Buaki-Sogo, M.; García-Carmona, L.; Gil Agustí, MT.; Zubizarreta Saenz De Zaitegui, L.; García Pellicer, M.; Quijano-Lopez, A. (2020). Enzymatic Glucose Based Bio batteries: Bioenergy to Fuel Next Generation Devices. Topics in Current Chemistry (Online). 378(6):1-28. https://doi.org/10.1007/s41061-020-00312-8

Enzymatic glucose-based bio-batteries: bioenergy to fuel next generation devices

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Abstract

This article consists of a review of the main concepts and paradigms established in the field of biological fuel cells or biofuel cells. The aim is to provide an overview of the current panorama, basic concepts and methodologies used in the field of enzymatic biofuel cells, as well as the applications of these bio-systems in flexible electronics and implantable or portable devices. Finally, the challenges needing to be addressed in the development of biofuel cells capable of supplying power to small size devices with applications in areas related to health and well-being or next generation portable devices are analyzed. The aim of this study is to contribute to biofuel cell technology development; this is a multidisciplinary topic about which review articles related to different scientific areas, from Materials Science to technology applications, can be found. With this article the authors intend to reach a wide readership in order to spread biofuel cell technology for different scientific profiles and boost new contributions and developments to overcome future challenges.

Keywords: glucose biofuel cells; energy harvesting; enzyme immobilization; bioenergy; implantable devices; flexible electronics.

1. Current panorama in fuel cell development.

Environmental concern and climate change have prompted the development of new energy sources [1]. These new energy sources should be able to support and endow autonomy to small devices present in daily tasks and activities. In this regard, the concept of energy harvesting is gaining interest as it allows energy to be supplied to such devices using fuel present in the surroundings without external power sources thus increasing their autonomy and versatility [2],[3].

Among energy harvesting systems developed in recent years, fuel cells appear as green and sustainable novel energy sources [4],[5],[6],[7],[8],[9]. This technology features systems capable of generating energy from electrochemical reactions. In addition to their capacity for

transforming chemical energy into electricity, these systems are sustainable with low greenhouse gas emission. Traditional fuel cells use metallic catalysts to obtain electricity by fuel oxidation-reduction reactions [7]. Fuels typically used are hydrogen [5],[8] and small organic molecules such as methanol or ethanol [4]. Once the fuel has been oxidized at the anode, an external circuit transfers electrons to the cathode, where they react with an oxidant (usually oxygen) giving rise to the electrical current together with H_2O and heat [9].

Fuel cell technology offers the advantage of high efficiency and high power density. However, high implementation cost, scarcity of metals from which catalysts are prepared and problems related with electrode passivation hinder its application on a broad scale [4],[5],[6],[7],[8],[9]. Development of alternative energy harvesting and storage systems such as biologic fuel cells (also known as biofuel cells) signify a promising approach especially for biomedical appliances.

Production of electricity from physiological fluids in living organisms using glucose as a fuel was first reported in the 1970s. However, the low selectivity towards glucose and the low power densities obtained from the implanted device together with the appearance of lithium ion batteries in 1972 hampered further development. The biocompatibility of metal catalysts was also an issue to be considered [10]. The need to overcome these drawbacks gave rise to the increasing interest of the scientific community with regards to enzymatic biofuel cells.

For many years research into enzymatic biofuel cell technology has been growing. The first implantable abiotic glucose biofuel cell described in the early 70s [11] was implanted in the flank of a dog and delivered a low power energy density of about 2 $\mu W/cm^2$. Another fuel cell able to deliver 40 $\mu W/cm^2$ was also implanted during the same period in a sheep's vein [12]. However, the problems associated with the use of noble metal catalysts hampered further technological developments. Nobel metal catalysts employed in abiotic fuel cells (Pt, Pd, Ir, Au and alloys) are expensive, scarce and suffer from poisoning. In addition, low catalyst specificity and power densities, along with inflammatory reaction of tissue and poor electro-catalytic activity at neutral pH, led technology to a dormant state for several decades [13]. With advances in nano-materials and nanotechnology during the 21st Century (especially those related with device miniaturization), research into biofuel cells has gained momentum. Taking advantage of nano-science advances biofuel cells are currently being developed to be used as energy sources for medical appliances and smart devices [14].

2. Enzymatic glucose biofuel cells: fundamentals and operation mode.

Biofuel cells could be described as systems able to produce electrical energy through the chemical transformation of a biologic substrate [15],[16]. In order to achieve this transformation, the electrodes forming anode and cathode are modified incorporating a catalyst in their structure [17],[18],[19]. Biofuel cells can be classified depending on the catalyst; thereby, there are three types of biofuel cells that can be found in literature: (1) abiotic biofuel cells (when the catalyst is not of biological origin, i.e. Pt, Pd, Au,…) [10], (2) enzymatic biofuel cells (when the catalyst is an enzyme) [16] and (3) microbial biofuel cells (when the chemical transformation is carried out by microorganisms) [20]; microbial biofuel cells find their main application in wastewater treatment with additional heat and energy generation [19],[20].

Enzymatic biofuel cells show the advantage of their ability to perform the chemical transformation to obtain electrical energy under mild reaction conditions [16],[17]. Enzymes are biological catalysts that can be found in the human body and are able to perform biochemical transformations of a target substrate in physiological conditions (37ºC and pH~7) [13]. Therefore, the main advantages of using enzymes in biofuel cells are their selectivity towards a target substrate, their activity under physiological conditions and the relative simplicity of electrode preparation [19]. These features highlight the potential of biofuel cells to power electronics such as implantable or portable next generation devices with potential application in biomedicine and other disciplines related to health and well-being.

In the last decade, interest in the development of devices with immobilized enzymes has increased rapidly attracting the interest of companies. Along with their potential application *in vivo* to medicine, biofuel cells (working with glucose or another biologic fuel) could be applied as an energy source to power devices with low energy requirements such as those for environmental control of animals and plants [21],[22] or sensors for disease control [23].

One of the most studied biological fuels is glucose. Glucose can be found in human blood in a concentration of about 5 mM [22],[23],[24]. In enzymatic glucose fuel cells, glucose oxidation takes place at the anode. Oxygen, which acts as a reducing agent at the cathode, is found in a concentration of 45 mM in human blood [22],[23],[24]. Therefore, glucose biofuel cells emerge as a promising technology since they have the potential to work indefinitely due to glucose and oxygen presence in blood and extracellular fluids in the human body. Figure 1 represents the transformation of glucose in an enzymatic biofuel cell to obtain energy.

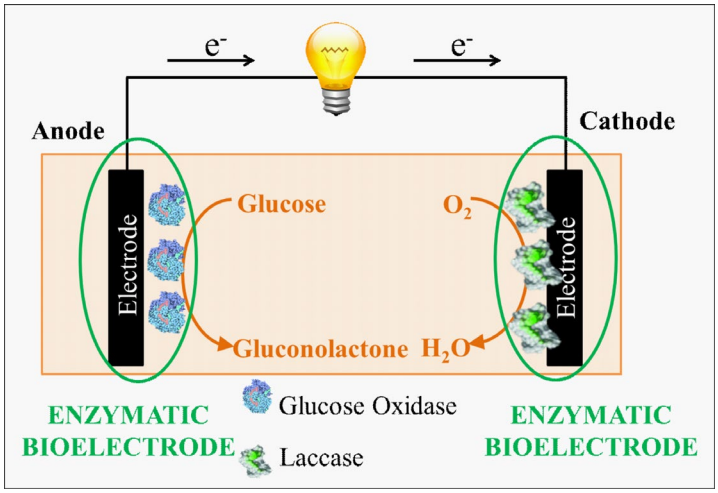

Fig. 1 Operation mode of an enzymatic glucose biofuel cell with 2 enzymatic bio-electrodes based on Glucose Oxidase and Laccase enzymes. Enzymatic glucose oxidation takes place in the anode by Glucose Oxidase while oxygen reduction is carried out in the cathode by Laccase.

2.1.Enzymes for glucose transformation and enzymatic electrode configuration.

2.1.1. Enzymes for glucose transformation

Enzymes are proteins widely used as catalysts in biochemistry. One of the main components in enzyme catalysis is the co-enzyme, which is a cofactor. In biochemistry, a cofactor is a non-protein the presence of which is required for enzyme catalytic activity [25]. The cofactor does not form part of the enzyme structure. The term cofactor applies to catalytic essential molecules or ions bound to the enzyme. Cofactors can be attached to the enzyme structure covalently (i.e. metal ions such as Na^{2+}, Ca^{2+}, Mg^{2+}) or non-covalently as is the case for most organic cofactors . They are known as co-enzymes when cofactors are not covalently bound to the enzyme structure [26]. When dealing with enzymatic bio-electrodes and biofuel cells, the location where the reaction takes place is referred to as the active site; for redox transformations, such as those performed in enzymatic biofuel cells, it can also be referred to as the redox site. Figure 2 shows a scheme of enzyme structure with the cofactor and the active site where the reaction takes place. These cofactors consist of low molecular weight organic moieties or inorganic compounds embedded inside enzyme protein structure as shown in Figure 3.

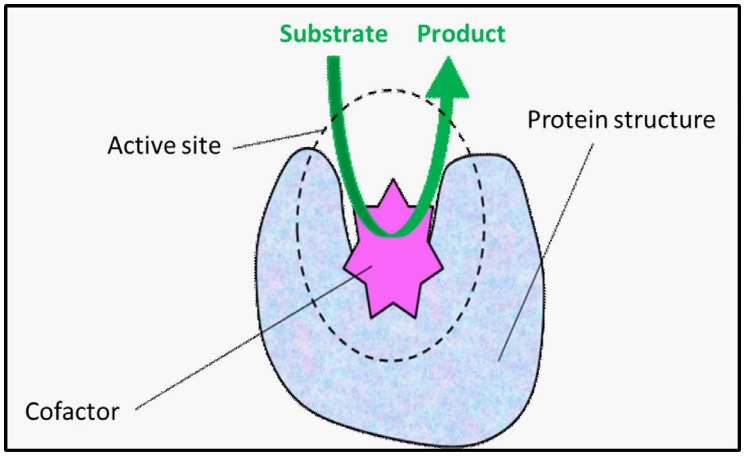

Fig. 2 Scheme of enzyme structure showing protein structure, cofactor and active site.

For bio-cathode configuration the enzymes commonly used are Billirubin Oxidase and Laccase. Both fit into the group of *multicopper oxidases* using coordinated Cu atoms to catalyze oxygen reduction to water [27]. In the anode oxidoreductase enzymes are able to oxidize electron donor compounds and to transfer such electrons to the electrode surface. In this category, various oxidoreductase enzymes capable of oxidizing glucose are included, with Glucose Oxidase being the one most studied [28]. These enzymes for glucose oxidation can be divided into two groups: *Glucose Oxidases (GOxs)* and *Glucose Dehydrogenases (GDH).* GOx enzymes are oxidoreductase enzymes using molecular oxygen as an electron acceptor, with the release of hydrogen peroxide. These are *Flavine Adenine Nucleotide (FAD) dependent* enzymes. GDHs enzymes do not use oxygen as an electron acceptor and can be classified depending on their redox active site. Classes include *Nicotinamide Adenine Nucleotide (NAD), Nicotinamide Adenine Nucleotide Phosphate (NADP)*, *Pyrrolquinoline Quinone cofactor (PQQ)* and FAD dependent enzymes [29]. The structure of cofactors for glucose oxidizing enzymes is shown in Figure 3.

The choice of a specific enzyme for electrode preparation is critical for the development of implantable biofuel cells [30],[31]. When biofuel cells are conceived to operate *in vivo*, the possibility of hydrogen peroxide formation exists and this by-product could be used as an electron acceptor [32],[33]. The main drawback with this approach is the toxicity of hydrogen peroxide and therefore, its presence should be avoided [30],[34]. This can be accomplished with the addition of catalase, an oxidoreductase enzyme that decomposes hydrogen peroxide into oxygen and water [34].

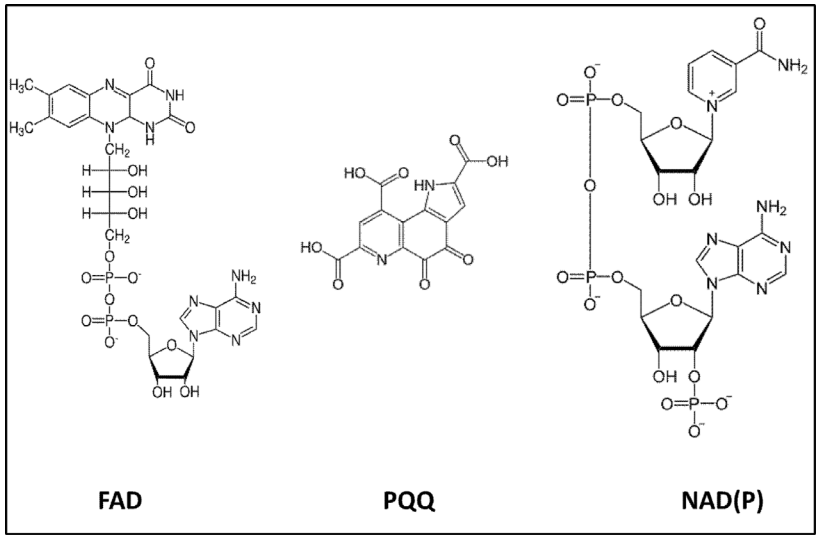

Fig. 3 Chemical structure of FAD, PQQ and NAD(P) cofactors usually found in GOx and GDH enzymes.

2.1.2. Enzymatic electrodes for glucose biofuel cells

Carbon-based nano-materials are widely applied as composites for energy storage [35], sensors [36] and controlled drug delivery, bio-imaging and other related medical disciplines [37]. They have also found applications in nano-electronics and nanotechnology [38],[39]. These materials can be modified with functional groups on their surface. These groups can then be used to bind enzymes. Most of the enzymatic bio-electrodes used in glucose biofuel cells are built up with carbon-based materials such as graphite, graphene or carbon nanotubes [34],[40],[41],[42],[43],[44]. Even though bio-electrodes are also prepared employing metallic supports, these kinds of electrodes are mainly used in non-enzymatic biosensor development [42].

Enzyme immobilization may be defined as confining the enzyme molecules to a solid matrix/support different from the one in which the substrate or products are present. This is achieved by attaching the enzymes to or within some suitable support material [45]. Enzymes are immobilized onto these materials via the following techniques: physical adsorption, covalent anchorage, entrapment, cross-linking and affinity (Figure 4). To carry out immobilization other reagents assisting in the immobilization process can be employed; polymeric matrices to trap or encapsulate biomolecules, cross-linking agents to connect the enzyme with the support or the addition of functionalities with an affinity for the functional groups in the biomolecule to the supporting material [43],[44]. In Table 1, the characteristics, advantages and drawbacks of the different enzyme immobilization strategies are presented.

Enzyme immobilization offers some advantages with respect to enzymes which are free in solution. Immobilized enzymes can display higher stability, are often reusable and are able to operate in continuous mode. Other advantages include resistance to changes in the environment, and low operation cost [46].

From all the techniques, those using covalent bond formation strategies show more advantages with respect to activity and support-enzymatic biocatalyst system stability. In the covalent immobilization of enzymes, stable complexes are formed between functional groups in the enzyme molecules and the supporting matrix. The enzymes are attached covalently to the support by means of a functional group present in the enzyme that is not essential for enzymatic activity. These functional groups usually involve lysine (amino groups) and cysteine (thiol group) residues or carboxylic groups from aspartic and glutamic acids in the enzyme structure. All of them can be used for enzyme covalent coupling [47]. Higher specific stability with controlled protein orientation can be achieved when peptide-modified surfaces are used for enzyme linkages [48]. Moreover, covalently binding enzymes to modified silica gel carriers have shown enhanced enzyme stability and also act as hyperactive biocatalysts [49],[50]. The maintenance of structural and functional properties of immobilized enzymes is very important and can be achieved with a cross-linking agent. Glutaraldehyde is one such cross-linking agent and is popularly used as a bifunctional cross-linker that can form stable inter- and intra-covalent bonds due to its solubility in water.

Covalent immobilization provides strong binding between the enzyme and support matrix and offers some advantages with respect to other methods such as physical adsorption or entrapment. When performing covalent binding, the effect of diffusion barriers is not observed since substrate accessibility to the active site is not hampered. Covalent bonds are stable to prevent enzyme leakage from the support matrices thus improving stability of the immobilized enzymes [51]. In addition, with this method it is possible to orient the enzyme on the surface to reach an efficient electron transfer and reproducibility is better than other reported methods such as adsorption. Recently, it has been proposed that the loss of enzymatic activity upon immobilization could be due to incorrect enzyme orientation with respect to the electrode. De Lacey and co-workers propose a strategy for covalent and oriented enzyme immobilization [52],[53]. In their work, De Lacey and co-workers carried out the covalent immobilization of Laccase following two different approaches. One approach deals with the formation of an amide bond from the reaction between carboxylic groups on the enzyme stucture and amine-functionalized support in the presence of EDC/NHS [54]. In the second approach hydroxyl groups from sugar residues in Laccase are reacted with $NaIO_4$ to transform them into an aldehyde; these aldehydes react then with the amino groups in the functionalized support by a Schiff base reaction with the formation of an imino bond [55]. By means of these strategies,

Laccase is oriented in such a way that direct electron transfer is favored. This approach has also been employed for the immobilization of Bilirubin Oxidase onto a gold electrode with the formation of amide bonds between the carboxylic groups in the modified gold support and the lysine residues of the enzyme in the presence of EDC/NHS [53].

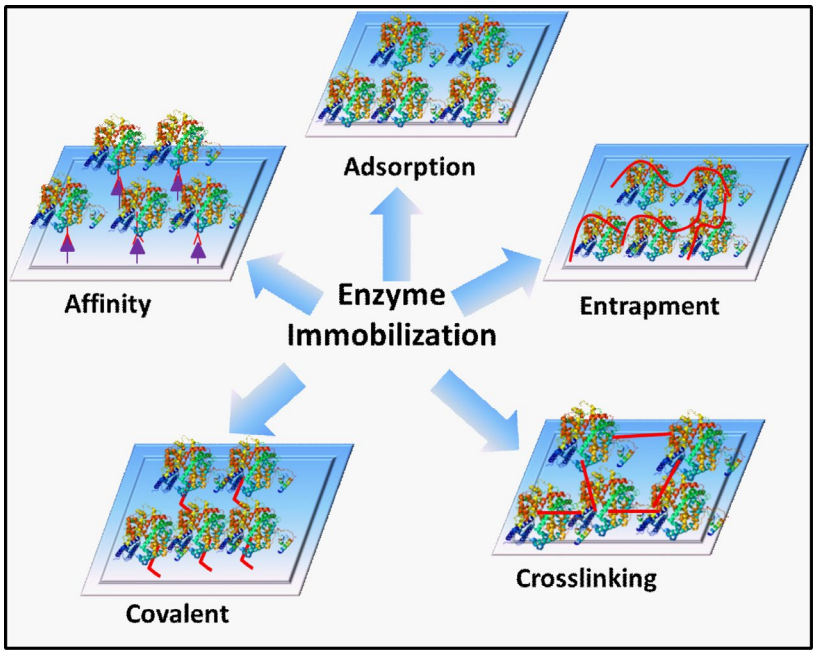

Fig. 4 Enzyme immobilization strategies in the preparation of bio-electrodes.

A broad variety of polymeric materials such as Nafion, chitosan, polypirrol, polyaniline, polyphenol, polyvinyl pyridine, polycarbonate and nylon, has been widely used in enzymatic electrode development. Moreover, these polymeric materials could also be used for other functions such as electron mediators, separation membranes or stabilizing agents [56],[57],[58],[59],[60].

Table 1. Main features of the different enzyme immobilization strategies. Kind of interaction, advantages and drawbacks are described for the 5 methods.

Adsorption	Weak Interaction	Easy and simple. Limited decrease in activity.	Desorption. Low specificity.	[45],[61]
Affinity	Bond between support functional groups and specific target in the biomolecule	Oriented and covalent immobilization.	Presence of specific groups and functionalities in biomolecule structure is highly desired.	[45],[62]

Covalent	Chemical bond between biocatalyst functional groups and support	No diffusion barriers. Short response times. Stable.	Loss of biocatalytic activity. Toxicity of reagents.	[18],[51]
Crosslinking	Biocatalyst/cross-linking agent/support functionalities bonding	Simplicity.	Loss of biocatalytic activity.	[63],[64]
Entrapment	Incorporation into polymeric matrix	Chemical reactions affecting biomolecule activity do not occur. One polymeric matrix can be used with different biomolecules.	Diffusion barriers. Biomolecule leakage. High concentrations of biomolecule are needed.	[65]

2.1.3. Electron transfer processes in enzymatic electrodes

Enzymatic biocatalysts show excellent selectivity with respect to target substrate [17]; this allows the enzymatic electrode to be used without the need for selective membranes or noble metals. However, one of the main challenges in enzymatic biofuel cell design lies in the achievement of an efficient electron transfer between enzyme redox site and electrode surface where the enzyme is immobilized [60],[66],[67].

Enzymatic biocatalysts immobilized onto electrodes do not usually show efficient electrical communication between the redox site and the conductive support. This electrical isolation is produced as a consequence of the protein structure surrounding the redox active site [46],[67],[68],[69]. The glucose oxidase enzyme structure can be seen in Figure 5. FAD cofactor is represented by colored spheres; as can be seen, the redox active site where co-factor is normally bound is not placed in the middle of the dimeric structure; the active site is deep within the tertiary/quaternary protein structure; therefore, the distance to the outer part of the enzyme structure is, except in one direction, higher than 14 Å; this is the distance considered to be optimum for efficient electron transfer [68],[69].

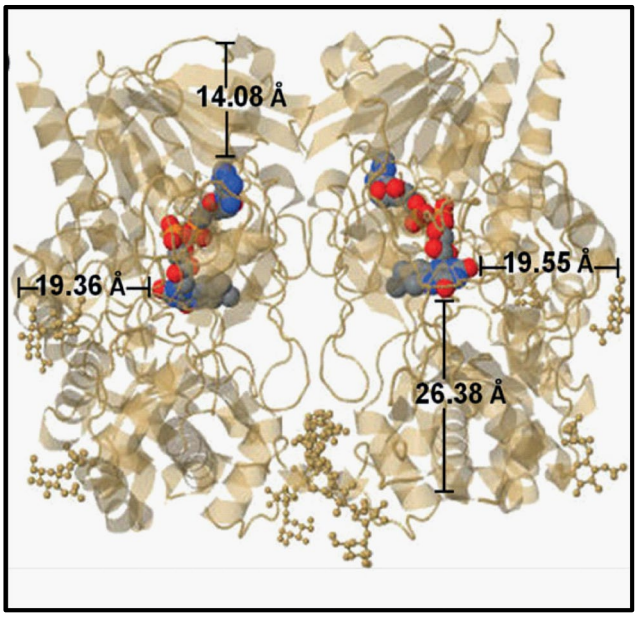

Fig. 5 Redox active site position (FAD) in the homo-dimeric enzyme Glucose Oxidase. Reproduced from reference [68] with permission of The Royal Society of Chemistry RSC 2020.

There are two types of electron transfer processes in enzymatic electrodes. One of them is based on the use of redox mediators when designing the bio-electrode (Mediated Electron Transfer, MET). In this case, the enzyme catalyses oxidation or reduction of the mediator which then returns to its initial redox state at the electrode surface. The main objective of using mediators is to increase electron transfer rate between the biocatalyst redox active site and the electrode [44],[46],[67]. Examples of mediator compounds include osmium-based polymers and polyamines (when there are problems related with bio-electrode stability), tricyclic basic dyes such as meldola blue for bio-anodes or ammonium salts such as ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) for biocathodes [46],[68]. Meldola blue, in the anode reaction, substitutes oxygen as an electron acceptor. This is interesting in bio-sensing applications where oxygen availability in the sensing medium is limited or to avoid the effect of interfering species modifying working potential [46]. ABTS in the cathode reaction returns the oxygen reduction enzyme such as Laccase back to its initial state after completing oxygen reduction to water [46].

The other electron transfer process in enzymatic electrodes is based on direct electron transfer between the redox active site and the electrode surface without the aid of mediator species. Direct electron transfer (DET) is achieved by means of immobilization strategies using covalent and non-covalent interactions giving rise to effective enzyme orientation for an efficient electron transfer [44],[46],[67].

Direct electron transfer in enzymatic electrodes has advantages when compared with transfer involving mediator species. Namely it avoids problems related with mediator stability, selectivity and mass transfer with the electrode. Moreover, the presence of the mediators adds one more step in the electron transfer sequence and this can diminish enzyme catalytic activity and reaction performance. In addition, most of the mediators used in enzymatic electrodes are based on compounds that might be avoided in the development of implantable systems that generate energy from living organisms due to their toxicity as is the case with osmium complexes and polymers from which osmium, a highly toxic compound, may leach from the corresponding complex or polymer[44],[70]. For these reasons it is important to focus on enzymatic electrode development using materials that will allow direct electron transfer processes between electrode surface and enzyme redox active site.

2.2.Operation mode in glucose biofuel cells

In general, energy generation by means of biofuel cells is similar to the process used in commercially available fuel cells since both involve redox reactions. As stated before, glucose biofuel cells make use of glucose oxidation in the anode and oxygen reduction in the cathode to produce electricity using an immobilized catalysts. The electrochemical reactions taking place could be written as follows:

$$Anode:\ C_6H_{12}O_6 + 2OH^- \rightarrow C_6H_{12}O_7 + H_2O + 2e^-,$$

$$Cathode:\ 1/2\ O_2 + H_2O + 2e\text{-} \rightarrow 2OH^-,$$

$$General:\ C_6H_{12}O_6 + 1/2\ O_2 \rightarrow C_6H_{12}O_7,$$

$$\Delta G^0 = 0.251 \times 10^6\ J/mol,$$

$$U^0 = 1.30\ V,$$

where ΔG^0 represents Gibbs free energy and U^0 represents theoretical cell voltage [16],[71].

Normally fuel cells require the anode and cathode to be separated by a membrane. While it is possible for a biofuel cell to be set up this way, it is not necessary due to the inherent selectivity of enzymes [25],[27],[28]. Therefore, in enzymatic biofuel cells the one-compartment configuration is most frequently used [46]

It is important to develop biofuel cells able to operate in living tissues and therefore material biocompatibility with the implantation site is an issue of concern for implantable enzymatic biofuel cells. Another issue of concern is fuel availability and the impact of concentration gradients on biofuel cell performance [72].

2.2.1. Fuel Availability

Glucose is the most common fuel for anode reaction due to its presence in most physiological fluids in moderate concentrations [22],[23],[24]. Table 2 shows the concentration of glucose in target physiological fluids. Glucose is an essential compound for humans and an energy source in various cellular processes. Blood is the main source of glucose in the human body. In the human body glucose mainly comes from intestinal absorption, glycogenolysis and glucogenolysis [72]. In the small intestine, food is broken down into carbohydrates such as glucose, fructose and galactose, with glucose having the fastest absorption rate. Fructose and galactose are also absorbed and transformed into glucose in the liver [72]. In contrast, endogenous glucose production is a continuous process that is regulated depending on glucose physiological need; this need is established depending on body condition (fasting and fed states) [74]. Plasma is another source where glucose can be found in a concentration of about 6 mM [75]. Plasma glucose homeostasis is maintained in healthy people by a balance with endogenous and dietary glucose. Glucose availability is not only critical in enzymatic biofuel cell performance, but there is also the question as to whether an implanted glucose biofuel cell will consume glucose causing a detrimental effect on health. Enzymatic glucose oxidation involves 2 electrons in comparison with the 24 electrons involved in the complete glucose oxidation. Thus, for a person at rest, the use of an enzymatic biofuel cell as an energy source for a device consuming between 1 mW and 100 mW, implies that glucose requirements will be between 0.1% and 10% of the total carbohydrate intake. This percentage will be lower for non-resting states. Therefore, there is a little or no impact on body metabolism [72]

Table 2. Glucose and Lactate availability in physiological fluids.

Physiological Fluid	Glucose (mM)[22],[23],[24]	Lactate (mM)[76],[77]
Blood	4.9 - 6.9	0.5 - 1.5
Sweat	0.06 - 0.11	8.0
Saliva	0.23 - 0.38	0.11 - 0.56
Ocular Fluid	0.05 - 0.5	0.02 - 5
Interstitial Fluid	3.9 - 6.6	1.2

Oxygen is the fuel for the bio-cathode reaction and is an essential component in the metabolism of living beings. An issue of concern in enzymatic biofuel cells is the effect of the enzymatic

biofuel cell on body oxygen levels and how this affects the operation limits of the biofuel cell [72]. One of the body fluids where oxygen can be found is blood in which oxygen is carried combined with Hemoglobin (Hb) although part of this oxygen is partially dissolved in plasma [72]. The maximum current density for the cathodes is limited by oxygen diffusion and this current density is estimated to be low. These low current densities imply that oxygen reduction at the cathode will be a limiting stage for the enzymatic biofuel cell operation due to the low concentration of oxygen in the human body. Thus, it is possible that an implantable biofuel cell has a small impact on the oxygen available for metabolic reactions. However, more factors must be kept in mind in order to maximize the production of electrical current from the reduction of oxygen in the bio-cathode. Such factors are oxygen mass transfer to reach the electrode surface; the total volume of the enzymatic biofuel cell that will determine the available surface area to produce electric current; and biofuel cell configuration to maximize cell voltage [72].

Another important fuel for enzymatic biofuel cells is lactate. Lactate is an intermediate metabolite of anaerobic glycolysis and is present mainly in blood, serum, tears and sweat as shown in Table 2 [76],[77]. Lactate is an interesting fuel for biofuel cells, since it is readily available in biological fluids for implantable applications and has a high energy density for portable power applications. Lactate is particularly attractive since its concentration in human sweat can rise as high as 50 mM with a median concentration of 14 mM [78]. Lactate biofuel cells act in the same way as glucose biofuel cells. In these systems, lactate is oxidized to pyruvate in the anode while oxygen is reduced to water in the cathode. Enzymes used for cathode development in lactate biofuel cells are the same as the enzymes used in bio-cathodes of a glucose biofuel cell namely Laccase and Billirubin Oxidase. Some studies also report lactate biofuel cells with platinum- or silver-based cathodes [79],[80]. Enzymes used for lactate oxidation in the anode are the oxidoreductase enzymes Lactate Oxidase and Lactate Dehydrogenase. Lactate biofuel cells are mainly used in the development of epidermal biofuel cells that will be discussed briefly in section 2.3.2.

2.2.2. Implantation site

In order to obtain optimum working conditions and good compatibility of enzymatic biofuel cells in living beings, the implantation site must fulfill the following requirements: it must allow for an adequate substrate supply and efficient removal of catabolites to avoid accumulation and prevent problems with local toxicity. In addition, the presence of the biofuel cell must not cause mechanical constraints that might lead to adverse physiological reactions such as structure compression or cavity obstruction. Moreover the implantation site must not promote biofouling processes which limit the exchange between the device and the body and which also compromise energy production [72]. Taking all this into consideration, the vascular system

appears to be an ideal site for biofuel cell implantation due to the fact that glucose and oxygen levels are high and catabolite removal would be quite efficient as suggested by Prof. Adam Heller [14]. However, biofuel cells implanted in veins only work in the short term, since biofouling phenomena and their presence in the blood flow can induce thrombosis [12]. Another possibility for the implantation of biofuel cells is the use of micro-needle networks [13],[81]. This approach has been implemented in order to design systems with minimum invasive capacity. Micro-needles are inserted in the skin and substrates are taken from the skin's interstitial fluid. While such minimally invasive biofuel cells have potential to be a viable wearable energy harvester, biofouling of the electrodes and potential leaching of reagents during the long term operation are additional challenges to be faced by these minimally invasive biofuel cells [78],[81].

A possible solution to avoid biofouling could be the implantation of the biofuel cell in the cerebrospinal fluid the main benefits of which in terms of biocompatibility arise from its limited cell content and low concentration of proteins [71]. Nevertheless, the possible appearance of inflammatory reactions, antibody recruitment, low glucose availability as well as the need for device miniaturization must be taken into consideration. It is also important to consider the presence of gluconic acid as a product from glucose oxidation and whether its presence can modify local pH. Changes in acidity of cerebrospinal fluid can affect regulation of breathing and as a consequence oxygen consumption by the cathode can also be modified [81],[82]. Figure 6 shows implantation locations used in mammals.

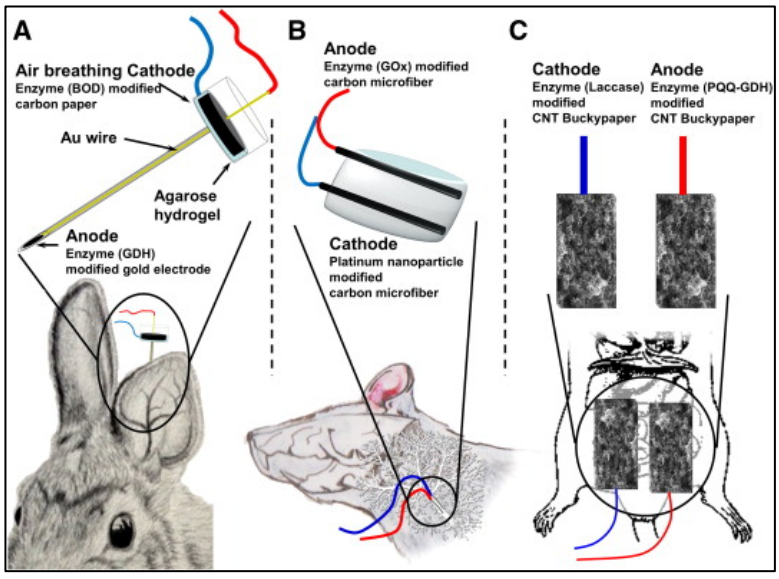

Fig. 6. Implantation sites for enzymatic biofuel cells in mammals. A) Enzymatic biofuel cell comprising a needle-based bio-anode and a paper-based cathode implanted in the blood vessel of a rabbit's ear. B) Carbon fiber-based enzymatic biofuel cell implanted in a rat's jugular vein. C) Buckypaper-based enzymatic biofuel cell implanted in the crestmaster muscle of a rat.

2.2.3. Consuming device integration

The design and development of enzymatic biofuel cells constitutes a challenge of great interest for the scientific community. However, their use in real environments is still far from being accomplished. Enzymatic biofuel cells are systems in the micron scale where power densities obtained are in some cases quite small and then, they can not be applied to power microelectronic devices such as those used in medicine. Moreover, device miniaturization decreases power output. Thus, the size and power output of the biofuel cells will determine the biofuel cell final application [82].

Nonetheless, in the design of biofuel cells the production of enough electrical current is not the main factor; integration of the biofuel cell with the consuming device is also a factor of great importance that needs the attention of experts from different areas such as electronics or medicine. One of the main problems in the integration of a consuming device with a biofuel cell is the mismatch between the voltage produced by the biofuel cell and the voltage required by the microelectronic device [82]. Voltage produced in a biofuel cell is limited thermodynamically by the potential difference between anode and cathode. In literature, the highest output voltage (open circuit voltage V_{OC}) that can be found for an enzymatic biofuel cell is about 0.8-0.9 V [34],[83]. However, many microelectronic devices require a voltage input in the range of 1-3 V for proper functioning [84]. A solution tested has been the serial connection of various biofuel cells [85],[86],[87]. This approach can only be used when working with individualized cells since implanted and wearable bio-electrodes show low resistances in biological tissues or skin. This low resistance between the bio-electrodes causes their short-connection preventing biofuel cells from operating independently and therefore output voltage can not be increased [85]. The increase in output voltage is only possible for biofuel cells operating in different individuals and this is not the most convenient strategy for powering implantable or wearable devices [82].

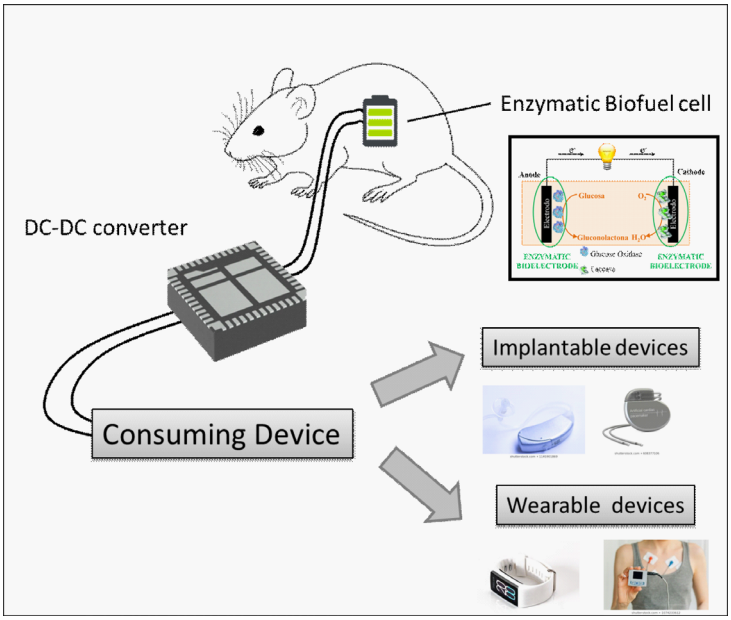

Fig. 7. Biofuel cell integration with the consuming device. In this example a DC-DC converter is used in order to increase the output voltage of a biofuel cell.

One of the solutions to the voltage problem could be the use of a DC-DC converter [87],[88] as shown in Figure 7. However, it must be considered that in this type of device, the increase in voltage comes with an increase in the current consumed; this involves an additional demand in the biofuel cell current output [14],[82]. When the electronic device to be powered does not work continuously other configurations can be applied. For example: a DC-DC converter combined with a capacitor (charge pump) [88] able to be charged up to a determined value and then release the energy stored when needed. This combination will allow the periodic activation of microelectronic devices when biofuel cell power output is small.

2.3. Enzymatic glucose biofuel cells for implantable devices and flexible electronics

2.3.1. Enzymatic glucose biofuel cells for implantable devices.

Biofuel cells employ biologic fuels such as glucose, an endogenous substance in biologic systems. This sets up the bases for the potential application of these systems as an energy source for implantable and portable devices [13],[30], [69]. To achieve this goal, biofuel cells must be biocompatible to allow them to be implanted in living organisms [71],[89],[90]. There are several works in literature related with this issue. However, two main problems arise related with short lifespan and low power density and these problems depend on enzyme stability, electron transfer rate and enzyme loading. These inconveniences must be solved in order to develop biofuel cells for more practical and real applications [13],[91],[92].

In literature there are reports of biofuel cells with the potential to be implanted in living organisms. In most of these studies, experiments are carried out in vitro in model solutions of serum or blood with different bio-electrode configurations [22][87],[93],[94],[95],[96],[97]. In recent years, it has been possible to implant glucose biofuel cells in living organisms such as insects [96],[97],[98],[99], molluscs [86],[100], lobsters [85] or mammals such as mice or rabbits [90],[91],[101]. Biofuel cells implanted in these living organisms were able to produce electric energy through modified electrodes with biocatalysts using the glucose present in body fluids and the oxygen present in body fluids and air as fuel. Figures 8 and 9 show examples of implantable biofuel cells and their performance in terms of power generated (Figure 8) and their capacity to power small electronics (Figure 9). Even though glucose is the most common fuel used due to its presence in blood and other body fluids, threalose (a physiologically produced sugar) is used as a fuel instead for insects [100].

The use of biofuel cells in energy supply for implantable devices such as pacemakers is possible but still requires improvement [85],[96]. Nowadays, some of the electrodes employed are too big to be implanted in the human body and this implies that current efficiency should be increased in order to develop smaller electrodes [13]. Biofuel cells used as an energy source in pacemakers are able to work for several hours and in some cases even for several days. However, the batteries currently employed in pacemakers last for approximately 10 years. Therefore, the biggest challenge for this kind of biofuel cell application is to achieve an operational stability that is greater than 10 years. There are, however, other kinds of *in vivo* applications not related with the use of pacemakers such as biosensors which do not require such long periods of stability [102]. Also, it is worth mentioning that the current observed varies depending on the living host (mammals, insects, molluscs) where the biofuel cell is implanted [13],[30],[34],[69].

Biofuel cell miniaturization could allow them to be implanted in the human brain. These biofuel cells would receive a constant glucose flow from cerebrospinal fluid. In this way, brain machines and the related interfaces could profit from having their implanted units fueled by glucose fuel cells. Then various biomedical technologies such as neuronal implants and systems to connect the brain with computers or electronic prosthetics could work with a continuous energy supply [71]. Efficiency of these biofuel cells could be affected by the decrease in oxygen concentration in both brain and CSF, as observed in the study carried out by Androlov and co-workers [103]. In this study a lethal dose of barbiturate was injected in the animal with an implanted biofuel cell in its brain. As the rat's heart stops beating, a drop in OCV values is observed. Then, efficiency of these biofuel cells could be affected when humans are asleep due to a lower availability of oxygen rather than due to the glucose flow from cerebrospinal fluid. Moreover, glucose concentration in cerebrospinal fluid is related with the level found in blood

that depends mainly on food intake [72]. Therefore, the decrease in glucose level in CSF at night might be considered for this application [104].

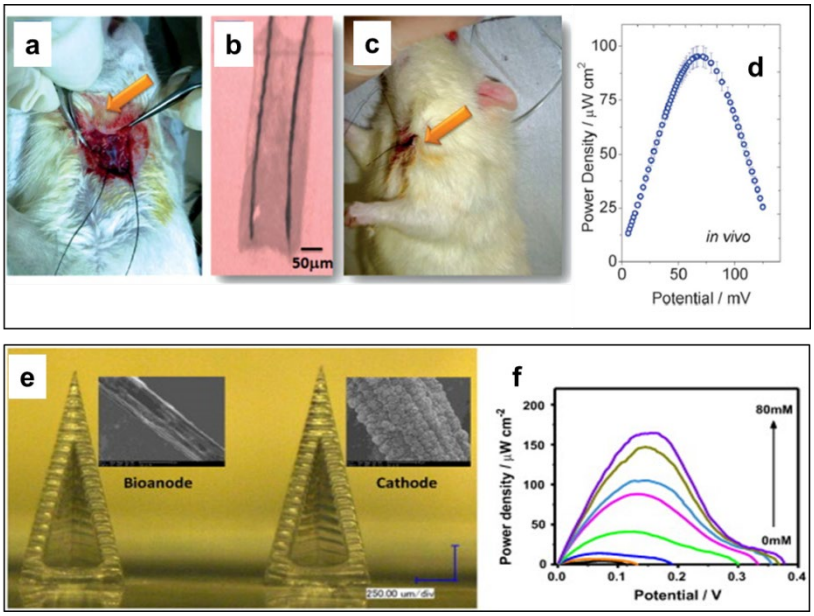

Fig.8 Examples of implantable enzymatic biofuel cells in operation: a) image of a carbon fiber-based biofuel cell implanted in the jugular vein of a rat; b) optical microscopy image of the implantable biofuel cell inside the catheter; c) surgical introduction of the catheter biofuel cell in a living rat; d) power density curve for the implantable biofuel cell operating in vivo; e) bio-anode and bio-cathode of a micro-needle glucose biofuel cell f) power curve in the presence of 0-80 mM glucose. Parts a-d are reproduced from reference [96] with permission of The Royal Society of Chemistry RSC 2020. Parts e-f are reproduced from [81] with permission of The Royal Society of Chemistry RSC 2020.

In recent years, enzymatic biofuel cells have been developed with improved properties. Professor Cosnier's group at the University of Grenoble has developed an enzymatic glucose biofuel cell able to operate stably for 1 year producing 30 mW·h. The configuration used in electrode development allows stability of the biofuel cell that is directly related with biocatalyst stability [95]. Furthermore, compact micro-fluidic biofuel cells able to operate with volumes in the micro-litre scale and to produce enough energy to fuel small electronics have also been developed [105]. Recently, a biofuel cell which had been implanted in a rabbit was able to operate stably for 2 months [91].

Pacemakers and defibrillators are examples of portable electronic devices in touch with biologic tissues for a better quality of life. However, modern lithium batteries are the most used energy sources for such devices raising concerns regarding leaching and poisoning [106],[107],[108]. Biofuel cells for energy supply to implantable devices have the features required to address this

issue. The incentive for biofuel cell development has been present during recent decades. However, the real potential has been evident due to important advances in Materials Science and nano-bioelectronics with remarkable examples of biofuel cells implanted in mice, cockroaches or lobsters as described previously. With further improvements, these systems could be used in the future as an energy source for implantable devices in living organisms, including humans [13],[97].

Finally, biofuel cells in mammals and invertebrates can be extended to plants as is the case for grapes [109] and oranges [88]. Advances in this field could contribute to remote sensor systems and environmental monitoring in the near future [21].

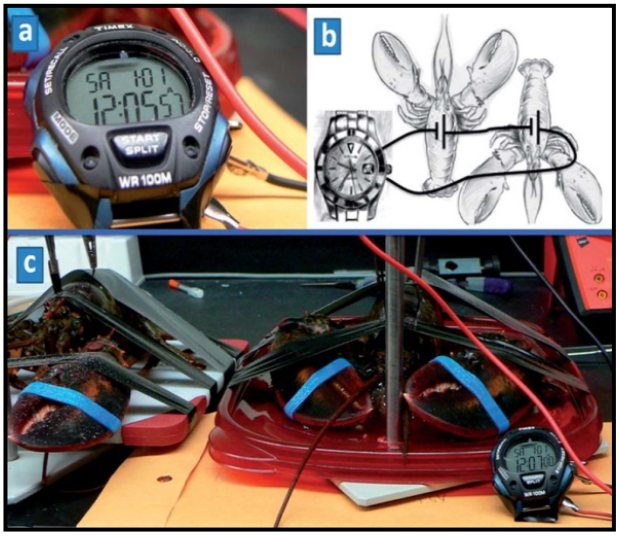

Fig. 9 Glucose biofuel cells connected in series implanted in 2 lobsters able to supply energy to a digital watch; (a) digital watch fueled by implantable glucose biofuel cells; (b) connection scheme for implanted biofuel cell assembly; (c) image of the experimental set up. Reproduced from reference [85] with permission of The Royal Society of Chemistry RSC 2020.

2.3.2. Biofuel cells for flexible electronics: epidermal and contact lens-based biofuel cells.

Recent advances in portable flexible electronic bio-devices providing information, entertainment and assistance with daily tasks appear as a result of the need for new technologies which can make life easier [110],[111]. The development of portable electronic devices is a promising discipline in its early stages and there are numerous challenges to be overcome in order for them to be adapted successfully to market needs [111],[112],[113]. Identification of suitable energy sources for portable devices is one of the biggest challenges present in most of the disciplines related with flexible electronics [92],[113],[114],[115].

One of the key parameters in energy sources for flexible electronics for portable devices lies in minimizing invasive capacity [92]. Most of the next generation portable devices are conceived

to be worn either body- or skin-attached. In most cases, the size of such portable devices is more related to battery than to electronic components. Implantable biofuel cells could provide a solution. However, it is preferable to avoid surgical intervention for biofuel cell implantation in non-medically related routine appliances, the reason being the inconvenience associated to wearing a sub-cutaneous device with the added problems of protuberances and biofouling [92],[96],[115]. Hence, in recent years biofuel cell development has been focused on the use of non-invasive configurations for epidermal skin-worn biofuel cells or contact lenses using lacrimal fluid as a fuel source [79],[80],[115]. These systems are briefly described in the following part:

Epidermal Biofuel cells- Epidermal biofuel cells are stuck on the skin surface and, as opposed to implantable biofuel cells, there is no need to pierce the skin and there are no protrusions outside the skin or on the skin's surface [78],[79],[116]. One of the most important requirements in epidermal biofuel cells is fuel selection considering that fuel must be supplied continuously and in the required amounts. For this type of biofuel cell, sweat from human perspiration is one of the most interesting fluids since it is produced in considerable amounts and is easily obtainable. Moreover, it consists of a mixture of substances, some of them being potential candidates as a fuel in biologic fuel cells [92],[78],[79],[116]. Among sweat components lactate stands out from the rest to be used as a fuel in epidermal biofuel cells. The reason for this is its abundance since we find lactate in the millimolar range in sweat. In addition, the enzymes lactate oxidase and lactate dehydrogenase show a remarkable ability to oxidize it [79],[80],[116]. Professor Wang's group at the University of California is working actively on epidermal biofuel cell development using lactate present in human sweat to produce electricity to supply energy for portable devices [78],[79],[80],[116].

Contact lens-based biofuel cells- Interest in smart sensor development based on contact lens and other optical devices has grown significantly during recent years [77],[117],[118],[119],[120],[121],[122]. The eye is one of the most complex and sensitive organs in the human body. Its soft nature, spherical form and confined structure hinder the development of contact lens-based devices. Inclusion of a reliable energy source is thus challenging and the implementation of a device with an integrated energy source appears to be the best option. Professor Shleev's group at Malmo University (Sweden) was one of the pioneering groups in establishing the need for ocular biofuel cells and in demonstrating its viability. In their research work they have used gold-supported enzymes to generate electricity from glucose and ascorbic acid in lacrimal fluid, for anodes and cathodes respectively [119]. The biofuel cells were capable of generating energy stably during several hours with the fuel concentration commonly found in tears. The main drawback of this system was related with the small size of the eye and low fuel concentration in tears. This problem was overcome by

Professor Minteer's group which built up enzymatic bio-electrodes using high surface area carbon to produce energy from lactate [120].

3. Future Challenges in Enzymatic Glucose Fuel Cells.

As with any emerging technology, biofuel cell research presents several challenges which need to be addressed; the success of developments related to this novel technology will be dependent on the manner in which these challenges are faced. The most important challenges discussed throughout this focus article are summarized below.

3.1.Enzymatic biofuel cell stability

One of the first challenges to be faced in enzymatic biofuel cell technology development is related with enzyme stability. Enzymes possess a marked tendency to lose activity when conditions are different from their ideal state working conditions or when they are immobilized onto a support. In these cases loss of activity critically affects their biocatalytic activity [123],[124],[125],[126]. When dealing with implantable device development, both stability and good biocatalytic activity become key parameters since the devices are usually employed in environments which are unfavorable for enzyme activity.

Enzyme activity is highly dependent on conditions such as pH and temperature, showing best catalytic action at specific conditions depending on the enzyme. Thus, changing conditions such as temperature, pressure, pH, ionic strength and humidity found in the different bio-fluids such as blood, sweat or tears can have a drastic effect on the enzyme's catalytic activity. Therefore the biofuel cell operation can be affected when the enzyme attached in the electrodic support operates in real environments. In addition, enzymes are not able to catalyze glucose transformation in changing environments during long term operation, which is also a major limitation. Another challenge to be faced by biofuel cell development technologies is related to the use of mediators that should be optimized or even avoided where possible. Instead, the use of glucose biofuel cells able to perform direct electron transfer with the electrode surface must be promoted [69].

Thus, the biofuel cell operation can be affected when the enzyme attached in the electrodic support operates in real environments [13],[72]. Protein engineering is addressing the issues related with the loss of enzyme activity when attached to electrodes or exposed to unfavorable conditions in long-term operations [125]. Thus, enzyme engineering could helpimprove enzyme attachment to provide better contact with the electrode surface or with the mediators for promoting electronic transference [125][126]. In this sense, modeling and docking techniques

can be useful to orientate the active site of the enzyme, promoting direct electron transference from the enzyme to the electrode.

On the other hand, specific activity, O_2 sensitivity and redox mediator interaction should be optimized if direct electron transference is not achieved. Protein engineering can be very useful to optimize enzyme structure without compromising its stability. Mutations in some amino acids in the glucose oxidase structure have demonstrated a significant impact on O_2 sensitivity and interaction with mediators. However there is still a long way ahead for researchers to detect which amino acids should be substituted taking into account the different enzymes and redox mediators used in electrode configuration [125]. This approach of protein engineering has been useful to improve the attachment of other enzymes such as bilirubin oxidase, allowing high retention of activity and perfect control of the attachment [127].

3.2.Power density supply and consuming device integration

Most of the electronic devices need a constant energy supply for optimal performance. In the case of enzymatic biofuel cells, a constant energy supply is difficult due to the fact that power obtained in these bio-devices depends on the fuel used. Fuel concentration for glucose biofuel cells or biofuel cells employing other metabolites depends on physiological parameters which will vary over time. This transitory concentration can have a detrimental effect on the biofuel cell capacity to produce energy [69]. Therefore, efforts in the field must address the development of systems able to reach a constant energy supply. There are several strategies that can be used to solve this issue; one being the development of micro-fluidic systems capable of bio-fluid storage and flow control [105]; another approach consists of bio-battery development able to be self-recharged continuously with bio-fluids [128].

In addition, sometimes substrate concentration is too low to supply power continuously. In these cases, biofuel cells could also be combined with other energy harvesting systems such as supercapacitors or charge pumps to store harvested energy and supply it later on in a controlled manner [129],[130],[131].

Glucose biofuel cells that have been developed in recent years have reached remarkable power densities and open circuit voltages in vitro [132]. This is the case with the glucose biofuel cell developed by Cosnier and co-workers based on carbon nanotube pressed disks with the enzymes Laccase and Glucose Oxidase. With this biofuel cell researchers were able to obtain a power density of about 1 mW/cm^2 with an open circuit voltage of 0.95 V [34]. In another study, Zhao and co-workers reported carbon nanodot –based glucose biofuel cell using Billirubin Oxidase and Glucose Oxidase as biocatalysts in the cathode and the anode respectively [133]. This biofuel cell was capable of delivering 40.8 $\mu W/cm^2$ with an open circuit voltage of 0.93 V

in vitro. Regarding the experiments performed in vivo, one of the best results reported for biofuel cell implantation in mammals was obtained using a carbon nanotube-based system. The biofuel cell was implanted in a rabbit during two months and was able to deliver a power density of 16 μW/ml with an open circuit voltage ranging between 0.58-0.65 V [91]. The appearance of problems related to biofouling and inflammatory processes hindered bio-device performance after 2 months.

In the case of lactate biofuel cells promising results have also been reported with a biofuel cell able to deliver 1.2 mW/cm^2 in the presence of 20 Mm lactate [134]. This biofuel cell was conformed onto a flexible support paving the way towards the development of novel energy sources for wearable technology. Other reported lactate biofuel cells were able to deliver about 50 $\mu W/cm^2$.

Biofuel cells developed to date appear to be useful to power wearable or implantable biomedical devices with the power consumption reported in Table 3 [131],[135],[136].

Table 3. Power consumption of some biomedical devices [135],[136]

BIOMEDICAL DEVICE	POWER CONSUMPTION
Pacemaker	10 μW - 30 W
Insuline Pump	70 μW - 400 μW
Neurostimulator	30 μW -3 mW
Muscle stimulator	1.3 mW
Cochlear implant	20 μW - 1 W
Artificial Organs	30 W
Hearing aid	50 μW

However, the restriction in the voltage needed for most of these kinds of devices must be taken into account. Implantable medical devices operate with a voltage of about 2-3 V [82],[84] and it would be necessary to interface with devices capable of storing and delivering the produced energy. Therefore, device interfacing and integration with the consuming device is a key point

to consider for the real application of enzymatic biofuel cells. In this way it will be possible to reach the required voltages for the performance of the consuming device.

3.3.Comfortability and mechanical properties

One of the requirements for the design and development of implantable or wearable enzyme-based biofuel cells is their comfortability and mechanical resistance. Common enzymatic biofuel cells are built using rigid electrodes with mechanical properties that differ from the ones found in biological tissues [30]. The incompatibility between biofuel cell materials and biologic tissue has a harmful effect on enzymatic biofuel cell efficiency and can also cause irritability problems in biologic tissues [13],[96],[105]. It is important that enzymatic biofuel cells have mechanical properties similar to those of the biologic tissues with which they will be in contact. In addition, biofuel cells must be tolerant of tissue deformations without biofuel cell performance being affected. At present, the strategies being followed to address this problem include the use of flexible and polymeric materials [69],[80],[105],[112],[113].

4. Conclusion and future perspectives

In summary, there are several challenges to be addressed in enzymatic glucose biofuel cell development before being competitive with currently used batteries. The main problems are related to:

- Immobilization strategies and exploitation of protein engineering to develop enzymatic biofuel cells for long term operation, suitable to supply energy to common electronic devices using an ideal combination of materials.
- Increase of enzymatic biofuel cell efficiency and performance. Device integration with energy storage systems in order to achieve sufficient voltage to power the consuming devices.
- Development of implantable biofuel cells comfortable and adapted to skin and human tissue morphology and resistant to tension and deformations caused by daily routine activities.

The field of implantable or portable enzymatic biofuel cells offers the possibility of facing challenges across different scientific disciplines. Application of the acquired know-how and knowledge of the state-of-the-art will help with identifying novel procedures in order to achieve advances and milestones to do more in-depth research into this technology.

Acknowledgements

Financial support from the Spanish Ministry of Science, Innovation and University, through the State Program for Talent and Employability Promotion 2013-2016 by means of Torres Quevedo research contract in the framework of Bio2 project (PTQ-14-07145) and from the Instituto Valenciano de Competitividad Empresarial – IVACE-GVA (BioSensCell project).

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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