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Enzymatic Electrosynthesis: An Overview on the Progress in Enzyme-Electrodes for the Production of Electricity, Fuels and Chemicals

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Abstract

Recent interest in the field of biocommodities production through bioelectrochemical systems has generated interest in the enzyme catalyzed redox reactions. Enzyme catalyzed electrodes are well established as sensors and power generators. However, a paradigm shift in recent science towards the production of useful chemicals has changed the face of biofuel cells, keeping the fuels or chemicals production in the upfront. This review article comprehensively presents the progress in the field of enzyme-electrodes for the production of electricity, fuels and chemicals with an aim to represent a practical outline for understanding the use of single or multiple redox enzymes as electrocatalysts for their electron transfer onto electrodes. It also provides the state-of-the-art information regarding the different existing processes to fabricate enzyme electrodes. Successfully-achieved electroenzymatic anodic and cathodic reactions are further discussed, together with their potential applications. Particular focus was made on the novel single/multiple enzyme systems towards product synthesis and other applications. Finally, techno-economic and environmental elements for industrial processing with enzyme catalyzed bioelectrochemical system (e-BES) are anticipated, in order to provide useful strategies for further development of this technology.

Keywords: Electrodes; Immobilization; Electrosynthesis; Biofuels; Redox enzymes; Electron transfer

Introduction

During the past decade, interest in bioelectrochemical systems (BESs) has significantly expanded. However, the major research focus is currently directed at microbial bioelectrochemical systems [1]. Enzymatic electrocatalysis involving energy applications has remained more discrete. However, the continuous search for highly selective, efficient and low cost non-precious catalysts, together with the recent advances in bioelectrochemistry and its related fields have already allowed further progress on enzymatic production of electricity from a wide variety of substrates or, the other way around, enzymatic applications of excess electrical energy for the production of chemicals and fuels [2].

Enzymes, contrary to microbes, have been the most important target for biosensor technologies. So, the electrochemical grounds for enzymatic applications are rather strong. Still, sensor operation is often desired at low current and potential differences, in order to avoid counter-reactions. On the other hand, energy applications demand maximum values of current and potential difference [3]. Furthermore, electricity-, fuel- or chemical-prospective devices are expected to have a stable and extended lifetime, which is still a crucial factor in the enzymatic-electrocatalysis-driven research of our days.

As an example of these technologies, Figure 1 presents a general scheme of current production in an enzymatic fuel cell. At the anode, enzymes can produce electricity and release protons from the oxidation of substrate fuels (e.g. glucose), while at the cathode substrate reduction (e.g. oxygen, carbon dioxide, volatile fatty acids) together with the use of electrons and protons can be completed for enzymatic electrolysis or electrosynthesis [4]. However, there are various critical fundamental challenges remaining unresolved in such processes. For example, the electron conduction between enzymes and electrodes still entails great improvement in the case of enzymatic bioelectrochemical systems (e-BES) [2].

At the anode, a reduced substrate (S_{red}) is oxidized (to S_{ox}) by means of an enzyme or enzyme-chain supported on an electrically-conducting material. At the cathode, a reduction reaction of an electron acceptor which may or may not be enzymatically-mediated e.g. O_2 reduction can

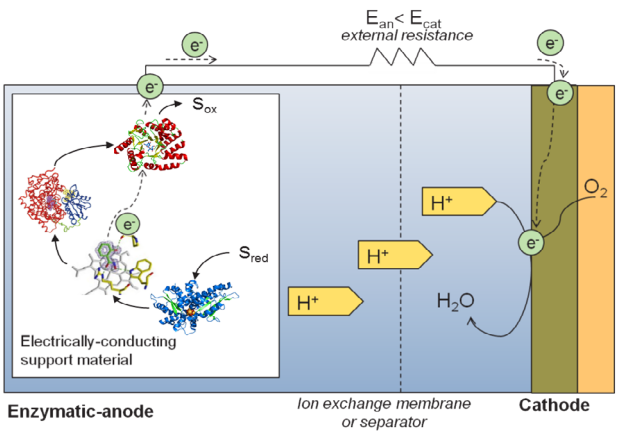

Figure 1: Schematic representation of a typical enzymatic fuel cell.

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be carried out over a Pt-based electrode or over a glucose-oxidase-based carbonaceous electrode, by using the electrons and protons released at the anode. Electricity is directly produced during this process. Otherwise, enzymatic conversion can be carried out at the cathode for electrolysis purposes when $E_{an}>E_{cat}$, for synthesis applications such as e.g. alcohol production from volatile fatty acid reduction. Paired electrolysis can also be achieved, producing other valuable compounds at both cathode and anode level. In some cases the anode and cathode compartments are separated by an ion-selective membrane, in others this component is not essential.

The major objectives of this review are (i) to present the progress of enzyme-electrodes for the production of electricity, fuels and chemicals, simultaneously understanding the use of single or multiple redox enzymes as electrocatalysts and their intrinsic electron-transfer mechanisms to electrodes; (ii) to provide state-of-the-art information regarding the different existing processes to fabricate enzyme electrodes (iii) to discuss successfully-achieved electroenzymatic anodic and cathodic reactions together with their potential applications. A special attention was focused on the possible enzymatic electrosynthesis mechanisms for the value-added product synthesis. Finally, techno-economic and environmental elements for industrial processing with e-BES are projected, in order to provide useful strategies for further development of this technology.

The ABCs of enzyme-electrodes

Enzyme organization: Enzymes can be classified in several ways. The highest classification level is related to their function: a) oxidoreductases (redox enzymes) catalyze oxidation or reduction reactions, b) transferases transfer functional groups, c) hydrolases catalyze the hydrolysis of various bonds, d) lyases cleave various bonds by means other than hydrolysis and oxidation, e) isomerases catalyze isomerization changes within a single molecule and f) ligases join two molecules with covalent bonds [5]. In this article, focus will be only on oxidoreductases, since they are the sole enzymes capable of catalyzing the transfer of electrons from one molecule to another or to electrodes.

Redox enzyme systems: Oxidoreductases are a group of enzymes that usually utilize nicotinamide adenine dinucleotide (NAD) or its phosphorylated analog (NADP) as cofactors. However, they can also act on other groups of electron donors such as CH-OH, aldehyde or oxo, CH-CH, flavine adenine nucleotide (FAD) or its phosphorylated analog (FADP), CH-NH_2, CH-NH, etc. Similarly, it also can act on the other compounds such as sulfur, heme, diphenols, peroxide, hydrogen as well as single or paired donors with incorporation of molecular oxygen, superoxide radicals, CH or CH_2, iron-sulfur proteins, reduced flavodoxin, phosphorus or arsenic and all the X-H and Y-H to form an X-Y bond among others. So far, the most relevant enzymes in e-BES have been oxidases, (de)hydrogenases and peroxidases. Redox enzymes typically contain one or more coenzyme structures that act as catalytic active centers. Flavins and Pyrroloquinoline Quinone (PQQ) are most commonly known coenzymes. All these enzymes typically undergo Mediated Electron Transfer (MET) or Direct Electron Transfer (DET). In addition to organic species, metalloproteins containing metal coenzymes are also used, e.g. copper, nickel-iron-sulfur, iron-sulfur, and heme-based. Cytochromes are commonly studied co-factors in BES and they are electron transport proteins containing heme groups [3].

Enzyme function in e-BES: Based on the function of enzymes, e-BES can be classified into two broad categories, direct energy producing and value-added product synthesizing. The enzymes that participate in the electron transfer chain between the fuel and the anode from oxidizing the organic matter with their simultaneous reduction at cathode in presence of an oxidant. This is the same principle as conventional or Microbial Fuel Cells (MFCs). On the contrary, the product synthesizing e-BES consists of enzymes that are involved in electroenzymatic synthesis of chemicals and fuels with the help of energy generated [6]. Although electricity generation in e-BES is rather interesting, up to date no commercial alternatives are available at the industrial scale. However, the use of enzymes in organic synthesis has shown great potential. So far, more than 150 industrial processes are known, where enzymes are used for the production of fine and commodity chemicals [4]. It is anticipated that enzymatic electrosynthesis will also rapidly expand to fulfill industrial needs in green chemistry.

Mono-enzyme vs. multi-enzyme electrodes: Compared to e-BES, the microbial Bioelectrochemical Systems (m-BES), using bacteria as electrocatalyst, already contain a wide variety of enzymes that allow complex oxidation or reduction processes for a great variety of substrates. On the contrary, most e-BES employs a single enzyme to partially convert a specific compound [7]. In general, a single enzyme can catalyze a simple chemical reaction, and approximately 4800 enzyme entries have been documented and classified to date [8]. Most single redox enzymes catalyze one- or two-electron reactions, and represent a single elementary step in more complex reaction mechanisms; although some enzymes (e.g. blue copper oxidases: laccase, ascorbate oxidase) catalyze four-electron reduction of oxygen to water, higher specificity is usually desired [3]. Relatively complicated chemical reactions can be mediated by multiple enzymes in one location (Figure 2). The use of multiple enzymes in one location has numerous benefits such as fewer unit operations, smaller reactor volume, high volumetric and space-time yields, shorter cycle times and less waste generation. Besides, with multiple enzymes working together, the equilibria among the reactions is usually regarded as unfavorable which can be driven to the formation of target products [8]. Multi-enzyme-bioelectrochemical systems (me-BES) can be considered a type of *in vitro* synthetic biology project, promising for the production of fuels, chemicals, biocommodities and bioelectricity [8].

Electron transfer mechanisms

Electrons generated during oxidation should reach the anodic electrode to enter into the power circuit. Similar to the m-BES known as MFCs, there are two different mechanisms that have been proposed for anodic electron transfer in e-BES (Figure 3), as earlier introduced, DET and MET (section 3.1.2) [9-11]. However, the enzymatic electron transfer processes have their singularities. In general, the enzymes will have two distinct sites, *viz*., the biocatalytic site (apoenzyme/protein

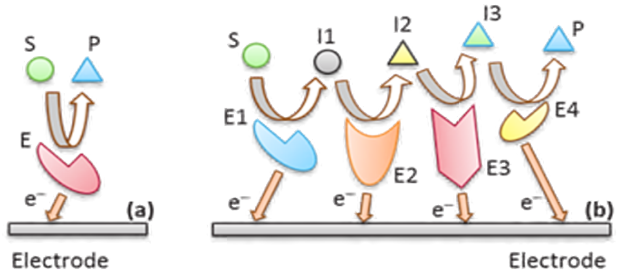

Figure 2: The functional difference between the mechanism of mono vs multiple enzyme electrodes [S: Substrate; P: Product; E: Enzyme; I: Intermediate; e-: Electron].

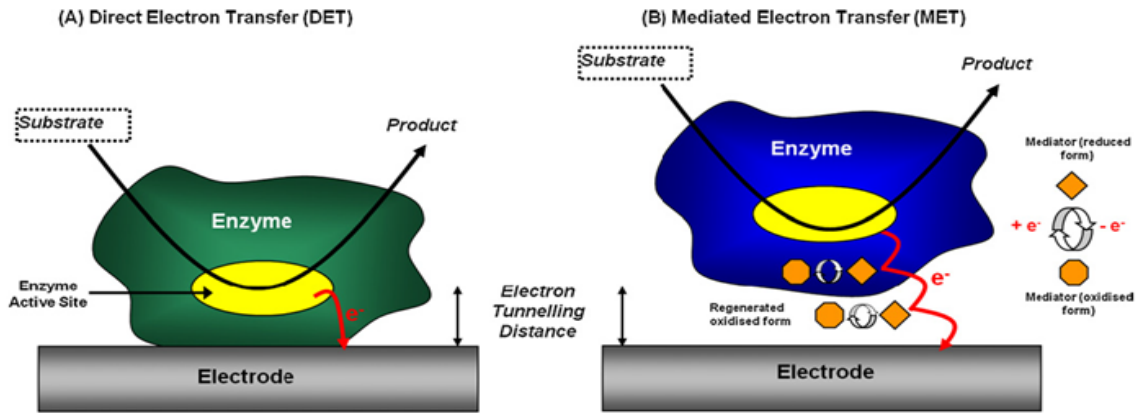

Figure 3: Schematic illustrating a simple arrangement for (A) direct electron transfer (DET) and (B) mediated electron transfer (MET), between the active site of an electrochemically-active enzyme and a solid electrode, during the oxidation of a substrate (Re-printed after [30] with permission from Elsevier).

part) for substrate recognition and the electrocatalytic site (prosthetic group/redox mediator) for electron transfer. The prosthetic group or internal redox site of the enzyme undergoes a conformational change during electron transfer, described as DET. However, some of the enzymes have only one site for both activities, which generally uses an external soluble redox carrier for electron transfer, described as MET. Overall, electrons derived from the enzymatically catalyzed oxidation of a substrate by oxidoreductases are transferred to the electrode through reduction of either a prosthetic group integrated within the enzyme (DET) or a co-substrate (MET), intermediately storing the transferred redox equivalents. However, the possibility to re-oxidize the prosthetic group or the co-substrate is crucial in order to regenerate the enzyme activity and make it available for further substrate recognition and conversion reactions [12]. Moreover, it is very difficult to clearly delineate the differences between MET and DET [12]. The detailed mechanism and existing challenges are discussed in the further sections.

Direct electron transfer (DET): The simplest and most appealing mode of interaction between electrodes and enzymes is DET, due to small number of transfer steps. In this form of electron transfer, the redox enzymes that possess tightly bound cofactors in the active site, can deliver the electrons directly at the electrode. The first reports on DET were published over 30 years ago [13] and recent reports are also available on a wide range of enzymes [14-18]. DET has been reported for about 40 redox enzymes, including laccase, peroxidases and complex multi-cofactor-containing enzymes [13]. Most extensively studied and best characterized enzymes for DET belong to the group of peroxidases [19-29]. Though there exist several mechanisms for the direct electrochemical communication between enzyme and electrode, several challenges need to be overcome to achieve significant rates of electron transfer, leading to appreciable current densities, between active sites and solid electrode surfaces. Major criteria for the effective electron transfer are shorter ET distance between enzyme and electrode and right orientation of the enzyme active site towards electrode.

The electrode surface itself is considered to be a "substrate" for the enzyme during DET, where the electron transfer kinetics are controlled by—at least—the electrode potential and by the distance between the surface and electron transfer structures in the enzyme [30]. DET involves the direct electrochemical recycling of the prosthetic group of the enzyme at the electrode surface, sometimes involving an electron tunneling mechanism. The mechanism of tunneling is based on a "bridge" molecule of complex structure (including different functional groups), that simply represents a barrier for ET which tunnels deep under the barrier. Superexchange is also a DET process similar to tunneling, but in a system with vacant electronic energy levels, higher than the energy of the tunneling electron. However, for this case, the electron transfer between two redox enzymes is not only dependent on the difference in potential between them and the distance between their respective redox centers, but also on the structural rigidity of the redox species involved [31,32]. The electron transfer distance between the prosthetic group of the enzyme and the electrode surface is obviously long due to the shielding provided by the protein shell, and this makes DET via tunneling a bit difficult. Although, immobilization of the redox enzyme on the electrode reduces the ET distance, negligible rates of ET have been observed for distances beyond 2 nm, which are very difficult to achieve. This indicates that DET can only take place when an electrode is placed within this distance to an enzyme cofactor in the active site [33]. Upon immobilization, the electrode can also block the access to the active site of the co-substrate/substrate resulting in no bioelectrocatalytic current for substrate electrolysis, even when an electrode can approach sufficiently close to an active site to achieve DET. Moreover, the denaturation of enzyme structure during immobilization with the consequential loss in activity, is another hurdle [12,13,34], caused by weak and unstable binding, random surface orientation, or impeded by the multiple redox sites present in a single enzyme [13,34]. Thus, an optimally designed electrode configuration has to ensure that the ET distance between an immobilized redox protein and a suitable electrode surface is made as short as possible but with favorable orientation. The enzyme molecules immobilized in the first monolayer on an electrode surface tend to show higher DET, but only a very small number of enzymes can be productively immobilized on the electrode surface which limits the overall electron transfer. In addition, proteins directly adsorbed on carbon, platinum or gold surfaces tend to denature, leading to electrode fouling and to unfavorable conditions for electron transfer [12].

Similarly, for enzymes that have their active sites sufficiently exposed to permit DET, the correct orientation of each enzyme at the electrode surface is a pre-requisite to keep the closest distance between enzyme and electrode. Design of suitable surfaces for the anisotropic and oriented immobilization of enzymes, such as immobilization on self-assembled monolayers (SAMs), is one approach to enhance

DET. Orientation of immobilized enzymes with the prosthetic group directed towards the electrode surface also drastically increases the rate of DET. The immobilization of an enzyme on a SAM also leads to the orientation of its prosthetic group towards the electrode surface and thus to a shortened electron transfer distance [12,35,36]. In addition, the SAMs also help in preventing denaturation of the proteins at the electrode surface [12].

Another approach for enhanced DET is the entrapment of enzymes in the conducting materials. These conducting matrices can also increase the "virtual" electrode surface by allowing the enzymes to immobilize at a fair distance from the electrode surface to take part in effective DET [12]. Entrapment into conducting materials such as sol-gel composites, polymers, etc., has been described previously [37-42]. However, the sensitivity of the enzyme has shown to become low in some cases after entrapment, and the ET mechanism could not be well defined as DET [43-45], except in very few cases [46].

Apart from these proposed mechanisms, another alternative for DET includes ET in a multi-cofactor enzyme with multiple subunits. This DET route is based on the pathway between the active site of the enzyme and the electrode surface, consisting of several steps between the different cofactors within the subunits of the enzyme. The studies related to multi-cofactor enzymes (mainly PQQ, FAD and heme-containing) reveal the priority of the distance separating the active site from the electrode [47-55]. However, proper immobilization of these enzymes on the electrode surface without losing any of the properties is a tough task. SAM-modified surfaces can also be used as a basis for the design of new ET cascades, as the distance between the active site and the electrode surface can be tailored. However, design of "molecular cables" by the integration of redox relays into the monolayer or by introducing the conducting oligomers with in the spacer chain to subdivide the overall ET distance, will result in enhanced electron recovery at the electrode surface through DET [12,56].

Mediated electron transfer (MET): Mediated electron transfer (MET) is an alternative to the DET, where a co-substrate or an electrochemically active chemical species (e.g. redox mediator) can be used to shuttle the electrons between the enzyme and the electrode. Mediators are artificial electron transferring agents that can readily participate in redox reactions with biological components. They form low molecular weight redox couples, which shuttle electrons from the active center of the enzyme to the electrode surface or vice versa [57]. Mediators are quite diverse in structure, properties and redox potentials (Figure 4). Their electron transfer, therefore, can be generally classified as homogeneous- and heterogeneously-mediated transfer. Homogeneous mediation occurs in solution, where both the mediator and the enzyme diffuse freely in the medium and after the electron transfer both the enzyme and mediator remains in the solution phase and then the free mediator interacts with the electrode. On the contrary, heterogeneous mediation implies diffusion of the mediator or the enzyme through an interface, keeping the other on the electrode, before and after achieving electron transfer between them.

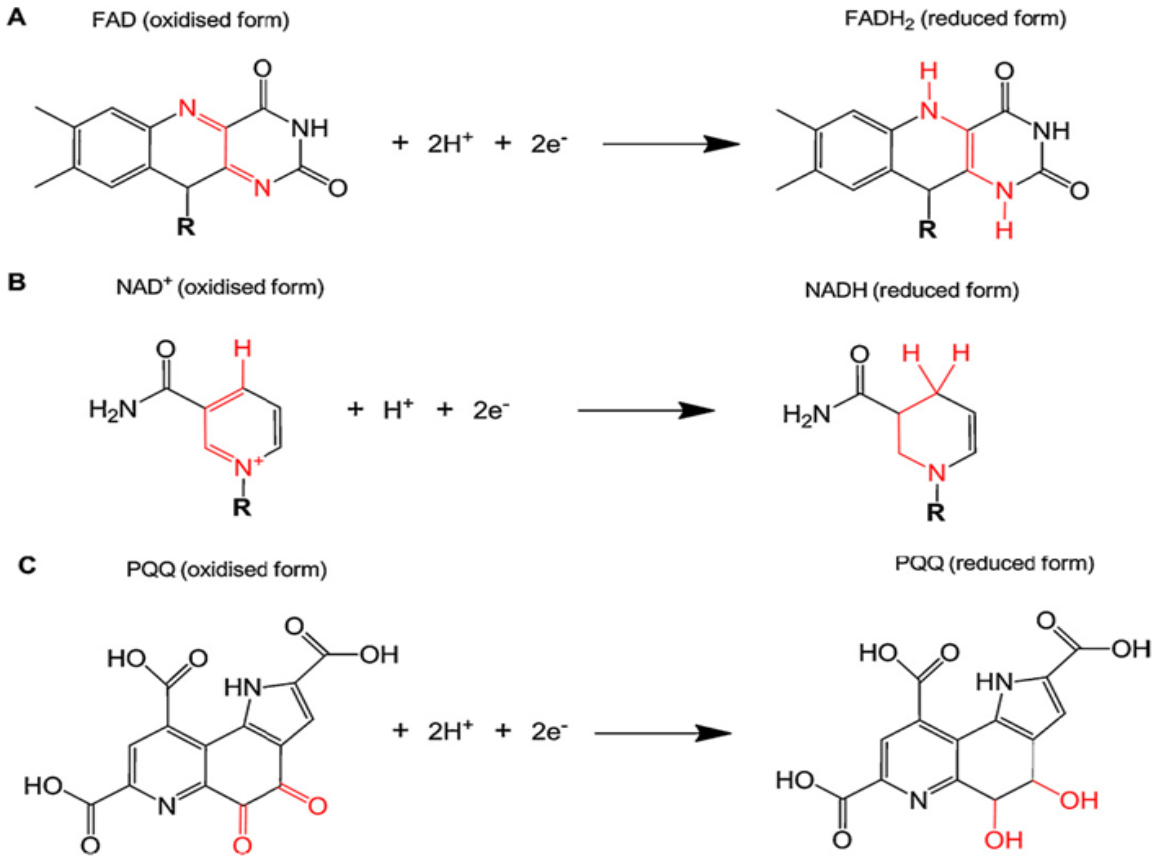

Figure 4: Cofactor structures and redox processes for: (A) FAD/$FADH_2$, (B) NAD^+/NADH and (C) PQQ, where R links adenosine diphosphate via ribitol to the flavin (A) or nicotinamide (B). (Re-printed after [30] with permission from Elsevier).

The latter occurs when the mediator is added to the bulk solution for reaching an immobilized enzyme or when the mediator is present on the electrode and not in the bulk solution, that contains the enzyme [3,57]. Initial studies on MET focused on the solution-phase mediators, which necessitate inclusion of a separating membrane between anode and cathode to prevent short-circuiting and cross reactions. Therefore, immobilization of mediator and enzyme onto electrode is preferable for the miniaturization of devices, permitting exclusion of the membrane. Initially, the freely diffusing natural co-substrate (NAD^+) was used as electron shuttle between the enzyme and the electrode (to recycle the prosthetic group of enzymes), based on the fact that these co-substrates can be reduced or oxidized at a metal-electrode interface [12]. However, the regeneration of the co-substrate is energy intensive (which decreases the cell potential) and also condition dependent. Introduction of artificial redox mediators was found as an alternative to this mechanism, as they lower the working potential resulting in decreased interference by other compounds that are directly oxidized/reduced at the electrode surface [12,58,59].

Ideal mediators should react rapidly with enzymes and exhibit reversible heterogeneous kinetics. Also, the overpotential for mediator regeneration should be low and pH independent. The mediator should have stable oxidized and reduced forms; the reduced form should not react with oxygen, while the oxidized form should not react with protons, if such are not the targeted reactions [57]. If these conditions are met, different mediators and prosthetic groups can be used for substituting or reducing expensive natural mediators of particular enzymes, allowing more economically efficient processes, if not also more kinetically favorable [60]. The artificial redox mediators are generally low molecular weight, soluble metal complexes with reversible electron transfer properties such as $K_4[Fe(CN)_6]$, quinones, Os-complexes, etc. [61-67]. These artificial mediators also help in the regeneration of co-substrates which cannot regenerate on the bare electrode surface (NADH), especially at lower potentials [12]. Further to this approach, the adsorption of soluble redox mediators on the electrode surface followed by the immobilization of the enzyme in a second layer has been carried out [59,68,69]. However, this mechanism is similar to the freely-diffusing soluble mediators mechanism as these mediators diffuse between enzyme and electrode. Moreover, leaching of the mediators, lack of long-term stability, and sample contamination, are the main disadvantages in this mechanism [12,70,71].

The free diffusional movement of the redox mediator is obvious and an indispensable prerequisite for a productive electron transfer. Henceforth, it is important to maintain a fast electrochemical communication between enzyme and electrode as well as to tightly retain the redox mediator at the electrode surface. One approach to satisfy these two conditions is known as "*hopping*", where electron transfer distances are reduced by dividing the overall ET process into a sequence of electron hopping reactions between redox mediators (relays) covalently attached to a matrix. The ET mechanism in hopping is dominated by a sequence of self-exchange reactions between adjacent redox mediator molecules. However, care should be taken so that the rate of these self-exchange reactions should not limit the electron transfer [12,13,72]. Similarly, covalent binding of the redox mediator via long and flexible spacer chains either to the electrode surface (seaweed mechanism) or to the outer surface of the enzyme itself (whip mechanism), has also been proposed as alternative [12,73]. Mixing the mediator into the carbon paste (graphite powder) is a relatively easy and effective method of mediator integration [74-81]. These carbon pastes can be further modified with stabilizers [82] or polyelectrolytes [75] to increase the long-term stability, response time, etc. The enzyme can also be mixed into the paste, but it is mostly immobilized on top of the carbon paste surface to increase the contact with the substrate. There are other approaches proposed to retain the redox mediator or enzyme at the electrode and prevent their leakage, such as trapping them within ion-exchange membranes [83-85], manufacturing them as colloidal particles [86], physical entrapment of the redox mediator into the matrix of composite electrodes [87,88], and entrapment into hydrogels [89] or in conducting polymers [90,91]. However, all these strategies have not completely solved the problem of mediator leakage and, in consequence; it has become indispensable to bind the mediators covalently in order to establish an electron-hopping mechanism instead of a shuttle mechanism [12]. Development of electroenzymes is another recent approach, where the protein itself is modified with covalently bound redox mediators at the outer surface [92-95] or at the inner surface of the protein, preferentially in close proximity to the active redox cofactor of the enzyme [96-99]. The covalently bound redox relays are supposed to shorten the ET distance between the deeply buried active site and the protein surface by allowing hopping mechanism via the enzyme-bound artificial mediators.

Irrespective of the mechanism, thermodynamic redox potentials of mediator(s) will dictate the power output during MET in fuel cell mode. More positive oxidative biocatalysis at anode and a more negative reductive biocatalysis at cathode drive the electron transfer between enzyme active site and mediator but will contribute to the loss in cell voltage. Therefore, achieving a best compromise between driving force and current generation is a major challenge in MET, in order to maximize the power output [12,13,100]. This can also be extrapolated as more energy consumption in electrolytic e-BES, which is quite relevant since more than 80% of the operational costs of electrolysis systems typically account for energy consumption. The electron transfer kinetics between the enzyme and the redox mediator should also be as fast as possible (i.e. high exchange current density) to compete with the regeneration of the enzymatic active site.

The absence of mediators is a big advantage when it comes to selectivity (there is less susceptibility to interferences due to lower electrode potentials) and because of the elimination of one reagent in the reaction sequence [13,57]. However, addition of a mediator may potentially increase the maximum rate of electron transfer, compared to DET. The mediators serve to facilitate a biological electron transfer, which is favorable thermodynamically but not kinetically [68]. Additionally, by using mediators the enzyme does not need to be in direct contact with the electrode surface, which minimizes enzyme denaturation possibilities. High electron transfer rate constant (k_{ET}) with the enzyme and accessibility in terms of steric effects, orientation and distance dependence, are two major factors to be considered while selecting mediators in order to obtain high currents [57,100]. Redox potential, electrostatic interactions, pH and ionic strength are also the other factors that play a major role on facilitating MET.

Development of enzyme immobilized electrodes

Enzymes are usually sensitive and their lifetime is limited but their stability over a long period of time is much crucial for their applications [101,102]. Immobilization of the enzymes onto the solid conductive matrices is an efficient and sustainable solution to confer a high stability and extended lifetime. It protects the enzyme from various external environmental conditions, such as shear forces, pH, temperature fluctuations, organic solvents, toxins, etc., [103]. Furthermore, immobilization affords a high concentration of enzyme moiety on the electrode, as well as easy handling of the e-BES along with the possibility of increased re-use of enzyme [104,105].

Henceforth, it can be concluded that immobilization of an enzyme offers a valuable solution for e-BES design and operation. In fact, the proper implementation of enzyme-electrodes relies mostly on the immobilization of enzymes or electrochemically active chemical species (e.g. mediators) and the chemical and physical properties derived thereof [6]. Immobilization of the enzyme on the electrode must be sufficiently strong to facilitate the transfer, but still suitable for not causing denaturation. Also, the enzyme must be properly oriented with respect to the electrode surface such that the active center is overlapped within an acceptable distance for the electron transfer to occur [3,72]. The electrode surface must be designed to resemble the surface characteristics of original environment (e.g. surface charge distribution or hydrophilic/hydrophobic properties) which facilitates the efficient interaction between enzyme and electrode without dramatic conformational changes [13].

Bare enzyme immobilization over electrodes has been the most common approach studied to achieve higher electron transfer. However, there are several other strategies, such as immobilization on the electrodes modified with promoters, SAMs of alkanethiols, polyelectrolytes, surfactants and ionic liquids, have also been used [13]. Enzymes with given surface groups suitable for direct chemisorption such as the thiolate and the disulfide group offer an attractive approach to protein-immobilization in well-defined orientations. The covalent, electrostatic or hydrophobic linking group may be close to the electrochemical redox center and this would support facile electrochemical electron transfer [72]. Despite the large record existing on enzyme-based electrochemistry (especially in the context of biosensors), the fabrication of bio-catalytically-modified electrodes with enzymes is still in early stages, particularly because of the difficulties in reaching high enzyme stability and power output. In recent years, studies on the development of new materials, enzyme modification, understanding the mechanisms of enzyme catalysis, enzyme immobilization methods, enzyme electrode structures and ways of preparation, have been carried out with the purpose of improving the performance of enzyme electrodes [6].

Numerous studies have been performed towards the development of enzyme-immobilized electrodes and their applications. The enzymes should be immobilized in such a way that electronic states in the surface material and enzyme active center overlap, increasing the probability of electron transfer across the interface [3]. Furthermore, care should be taken during immobilization in aspects such as enzyme orientation with the electrode, stability and activity after immobilization, possible denaturation during immobilization, etc. The methods of immobilization (Figure 5) developed over years can be broadly classified into three major groups, i.e. adsorption, covalent binding and entrapment [106-108]. Some of the previous studies reported about these methods in detail [2,109,110]. These immobilization methods are commonly used to construct bioactive hybrid materials and devices, including bioelectrodes for biofuel cell applications. However, there are certain challenges still needs to be addressed in the methods of immobilization. This part of the review mainly focuses on these existing challenges and various materials used for immobilization in a broader context.

Challenges in Immobilization: Though, there is enormous literature available on the immobilization technology, still there are certain constrains to be overcome to make it industrially applicable. The major challenges can be summarized as, maintenance of enzyme activity, stability over changes in physico-chemical factors, enzyme life-time, right orientation of enzyme active center on the electrode surface, synergistic interaction of the enzyme with the electrode after immobilization, among others [103]. A recent review on immobilization technology by Yang and co-workers has thrown some light on the existing methods and the materials for enzyme immobilization including the challenges and the future scope [111]. Adsorption is very easily adapted and well-studied method but has the enzyme leaching problem. Similarly, covalent binding is known for enzyme stability but has a problem of lower activity after immobilization. Arrangement of enzymes in different layers and synthesis of enzyme-electrodes are the two major approaches that can be pursued for the better immobilized moieties. Immobilization structures strongly influence mass transfer in the e-BES in terms of substrate diffusion through the active sites, the electron transfer and diffusion of redox mediators [111]. High resistance for the mass transfer process in biofuel cell necessitated the development of designed immobilization structures which may help to alleviate the mass transfer problem. The increasing interest in nano materials and the involvement of multiple disciplines towards biofuel cell development has put forward the progress in using various nanostructures, *viz*., nanoparticles, nanofibers, nanowires, nanotubes, nanosheets, nanopores and nanocomposites, because of their larger surface areas, short charge diffusion lengths, and fast diffusion rates, etc. In general, the large surface area of nano structures leads to possibility of high enzyme loading and thus resulting in improved power density. Furthermore, these nanostructures also help in extending the lifetime of the biofuel cells by increasing the enzyme stability and activity under higher mass transfer rates. However, these nano-materials can be arranged into different layers for further enhancement in the process.

The direct usage of nanoparticles, which have high electronic and catalytic properties for the enzyme immobilization for effective synergetic functions. For example, Au nanoparticles have been used to prepare biocatalytic electrodes for biosensor applications via a co-deposition approach with redox enzymes/proteins on electrode supports [112-123]. One-dimensional nanostructures such as fibers and tubes

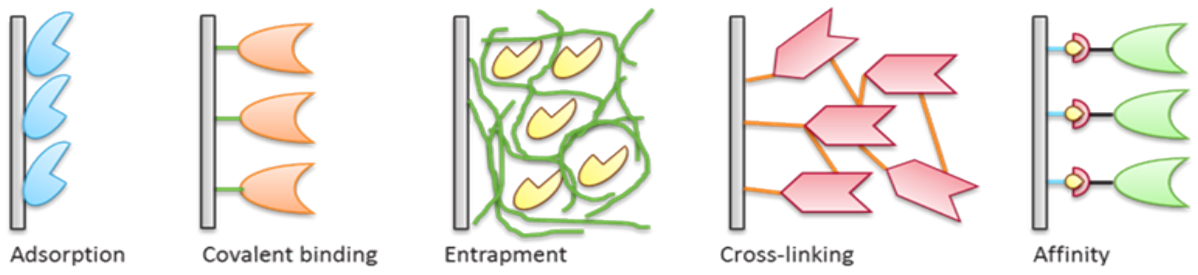

Figure 5: Schematic representation of various immobilization methods of enzymes into electrode materials.

have been also studied recently for their potential in nanoelectronic devices. For example, electrospun nanofibers provide a large surface area for the attachment or entrapment of enzymes and reduce the substrate diffusional path leading to better enzyme activity [124-136]. These electrospun carbon nanofibers were also studied in MFC, for their efficiency, and showed higher electroactive microbial biofilm growth [137]. Similarly, carbon nanotubes (CNTs) also made a great impact in the field of e-BES for stable and active enzyme immobilization systems with its high specific surface area which helped in effective adsorption of the enzyme molecules [138,139]. Multilayered enzyme assemblies on solid conductive supports (electrode) are another approach using nanolayers and sheets. Initially, enzyme layers were made in contact with nanolayered immobilization-support materials under controlled enzyme deposition, through electrostatic interactions or through cross-linking (affinity interactions) or through encapsulation in matrices (tailored organized biomaterial layers) [111,140-147]. For example, reconstitution of the apo-enzyme on the electrode surface with surface-bound and electrochemically active prosthetic groups was shown to be an effective way to achieve higher electron transfer rates by glucose dehydrogenase on PQQ-modified surfaces [13,141,142]. Apart from these, nanoporous materials with high specific surface areas, multi-scale porosity, tunable pore sizes, interconnectivity and rich surface chemistry were also have been studied for enzyme immobilization [111,148,149]. For example, enzymes immobilized on mesoporous materials and their applications in e-BES [2,150-152].

Even if layered structures for e-BES have shown efficient electron transfer, they are also not ideal because the amount of immobilized enzyme is small, due to monolayer covalent binding. Moreover, the thicker or higher the number of monolayers, the more elevated electric resistance develops on the interface, which adds to the ohmic drop within the system. Similarly, the catalytic activity will depend on the orientation of the enzymes and distribution of mediator molecules, if the latter are used in the system [6]. Conducting redox polymers can be a solution to overcome these limitations. Polypyrrole and polyaniline (emeraldine-base) are commonly applied in BESs [153]. They have unique properties for facilitating electron transfer, even over insulating basal nature of the materials, and were proven to enhance the current densities in both e-BES and m-BES systems [6,153]. They also showed an improved selectivity and stability over the others. However, the use of polymer-mediators has raised concerns on biocompatibility for implantable-device applications [6]. Besides, these immobilization methods essentially involve the use of chemicals, which add costs to the fabrication process. Similarly, the synthesis of electrodes (entrapment) is generally considered a rather expensive process and not easily scalable. Therefore, it is important to put some effort on the elaboration of new and more efficient means for their production. Moreover, enzyme electrodes become even more difficult, since they generally involve (as described before) the use of a variety of chemicals, high temperatures, among other difficulties. From this perspective, low-temperature calendaring (cold rolling) appears as an interesting alternative because it is simple, low cost, well known in the industry and easily scalable [154,155]. Surprisingly, its application on the fabrication on enzyme-electrodes is minimally used although ongoing research on the group of the authors of the present manuscript aims to confirm this direction.

Immobilization materials: The materials used for immobilization in e-BES are also considered to be critical and they must be capable of extracting or bringing the electrons from or towards the active site of enzyme, respectively. The bioelectrocatalytic efficiency of an immobilization material is largely governed by the electrical conductivity and hence it is the primary concern for the selection of an immobilization material along with the hardness of the material. In general, solid supports such as gold and platinum are considered as immobilization materials but with advancement in materials sciences, the application of polymers, carbons, oxide and metallic nanomaterials, sol–gel based materials and composite materials are also being used as efficient immobilization materials in e-BES.

Polymers

A variety of polymeric materials, *viz*., Nafion, chitosan, polypyrrole, polyaniline, polyphenol, polythiophene, poly-1,3-phenylenediamine, polyvinyl pyridine, polyvinyl alcohol, polycarbonate, and nylon, have been studied as immobilization materials in e-BES [156-161]. Conducting electro-active biocompatible polymers are widely used as immobilization materials because of the guaranteed electron transfer which sustains the electrocatalytic reaction of the enzyme [162]. Similarly, use of efficient mediators such as osmium containing redox polymers has also been studied for effective electron mediation at anodes as well as cathodes [163-168]. Although there are limitations for this kind of approach in human applications due to the toxic effects of the metals, perhaps there is room for application of these materials at industrial level for the enzymatic electrocatalysis for the synthesis of commodity and fine chemicals.

Similarly, some functionalized polymers have been studied for immobilization of electrochemically-active enzymes. Nagel et al. studied electro-deposition of the polymer over a support which was functionalized by amino groups, followed by subsequent coupling of the biomolecules via the carboxylic group of the protein cross-linked by carbodiimide. They studied their function as enzyme immobilization matrix as well as binder and electron transporting mediator [169]. Electrospun nanofibers are also proved as promising material for the encapsulation of nanoparticles, enzymes, proteins and whole cells. Cells and enzymes encapsulated within electrospun nanofibers can be a straightforward and cost-effective method as well as they can play a role on controlling the viscosity of the electrolyte solution [170-173]. Polymer-brush-modified electrodes were also studied for their application in e-BES but the activity is dependent on the pH of the solution [174]. The polymeric materials are also employed to achieve additional functionalities such as receptors in the form of a polymer matrix, mediators, or as ion-selective membranes [175].

Carbon based materials

Carbon based materials are attractive electrode supports because of their unique properties such as large surface area, high electrical conductivity, high electron delocalization, and high chemical and thermal stability [111,176]. Immobilization of enzymes has been studied on a wide variety of high-surface area carbon materials, including carbon blacks, carbon pastes, nanotubes, nanofibers, graphite, carbon fibers, clothes and paper, glassy carbon, carbon aerogel, mesoporous carbon and reticulated vitreous carbons. Carbon nanofibres showed as good electrode supports in e-BES for effective enzyme immobilization and their conductivity facilitates diffusion of free electrons [177,178]. CNTs with high electrical conductivity have been also studied as electrode materials and showed to help in mediating electron transfer reactions. CNTs can also be introduced as efficient molecular-scale "conductive wires" between the electrode surface and redox enzymes, such as GOx and NiFe-hydrogenase to increase electron transfer [72]. Alternatively, a free suspension of support materials with enzyme (and mediator, if necessary) can be deposited on a porous support. This procedure allows profound mixing of enzyme with carbon nanotubes prior to deposition, but it is limited regarding the types of structures

that can be achieved after introduction of the enzyme. Majorly, two applications in the utilization of CNTs are commonly studied i.e. CNTs with metals (Pt/Au) and CNTs with polymers [179-182]. Carbon aerogel possesses high porosity, a large surface area and is extensively utilized as an enzyme adsorbent and electron conducting material [183]. For example, a laccase-adsorbed carbon aerogel electrode remained very stable without loss of the enzyme activity. Nanoporous structures of carbon aerogel have the advantage of stabilizing the enzyme electrode and they hold great interest in wastewater treatment [184,185]. On the other hand, graphene has several advantages for its application in e-BES, *viz*., superior electrical and heat conductivity, mechanical strength and unique optical absorption, and can thus be used as a novel class of electrode material [186,187]. However, graphene is hydrophobic and easily forms agglomerates irreversibly which is the limiting factor for its exploration as graphene-modified electrode. Similarly, mesoporous carbon with controlled porosity, high pore volume and large surface area, was also given much attention for e-BES applications [188,189]. Mesoporous carbon materials can be used in biofuel cell by enzyme cross-linking or by highly ordered mesoporous structures [190-196]. It is also noteworthy that mesoporous carbon is also recommended for bacterial adsorption [197]. The application of mesoporous carbon modified electrodes is extended in MFCs, where electron transfer rates have significantly increased [197,198]. Decreasing surface area and increasing pore size and distribution are two morphological parameters that substantially influence macroscale catalytic activity and reactant transport [3]. Activated carbons have surface area over 1000 m^2g^{-1} but are generally unsuitable for supporting biocatalysts because most of this area exists in micropores that are inaccessible to catalysts or even to electrolyte solution [3,199]. In general, enzyme immobilization in nanostructured electrodes extends their lifetime and improves their activity, due to relieved mass transfer limitation of substrates in the nanostructures as compared to macro-scale diffusion, especially when the size of the nanopores is slightly larger than the size of the enzyme.

Metallic oxides and magnetic nanoparticles

Apart from polymers and carbon based materials, oxides and metallic nanomaterials have also been studied extensively for their application in e-BES due to their unique physical and chemical properties [200,201]. Most metallic nanoparticles that have been the focus of research are based on Au, Ag and Pt, due to their high thermal stability, electronic properties and promising applications [200,202]. Similarly, some oxide nanoparticles such as Fe_2O_3, Al_2O_3 and CO_3O_4 [203-205] are well studied in enzymatic fuel cells for valid electron transfer. In comparison with conventional immobilization methods, nanoparticle level immobilization involves three important benefits, *viz*., ease for synthesis in high solid contents without using surfactants or toxic reagents, homogeneous and well defined structure and distribution can be obtained, and particle size can be conveniently controlled. In addition, with growing attention on multi-enzyme systems, co-immobilization thereof can be achieved in such nanoparticles [206]. Furthermore, magnetic and paramagnetic nanoparticles favor high enzyme-binding capacity and high catalytic specificity along with enhanced stability, due to their surface to volume ratio. Moreover, magnetic nanoparticles can be separated from the reaction medium simply by using a magnet, which was demonstrated in a study with lipase attached to γ-Fe_2O_3 nanoparticles by covalent bonds [204]. This allows enzyme reuse over a longer period than that for free or physisorbed enzymes. GOx, peroxidases, β galactosidases, lipases, cholesterol oxidase, trypsine, laccase, α amylase, hemoglobin, cellulase multienzyme mixtures, among others, have also been successfully immobilized (covalently) in this kind of particles, using several ligands. pH, temperature and substrate concentration-stability are also attained for periods as long as three months of continuous operation. Binding efficiency has also shown to increase; moreover, enzyme properties after storage within these particles are also enhanced [206]. Consequently, the use of magnetic nanoparticles represents an innovative approach for enzyme-electrode fabrication without using strong chemicals or high temperatures. Likewise, such particles allow easy-enzyme recovery and reuse which is important for reprocessing, especially when waste or high substrate content streams are considered. Recently, TiO_2 based nanotube arrays, also demonstrated their remarkable charge transfer and photocatalytic properties with enzyme electrodes [207-209]. The application of these novel particles has been demonstrated, as well, in MFCs [205].

Mesoporous and sol–gel based materials

Micellar or mesoporous phases are usually added to the enzyme electrodes to provide an immobilized ion exchanger, buffer, prevent access of poisonous or competitive species, or enhance stability. Such materials are usually polymer or silica-based. Nafion, doped-Nafion, chitosan, have been used on the polymeric approach. In this way reactant permeation is allowed. The process is considered gentle enough that enzymes may be co-casted to form composite membranes [3]. Porous silica structures can encapsulate enzymes by gelation of sol-gel precursors surrounding biomolecules or by adsorption of the enzyme after gelation. The presence of the enzyme restricts the location in which the gel can form [3]. Mobile Crystalline Material MCM-41 (pore size: 4 nm) is a silicate obtained by a templating mechanism, that was the first used for enzyme-electrode immobilization. After it, ordered mesoporous silica (e.g. SBA-15, pore size: 5-13 nm), mesocellular foam (MCF, pore size: 15-40 nm) and mesoporous carbon, have been also applied. Modifications on these materials have also been achieved, such as enlargement of pore size and modified morphologies, for successful enzyme quick adsorption [2]. Sol-gels are porous polymeric matrices with increased surface area which resulted in the development of innovative advanced materials for the immobilization of biological receptors within silica, metal oxide, organosiloxane, and hybrid sol–gel polymers [210-212]. These sol-gel based materials are environmentally friendly and biocompatible and can be combined with many biological systems from molecules to single cells [111]. Sol-gel based materials and their uses for construction of enzyme electrodes have been extensively reported based on their biocompatibility which gives a stable environment for the enzyme function [211,212].

One frequent approach for enzyme immobilization in mesoporous materials is simple adsorption. The stability on the enzymes in such material depends mainly on pore size and charge interaction. It is considered that the pore size of mesoporous materials should be similar to or larger than of enzymes for achieving successful adsorption. The relationship between pore size and molecular diameter is important. Larger pore size, usually leads to poor enzyme stability. If the charge of mesopores is opposite to the net surface charge of the enzymes, it will make a stable system. On the other hand, when the charge is the same, there is repulsion between the enzyme and the surface of mesopores. Charge can be controlled by changing pH, adding buffers or by mesopore functionalization (e.g. with amino or carboxyl groups) [2]. Enzymes covalently attached to mesoporous materials have longer half-life (e.g. 1000 fold higher than that of native enzyme). Beside adsorption and covalent attachment, other approaches can be used for mesoporous material enzyme immobilization, such as partial closure of micropore inlets, nanocomposite shell on the particle surface, and cross-linked enzyme aggregates via a ship-in-a-bottle approach [2].

Ionic liquids

Non-aqueous biocatalytic systems have acquired recent interest because of their unique synthetic opportunities. For example, hydrolytic enzymes (e.g. lipases and esterases) in non-polar, organic solvents with low water content have been shown to carry out reversed hydrolytic and transferase-type reactions in such media. Recently, ionic liquids (ILs) have emerged as an alternative media to non-polar, hydrophobic solvents for supporting biocatalysis [213]. Electrode modification with ILs is interesting due to their hydrophobicity, high viscosity, ionic structure, ionic conductivity, low-volatility and biocompatibility. The electrochemical properties of ionic liquid-modified electrodes (ILME) are determined by the presence of a well-established ionic liquid/liquid interface and three-phase junction electrode/ionic liquid/liquid where in most cases ET starts [214]. Ionic liquids are entirely comprised by anions and cations. A myriad of organic cation and inorganic or organic anion combinations are possible [213,214]. Therefore, the toxicological and pharmacological effects of most ILs have still to be defined. The melting point of most ILs is below room temperature. Their conductivity can be as high as 100 mS cm^{-1} and bulk electrode materials comprising this liquid acquire this property [215]. Viscosity of ILs is typically in the range of hundreds of mPas (at 25°C). The wide potential window is considered one of the major advantages regarding ILs in electrochemical systems. Indeed, ILs has been used for enzyme electrodes and was reported to increase enzyme stability and activity and for this reason they have become popular as a novel route for enzyme-electrode manufacturing [214]. The presence of specific functionalities in ILs permits their application for electrode modification. For example, the use of amino acid-functionalized IL also provides stable enzyme immobilization [216]. Nonetheless, to date relatively little data exist on the enzymatic activity of oxidoreductases in ILs. Recent studies have shown that the classic redox-active hemoprotein, cytochrome c (cyt-c), and analogues of its active site, conditionally retain peroxidase activity in some ILs, but they must be coordinated with a ligand for this purpose [213].

IL-modified electrodes can be divided into five major categories. 1) Electrodes modified with ionic liquid droplets or films: electrochemically generated ion transfer across IL/aqueous solution interface is observed, and their electrochemical behavior seems more complex than other ILs; however, these ILs produce suitable configuration for enzyme-electrode electron transfer. 2) Film electrodes with ionic liquids as one of the components: they are considered important supports for enzymes, because of its stabilizing properties and because they have supported DET of glucose oxidase, horseradish peroxidase, hemoglobin, myoglobin, cytochrome c, catalase and chloroperoxidase; still, the ET mechanisms with such ILs and enzymes are not yet understood. 3) Carbon paste electrodes with ionic liquid as a binder: they have higher viscosity; complex enzyme-electrode architectures can be built with such ILs.4) Electrodes prepared of ionic liquid-carbon nanotubes gel: GOx has been successfully immobilized in this type of IL-based electrodes; however redox reaction with other enzymes has appeared difficult to achieve, but metallic and magnetic nanoparticles seem an interesting possibility to explore for this type of IL-electrodes. 5) Electrodes modified with appended ionic liquids: contrary to the other ILs presented, these do not consist of the ILs as they are, they are prepared from imidazolium cation ILs with different functionalities related to their immobilization procedure; this is, they are just ionomers with immidazolium functionalities, therefore their wettability can be controlled by electric field. Application of the latter type of ILs to enzyme electrodes has been carried out, however, low current has been observed when compared to bare electrodes, possibly due to ion preconcentration effects [214]. For sure, the presence of ILs affects the ET processes and mechanisms and they bestow numerous unexplored possibilities for enzymatic electrocatalysis.

Composite materials

Apart from all these materials, there are numerous studies based on the combination of two or more kinds of them with resulting significantly different physical or chemical properties. Such materials can be termed as composites. Composite materials have the advantage of combining different structures and their native properties at the macroscopic or microscopic scale [111]. However, they may acquire all the native properties of the different combined materials or may possess unique hybrid properties of neither the incorporated components nor the host matrixes. Though there are several combinations, three major combinations were reported based on immobilization materials by Yang et al. [111]. These include polymer-based composite materials with carbon, nanomaterial and sol–gel, carbon-based composite materials with nanomaterial and sol–gel, and composite of sol–gel materials with metallic oxides and novel nanoparticles [111]. A few examples of these composites include novel metal/CNT/polymer composite electrodes which have presented significantly improved electron transfer properties [217]. Similarly, a new kind of electroactive nanocomposite formed by methylene green, that noncovalently functionalizes chemically reduced graphene, has been applied to e-BES [2,188,189,218-221]. Sol–gel materials incorporating other constituents have also been extensively investigated, with the combinations of biopolymer chitosan [222], CNTs [223], etc. Overall, the combination of two or more compounds can bring some of new properties to the immobilization matrix which helps to support the enzyme activity, stability as well as the electron transfer rates to the electrodes. The incorporation of metallic or semiconductive nanoparticles into conductive polymers can be a typical example, which helps in increasing the electrocatalytic properties of nanoparticles and in return the conductivity of hybrid systems is enhanced with the metal nanoparticles [224].

Electroenzymatic reactions and applications

Enzymatic reactions on electrodes can be applied for anodic oxidation or cathodic reduction reactions, based on its functionality. The enzyme in the anodic compartment oxidizes the substrate, while transferring electrons to the electrically contacted electrode. Similarly, the cathodic enzyme reduces the available oxidizer compound with the help of electrons coming from the anodic oxidation. The electrons will flow through the external circuit, while the protons transfer through the electrolyte. Several enzymes belonging to the family of oxidases and dehydrogenases were studied in fuel cell for anodic oxidation of diverse compounds, for instance, saccharides, alcohols, acids and amino acids, etc. The most studied enzymes at cathode are based on oxygen reduction, such as laccase, peroxidases, etc., [225]. The difference in the thermodynamic redox potentials of the redox enzymes used at anode and cathode, determines the maximum voltage of the biofuel cell. However, the incorporation of redox relays to increase the electrochemical communication between electrodes and enzymes will lead to a potential drop, conducting to lower power outputs. Therefore, it is important to select the mediators with redox potentials close to the thermodynamic potentials of the enzyme. The turnover number of enzyme also plays a crucial role in current generation, which is determined by the rate of reaction occurring between the enzyme and the fuel/oxidizer. Though, two enzymatic electrodes are coupled and operate in a fuel cell, the cathodic reduction reaction is the major rate limiting step determining the current generation, similar to the MFCs. Over 1400 oxidoreductases are known to date (www.enzymedatabase.

org), any of which could possibly be utilized as catalyst in an enzymatic FC. In the majority of cases, the use of mediators is needed to electrically connect the enzyme to the electrode, since only less than about a hundred of the known oxidoreductases are capable to communicate with an electrode surface via a DET mechanism [225]. Many redox enzymes have their catalytic sites buried deeply within the protein matrix, which acts to insulate the redox site and will eventually prevent DET.

Anodic oxidizing reactions: The most studied anodic reactions are based on glucose oxidation using oxidases and dehydrogenases. GOx is a well characterized and stable enzyme studied as anodic biocatalyst of choice for many e-BES [30]. Similarly, the other one is glucose dehydrogenase (GDH), catalyzing a similar reaction, oxidation of glucose to gluconolactone, thereby liberating two electrons. These two enzymes have been studied in the glucose/O_2 system combined with the oxygenases such as laccases, bilirubin oxidases, peroxidases, etc., at the cathode. However, there are numerous other enzymes studied in the e-BES for their catalytic efficiency of oxidation or reduction, which are represented in Table 1.

Indeed, glucose oxidation has been extensively studied in the context of biosensing devices and does not provide a major reaction of interest for industrial exploitation; however, the fundamentals and practical experiences derived from the immoboilization, characterization, analysis and optimization of such enzymatic systems remain important due to their contributions to the overall field of enzymatic electrocatalysis. Apart from the glucose, alcohols were also extensively studied as fuel at anode in e-BES catalyzed by non-specific or specific alcohol dehydrogenases [240,241]. Similarly, cellobiose [229-233], fructose [234,235], pyruvate [227], glycerol [227], hydrogen [238,239], etc., were also studied as fuels in e-BES. Various studies have been performed to enhance the current densities, to meet the complete oxidation of the fuel, to increase the stability and longetivity of the enzyme properties. GOx is a well characterized and stable enzyme which catalyzes the oxidation of glucose to glucanolactone [242]. Several studies were performed to increase the electron transfer in GOx, especially based on using carbon nanotubes (CNTs) and gold nanoparticles [30,36,99,109,156,225,228,243-247], by using mediators such as ferrocene derivatives [59,248,249], by coordination complexes of osmium with polymers [243,250-257], by protein modification and engineering to achieve improved GOx properties [258-260] etc. Likewise, the other enzymes such as GDH, alcohol dehydrogenases were also studied from different sources and with various enhancing strategies in e-BES [14]. Oxidation of H_2 is another potential application in e-BES, where different types of hydrogenases were studied for their function [14,238,239]. [Ni-Fe] hydrogenases with Fe-S cluster were found to be more efficient among these hydrogenases [14]. However, the complete oxidation of any fuel is not feasible in single enzyme catalyzed systems. Few studies on multi-enzyme cascade systems were also reported for the complete oxidation of fuels such as glucose, ethanol, methanol, pyruvate, etc., (see section about Multi enzyme cascades).

Cathodic reduction reactions

Similar to anode, there are several enzymes studied at cathode for completing the reduction reactions. Most of these cathodic enzymes are typically multi-copper oxidases, such as laccases [163], bilirubin oxidase [167], peroxidases [225], etc., which are capable of four-electron O_2 reduction [261]. Laccases are generally employed under slightly acidic conditions, while bilirubin oxidase has its activity in more alkaline media which allows it to be used at neutral pH. Apart from these, several other enzymes such as cytochrome oxidase and cytochrome c, have also been employed at cathodes in e-BES. In the case of H_2O_2 reduction, microperoxidase [99,225] and horseradish peroxidase [262] are commonly used as electrocatalytic enzymes. A comprehensive list enzymes used at cathode in e-BES was depicted in Table 2.

The cathodic reduction reaction is as crucial as anodic oxidation for the completion of the circuit in the electrochemical cell, and needs a terminal electron acceptor, such as oxygen. Various metallic catalysts, such as platinum, have been used to increase both selectivity and electrode kinetics towards reduction reactions at cathodes. However, enzyme-catalyzed cathodes are more efficient [266]. Multiple copper oxidases appear highly relevant in scientific literature, especially laccases, due to their high reduction potential, capacity to utilize multi-atomic reaction sites, flexibility of interatomic distances and the positive influence of residues adjacent to the active site on reaction mechanism [263,267-270]. Laccases from fungal origin are most extensively studied due to the higher redox potential (~0.58 V vs Ag/AgCl), ligand co-ordination geometry, and the presence of weakly axially co-ordinated residues contributing to the difference in redox potential [30]. However, these fungal laccases are inhibited by hydroxyl ions and, to a lesser extent, by chloride [271], which limits their usage at biocathodes. Apart from this, laccases from plants [263] and bacteria [272,273] have been also studied, but they have a low reduction potential (~0.23 V vs Ag/AgCl). The higher reduction potential of some laccases close to the thermodynamic potential for oxygen reduction enables the effective reduction reaction at cathode. Laccases were adsorbed on graphite [263,274], carbon aerogel, HOPG [183], carbon nanotubes [184,275-277], nanoparticles [278-280], gold nanoparticles [281] to enhance electron transfer rates from laccases. Alternatively,

Enzyme	Substrate/ Fuel	Natural/ Artificial electron acceptor	Co-factor	Half-cell reaction	Reference
Glucose oxidase	Glucose	O_2	FAD	Glucose → Glucono-1,5lactone+$2H^+$+$2e^-$	[226-228]
Glucose dehydrogenase	Glucose	NAD	NAD	Glucose → Glucono-1,5lactone+$2H^+$+$2e^-$	[227]
Glucose dehydrogenase	Glucose	Quinone	PQQ	Glucose → Glucono-1,5lactone+$2H^+$+$2e^-$	[227]
Cellobiose dehydrogenase	Glucose	FAD	Heme	Glucose → Glucono-1,5lactone+$2H^+$+$2e^-$	[229-232]
	Cellobiose	FAD	Heme	Cellobiose → Cellobiono-1,5lactone+$2H^+$+$2e^-$	[233]
Fructose dehydrogenase	Fructose	FAD	Heme	Fructose → 5-dehydrofructose+$2H^+$+$2e^-$	[234-235
Succinate dehydrogenase	Succinate	FAD	Fe-S	Succinate → Fumarate+$2H^+$+$2e^-$	[236]
Alcohol dehydrogenase	Ethanol	PQQ	Heme	Ethanol → Acetaldehyde+$2H^+$+$2e^-$	[237]
Oxalate oxidase	Glycerol	O_2	FAD,Mn	Oxalate → $2CO_2$+$2H^+$+$2e^-$	[227]
Pyruvate dehydrogenase	Pyruvate	NAD	NAD	Pyruvate+SCoA→acetylCoA+$2H^+$+$2e^-$	[227]
Hydrogenase	Hydrogen		Fe-S	H_2→$2H^+$+$2e^-$	[238]
Membrane-bound hydrogenase	Hydrogen		Fe-S	H_2→$2H^+$+$2e^-$	[239]

Table 1: Some of the most studied enzymes for anodic oxidation and their respective reactions.

retention of the enzyme behind a membrane at electrode surface [282] and chemical derivatisation to retain laccase through hydrophobic pockets were also studied to enhance the electron transfer rates [283]. Laccases cross-linked with an osmium-based redox polymer on carbon electrodes were shown to provide steady-state current densities [284]. Bilirubin oxidase [229,231,232] and cytochrome oxidase [285] were studied as alternative to laccases at cathode for O_2 reduction and peroxidases have been well studied for the application in e-BES.

Hydrogen peroxide (H_2O_2) is considered as a stronger oxidant than O_2 at cathode in e-BES which can be oxidized by highly active peroxidases as the cathodic electrocatalyst [286,287]. Horseradish peroxidase, cytochrome c peroxidases and microproxidases are the common peroxidases used in e-BES [19,28,237,262,264]. Apart from these electron acceptors, few other oxidized substrates such as formate, fumerate, aldehydes, etc., were also being studied recently at the cathode of e-BES [54,238,265]. Considering these substrates for their reduction at cathode has opened new windows in the electrosynthesis research where single/multiple enzymes can be used to reduce the unwanted/waste substrates to potential value added products with the help of in situ generated reducing equivalents (H^+ and e^-) (see section about Enzymes for electrosynthesis).

Novel enzymes and their applications: Apart from these well studied enzymes, there are several enzymes being studied for their application in the enzymatic electrosynthesis as depicted in the Table 1 and 2. However, certain enzymes should be mentioned here for their novel applications. Peroxidases such as horseradish peroxidase, chloroperoxidase, lignin peroxidase, etc., are well known for their application in treatment of complex and toxic pollutants. For instance, chloroperoxidase has proven highly valuable in the catalysis of epoxidations, hydroxylation, and oxidation of alcohols and indole. Heteroatom oxidation (N^- and S^- oxidation) has also been achieved; therefore, oxidation of heterocyclic sulfur compounds present in fuels and the modification of petroporphyrins and asphaltene molecules are two promising large-scale applications for e.g. petroleum industry, in order to improve the quality of petroleum and petroleum fuels, as well as for reducing their environmental polluting effects [288]. Studies on this enzyme were also extended to understand DET [215], immobilized on mesoporous materials [288], ionic liquid-modified carbon electrodes [215] and with conductive polymers [289]. Moreover, this enzyme can also be applied at anodes for electrochemical heteroatom oxidation (N^- and S^- oxidation) with simultaneous cathodic reduction of O_2 and H_2O_2 [215,289].

Multi enzyme cascades: The combination of two or more enzymes at the anode or cathode as cascade has shown higher anodic oxidation and cathodic reduction reaction rates in e-BES [30,290]. The first and one of the simplest me-BES that have been described is relative to the oxidation of methanol to CO_2 and water by a three-step mechanism catalyzed by NADH-dependent systems [290]. Similarly, the two-step oxidation of ethanol to acetate was also studied in a novel membrane (Nafion) assembly [291]. A polymer-based electrocatalyst (poly-methylene green) was used to regenerate NAD^+ and to shuttle electrons from NADH to the electrode [292].

The complete oxidation of methanol to CO_2 using solution phase dehydrogenases [290] and the reduction of CO_2 to methanol using the enzyme cascade [265] are novel examples of this kind of reactions. Similarly, in some of the studies multi enzyme cascade was used for the complete oxidation of the substrates such as glucose [293], methanol [294], ethanol [295], pyruvate [296-298], glycerol [299-301], etc., to CO_2 generating higher number of electrons. Modified electrodes combining cellobiose dehydrogenase and pyranose dehydrogenase have shown to be capable of extracting up to 6 electrons from one molecule of glucose [302].

The single enzymes in e-BES, studied over the years are, peroxidases, the multicenter redox enzymes hydrogenases, multiheme nitrite reductase, large membrane-bound enzymes including fumarate reductase, succinate dehydrogenases, Mo-containing nitrite reductases, sulfite oxidase and lacasses [72]. The current densities achieved in all these cases are much lower than those achieved by me-BES. Most e-BES implies low efficiency due to single-step redox reactions, but efficiency can be improved by using me-BES. In conclusion, one of the key issues to develop effective and efficient e-BES is the successful immobilization of multi-enzyme systems that can completely oxidize organic compounds to CO_2 or vice versa, in order to increase the overall substrate-to-electrons efficiency of the cell.

Enzymes for electrosynthesis: Electrosynthesis is one of the emerging applications of BES where the negative valued substrates can be converted to commercially viable substrates under small applied potentials in presence of a chemical/biological catalyst. However, biologically catalyzed systems have more significance due to the renewability of the process and recyclability of the catalyst. Several researchers across the globe are currently working on the microbial electrosynthesis process in BES for the production of chemicals and fuels apart from electricity [10-11,303,304]. Bioconversion of fumarate to succinate [305], CO_2 to acetate [306], acetate and butyrate to alcohols, acetone and elongated CFAs such as caproate [1], methane [308,309], volatile acids to polyhydroxyalkanoates [309], etc., have been reported

Enzyme	Oxidizer/ Electron acceptor	Natural/ Artificial electron donor	Co-factor	Half-cell reaction	Reference
Laccase	O_2		Cu	$O_2+4H^++4e^-\rightarrow 2H_2O$	[226,228,239,263]
Bilirubin oxidase	O_2		Cu	$O_2+4H^++4e^-\rightarrow 2H_2O$	[229,231-232]
Chloroperoxidase	H_2O_2		Heme	$H_2O_2+2H^++2e^-\rightarrow 2H_2O$	[21]
Cytochrome c peroxidase	H_2O_2		Heme	$H_2O_2+2H^++2e^-\rightarrow 2H_2O$	[19]
Microperoxidase	H_2O_2		Heme	$H_2O_2+2H^++2e^-\rightarrow 2H_2O$	[28,237,264]
Horseradish peroxidase	H_2O_2		Heme	$H_2O_2+2H^++2e^-\rightarrow 2H_2O$	[262]
Fumarate reductase	Fumarate	FAD	Fe-S	Fumarate$+2H^++2e^-\rightarrow$ Succinate	[264]
Alcohol dehydrogenase	Formaldehyde	Neutral red	NAD	Formaldehyde$+2H^++2e^-\rightarrow$ Methanol	[265]
Formaldehyde dehydrogenase	Formate	Neutral red	NAD	Formate $+ 2H^++2e^-\rightarrow$ Formaldehyde $+ H_2O$	[265]
Formate dehydrogenase	HCO^{3-}	Neutral red	NAD	$HCO^{3-}+2H^++2e^-\rightarrow$ Formate	[265]
Carbonic anhydrase	CO_2	Neutral red		$CO_2+H_2O\rightarrow HCO^{3-}+H^+$	[265]
Hydrogenase	H^+	Methyl viologen	Fe-S	$2H^++2e^-\rightarrow H_2$	[238]

Table 2: Some of the most studied enzymes for cathodic reduction and their respective reactions.

so far. Still further research is on and going towards the production of various commercially viable products in renewable and sustainable way through BES. On the contrary, the research in the direction of enzymatic electrosynthesis has started more recently and very few articles are available in the literature. The major hurdles in this area is requirement of single substrate, stability and longetivity of enzymes, interference reactions, end-product inhibition, etc. Recent report on the methanol production from CO_2 [265], has shown the possibility of using CO_2 as precursor for the synthesis of useful chemicals and fuels at cathode in e-BES. Various novel routes for the product synthesis through enzymatic/chemo-enzymatic processes were reported extensively [310]. Adapting these enzymatic processes at e-BES cathode will have an added advantage of simultaneous power generation and co-factor regeneration. Electrosynthesis of methanol, ethanol, butanol, acetate, fumerate, etc., using enzymes cascades should be focused. Similarly, the conversion of waste glycerol, a major intermediate from biodiesel industry [310], to dihydroxyacetone phosphate, synthesis of polyhydroxyalkanoates using volatile acid straems also has higher commercial viability.

Criteria and cost elements for industrial processing with e-BES

According to the state of the art literature, industrial biotransformations must minimally consider three key factors:1) product yield (gram product per gram substrate/enzyme), 2) product titer (gram product per liter), and productivity (gram product per liter per hour) [8]. However, electrochemically-mediated enzymatic processes and biological in general—must be seen from additional perspectives, due to their heterogeneous-catalytic nature. Current density (amperes per square meter of electrode), faradic efficiency or fuel utilization efficiency (% of substrate to electrons conversion, from bioelectrochemical reactions) and energy efficiency (useful power output per total power input), are other core parameters related to production within these systems.

Product yield and faradic efficiency are considered the most important for the production of biocommodities by e-BES, since a large fraction (30-70%) of the conversion step costs come from feedstock [8]. Product titer and current density come next in importance. Especially high-value products are habitually present in rather diluted concentrations and require intensive separations. Besides, reduced quantities are obtained from large electrode surfaces, which represent a significant extent of the processing costs [8]. Product yield is not significant for final product production costs. However, energy efficiency becomes important when enzymatic electrolysis or electrosynthesis are anticipated, since generally about 70-80% of the costs in electrochemical processing account for electricity [311,312].

In their application as enzymatic fuel cells, e-BES must have a positive energy budget. This is the power relative to consumption, i.e. pumping, must be lower compared to the power output from the electrochemical cell. In addition, especially if such enzymatic power source is meant to be applied as implantable or portable power source, economy of scale becomes highly relevant for comparison to batteries, as the latter have no pumping costs and relies only on diffusion. Volume and weight become critical for such comparison [313].

Compared to metallic electrocatalysts, enzymatic electrocatalysts offer competitive advantages. For instance, since biocatalysts are nowadays produced industrially, production costs are rather low, while this is opposite for the case of transition metal catalysts [313]. Still, there is further work needed to understand important enzymatic and engineering issues, especially when scale-up for industrial bioproduction is foreseen. So far, for such applications, there are no electroenzymatic pilot studies available. For this reason, scaled-up demonstration projects are required in order to prove the reliability and cost-effectiveness of enzymatic electrocatalysis. Although initially enzymatic electrocatalysis has evolved in the context of biosensors and now it has been moving to the field of miniaturized power devices, it is likely that it finds soon an industrial niche on the synthesis of fuels and chemicals as well. Given that electricity costs are relatively low compared with the value of such product chemicals, enzymatic electrosynthesis will become a key process for chemical synthesis if lifetime of enzymatic electrodes and reactor engineering issues are soon overcome.

On the other hand, enzymatic systems involve additional costs associated to their production. Specifically, separation and stabilization costs, as well as addition of coenzymes, are critical factors. For example, the cost factor to purify stabilized enzymes aimed for sugar oxidation (C_E) can be calculated as [8]:

$$C_E = F_M C_S F_P F_S / Y^E_{X/S} Y_{E/X}$$

Where F_M is a cost correction coefficient for fermentation relative to sugar, C_S is the cost of sugar ($ per kg of sugar), FP and FS are coefficients concerning the ratio between pure and crude enzyme and stabilized to free enzyme, respectively. $Y_{E/X}$ is the yield of desired enzyme based on microbe mass (kg enzyme per kg cell mass). $Y^E_{X/S}$ is the cell mass yield, based on sugar (kg cell mass per kg sugar), being 0.5 for aerobic fermentations for the production of the desired enzymes. Processes for enzyme overproduction typically haave have values of $Y_{E/X}$ ranging from 0.1 to 0.4 [8].

The total enzymatic turnover based on product weight (TTN_W) is also an important parameter, especially when compared to microbial electrocatalytic systems. The TTNW is typically 1 to 7 orders of magnitude higher for enzymatic systems than for microbial systems [8]. This can be extrapolated to enzymatic electrocatalytic systems, as they have higher reaction specificity and due to their possibility of direct electron transfer when immobilized which also confers longer-term stability as compared to free enzymes.

Of course, the development of ready-to-use and stable low-cost enzyme electrodes is anticipated to become the most critical factor in order to enable enzymatic electrocatalysis for bioproduction at industrial level. The research group oject of the present review is taking pioneering actions in this direction, being one of the leading groups in the fabrication and optimization of good performing electrodes for microbial electrochemical and classical electrochemical systems [1,155,314].

Conclusion

Though the concept of enzyme catalysis has existed since long time, its potential in the production of bioelectricity and biocommodities has recently emerged due to the discovery of bioelectrochemical systems (out of the biosensor applications). Some major bottlenecks that could hinder its industrial application are: the lack of long-term stability of single and multi-enzyme systems, non-uniform enzyme distribution on enzyme-electrodes, aggressive manufacturing procedures (pH, T, strong chemicals), inefficient electron transfer due to enzyme-electrode weak contact, reactant interference and contaminants which leads to the low productivity, current density and coulombic efficiencies. Enzymatic electrosynthesis and paired electrolysis are barely explored fields that offer a chance for industrial innovation towards green

chemistry applications. Success in this direction strongly depends on the advancement made in the enzyme immobilization methods, as well as on the right choices on enzymes, target reactions, materials and composites. Composites with performing electrode material and ionic liquids, magnetic nanoparticles, carbon nanotubes or mesoporous materials seem promising approaches for increasing the enzyme to electrode wiring potential.

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