel, and rGO based multi-layered immobilization matrix (denoted as CS@TiO_2-CS/rGO) [130]. In another work performed by Syshchyk group, a novel enzymatic biosensor was developed for direct determination of glucose and urea as well as heavy metal ions (by means of inhibitory effects) using porous

silicon layers. Porous silicon was used as a highly effective transducer, based on the effect of changing its photoluminescence at varying pHs in a solution caused by the enzymatic reactions [131].

Food and drink sensors. Rapid and accurate analysis of food and drink products is essential, and electrochemical biosensors can be applied to meet those needs. Biosensors have been usually used to determine glucose and other sugars in different foods and drinks [132-137]. In addition to the advantages mentioned in the previous sections, biosensors are very easy to use and do not require highly trained personnel, and therefore, commercially available devices can be easily launched in the consumers market. López-Ruiz *et al*. have designed enzymatic electrochemical biosensors for tyramine detection using tyrosinase as biological material and orthophosphate calcium materials as enzyme-immobilizing substrate [138]. These inorganic materials have proven to be highly effective enzyme host matrices in biosensing applications [139,140]. Finally, the designed biosensor was used for monitoring of tyramine in cheeses. Over the past two decades, electrochemical biosensors have been developed to measure a wide range of ingredients in foods such as cholesterol [141,142], vitamin C (ascorbic acid) [143], amino acids such as lactate [144], malate [145], glutamate [146] and lysine [147,148] and were used in beverages such as wine [149,150], beer [151] and tea [152] to identify alcohols and polyphenols. Also, electrochemical biosensors have been reported for the identification of bacterial contamination in food [153]. The principle of the specific detection is the hemolytic action of the bacteria on various types of liposomes, coupled to the reduced action of bacteria on a liposomal mediator in an electrochemical cell.

Biofuel Cells

Enzymatic BFCs are a specific type of fuel cells, in which the enzymes are used as the biocatalysts to catalyze the oxidation of fuel and/or reduction of oxygen or peroxide for conversion of chemical energy to electricity. In other words, enzymatic fuel cells transform the chemical energy of a biological catalytic reaction into electricity by oxidizing a fuel at the anode and reducing an oxidant at the cathode. In the mid-1780s, Italian physician Luigi Galvani discovered the connection between biology and electricity when generated current from a static electric generator caused a frog's leg to twitch, revolutionizing the understanding of the nervous system [154]. In the late 1950s and early 1960s, the increasing interest in fuel cells triggered by the USA space program, led to the development of microbial BFCs as a novel technology for a waste disposal system for space flights that would also generate power. Also, in the late 1960s, several reports of biofuel cell using oxidoreductase enzyme were made and a mediator for performing MET to the electrode surface was found [155,156]. In a conventional fuel cell, an oxidation reaction occurs at the anode which releases electrons and moves to the cathode by the external circuit and the reduction reaction takes place at the cathode. Compared to the conventional fuel cells, the enzymatic BFCs are more complex because potentials of the open circuit are significantly lower than the theoretical potentials due to cofactor redox, enzyme redox and mediator redox potentials [11]. Typically, DET is for enzymatic biofuel cells, but most BFCs use MET because DET is difficult to achieve and produces lower current densities when compared to BFCs based on MET. Enzymatic BFCs have many applications. The first application considered by researchers was powering sensors. Enzymatic BFCs that are fuelled by glucose and utilize oxygen as the oxidant and electron acceptor are commonly reported. Other enzymatic BFCs have been also reported that can operate on various fuels such as sucrose [157], trehalose [158], hydrogen [159] and short-chain alcohols [160].

In recent decades, extensive research activities have been performed on the transfer of enzymatic BFCs from the lab bench to implantable systems. Mano and Heller worked in 2003 on the first biofuel cell operating in living organisms by implanting their biofuel cell in grape [61]. In 2010, Cosnier *et al.* implanted the first glucose biofuel cell in a rat [161]. In 2012, Rasmussen *et al.* [158] implanted atrehalose biofuel cell in a cockroach and Katz *et al.* implanted a glucose biofuel cell in a snail [162] and a clam [163]. In 2013, Katz *et al.* scaled up to implant their glucose biofuel cell in a lobster, and Cosnier's group made further developments in rat implantation [164]. Most studies are carried out to use enzymatic BFCs as powering portable devices. This research leads to the production of various enzymatic BFCs cells such as bio-batteries [165,166],

microfluidic prototypes [167,168], and paper-based BFCs [169-171].

TRENDS IN BIOSENSING TECHNOLOGY

In the following section, some publications appeared in the last decade are briefly described and trends in the development and main achievements in biocatalytic systems for health monitoring are reviewed.

Miniaturization

In recent years, there have been prominent trends in developing biosensing systems that combine high sensitivity and specificity with rapid sample-to-response times, portability, the possibility of multi-analyzing, and ease of use. The successful applications of microelectronics in the industry and the abundant use of chip transistors by the researchers have led to miniaturization of biosensors. In order to miniaturize these systems, the micro- and nanometer-size electrodes can be easily fabricated to use, but electrochemical detector and other required instruments should be replaced by microelectromechanical (MEMS) and nanoelectromechanical (NEMS) systems designed for biosensors, leading to reduce their efficiency [172-175].

Reducing the electrode size to micro dimensions brings significant advantages for the biosensing systems; for example, high selectivity, low detection limits, rapid analytical response and enhanced mass transport at the electrode surface because diffusion of the species at the electrode surface becomes predominantly radial [172,176].

Screen-printing as a standard technology for the fabrication of miniaturized biosensors seems well suited for the analytical systems in various applications. Banks group designed the 2D-MoS_2 screen-printed electrodes (2D-MoS_2-SPEs) for the electrochemical oxygen reduction reaction (ORR) within acidic aqueous media [177]. 2D-MoS_2-SPEs exhibited an excellent electrocatalytic behavior towards the ORR in comparison to other electrodes with no visible degradation in the signal output over the course of 1000 repeat scans. The ORR is usually the rate-limiting reaction in the generation of energy by proton exchange membrane fuel cells. This considerable kinetic inhibition is attributed to the strength of the (di)oxygen double bond. Finally, the designed 2D-MoS2-SPEs can be developed as electrocatalytic fuel cell electrodes due to their economies of scale and inherent compatibility. Microelectrode arrays (MEAs) (also referred to as multielectrode arrays) are devices consisting of multiple (tens to thousands) microelectrodes [178,179] or interconnected electrodes [180,181]. The fabrication of interconnected electrodes is technically simple, and also individual MEAs offer many benefits to biosensing technology such as high spatial resolution, low ohmic potential drop and the possibility of multi-analyzing measurements. Using MEAs allows integrating biological catalytic systems into lab-on-a-chip devices applied to the field of health care. Compton's group and other groups have published the theory and methods for fabricated microelectrodes arrays [172,174,182]. Ross and coworkers introduced the first MEA-based biosensor in 1994 [183]. They designed a micro biosensor on the basis of amperometric enzyme MEAs using immobilization of different enzymes like GOx, choline oxidase and lactate oxidase in a conducting organic polymer; *e.g.*, polypyrrole for environmental analysis [183].

Buk *et al.* introduced a highly sensitive glucose biosensor based on a microdisk array electrode modified with carbon quantum dots and gold nanoparticles for glucose detection (CQDs/AuNPs-GOx) [184]. Each gold microdisk array electrode with 20 mm diameter contained 85 disk electrodes that were fabricated using electronics industry-standard lithography, deposition and etching technologies. The CQDs/AuNPs-GOx microdisk array electrodes were made using several immobilization techniques and were developed as the GOx biosensor, which exhibited excellent analytical performance in comparison to the counterpart planar biosensor.

Paper-based Devices

Paper-based analytical devices (PADs) have attracted much attention in biosensor technology for developing countries because they offer clinicians the ability to deliver point-of-care testing, equipment-free and onsite analysis. In 1956, the first paper device for the semi-quantitative detection of glucose in urine was demonstrated [185]. The human pregnancy test kit is another well-known example [186], which further developed into immunochromatographic paper test strips in the form of dipsticks and lateral-flow analysis. The paper-based devices

can be coupled with instrumental detection techniques, such as mobile phone colorimetric detection, fluorescence, chemiluminescence and electrochemistry [187]. A large number of articles have focused on paper-based diagnostics and advantages of electrochemical detection for miniaturization [188,189]. There are many published studies related to the paper-based diagnostics [187,189]; so, the focus of this chapter is on the paper-based biosensors and their applications in the health diagnosis fields, with an emphasis on electrochemical detection rather than the microfluidic component, and on fully integrated paper-based devices.

The first bioelectrocatalytic systems integrated with electrochemical paper-based analytical devices (EPADs) was introduced by Medisense (Abbott) in 1995 (Precision QID™) by introducing glucose sensors. Afterward, Dungchai groups fabricated the first paper-based microfluidic electrodes directly on paper using printing techniques in 2009. In this work, determination of glucose, lactate, and uric acid was performed in biological samples using oxidase enzyme (GOx, lactate oxidase, and uricase, respectively) reactions without using additional chemicals [190]. Screen-printed electrodes were prepared on the surface of a filter paper with microfluidic channels by the photolithography technique. According to the literature, various techniques for fabricating EPADs are currently available such as photolithography [190,191], wax screen-printing [192], wax dipping [193,194] and wax printing [195]. In addition, Dungchai group in 2011 designed a similar paper-based device to measure blood glucose based on wax screen-printing method, which could be attributed to the paper of Müller and Clegg in 1949 for the paraffin impregnation method [196]. Due to the properties of paper-based microfluidics, this technique can be used as an alternative way to develop single biosensors in different environments. Among the different printing techniques, wax screen-printing is one of the most widely used techniques, because it is a cost-effective and straightforward technique [196]. To increase the efficiency of screen-printed devices, the physicochemical properties of the ink can be modified by using nanomaterials, such as metal nanoparticles (Au, Pt, Ag, *etc.*), carbonaceous nanomaterials (graphene, CNT, carbon black, *etc.*), or conductive polymers (polypyrrole, polyaniline polythiophene, *etc.).* The combination of nanomaterials with inks increases the conductivity, specific surface, but decreases defective sites and boosts the analytical properties of the sensors [197-200].

Ruecha *et al.* prepared the screen-printed carbon electrodes by wax-printing to create microfluidic channel using a paper-based electrode modified with a novel nanocomposite based on graphene/polyvinylpyrrolidone/polyaniline (G/PVP/PANI). Graphene significantly enhanced the high conductivity and large surface area of paper-based biosensor, and PVP increased the dispersibility of graphene in the nanocomposite , which ultimately improved the sensitivity of the biosensor in cholesterol detection [201]. This modified paper-based electrode shows excellent electrocatalytic activity towards the oxidation of hydrogen peroxide (H_2O_2). Furthermore, cholesterol oxidase (ChOx) is attached to G/PVP/PANI-modified electrode for the amperometric determination of cholesterol.

Liu *et al.* developed an electrochemical microfluidic paper-based analytical devices (μPADs) based on Au nanorods (NRs) for detection of microRNA (miRNA) by using cerium dioxide-Au@glucose oxidase (CeO_2-Au@GOx) as an electrochemical probe for signal amplification [202]. In this work, Au NRs were used to increase the conductivity and accelerate the electron transfer between materials and electrode surface. Au NRs fixed onto CeO_2 surface via DNA hybridize enhanced the catalytic performance [203], anti-inflammatory [204], superior biocompatibility, and high adsorption capability [205] of the biosensor. In addition, GOx was loaded on Au NRs and catalyzed the production of H_2O_2. The designed biosensor provides a platform for miR-21 detection and point-of-care diagnosis for clinical samples.

Wearable Bioelectrocatalytic Systems

The development of wearable sensors for health monitoring has attracted a lot of attention in recent years, and the research published in this area is exponentially increasing [206-208]. Wearable sensors have the ability to provide useful insights into the performance and health of individuals, and change the monitoring process of centralized hospital-based systems to home-based personal medicine because it can reduce the cost of health care.

Many wearable sensors have been developed to control parameters such as heart rate, blood pressure, respiration rate, brain activity, skin temperature, body movement, etc. [209]. Nonetheless, non-invasive wearable biosensors are confronted with challenges such as low detection limit, small sample size, and sensor compatibility with monitoring the chemical information about patient health. Today, the wearable biosensors are available in various forms such as hats, shirts, rings, belts, bracelets, shoes, socks, glasses, contact lenses, necklaces, and watches.

The electrochemical transducers are very useful for wearable biosensors due to the advantages of small sample volume sensitivity and integration into textile materials or directly on the epidermis for different monitoring applications [208]. Many studies have been carried out on wearable electrochemical biosensors for real-time non-invasive monitoring of electrolytes and metabolites in sweat, tears, or saliva as the indicators of a wearer's health status [206,210,211]. Wang's group reported a wearable electrochemical biocatalytic sensor for continuous monitoring of salivary metabolites [212]. In this work, they integrated a printable amperometric enzymatic biosensor into an easily removable mouthguard platform toward non-invasive monitoring of lactate. The use of a mouthguard can minimize the risks of sustaining oral injuries during participation in sports. Since salivary lactate concentrations well correspond to blood lactate levels, the mouthguard enzymatic biosensor was designed based on an immobilized lactate oxidase in the low potential detection of the peroxide product for human saliva samples. The wearable biosensor was made by screen-printing three separate layers on a flexible PET substrate so that conductive ink of Ag/AgCl was printed to provide reference electrode and Prussian blue-graphite ink was applied to obtain the working and auxiliary electrodes. The insulator layer was printed on the surface using the DuPont 5036 Dielectric paste (Wilmington, DE, USA) and after each printing process, the electrode was dried in air. Finally, the printed electrode system was attached to the mouthguard body using a double-sided adhesive, and Lactate oxidase was immobilized on the working electrode surface by electropolymerized entrapment in a poly(ophenylene-diamine) (PPD) film. Hydrogen peroxide generated by the enzymatic reaction was monitored amperometrically at 0.042 V *vs*. a printed Ag reference. This mouthguard biosensor has some disadvantages in that saliva is continuously exposed to food contamination and the amount of saliva varies from one person to another, which affects the results. By overcoming the challenges, salivary analysis can be used to diagnose lactate, salivary amylase, and other protein markers.

Wearable biosensors for sweat analysis are divided into two categories of textile devices and epidermal based sensors [206]. Epidermal sensors are more applicable in comparison to textile-based biosensors because they facilitate prolonged and strong contact with the skin, and therefore, are capable of 'wear-and-forget' functionality [208,213]. Wang *et al.* reported the first example of a noninvasive enzymatic temporary-transfer tattoo electrochemical biosensor for the continuous determination of lactate amount in human perspiration [214]. The goal is to develop epidermal biosensors to control sweat lactate during physical activity. This epidermal lactate biosensor is fabricated using commonly screen printing techniques that conform to body lines and the sensor responds to mechanical stresses due to the presence of scattered carbon fibers within the screen printed inks. Finally, the lactate biosensor is placed on the skin of the person who has been exercising for a long time, and a diagram of sweat lactate temporal is recorded with an amperometric method.

In another work, the Wang group reported the flexible epidermal tattoo and textile-based electrochemical biosensors for detection of vapor-phase OP nerve agents [215]. In this work, OP-based wearable sensing platform was intergraded with the stress-enduring flower-like printed electrode system and the flexible electronic interface.

This wearable electrochemical biosensor is fabricated based on elastic conducting inks printed on the tattoo paper and transferred onto the skin. The Ag/AgCl ink combined with the Ecoflex elastomer is used for printing reference electrode, and a tensile layer consisting of a carbon ink modified with polystyrene-block-poly-isoprene-block-polystyrene was used to print the working and counter electrodes while providing a resistance to mechanical switches. In this work, the skin mounted flower-like tattoo and textile-based electrochemical biosensors are made using elastomeric inks and is resilience against mechanical stresses expected from the wearer's activity without

damaging the biosensing performance. These biosensors are able to detect the OPs compounds by measuring the *p*-nitrophenol generated from the enzymatic reaction by square wave voltammetry method. The voltammetric responses in these biosensing systems were controlled using a wearable and flexible electronic board for the skin tattoo and textile printed biosensors, respectively. The obtained voltammograms are transmitted wirelessly to a mobile device (*e.g*., phone or laptop) via a Bluetooth connection. The rapid and accurate detection of OP compounds by this wearable sensor system could help the monitoring-decontamination system to protect our soldiers, civilians, and farmers via effective and timely countermeasures upon detection of skin exposure.

Self-powered Biocatalytic Sensors

One of the disadvantages of wearable biosensors is their power source and electrical circuits needed for data recording and transmission. BFCs based on enzymes and whole cells are able to produce and store energy from the body and thus provide a suitable power supply for future wearable biosensors. The self-powered biosensors are based on enzymatic fuel cell technology, thus, do not require an external electrical energy source and generate current proportional to the concentration of the analyte.

The BFCs were introduced from 1911 to 1931 and a detailed report was provided in 1964 [156]. The self-powered biosensors were mostly taken into consideration after publishing the Willner *et al.*'s paper, in which a novel glucose/O_2 biofuel cell element was designed by assembling the layered bioelectrocatalytic electrodes [216]. Ramanavicius's group designed a self-powered biosensor based on an anode and a cathode powered by the same fuel glucose and GOx, which was used as a glucose consuming biocatalyst for both poles [217]. The 5-amino-1,10-phenanthroline (5AP)-modified graphite rod electrode (GRE) with crosslinked GOx was applied as the anode while GRE with co-immobilized horseradish peroxidase (HRP) and GOx was used as the cathode. By adding glucose to the biofuel cell, oxidation occurs at the anode, followed by the reduction of hydrogen peroxide in the cathode, resulting in the production of HRP with the generation of a maximum power density that is proportional to the concentration of glucose. In this work, although a similar enzyme was used in the anode and cathode, there is a need for both of the enzymes. In the work of Sekretaryova and his co-workers, they used a single enzyme-based self-powered biosensor; in other words, both cathodic and anodic bioelectrocatalytic reactions are powered by the same substrate [176]. The ChOx enzyme was immobilized in a sol-gel matrix on carbon cloth electrodes. The biocatalytic oxidation of cholesterol occurred at the anode and hydrogen peroxide, which is the product of the enzymatic conversion of cholesterol, was reduced by the use of Prussian blue at the cathode. The sensitivity of self-powered biosensor that is made of two electrodes was much higher than either of the two individual electrodes and power density generated was proportional to the concentration of cholesterol.

Other important strategies for self-powered biosensors are based on the inhibition effect, so that the enzyme inhibitors can be detected by their influence on the output of the BFC. In 2016, Minteer's group reported the first experimental evidence of the enzymatic inhibition of laccase by both arsenite and arsenate. The laccase biocathodes were applied within a glucose/O_2 enzymatic fuel cell, yielding a self-powered arsenite/arsenate biosensor [218]. In this work, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as a substrate for colorimetric assays and laccase was combined with flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) to generate a glucose/O_2 enzymatic fuel cell. The BFCs consisted of FAD-GDH bioanode and a laccase based biocathode. Both poles were prepared on Toray carbon paper electrodes, and biocathode acted as a limiting component. The oxidation of glucose *via* FAD-GDH in the presence of arsenite and arsenate did not affect enzymatic activity while a decrease in O_2 reduction on the biocathode resulted in a decrease in the power output. With the injection of arsenite or arsenate, a decrease in power densities was observed with increasing the concentrations from 1-20 mM arsenite and 1-8 mM arsenate. Furthermore, the device only operated at 10% current draw of the maximum enzymatic fuel cell current density.

Renata, in 2018, introduced an integrated self-powered sensing system fabricated by hybrid biofuel cell (HBFC) and a small three-electrode sensing device [219]. This system consists of a zinc-plated anode, and a

biocathode is made from nine carbon paper discs, which each disc is modified with CNTs and BOX or laccase and then pressed together. The self-powered three-component analytical biodevice was used for detection of catechol and oxygen sensing by chronoamperometry. Determination of the catechol is very important because it played an important role in the function of central nervous, renal, hormonal, and cardiovascular systems. The purpose of this work is to design and construct a portable and small device, a micro-sensor powered by HBFC that can be a useful alternative application for environmental analysis, medical diagnostics and chemical analysis where a wireless system is desirable.

CHALLENGES AND FUTURE OUTLOOK FOR BIOSENSORS

Biocatalytic systems play an important role in measuring the safety of food, personal safety and environmental monitoring. Here, we have reviewed the principles of biocatalyst and their technology trends. The considerable progress in the development of biosensors and the increased demand for personal analysis inspire researchers to develop inexpensive and portable biosensing systems for point-of-care testing. The increasing use of mobile health applications dictates a key direction for future biosensor development towards systems that can be combined with modern telecommunications. Today, to achieve these goals, developed biosensors such as screen-printed, paper-based, wearable biosensors and self-powered biosensors are integrated with wireless communication technologies. These tools could have a significant positive impact on health and health care.

In spite of a considerable progress in the development of biocatalytic sensing, several challenges needed to be addressed so that the novel bioelectrocatalytic systems can be used in real-life applications. To overcome defects and future development of biosensing systems, more optimization of biosensor architecture is yet required in terms of simplicity, sensitivity, repeatability, and stability. Therefore, immobilization techniques of enzymes or other biocompatible compounds should be investigated in order to increase the electron transfer rate and long-term stability. Further research must be done to clarify the direct or intermediate electron transfer systems in order to increase the sensitivity and commercial success of novel biosensing devices.

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