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Review

Enzymatic Biofuel Cells: A Review on Flow Designs

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Abstract: Because of environmental concerns, there is a growing interest in new ways to produce green energy. Among the several studied applications, enzymatic biofuel cells can be considered as a promising solution to generate electricity from biological catalytic reactions. Indeed, enzymes show very good results as biocatalysts thanks to their excellent intrinsic properties, such as specificity toward substrate, high catalytic activity with low overvoltage for substrate conversion, mild operating conditions like ambient temperature and near-neutral pH. Furthermore, enzymes present low cost, renewability and biodegradability. The wide range of applications moves from miniaturized portable electronic equipment and sensors to integrated lab-on-chip power supplies, advanced in vivo diagnostic medical devices to wearable devices. Nevertheless, enzymatic biofuel cells show great concerns in terms of long-term stability and high power output nowadays, highlighting that this particular technology is still at early stage of development. The main aim of this review concerns the performance assessment of enzymatic biofuel cells based on flow designs, considered to be of great interest for powering biosensors and wearable devices. Different enzymatic flow cell designs are presented and analyzed highlighting the achieved performances in terms of power output and long-term stability and emphasizing new promising fabrication methods both for electrodes and cells.

Keywords: enzymatic biofuel cell; electron transfer; flow design; microfluidic cell; material engineering

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1. Introduction

Due to the current environmental policies, there is a growing interest in alternative ways to produce green energy with no pollutants and emissions. Among the several developed applications, fuel cells represent a promising technology. In detail, fuel cells transform chemical reactivity into electricity by oxidizing fuel at the anode and reduce oxidant at the cathode, using noble metal catalysts, in order to provide an electrical power output, according to the fuel and oxidant availability [1]. In this research field, biofuel cells are an interesting application involving biological catalytic reactions at low temperature, in place of metal catalysts, to generate electricity from electrolysis of fuel and oxidant. Hence, a possible classification can be made considering the biological catalyst:

- Microbial fuel cells (MFCs), using living cells as catalysts;
- Enzymatic biofuel cells (EFCs), having enzyme catalysts at both anode and cathode.

Nowadays EFCs are recognized as renewable and eco-friendly technologies, thanks to their peculiar features as easy miniaturization, portability, potential to produce renewable and sustainable energy [2]. Moreover, enzymatic biofuel cells present, as benefits, the possibility of operating at room temperature, high conversion efficiency, scalability and great versatility, because they can produce electrical power from a wide range of organic substrates.

Over the last decades, many studies have been carried out on the MFC and EFC development. In particular, as described in [3], MFCs possessing lifetimes of up to five years have been developed, and many of them can completely oxidize their fuel but have been limited by low current and power densities. On the other hand, EFCs show higher

current and power densities, whilst limiting by fuel incomplete oxidation and lower lifetime stability. EFCs, first introduced in 1964 by Yahiro et al. [4] are expected to be better candidates as biocatalysts than microbes, not only because of their excellent intrinsic properties, such as specificity toward substrate, high catalytic activity with low overvoltage for substrate conversion, and mild operating conditions like ambient temperature and near-neutral pH, but also due to their low cost, renewability and biodegradability. Possible applications range from miniaturized portable electronic equipment, sensors to integrated lab-on-chip power supplies and advanced in vivo diagnostic medical devices that use reactants available in the ambient environment [5]. The low current density characteristic of EFCs is suitable for a wide variety of self-powered biosensors. Moreover, multiple enzymatic cells can be arranged in series or in parallel configurations, in order to generate enough energy to obtain the desired electrical parameters, thus expanding significantly the possible application areas. EFCs can be also integrated into implantable bioelectronics in living systems. An increasing interest was recently addressed to in vivo tests in many animal species, including mammals. EFCs application in human bodies through minimally invasive devices has also been investigated, reporting examples including contact lenses, which exploit some transparent and flexible materials and use lachrymal fluids, transdermal patches or even tattoos [2]. Despite all the EFCs advantages described above, research efforts are still directed to overcome the main current issues: long-term stability and high power output. Moreover, although enzymes increase the limited output performance of MFCs related to the mass transfer resistances across the cell membranes [6], their high selectivity avoids fuel complete oxidation. Several comprehensive reviews on EFCs, focusing on the materials and techniques to enhance electron transfer mechanism and the possible applications relating to wearable and implantable devices have been realized [1,2,6–13]. Nevertheless, to the best of our knowledge, no review takes into account the performance of flow-based cell designs and the most promising techniques used for electrodes and cell fabrication. Only a mini review exclusively concerning microfluidic EFCs is presented in literature to date [14]. Therefore, the main aim of this review concerns an in-depth analysis on flow-based configurations for enzymatic biofuel cells, assessing and comparing both microfluidic and non-microfluidic designs in terms of electrochemical performance and stability over the time. Specifically, a great emphasis has been placed on several promising cell fabrication methods and cell power output performance.

The review is organized as follows: Section 2 discusses the fundamentals of EFCs, also focusing on the several possible used substrates. Section 3 describes the typologies of the typical enzymes employed in the EFCs and the distinction between direct and mediated electron transfer, emphasizing their advantages and limits. Moreover, the main immobilization techniques and the electrode material engineering are illustrated. In addition, the standard electroanalytical techniques used to define the electroactivity of the electrodes and the cell are presented. Section 4 deals with the analysis of different cell designs for flow-based enzymatic biofuel cells, making a distinction between microfluidic and non-microfluidic EFCs and according to the employed fabrication technique. At the end, the main conclusions and future outlooks concerning the challenges to overcome the current limits of the aforementioned technologies are discussed.

2. Enzymatic Fuel Cells: Fundamentals

Enzymatic biofuel cells represent a particular fuel cell in which biological catalysts (enzymes) are used for fuel oxidation at the anode and oxidant reduction at the cathode [1]. An EFC can directly transform chemical energy into electricity through reactions involving biochemical steps [15]. The operating mode of an EFC resembles the functioning of conventional fuel cell: first, a fuel undergoes an enzyme-catalyzed oxidation at the anode side. This reaction releases electrons that reach the cathode side through an external circuit. In the cathode, an oxidant (usually O_2) is reduced (Figure 1). Thus, the electric current flows according to a potential difference, and subsequently, an enzyme-catalyzed reaction involving a fuel (substrate) generates electrical power.

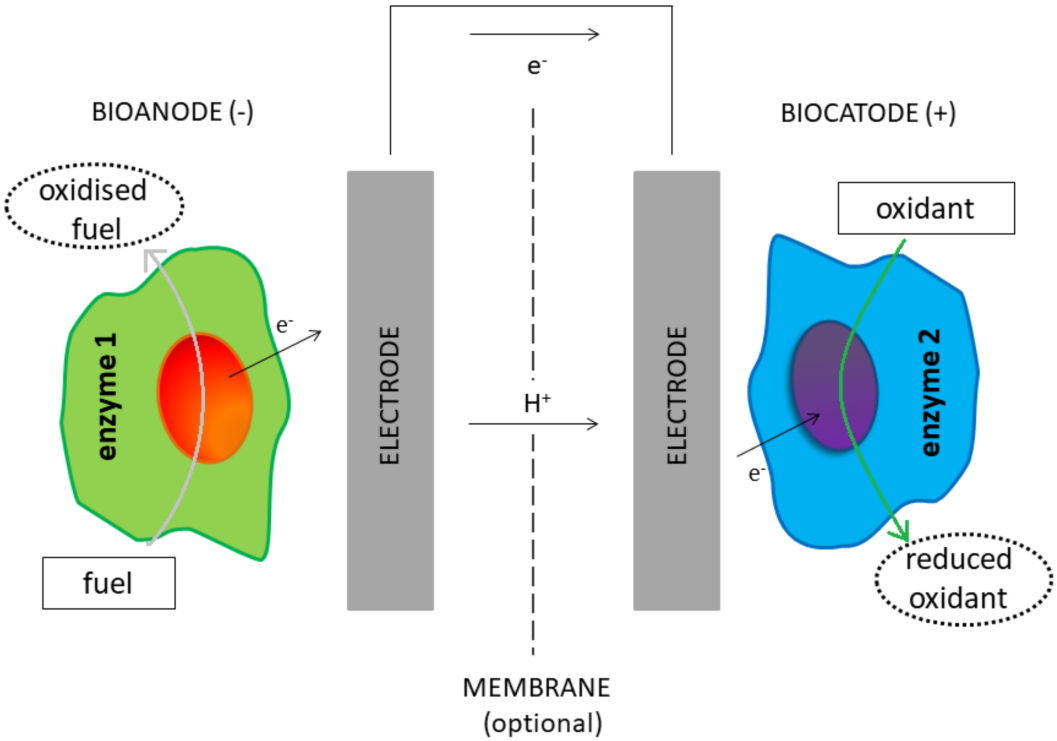


Figure 1. Schematic representation of an enzymatic biofuel cell (EFC) device.

In detail, as mentioned in [1], the power output of an EFC is obtained by the product of the cell voltage and the current. Cell voltages depend on many parameters, as the fuel and oxidant selected, the rate of electron transfer, the resistances within the cell (Ohmic losses) and the mass transport processes. Nowadays, the most common fuel sources are sugars. Among them the most investigated fuel is glucose, thanks to its high energy density (4430 Wh/kg) [11,16] and its wide abundance both in environment and human blood. The estimated thermodynamic reversible cell voltage for the complete oxidation of glucose to carbon dioxide and water, according to Equation (1), is 1.24 V at 298 K, considering the standard Gibbs free energies of formation of all the reaction components. This value corresponds to the maximum cell voltage expected from an enzymatic biofuel cell under standard conditions, considering a coulombic efficiency of 100% without overpotentials or ohmic losses. The complete oxidation of glucose for the extraction of 24 electrons can be only realized by multiple enzymes operating in sequences. For this reason, scientific research has mainly focused on the use of a single enzyme at the anode to oxidize glucose to gluconolactone, as shown by Equation (2), extracting only 2 electrons per mole of glucose and obtaining a maximum reversible cell voltage equal to 1.18 V. The maximum cell voltages of EFCs are commonly calculated by the difference between the formal redox potentials of the enzyme cofactors, in the active site, for anode and cathode.

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O + 24\,H^+ + 24\,e^- \quad (1)$$

$$C_6H_{12}O_6 + \frac{1}{2}O_2 \rightarrow C_6H_{10}O_6 + H_2O + 2\,e^- + 2\,H^+ \quad (2)$$

Beside fully enzymatic cell devices, hybrid systems constituted by the combination of a biocatalysts at the anode and an inorganic catalyst (mainly a noble metal, as Platinum) at the cathode have also been developed. Hybrid cells produce higher power density with respect to enzymatic cell operating in the same conditions. The use of metal catalysts at the cathodic side leads to overcome the main issues related to the low activity of enzymatic biocatalysts for the oxygen reduction reaction (ORR), which constitute one of the most important limiting factors for EFCs application [17].

Substrates

Enzymatic fuel cells are able to harness power from a wide variety of renewable biological sources, employing many organic compounds derived from biomass or intermediates metabolized in living organisms. This fuel diversity represents a substantial difference between EFCs and the traditional fuel cells catalyzed by rare metals, which are predominantly powered by hydrogen or methanol. Among the broad variety of fuels potentially used in EFCs, many factors have to be taken into account in terms of EFCs development, such as their availability, possible toxicity, energy density and cost [18].

The fuels most commonly used in EFCs are different types of sugars derived from lignocellulosic biomass (xylose, fructose, sucrose and polysaccharides), since they are particularly abundant, renewable, inexpensive and safe to handle. Among them, glucose is widely employed in EFCs, due to its high theoretical energy density (4125 Wh L^{-1}), released in the case of complete oxidation to carbon dioxide and water, resulting in the production of 24 electrons per glucose molecule. Moreover, since the concentration of glucose in human blood is enough to supply an enzymatic cell, glucose-based EFCs are particularly suited for implantable applications, as potential alternatives to some traditional medical devices. The concentration of fuel is considered a key issue for a correct EFC operation: high substrate concentration is often a limiting factor for EFCs operation, due to possible severe crossover problems which lead to a decrease in system performance [18].

Other fuels are also appealing for EFCs. Renewable hydrogen produced from biomass or water splitting, characterized by one of the highest energy density values per mass, can be employed in EFCs catalyzed by hydrogenases. Methanol and ethanol derived from biomass degradation are also promising power source alternative to hydrogen in enzymatic fuel cells, thanks to some advantages, such as wide availability and low cost. Moreover, the energy densities of methanol (4047 Wh L^{-1}) and ethanol (5442 Wh L^{-1}) are, respectively, comparable and even higher than glucose. Glycerol (energy density 6260 Wh L^{-1}) is also appealing as fuel for EFCs: it is abundant, since it is a by-product of biodiesel production, and possesses important properties, as high energy density, low toxicity, low flammability and very low vapor pressure. Pyruvate (energy density 4594 Wh L^{-1}), an intermediate of the glycolysis pathway, has also been used to power EFCs. Moreover, it requires fewer enzymes than glucose for the complete oxidation to CO_2 and water.

3. EFC: Electrodes Design

3.1. Enzyme Typologies

Different types of enzymes can be employed in EFCs, depending on the substrate and the degree of degradation to be obtained. The majority of them work at ambient temperature and pH value close to the neutrality, thus allowing their exploitation at physiological conditions in medical devices. Nevertheless, a class of enzymes, as thermostable bilirubin oxidase (BOD) and hyperthermophilic O_2-tolerant hydrogenase, is able to deliver energy in a wide range of temperatures (30–80 °C) when immobilized on carbon nanofibers [19].

The most commonly used enzyme for glucose oxidizing bioanodes is glucose oxidase from Aspergillus niger. This biocatalyst provides very high specificity, activity and stability towards β-D glucose, present in biological fluids, compared to other glucose oxidizing enzymes [20]. Furthermore, the intrinsic co-factor, Flavin adenine dinucleotide (FAD), has an advantageous redox potential (around −0.45 V vs. Ag/AgCl) for use in the design of bioanodes. However, some concerns are associated to the use of GOx, which is a particularly large enzyme, characterized by a molecular weight of around 160 kDa and an average diameter of 8 nm. As a consequence, the active site of the enzyme and the FAD cofactor are deeply embedded inside the protein matrix. The large enzyme size assures high stability in terms of catalytic activity but causes, at the same time, steric constraints, since the deeply embedded active site results in a long electron tunneling distances, which makes difficult the electron transfer mechanism.

The enzymes mostly used in oxygen-reducing biocathodes in EFCs belong to the multicopper oxidase (MCO) family. This category of enzymes is characterized by a set

of four copper centers, which act as active sites for electron transfer. Specifically, since T1 copper center is located near the surface of the protein, many strategies in biocathode fabrication are directed to minimize the distance between the electrode and the T1 center through a correct orientation of the immobilized enzymes. In particular, Laccases and bilirubin oxidases (BOD) from specific organisms have been selected for the high redox potential of their T1 copper center, which determine low-overpotential in oxygen reduction electrocatalysis. However, some drawbacks are associated to the use of these enzymes, especially related to in vivo applications: low catalytic stability and inhibition by common substances present in physiological fluids, such as chloride (for Laccase) and urates (for BOD). Furthermore, Laccase shows low activity at neutral pH. Compared to Laccase, BOD is more resistant to chloride and it has also higher activity towards oxygen reduction in neutral solution [21]. Many efforts in the research activities are recently focused to find different enzymes derived from other organisms or innovative combinations of them to overcome these important issues.

EFCs mostly employ a single enzyme to perform the partial oxidation of a fuel (i.e., glucose, lactate, pyruvate, ethanol). The complete fuel oxidation requires several enzymes, in order to use the total energy content available in the fuel, increasing the overall energy efficiency and power output [18]. Therefore, one key issue in the development of EFCs characterized by high energy density is the effective design of multi-enzyme systems that can completely oxidize the fuel. Depending on the substrate, different pathways can be followed for its complete degradation to carbon dioxide and water. As an example, glucose can be oxidized through glycolysis to pyruvate, which is subsequently oxidized to acetyl-CoA by pyruvate dehydrogenase. Therefore, EFC design has to simulate the natural substrate degradation process on the basis of the metabolic pathways in living cells, thus requiring specific sets of enzyme cascades.

Enzymes are particularly selective to substrate. The majority of EFCs fed by glucose are based on a single oxidoreductase enzyme (i.e., glucose oxidase or nicotinamide adenine dinucleotide (NAD)-dependent glucose dehydrogenase), generating only 2 of the total 24 electrons per glucose molecule. In order to achieve more complete oxidation of glucose, highly efficient anodes for glucose-based EFCs are developed, as an example by combining pyranose dehydrogenase and cellobiose dehydrogenase, resulting in up to six electrons obtained by the oxidation of one glucose molecule. Moreover, a bioanode following a six-enzyme cascade was proposed to glucose full oxidation to CO_2.

However, the use of enzyme cascades leads to an increase in system complexity and introduces several problems. Firstly, the amount of each type of immobilized enzyme is limited by a fixed number of anchoring points on the surface of the electrodes. Moreover, due to the marked enzyme specificity, it is difficult to find optimal operating conditions, specifically in terms of temperature and pH values, for all the steps of the process, thus reducing system efficiency. Additional concerns for the employment of enzyme cascades regard the overall stability of the enzymatic fuel cell, which is limited by the enzyme that possesses the lowest stability and the electrode fouling, depending on both enzyme and cofactor degradation.

3.2. Electron Transfer Mechanism

One of the most crucial aspects regarding EFCs operation and the optimization of performance parameters concerns the realization of an efficient electrical wiring of biocatalysts with the electrodes. The electron transfer depends on many factors, as the structure of the enzyme, the location of the active site, the material constituting the electrodes and the typology of the connection between the enzyme and the electrode. Specifically, in flow systems design, particular attention has to be devoted to the effectiveness of electron transfer mechanism, due to the potentially lower contact time between the fresh substrate and the enzyme, related to the catalytic turnover number. The electrical wiring refers to the electrons transfer (ET) involved in the redox process from the active sites of the biocatalyst to the electrode and vice versa, at anode and cathode sides, respectively. Indeed, according

to [22], assuming an enzymatically catalyzed oxidation of a substrate (e.g., alcohol, lactate, glucose, etc.) using an oxidoreductase as biological recognition element, either a prosthetic group integrated within the enzyme (i.e., FAD, pyrroloquinoline quinone (PQQ), transition metals, etc.) or a co-substrate (e.g., NAD+, FMN, etc.) has to be reduced, immediately storing the transferred redox equivalents. Consequently, a signal is only produced when the ET between the intermediately reduced enzyme (i.e., the prosthetic group or the co-substrate) and the electrode is allowed. It implies a high kinetic ET mechanism in order to develop high sensitivity and fast response EFCs. Concerning the process discussed above, ET can be distinguished in direct electron transfer (DET) and mediated electron transfer (MET).

As described in [22], Marcus theory explains that ET kinetics between two redox species are driven by the potential difference, the reorganization energy and the distance between the two redox centers. Hence, DET mechanism, which involves electron tunneling mechanism, is often hindered by the long distance between the prosthetic group, shielding by the protein shell, and the electrode surface. Nevertheless, 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 [23]. In order to overcome this issue, MET mechanism has been implemented and studied in several papers. MET consists in the use of small, artificial substrate/co-substrate, electroactive molecules (i.e., mediators) that are able to extract electrons from the active sites of the enzyme and shuttle them between the enzyme and the electrode [1]. Anyway, in MET the thermodynamic redox potential of mediator(s) contributes to the reduction of EFC cell voltage. Thus, a more positive redox potential for oxidative biocatalysis (at anode) and more negative for reductive biocatalysis (at cathode) is needed with the purpose to provide a driving force for ET between enzyme active site and mediator, both contributing to EFC voltage losses. Although the choice between MET and DET is strictly dependent on the type of used enzymes, the challenges and complications concerning the rely on electron shuttles in MET are pushing research to look for enzymes capable of DET [24]. The direct electron transfer represents an ideal case that results in optimal cell voltages and maximum current outputs. DET needs of a direct connection between the enzyme and the electrode, which depends on active site position and orientation inside the protein. Specifically, the electronic contact between the enzyme and the electrode can be realized only when the distances between the active site and an electron relay in the enzyme and between the electron relay and the surface of the electrode are lower than the tunneling distance (about 1.5 nm). Consequently, despite the advantages related to DET configuration, current densities are usually lower than the ones associated to MET mechanism [25].

3.3. Immobilization Techniques

At first, enzymatic biofuel cells have been mostly developed using enzymes placed in solution, enabling the electron transfer from the enzyme biocatalysts to the electrode thanks to redox couples acting as mediators. In this case, the rate of electron transfer is generally limited by diffusion properties of the redox species [5]. Enzyme immobilization on electrode surfaces is currently the most investigated method to improve electron transfer rates, thus playing a significant role in the long-term stability of both DET and MET mechanisms. Several studies have been carried out on the different enzymes immobilization ways; according to the immobilization typology, the proposed techniques can be classified as follows [12]:

- Physical Immobilization;
- Chemical immobilization.

Physical immobilization is characterized by weak interactions between the enzymatic molecule and the material acting as support. As illustrated in Figure 2, this type of immobilization can be realized by adsorption on a solid support or through entrapment within a matrix.

(**a**) (**b**)

Figure 2. Enzymatic physical immobilization techniques: adsorption (**a**) and entrapment (**b**).

Physical adsorption is the simplest technique for enzyme immobilization. Adsorption phenomenon relies on the formation of surface interactions, mainly Van der Waals or ionic forces, between enzymes and support. Specifically, the ability of an enzyme to be adsorbed depends on its affinity for the support, mostly related to the presence of specific chemical groups exposed on the material surface. This immobilization method is often obtained maintaining the support immersed in an enzymatic solution. Enzymes physical adsorption can be realized on metal electrode planar surfaces or by using 3D structured bioelectrodes made of nanomaterials to improve electrocatalytic activity and operational stability. Nano-structure-based bioelectrodes provide also a suitable microenvironment for enzyme immobilization, preventing effectively both enzyme desorption and denaturation. Moreover, surface properties as hydrophobicity or the presence of surface charges and particular functional groups can affect bioelectrode activity [18]. The enzymes entrapment into a matrix can be realized by forcing free enzymes to be included into a not reactive porous media, usually polymer or membrane-like matrices, inorganic frameworks, or in the meshes of a gel. As with adsorption technique, only weak surface bonds are formed between the enzyme and the matrix. With respect to physical adsorption, the amount of leaching is reduced, and denaturation or conformational changes can be avoided. Nevertheless, since some matrices are characterized by poor conductivity, other materials with high conductivity, such as carbon nanotubes (CNTs), or redox mediators, such as ferricyanide or 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), can be co-encapsulated, thus obtaining stable bioelectrodes with high electroactivity [18].

On the other side, chemical immobilization is achieved when the enzymes are linked to the support through chemical bonds, thus involving strong interactions. In this case, a stronger degree of interaction between the enzymes and the electrode surface is realized, leading to higher stability of the bioelectrodes. The mechanisms of chemical immobilization are based on covalent and cross-linking methods (Figure 3).

(**a**) (**b**)

Figure 3. Enzymatic chemical immobilization techniques: covalent bonds (**a**) and cross-linking (**b**).

Covalent immobilization implicates the formation of covalent bonds between the enzyme and an activated support. In particular, the use of suitable linkers functionalizes the support, so that it can expose specific functional groups, able to react with the enzymatic molecules. Frequently, the NH_2 amino groups of the side chains of the amino acids peculiar to the enzyme to be immobilized are involved in the chemical bond. The main advantage of this type of immobilization is the greater enzyme stability towards the variation of the operating conditions, as temperature, pH and the concentration of organic solvents, thus drastically reducing the loss of enzymes. Moreover, the covalent binding prevents enzymes orientation changes, thus improving the electron transfer at the electrode interface [18]. Enzyme leakage is also strongly reduced, and an increase of bioelectrode

lifetime is consequently expected. On the contrary, the formation of covalent bonds can provoke important conformational changes in the enzymatic molecules, which can affect the correct orientation of active sites, reducing the catalytic activity of the enzyme [26].

Cross-linking is a simple and effective method for enzymes immobilization. The cross-linking technique involves the creation of covalent bonds between the proteins through the use of bi or multi-functional chemical species, resulting in the formation of rigid and insoluble enzymatic aggregates that significantly reduce enzymes mobility, thus improving system stability. Differently to the covalent immobilization, the strong interactions enzyme–electrode are realized without the use of a support [27]. Glutaraldehyde is frequently employed as bifunctional group to favor enzymes reticulation, inhibiting enzyme leaching and enhancing enzyme stability [21]. However, a drawback of this method is the heterogeneous distribution of enzymes orientation, which can determine slower electron transfer rates [18].

3.4. Electrode Materials Engineering

As described in [28], major improvements in EFCs have actually been due to the incorporation of new materials, as well as the engineering concerning the electrochemical cell design. This paragraph focuses on materials engineering, while cell design is discussed in the following Section 4. Materials development paths can be classified according to four approaches: (1) nanomaterials improving the electron transfer mechanism from the biocatalyst to the electrode surface, (2) materials that offer greater stability and immobilization of the biocatalyst, (3) materials that increase the conductivity and surface area of the electrodes and (4) materials able to improve mass transport phenomenon.

As reported in [29], the need for delivering enhanced power has resulted in high surface/volume ratio conductive materials as enzyme host matrices. With this aim, in the early 2000s, nanomaterials ranging from carbon nanotubes to gold nanoparticles started to be synthetized and employed [13]. Gold nanoparticles can be used as a conductive substrate to immobilize biomacromolecules, mainly through their interaction with the amino acid ligands in the vicinity of active site in redox proteins. Some distortions in the micro-environment of enzyme cofactors can be observed, resulting in catalytic activity reduction. Suitable modification of aromatic heterocyclic groups or aromatic rings on the interface of nano-gold particles can favor bio-molecules linkage on nanoparticles surface and support DET mechanism inside the electrode [30]. On the other hand, carbon nanotubes and other nanostructured carbon materials have also been used to decrease the overpotential for NADH (reduced form of NAD) oxidation. In the EFCs field, crucial aspects relating to protein interaction and orientation at the nanoscale can be overcome by means of nanomaterials [28]. Indeed, their aspect ratio approaches the molecular scale, making it possible to establish a close association with the material, decreasing the electron tunneling distance. Many research activities have involved the use of covalent and non-covalent carbon nanomaterials focusing on DET improvement for biocathodes, as reported in [31–33]. Reference [29] also proposes new concepts to overcome issues such as the use of new nano- and meso-porous materials for enzyme hosting, their functionalization to improve the wiring of enzymes, the modelling of pore structure and its consequences on enzyme electroactivity and loading, and the role of high surface area materials (HSM) in improving bioelectrode stability and enzyme resistance to inhibitors. According to [24], nanostructured materials can improve the electron transfer by shortening the distance between the deeply buried redox center and the electrode surface, and they can be modified to be more biocompatible in order to improve the stability of enzymes. Specifically, carbon has been widely employed as electrode material, due to its good biocompatibility, high electrical conductivity, and high number of docking sites [34]. In detail, several carbon materials have been explored, such as carbon nanotubes, graphene [35] and pressed carbon nanotubes in buckypaper [36].

3.5. EFC Electrode Performance Assessment

Bio-electrode characterization in terms of electrochemical behavior can be performed by using different standard techniques, mainly including linear sweep voltammetry (LSV), cyclic voltammetry (CV), and amperometry. Researchers employ these methods to evaluate both semi-cell and complete biofuel cell activity [15]. The main goals are the estimation of biocatalyst loading on the electrode surface, the validation of the strategy used for enzyme immobilization, the study of the enzymatic kinetic mechanism, the quantification of the amount of substrates, as well as the presence of possible inhibitors for the enzymatic reaction [37]. These tests can be very helpful to compare the effectiveness of the different enzyme methodologies and to calculate the amount of enzyme that remains active after the anchoring procedure during the immobilization processes. In EFCs performing MET, electrochemical characterization allows also to assess the operation of mediator species and their diffusivity, since, in this case, the thermodynamic driving force depends on the difference between the redox potentials of the enzyme redox center and mediator species.

Linear sweep voltammetry is a voltammetric method that employs the traditional three-electrode setup (working, reference and auxiliary electrodes) and consists in the measurement of current at the working electrode, while a linear variation with time of the potential between the working and reference electrodes is produced. A peak in the current signal is registered corresponding with the potential at which the species begin to be oxidized or reduced. The sensitivity of current vs. voltage variations can be enhanced by increasing the scan rate, since higher potentials per second result in a deeper oxidation or reduction of species on the working electrode surface. Both the bioanodic and biocathodic current peaks increase with scan rate; generally, the current is proportional to the square of the scan rate. The bioanodic and biocathodic current peaks tend, respectively, toward a less positive and more positive voltage potential [38].

Cyclic voltammetry is commonly applied to study reversible systems, in order to obtain data about both the forward and the reverse reactions, examining the redox-reaction mechanism of the electrode. A linear potential variation over time is applied but, differently to LSV, at a desired point, the potential reverses and sweeps back to the starting value. Therefore, CV is a simple and fast technique, useful to initially characterize the reversibility of redox processes occurring in the enzymatic cell. Generally, the analysis of the voltammetric profile of a given system leads to the estimation of the amount of electrons transferred and provides information about anodic and cathodic processes. A sigmoidal-shaped slow-scan rate cyclic voltammogram characteristic of electrocatalytic oxidation of glucose is usually obtained for all the enzymatic electrodes [39]. CV electrochemical tool can be employed on enzymatic cells performing MET to characterize different mediator species with distinct voltammetric behavior (ferrocene, osmium, ruthenium-based complexes, etc.) In the case of DET, cyclic voltammetry is used to confirm the direct electrochemistry of redox proteins on different electrode materials (carbon, gold, etc.).

Chronoamperometry is an electrochemical technique consisting in potential step variation on the working electrode, while the resulting current from faradaic processes is monitored as a function of time. The functional relationship between current response and time is usually measured applying a single or double potential step to the working electrode of the electrochemical system.

Beside electrochemical techniques, many physicochemical methods are often employed in studies involving enzymatic biofuel cells. As an example, fluorescence measurements give important data to understand the interaction between enzyme and support, providing information about enzyme occupation, distribution and stability, especially in the case of polymeric matrixes. In some experiments, the fluorescent emissions are related to the changes in the dipole moment, indicating variation in the hydrophilic character of the local microenvironment, directly linked with the enzymatic activity.

Regarding the bioelectrodes surface morphology, Brunauer–Emmett–Teller (BET) and Langmuir isotherms represent the main methodologies employed in enzymatic biofuel cell to investigate the coverage profile, particularly useful in the case of electrodes based on

porous structures. Moreover, scanning electron microscopy images and X-ray diffraction analysis are usually performed to evaluate the thickness of the immobilized species and the film formation capability.

4. Flow-Based EFC: Cell Design

One of the most crucial aspects relating to EFC development concerns design engineering. Since the first enzymatic fuel cell prototype, researchers have spent many efforts in optimizing cell design, with the purpose of reducing the distance between anode and cathode to increase power output. Main objective of this work is to present a comprehensive review on flow-based EFCs, which result to be promising applications for powering biosensors and wearable devices.

4.1. Microfluidic Configurations

Microfluidic enzymatic biofuel cells (MEFCs) are currently arousing great interest in the scientific world thanks to the continuous operating mode and the possibility to be integrated in various low-powered miniaturized bio-devices [40]. Examples of application areas are in vivo operation to power sensing or micro-recording systems (as, for example, self-powered glucose biosensor), supercapacitors for energy conversion and storage or power generation in remote areas through wireless networks. The advantages obtained at the micro-scale with miniaturized EFCs include higher mass transfer and reaction rates, higher surface to volume ratio (SVR), lower reagent volume and power consumption, automated fluid delivery, faster response times and reduced operational costs.

Microfluidic technology uses fluids working in co-laminar regime: the Reynolds number (Re) related to fluid flow, which equates the inertial force to the viscous force, is typically lower than 2000. By establishing a co-laminar flow, the streaming interface between the fluids inhibits anolyte and catholyte mixing, allowing the development of membraneless configuration (M-MEFCs). In enzymatic fuel cell technology, there is a great incentive to eliminate the separator membrane, which induces high internal resistance, makes the cell bulky and costly and causes often the reduction of cell lifetime. Moreover, membrane can be toxic towards redox enzymes. Although the presence of a membrane separator can be generally avoided due to the specificity of redox enzymes, possible crossover reactions may induce enzyme inhibition by generation of reactive oxygen species, thus reducing the power output. On the other hand, the presence of a physical barrier impermeable to gases is strictly required in the particular case of H_2/O_2 EFCs, in order to avoid the risk of explosion.

In a microfluidic fuel cell system, the processed fluid volumes are very small, usually in the range from femtoliters (10^{-15} L) to microliters (10^{-6} L). The fluids are guided in micro-channels (diameter 1–1000 μm), where parameters, as capillary forces, surface and interfacial tensions, play important roles. Specifically, the viscous forces dominate the inertial forces: the fuel and oxidant flows can be conducted as parallel streams, establishing a virtual interface that physically separates the fluids, enabling at the same time the ionic exchange along the micro-channel. Fluids mix, due to diffusion phenomenon, is limited to a narrow interfacial zone, whose thickness can be controlled by the microchannel dimensions and flow rates [5].

The distance between the two electrodes can be reduced to overcome problems related to the low diffusion of protons; moreover, different electrolytes can be used on the anode and cathode sides, optimizing pH value for each enzyme.

The specificity of enzyme catalysis allows the combination of fuel and oxidant streams in a single manifold [5], with multiple benefits in terms of fuel cell design and operation. Firstly, the use of a proton exchange membrane can be avoided, thus eliminating the water management issues associated with PEM (Proton Exchange Membrane) fuel cell technologies. Furthermore, requirements related to sealing, manifolding and fluid delivery are significantly reduced [10]. The optimization of electrical parameters leads also to the decrease of internal ohmic losses. The reaction kinetics for both anode and cathode can

be improved by adjusting the fuel and oxidant streams composition, in order to obtain optimal enzymatic activity and great stability [41].

An important feature of this technology is the reduced costs, also related to the absence of membranes, thus making enzymatic fuel cells competitive for small-scale power supplies with other conventional systems. On the other hand, biological enzymes are abundant, since naturally derived from organisms or produced using low-cost fermentation techniques. Other advantages are the possibility of operation at room temperature and the realization of compact units produced by well-established and often inexpensive microfabrication techniques [41].

Microfluidic EFCs are compatible with simple microfabrication methods, such as soft lithography, prototyping xurography and paper-based technology. The fabrication process mainly employed to realize single microchannel is the soft lithography, while multi-level and 3D microfluidic systems can be based on the stacking of different technologies [41]. Microfluidic prototypes manufactured with soft lithography technique are usually made in poly-dimethylsiloxane (PDMS), an inert elastomeric liquid organic polymer easy to handle and cost-effective, characterized also by important features for potential implantable devices, as biocompatibility, flexibility and transparency. Other polymers sealed to solid substrate, as glass or silicon, can be also used. Soft lithography procedure allows the design of microfluidic enzymatic cells with different geometries: T-shaped, Y-shaped or I-shaped. For what concerns soft-lithography, a sustained power production from continuous flow-through EFC up to one month without the use of external redox mediators has been demonstrated in [42].

Xurography technique allows the fast realization of very thin microstructures (down to 20 μm) using various flexible polymer films. In paper-based devices, fluids flow via capillary action through a passive liquid transport without the need of an external pressure source. A fully wetted flow regime is established whose flow rate depends only on the paper type. Porous filter papers are used as matrix, frequently by attaching an absorbent pad at one end of a paper strip in order to realize a self-pumping. These microfluidic systems are biocompatible, disposable and cost-effective. They also present good compatibility with many chemicals and can be combined with other low-cost materials, such as plastics, to offer additional mechanical support.

4.1.1. 3D-Printing MEFCs

3D-printing technology has been recently applied to MEFC systems. This fabrication method is particularly suited for the realization of micro-channels for miniaturized devices and allows also the production of many types of composite materials. It also offers many advantages, since is simple, cost-effective and time-efficient, eliminating the requirement of post-processing activities [43]. In [42], the authors demonstrate a sustained power production from continuous flow-through enzymatic biofuel cells for periods of up to one month without the use of external redox mediators. Two different designs carried out by means of 3D printing were compared for use in blood vessels (Figure 4). Indeed, the continuous flow through operation leads to the benefit by having higher concentrations of glucose and oxygen by means of uninterrupted supply of blood. The electrodes were made by highly porous gold (hPG) because of their remarkable properties, such as high conductivity, non-toxicity, large surface area, three-dimensional open porosity and biocompatibility. In the first design, the anode and the cathode are fit in two parallel channels separated by a PDMS wall (Figure 4A). The second design is characterized by a single channel containing the two electrodes, which are positioned so that the electrolyte would flow over the cathode and the anode sequentially (Figure 4B). Nevertheless, these designs show, with reference to the polarization curves in Figure 5, an OCV 90 mV lower, due to the back diffusion of H_2O_2 to the cathode that causes an electrochemical short-circuit at the Laccase electrode. Both configurations were tested by continuously feeding with an aerated PBS (phosphate buffered saline) solution containing 27 mM glucose, at a rate of 0.35 mL/min and under the constant temperature of 37 °C.

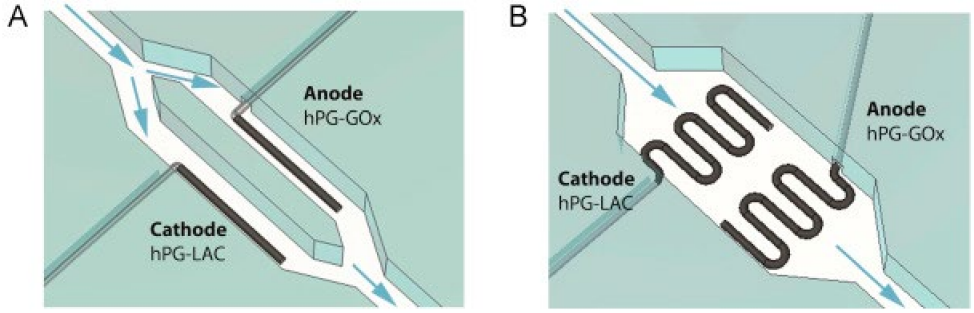


Figure 4. The two 3D-printing studied microfluidic enzymatic biofuel cells (MEFCs). (**A**) configuration with two parallel flow channels, (**B**) configuration with one single flow channel [42].

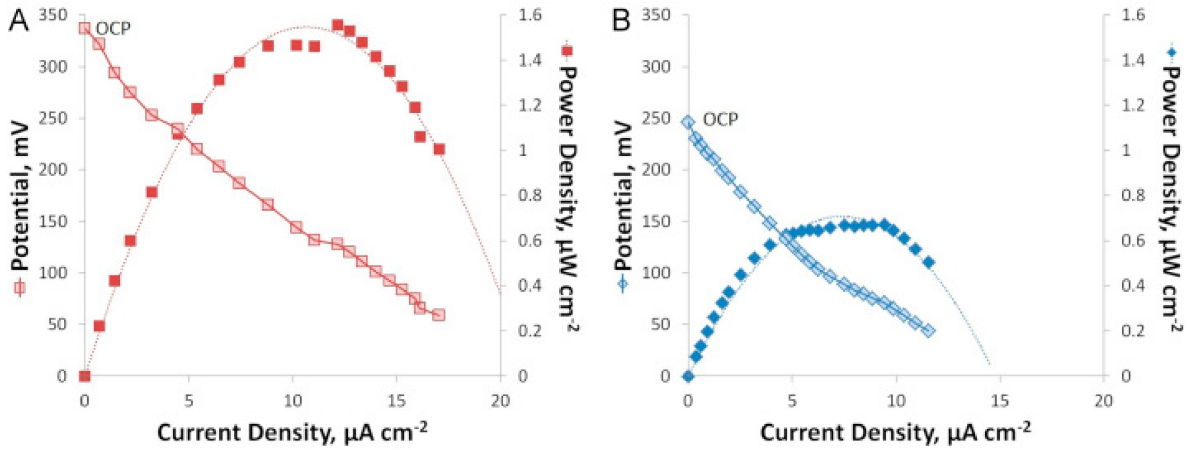


Figure 5. Potential and power density as function of the current density for the two investigated cells, indicated in Figure 4. (**A**) Open circuit potential and power density for cell with two parallel channels, (**B**) Open circuit potential and power density for cell with one single channel [42].

Moreover, configuration A was assessed as more stable during the time, exhibiting continuously an enzymatic activity for a period up to 1 month, as evident in Figure 5 in terms of registered power output.

The power output of miniaturized MEFCs can be improved through cell stacking. In [44] a micro-EFC stack composed of 3D printing four cells is realized with an air-breathing cathode design: the channels, made of silicone elastomer film, are contained between two plates made of poly-methyl methacrylate (PMMA), with two separated inlets for the anolyte and the catholyte (Figure 6). The same electrolytes and fuel are reused for all the cells, obtaining a cascade-style microfluidic device: the resulting decrease in glucose concentration doesn't affect system performance, since a very low amount of fuel is used in each enzymatic cell, limiting, at the same time, the cross-over phenomenon. The cells in the stack are connected employing different configurations, as series, parallel or combined series/parallel, as indicated in Figure 6.

The maximum open circuit potential (OCP) (1.27 V) was achieved in series configuration, while the highest current density (2007 μA cm^{-2}) and power density (579 μW cm^{-2}) are obtained in the case of parallel connection. As interesting output of this research activity, the parallel-series (P-S) connection is characterized by a voltage close to the serial configuration (1.23 V). Generally, the OCP values obtained were lower than the expected ones according to Kirchhoff's law, due to the different performance and the high total resistance of each individual cell and the presence of shunt currents between cells. Potential applications of the system are devices that demand low currents and a voltage above 1.2 V, as an alternative to the use of boosters or current transformers.

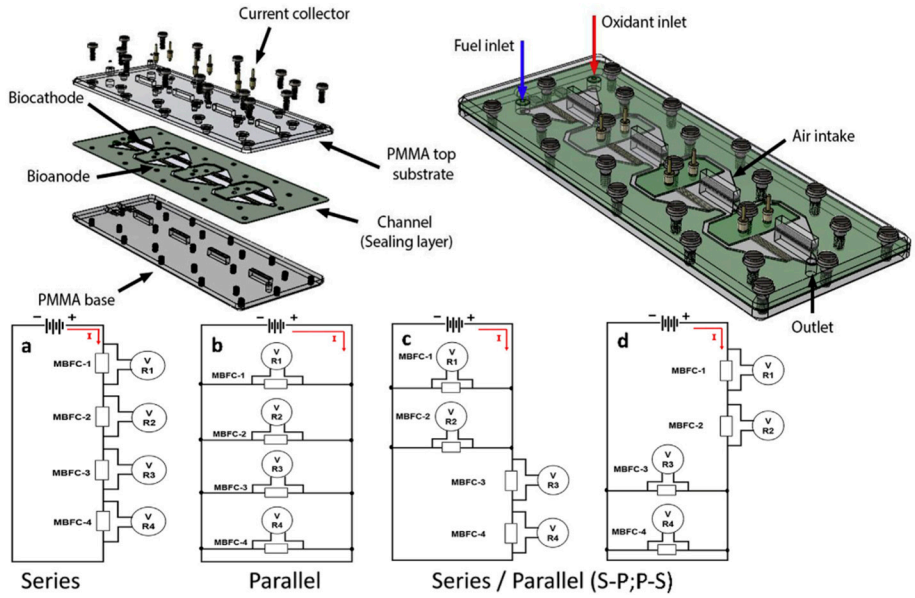


Figure 6. A schematic illustration of the micro-EFC stack realized by the integration of four individual cells and diagrams of the investigated cell connections. Configuration (**a**) corresponds to series connection of the cells, (**b**) to parallel connection, (**c**,**d**) to a mix of series and parallel electrical connections [44].

4.1.2. Soft Lithography MEFCs

Another design based on laminar flow is illustrated in [45]. The authors developed a device fabricated using a two-part PDMS elastomer and a standard soft lithography method (Figure 7). This cell consists of a Y-shaped microfluidic channel in which fuel and oxidant streams flow laminarly in parallel at gold electrode surfaces. At the anode, the glucose is oxidized by the enzyme GOx whereas at the cathode, the oxygen is reduced by the enzyme Laccase, in the presence of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) redox mediators. In this work, a sensitivity analysis was carried out concerning the evaluation of the optimal flow rate in order to enhance the current density of the cell by limiting the mass transport effect, while accepting a convective mixing in a narrow depletion boundary layer. Specifically, the OCV of 0.55 V and a current density up to 0.69 mA cm^{-2} were registered. The maximum power density (obtained with a flow rate of 1000 µL/min) delivered by the assembled biofuel cell reached 110 µW cm^{-2} at 0.3 V with 10 mM glucose at 23 °C.

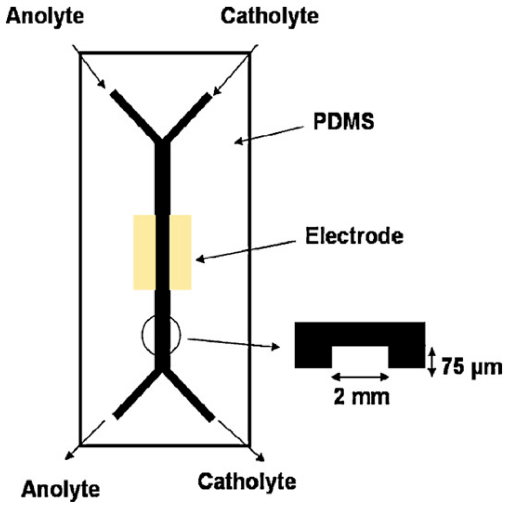


Figure 7. Scheme of Y-shaped microfluidic EFC. Reprinted from [45], with permission from Elsevier.

4.1.3. Xurography MEFCs

Xurography technique, frequently used for MEFCs manufacturing, is based on the use of a cutter plotter applied on thin and flexible, adhesive or double adhesive, polymer films. The width and length of the microfluidic channel is defined by the patterning process, whereas the height of the channel is related to the thickness of the film. Therefore, this fabrication process is easier and significantly less expensive than conventional photolithography-based methods, avoiding clean room facilities, costly photomask and photoresists [41]. The related microfluidic EFC devices are compact micropower sources, able to deliver power output in a minimum volume, thus favoring the scale-up of the manufacturing process. Moreover, xurography method is very fast, allowing the production of microfluidic chips in a few minutes. The first developed membraneless glucose/O_2 microfluidic biofuel cell with laminated materials based on adhesive polymer led to the realization of a device constituted by a single Y-shaped microchannel with immobilized enzymes on pyrolyzed photoresist film electrodes.

By using the xurography methodology, it is also possible to realize 2D and multi-level microfluidic enzymatic cells, constituted by arrays of microchannels both in series or parallel configurations [41]. The fabrication process of the microfluidic device implicates the stacking of double-sided adhesive tape layers alternated to transparent sheets and equipped with appropriate holes to connect the microchannels for substrate feeding. The multi-level microfluidic enzymatic cell is developed in order to increase the power delivered by a single device in a minimum volume, operating with glucose and oxygen solutions (Figure 8). Two electrode configurations are used, with the electrodes connected in series, to maximize the voltage (Figure 8a) and in parallel, to increase the electrical current (Figure 8b). Two layers of double-face adhesive film are successively deposited, one to form the microchannels in contact with the electrodes and the other to obtain the correct distribution of fluids, which flow both vertically and laterally without mixing (Figure 8c). The two double-face adhesive films are then sealed by means of two semi-rigid transparent films, obtaining the final structure of the enzymatic device shown in Figure 8d.

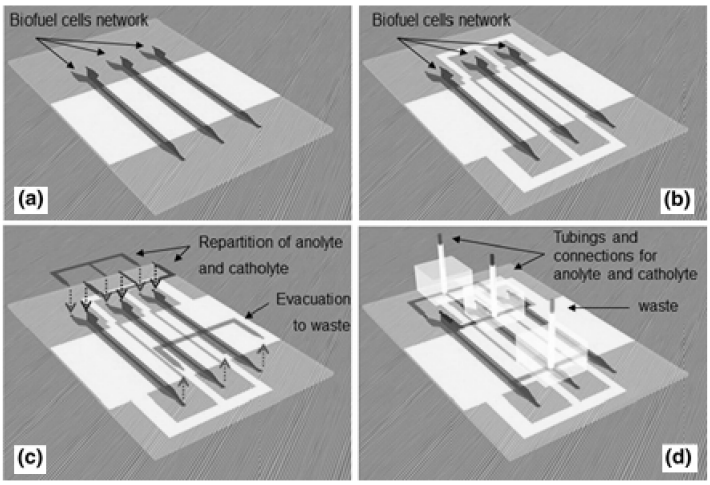


Figure 8. Multilayer microfluidic biofuel cell fabrication based on xurography technique used in [41]; (**a**) serial configuration of electrodes; (**b**) parallel configuration of electrodes; (**c**) schematic view of repartition layer; (**d**) final structure of the enzymatic microfluidic chip.

The experimental results show that the system is 183% more efficient, in terms of maximum delivered power, than the 2D device based on a single microchannel with the same chemical energy and the same number of inlet and outlet holes. The interest on this multi-layer system relies on the maximization of power output in a minimum volume, which is an important challenge for microfluidic biofuel cells operation. The choice between the two configurations depends on the target application and the input resistance of the system supplied by the EFC.

4.1.4. Paper-Based MEFCs

A different approach towards the development of a cost-effective enzymatic glucose/O_2 microfluidic fuel cell is the realization of a paper-based system, in which the fluid transport is based on capillary action [40]. An advantage of paper-based matrixes as substrates for microfluidic fuel cells is their intrinsic capability of establishing laminar flow, so that fuel and oxidant streams can flow in parallel without mixing. The capillary flow of reactants allows the movement of liquids without outer pressure sources, thus avoiding the power requirements of external equipment. An important property of the papers selected as substrate for the systems is a high wicking rate, normally obtained by attaching an absorbent pad at one end of a paper strip: the liquid moves through the strip and fills it, reaching the wicking pad and establishing a fully wetted flow regime. In paper-based fuel cells, the increase of cathode dimension with respect to the anode, to compensate the lower activity of oxygen reduction enzymes and the low solubility of oxygen in liquid media, is not feasible, since the planarity of the electrolyte moving in a thin layer of paper imposes a reduced distance between the electrodes, in order to minimize the resistance of the electrolyte [46].

Regarding system design, in [40] the enzymatic cell is supported on a glass slide: the electrodes of the microfluidic cell are made of carbon paper (active area 0.10 cm^2), and their outer parts are connected using a conducting copper foil (Figure 9). Two possible configurations, Y-shaped and I-shaped, can be implemented. The most common paper-based system typology is the Y-shaped cell, constituted by two separated inlets for the reactants, resulting in two parallel flows. In order to achieve simplicity in use, in the I-shaped configuration both fuel and electrolytes are added together in the inlet stream, combining in a single flow anolyte and the catholyte components. Therefore, the fuel cell has to work with a single electrolyte using a specific pH value (sodium phosphate buffer solution at pH 5.5), which represents a compromise between anolyte (pH 4.5) and catholyte (pH 7.4) requirements. Glucose oxidase from Aspergillus niger and Laccase from Trametes Versicolor are selected as anodic and cathodic enzymes respectively.

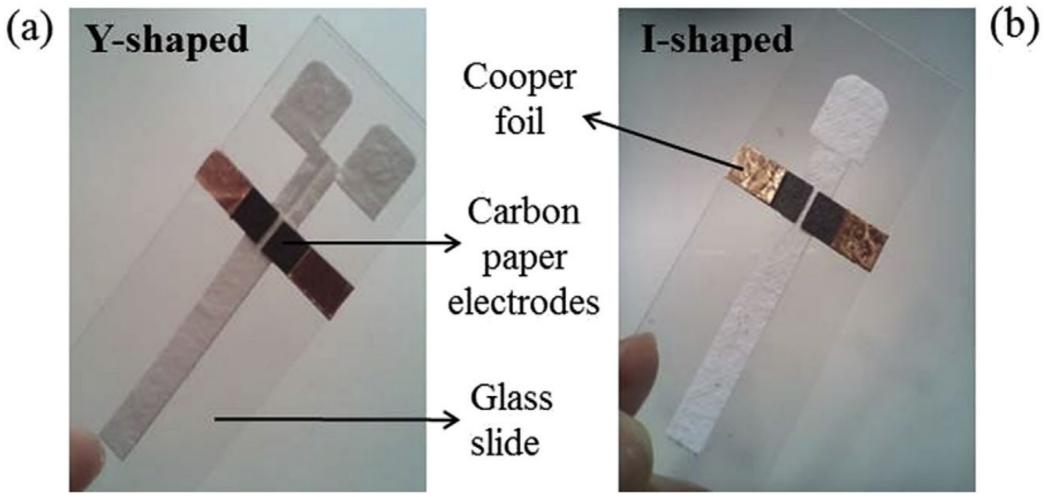


Figure 9. In detail, (**a**) represents Y-shaped paper-based fuel cell and (**b**) I-shaped paper-based fuel cell, as reported in [40]: the system is fixed on glass slides, displaying carbon paper electrodes attached to a piece of cooper foil. Reprinted from [40], with permission from Elsevier.

The experimental results evidence, for the I-shaped cell, a maximum open circuit voltage of 0.55 V and maximum current and power density of about 225 μA cm^{-2} and 24 μW $cm^{-2,}$ respectively (Figure 10). Even if system performance decreases compared to the Y-shaped configuration, the single-stream microfluidic cell can be easily implemented in a real application. These paper-based fuel cells can become an alternative for supplying energy to power microelectronics with low power consumption demand, for example small single use point-of-care devices.

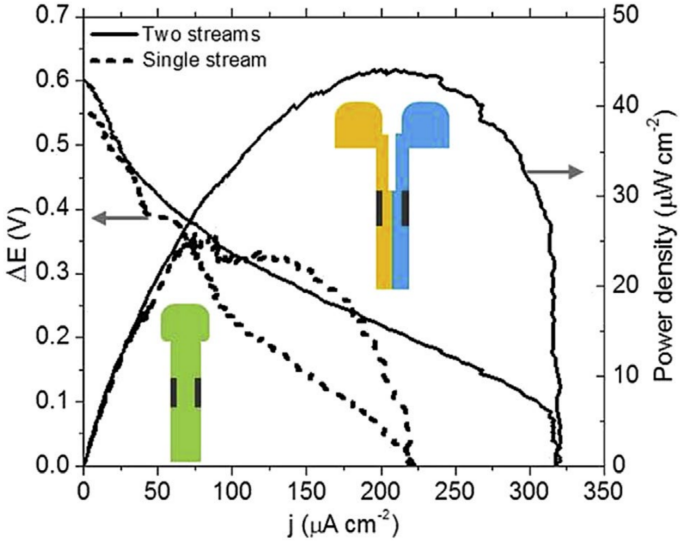


Figure 10. Polarization and power curves of two enzymatic paper-based fuel cells: single stream I-shaped (dotted lines) and two streams Y-shaped (solid lines) configurations. Reprinted from [40], with permission from Elsevier.

Another interesting system design is reported in [46]. The device consists of a thin paper strip acting as flow channel and a circular paper piece, constituting the absorbent pad, placed at one end of the paper strip (Figure 11). Glass fiber characterized by uncompact filaments is used as channel material, thus realizing a capillary flow with very low fluidic resistance. The absorbent pad is made of cellulose: a constant capillary pressure is established, thus obtaining a homogenous fluid front. The flow channel and the absorbent papers are assembled on a transparent poly methyl methacrylate (PMMA) holder, used for fluid storage. In this microfluidic system, operating in physiological conditions (5 mM of glucose and pH 7.4), the liquid moves by capillarity through the first paper strip, in contact with the electrodes, and then reaches the absorbent pad. When the wet volume of paper increases, the fluid front moves from the reservoir; at the same time, the increase of viscous forces decelerates the movement of the fluid. On the other hand, the circular geometry of the absorbent pad enhances the total fluid front surface area as it is pulled into the absorbent material, thus improving the capillary driving force. These two opposing forces allow the realization of a quasi-steady flow rate inside the system. Different flow rates have been obtained by modifying the paper materials that constitute the adsorbent pad of the device. The experimental results demonstrate that an increase in the amount of power and current extracted in a paper-based fuel cell can be attained establishing a quasi-steady capillary flow. Moreover, it has been proved that the convective mass transport induced by the capillary flow improves the overall fuel cell performance.

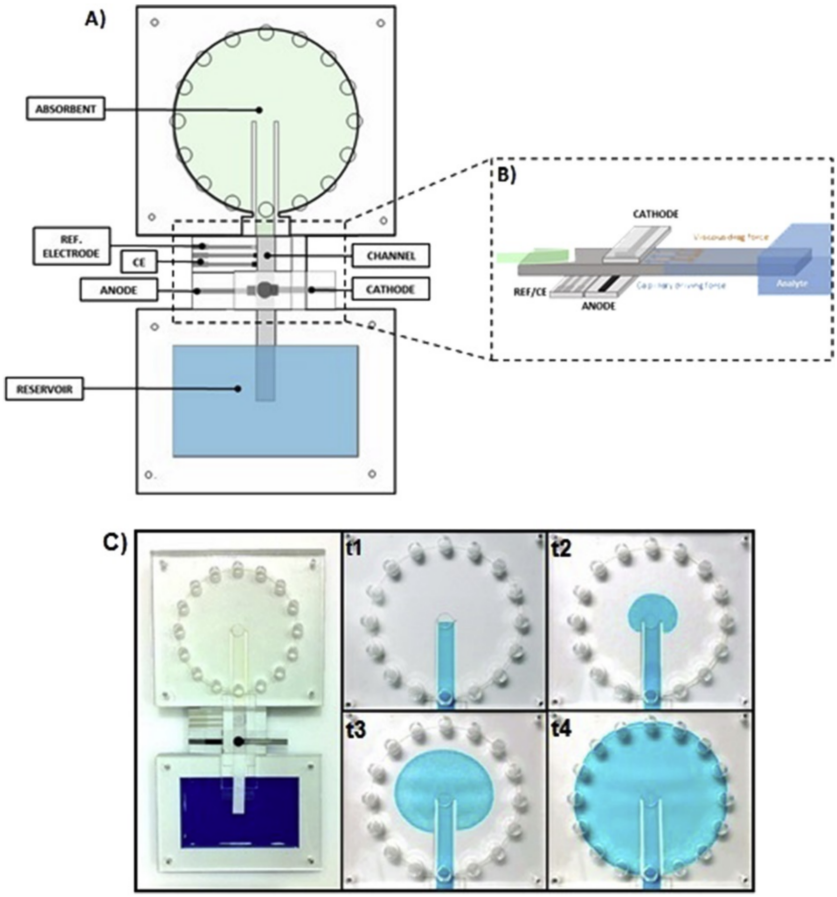


Figure 11. Schematic drawing of the glucose paper-based enzymatic cell described in [46]: (**A**) and cross-sectional view of the individual components (anode/cathode, reference/counter electrodes) (**B**); absorbent pad filling at four different times generating the quasi-steady flow of the device (**C**). Reprinted from [46], with permission from Elsevier.

4.1.5. Microfluidic Fuel Cell Modeling

Mathematical models are often used to understand, predict and optimize the performance of enzymatic electrodes and complete biofuel cells as a function of main experimental parameters, such as the amount of substrates, the loading of biocatalysts, the diffusivity of the different species, the effectiveness of the used strategy for enzyme immobilization and the influence of mediator species or possible inhibitors [37]. The aim is the enhancement of microfluidic enzymatic fuel cell structure, in order to provide guidelines for the realization of novel architectures in the design and fabrication techniques and to reduce the time involved in prototyping, building and characterizing the actual devices [6].

Numerical CFD models of microfluidic enzymatic fuel cells are present in literature, including Navier–Stokes equations for conservation of mass and momentum, the equation for mass conservation of solute species and the equation for the current. The first computational study related to the MEFC technology investigated the complex mechanism involving species transfer, heterogeneous chemical reactions and enzyme kinetics based on microchannel geometry [5]. The authors tested different enzyme patterning strategies, involving both spatially distributed or mixed enzymes on the electrode surface, with the purpose to optimize overall current density and fuel utilization. According to the model, a decrease of flow rates leads to fuel utilization improvement, while higher enzyme turnover numbers are responsible for the enhancement of system performance. Enzymatic fuel cell activity was shown to be limited by the reaction rates associated with enzyme kinetics, rather than by diffusion phenomena. Consequently, system optimization can be achieved

implementing mixed enzyme patterning tailored with respect to individual turnover rates, enabling high current densities combined with nearly complete fuel utilization.

Regarding oxygen reduction reaction in MEFCs, an interesting simulation study, realized by solving the governing 3-D conservation equations related to flow and species transport, reveals that oxygen availability limits the performance of the cathode [47]. Specifically, an exponential decay in oxygen availability is observed along the length of the microchannels, consistently with experimental observations. The increase of electrolyte flow rates leads to the reduction of the diffusion boundary-layer thickness. Consequently, a decrease of oxygen mass transfer resistance is attained, thus improving mass transport phenomena at cathodic side. However, disparity between anolyte and catholyte flow rates can induce wastage of dissolved oxygen.

Modeling can also be helpful to clarify the complex effects regarding electrochemical aspects and mass transfer in MEFC components. The first developed CFD based model for microfluidic fuel cells considered a T-shaped microchannel using tapered electrodes and proposing methods to improve fuel utilization. Theoretical models for the electrochemical kinetics have also been proposed, considering Y-shaped or F-shaped microchannels. In [48], the work focuses on the development and validation of a complete computational model applied to a microfluidic fuel cell with flow-through porous electrodes. The model takes into account the main phenomena present in a microfluidic fuel cell, including fluid flow in microchannels and porous media, electrochemical kinetics and mass transport, investigating both individual half-cells (anode and cathode) and the entire fuel cell.

4.2. Non-Microfluidic Configurations

A particular membraneless fully enzymatic cell integrating a flow-through anode and an air-breathing cathode (Figure 12) was described in [49]. Fuel was pumped within the cell through a peristaltic pump at a flow rate of 3 mL/min. Specifically, two anodic NAD+-dependent dehydrogenase enzymes (MDH-malate dehydrogenase and ADH-alcohol dehydrogenase) were compared in continuous flow-through operation with the same cathodic enzyme (Laccase).

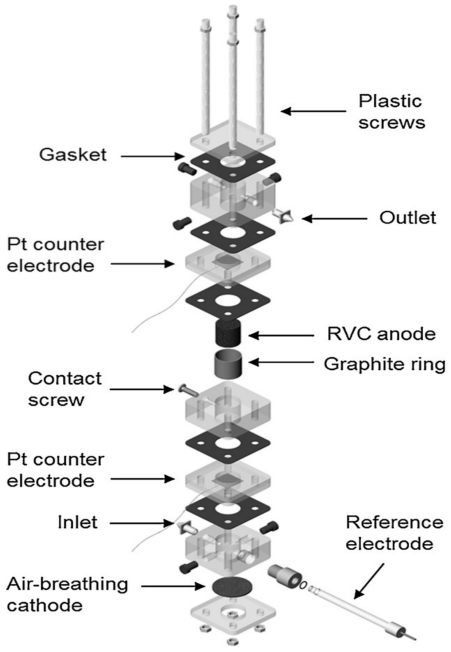


Figure 12. Schematic view of the EFC reported in [49].

For ADH-Laccase EFC, the biofuel cell sustained an OCV of 0.618 V, slightly higher than that of the MDH-laccase biofuel cell (0.584 V). The maximum power density was determined to be about 26 μW cm^{-2} at 0.372 V, which is almost three times higher than

that obtained for the MDH-laccase biofuel cell (9 μW cm^{-2}). Furthermore, MDH-Laccase demonstrated some limitations in the anode performance that were reflected in a lower limiting current (~65 μA) with respect to the ADH-Laccase (~160 μA). The higher performance of the ADH-Laccase is attributed to higher enzymatic activity of ADH.

A particular glucose/O_2 biofuel cell system was also realized using a concentric configuration [21], constituted by two tubular electrodes with the cathode inserted in the anode (Figure 13). The electrolyte (10 mM of glucose in a Nitrogen saturated PBS solution, pH 7.4) is contained in the annular area between the electrodes and glucose oxidation is performed in the inner surface of the anode by glucose oxidase (GOD). At the same time, an O_2 saturated solution continuously circulates through the internal cavity of the biocathode, where oxygen is reduced by BOD. The peculiar design allows anode and cathode chambers separation: the dissolved oxygen flows without a direct contact with the electrolyte, thus avoiding the undesired hydrogen peroxide formation due to secondary reactions at the anode side. The electron transfer was assisted by anodic and cathodic mediators (8-hydroxyquinoline-5-sulfonic acid hydrate, HQS and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate, ABTS2-diammonium salt respectively) co-immobilized with the corresponding enzymes on carbon-based electrodes by means of polypyrrole films obtained by electropolymerization.

The overall performance of enzymatic biofuel cells is the consequence of the interaction between several physical and bio-electrochemical phenomena, as species transport, enzymatic reactions and heterogeneous electron transfer processes between the electrode and the enzyme or a mediator, in the cases of DET and MET, respectively. Modeling and simulation of these processes allows understanding and optimizing the performance of enzymatic electrodes and consequently of the entire fuel cell. Specifically, the focus involves the mathematical resolution of the corresponding non-linear reaction–diffusion problems [38,50], including reaction and transport kinetics, statistical analysis and metabolic control analysis. Theoretical, numerical and experimental methods for estimating the biofuel cell performance was discussed by various authors. In [51], the authors modelled the effects of convective flux and temperature on the performance of an enzymatic glucose fuel cell based on flow design. The cell employs a cation exchange membrane and glucose oxidase enzyme at the anode. The model schematizes the cell as a plug flow reactor, assuming total lateral mixing for glucose and hydrogen ion transfer through the cell. The glucose fuel cell domain is consequently reduced to one dimension and is divided into five sections: the anode diffusion layer (ADL), the enzyme layer (EL), the anion exchange membrane, the cathode catalyst layer (CCL) and the cathode diffusion layer (CDL) (Figure 15). The model assumes that the membrane is only permeable to hydrogen ions and not to glucose; moreover, glucose reacts exclusively in the enzyme layer at fuel cell anodic side in presence of GOx enzyme, and the transport of glucose is only due to diffusion and convection.

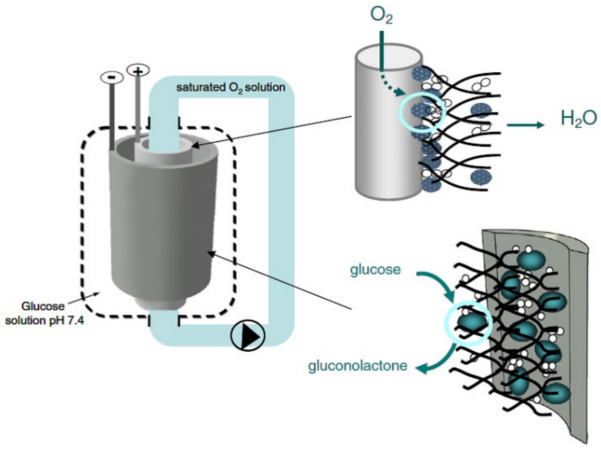


Figure 13. Schematic layout of the concentric glucose/O_2 biofuel cell. Reprinted from [21], with permission from Elsevier.

The optimized biofuel cell (bioanode modified by a 2 µm thick polypyrrole film followed by glutaraldehyde treatment to favor enzyme cross-linking and biochatode modified by a 1.4 polypyrrole film without enzyme cross-linking) was operated at OCV 0.44 V, obtaining a maximum power density of 42 µW cm^{-2} at 0.30 V. The experimental results obtained for the optimized biofuel cell are indicated in Figure 14.

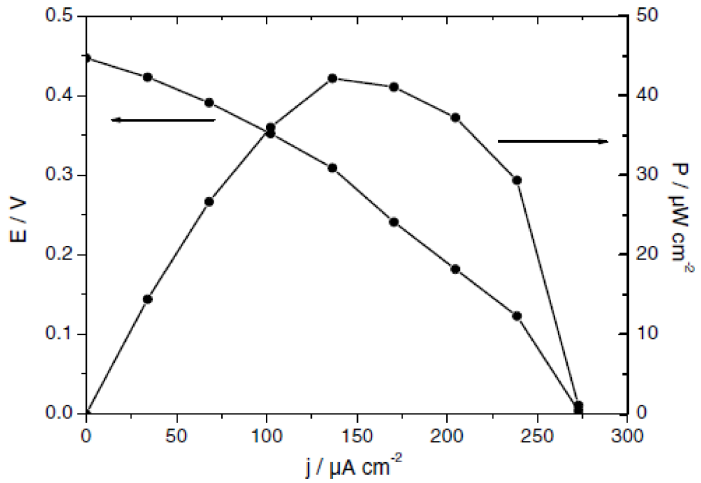


Figure 14. Experimental results obtained in [21] with the optimized enzymatic cell configuration. Reprinted from [21], with permission from Elsevier.

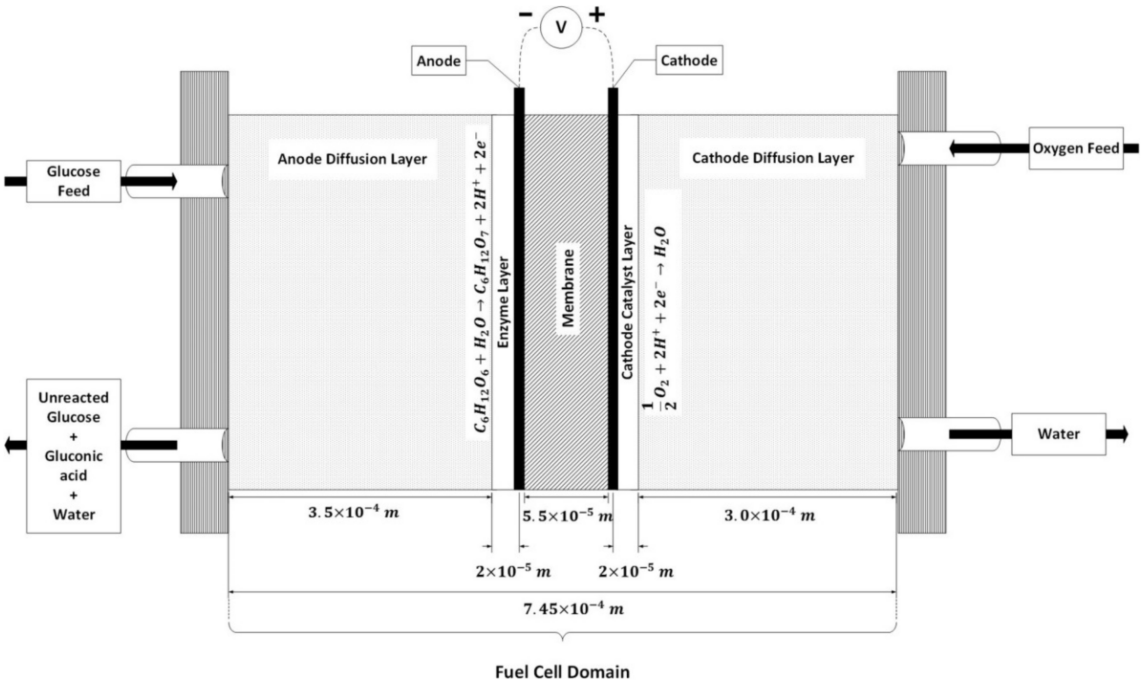


Figure 15. Schematic representation of the glucose enzymatic fuel cell used for the model reported in [51]. Reprinted from [51], with permission from Elsevier.

Figure 16a shows the variation of glucose concentration with time across the half-cell composed by anode diffusion layer, enzyme layer and membrane, as a function of inlet glucose concentration and inlet flow rate. At the first time step, a rapid drop in glucose concentration profile across the ADL occurs, due to limitations to diffusion phenomenon. Then, the concentration profile across the ADL increases with time and becomes stable. Figure 16b shows the variation of glucose concentration across the ADL, the EL and the

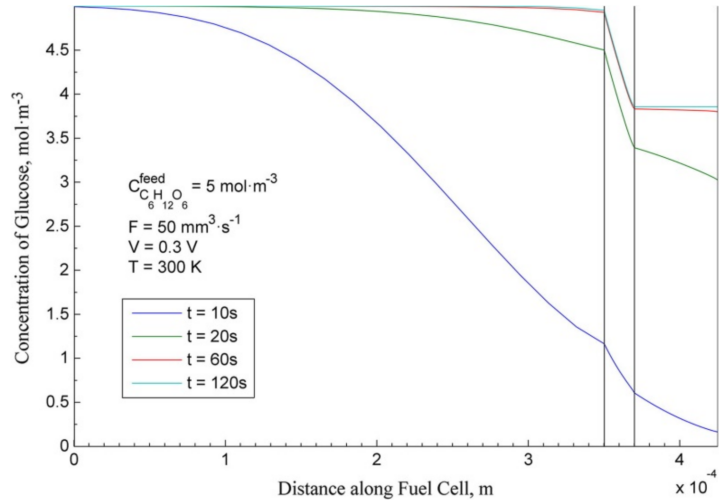
membrane as a function of inlet glucose flow rate, revealing a drop in glucose concentration in the enzyme layer, due to the reaction with glucose oxidase enzymes. At the increase of flow rate, the diminution in glucose concentration in EL is less marked, depending on the reduced contact time between the enzyme and the substrate. On the contrary, across the ADL, no drop is observed in the concentration of glucose species, since the high flow rates determine a well-established convective flux that overcomes the diffusive one. Figure 16c illustrates the variation of glucose concentration across the ADL, the EL and the membrane as a function of temperature. The concentration of glucose remains almost constant in ADL, depending on the high value of the diffusion coefficient established in the range of the investigated temperatures. Differently, the glucose concentration drops across the EL at operating temperature increase: Increasing system temperature, the reaction rate in the enzymatic cell is strongly enhanced and, consequently, higher consumption rate of glucose molecules is observed.

The variation of hydrogen ions across the enzymatic cell (anode diffusion layer, enzyme layer, membrane, cathode catalyst layer and cathode diffusion layer) can be seen in Figure 17, assuming a complete consumption of hydrogen ions at the cathodic side (CCL and CDL). In Figure 17a, the concentration of hydrogen ions increases in the enzyme layer, since they are generated by the glucose oxidation reaction. Then, hydrogen ions concentration sharply decreases in the cathode catalyst layer, due to H- consumption for the cathodic reduction reaction. The occurrence of hydrogen ions in the anode diffusion layer evidences also a backward diffusive flux, caused by the presence of a potential across the cell. The peak concentration of hydrogen ions is observed roughly near the enzyme layer at steady state, since migration and cathode kinetics become dominant factors, reducing the effect of diffusion phenomena and generation of hydrogen ions in the enzyme layer [52].

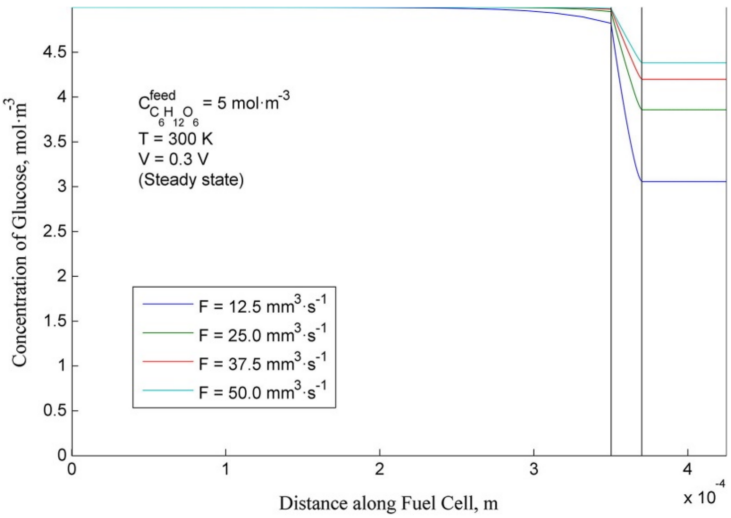
Figure 17b shows the variation of hydrogen ions across the enzymatic cell as a function of temperature: Higher operating temperature results in higher concentration of hydrogen ions in the enzyme layer, depending on the enhanced diffusive flux and reaction rate, as in the case of glucose concentration variation along the cell. Hydrogen ions concentration is also improved at the increasing of enzyme layer thickness, because, in this case, more sites for oxidation are available, resulting in higher H- generation.

Near the cathode region, the concentration of hydrogen ions is higher initially, mainly due to slower migration rate. When the system approaches steady state, migration and cathode kinetics become dominant factors, reducing the effect of diffusion and generation of hydrogen ions in the enzyme layer. Due to the interplay of these competing phenomena, the peak concentration of hydrogen ions is observed roughly near the enzyme layer at steady state.

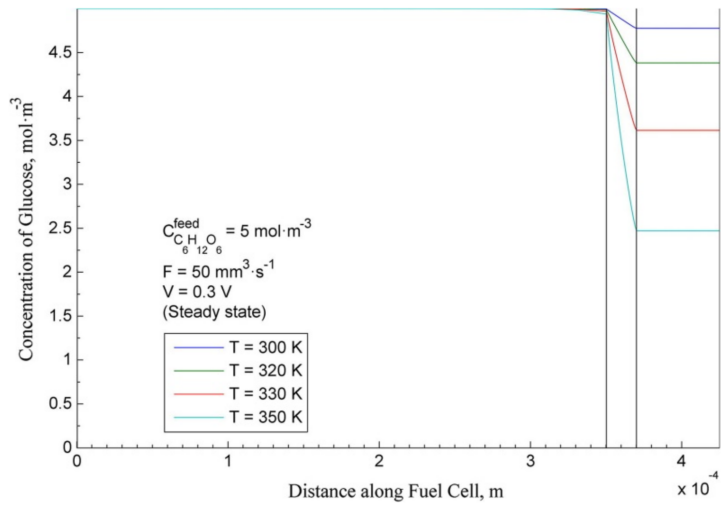
Another interesting approach for modeling enzymatic fuel cells was presented in [53]. In a theoretical study, the authors investigated possible kinetic limitations in an osmium-mediated glucose oxidase/laccase enzymatic biofuel cell by means of metabolic control analysis (MCA) methodology. Oxygen concentration in the cathodic solution represents a crucial parameter for enzymatic fuel cell operation, since oxygen is required at the cathode, but it participates in a non-productive reaction at the anode. The total mediator and oxygen concentrations have opposing effects on the distribution of control between the two electrodes. Increasing the total mediator concentration shifts the control to favor the GOx anode under most operating conditions, so that fuel cell performance appears to be dominated by the anode. On the contrary, the increase of oxygen concentration shifts the control to favor laccase at the cathode. Between these two limiting cases, a distribution of control between the two enzymes can be observed. Therefore, selecting specific conditions, the enzymatic biofuel electrodes can operate under a balanced control distribution over the current production, thus improving fuel cell stability.



(**a**)

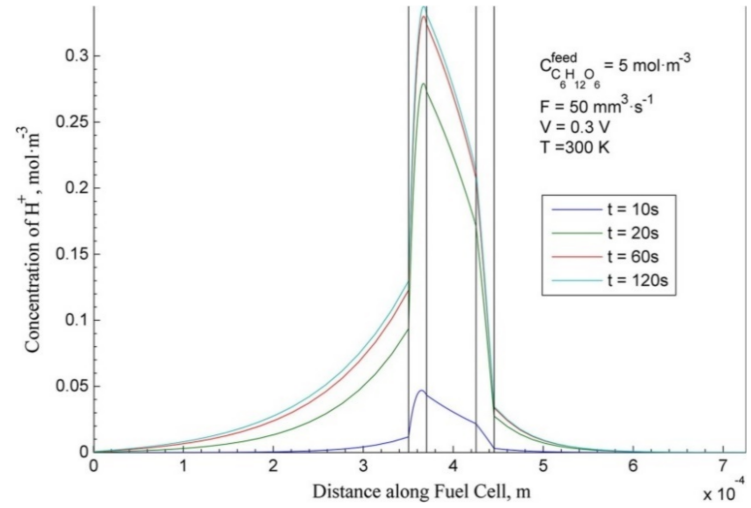


(**b**)

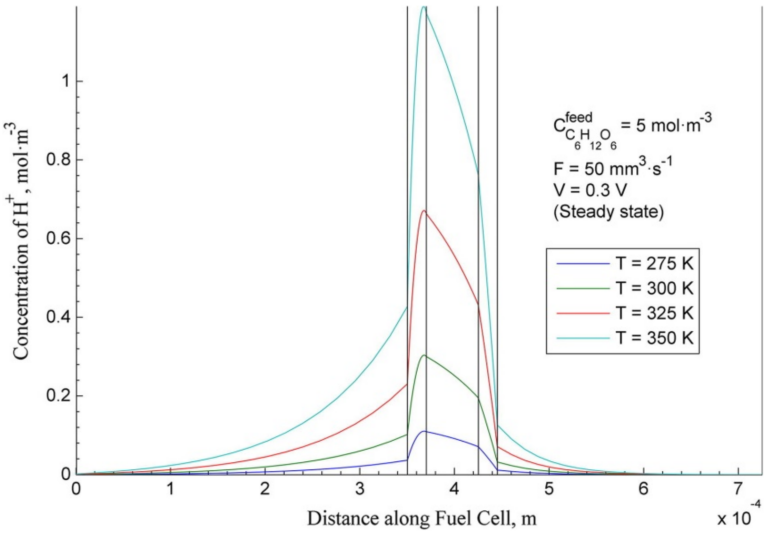


(**c**)

Figure 16. Variation of glucose concentration in the anodic enzymatic half-cell as function of time (**a**), flow rate (**b**) and temperature (**c**). Reprinted from [51], with permission from Elsevier.



(**a**)



(**b**)

Figure 17. Variation of hydrogen ions concentration along the enzymatic cell as function of time (**a**) and temperature (**b**). Reprinted from [51], with permission from Elsevier.

A mathematical modeling based on Homotopy perturbation method is reported in [50] for an enzymatic glucose membraneless fuel cell with direct electron transfer. The solution of the time independent non-linear reaction-diffusion differential equations describing glucose concentration and hydrogen ions concentration, both inside and outside the enzyme layer, led to the estimation of fuel cell kinetic parameters and their effect on power density. Moreover, these analytical solutions are compared with zero order ones.

5. Conclusions and Future Outlooks

The review paper reported the main features regarding enzymatic fuel cell systems, specifically focused on flow design configurations, considered of great interest for the future development of this technology. The recent research activities on EFCs are mainly oriented on implantable applications and biosensors realization, due to the possibility to exploit the natural fluids flows circulated in living organisms or connected to some industrial processes involving residual biomass.

Different microfluidic and non-microfluidic enzymatic flow cell designs are reported, describing also promising fabrication methods for electrodes, single cells and stacks that

allow simple, fast and low-cost EFC manufacturing. Microfluidic enzymatic systems were recently investigated in depth, due to the possibility to be easily integrated in miniaturized devices. In this field, many techniques, such as 3D-printing, soft-lithography and xurography, can be effectively applied to fast realize compact and low cost systems. These methodologies are also oriented to the use of innovative materials, in some cases employed as composites, in order to obtain suitable properties (flexibility, transparency, etc.), depending on the desired application. Among microfluidic configurations, paper-based design is particularly highlighted, due to its intrinsic capability to realize fluid flows exclusively based on paper capillary action, without the need of pressure sources. These systems represent an interesting approach towards the development of cost-effective enzymatic microfluidic fuel cells, reducing, or even avoiding, the power requirements for external equipment.

Research activity regarding the development of mathematical models, both on microfluidic and non-microfluidic systems, is also discussed, in order to understand, predict and optimize the performance of enzymatic electrodes and complete biofuel cells, as a function of main operative conditions, regarding the used substrates, the biocatalysts and the diffusivity of different species. The final goal is to assess the effectiveness of the used strategy for enzyme immobilization, the influence of mediator species or possible inhibitors and the evaluation of the power output, providing useful guidelines for the realization of novel architectures in the design and fabrication techniques, in order to reduce the time involved in prototyping, building and characterizing EFC devices.

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