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Electrochemical and electrophoretic deposition of enzymes: Principles, differences and application in miniaturized biosensor and biofuel cell electrodes

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ABSTRACT

Recent advances in nano-biotechnology have made it possible to realize a great variety of enzyme electrodes suitable for sensing and energy applications. In coating miniaturized electrodes with enzymes, there is no doubt that most of the available deposition processes suffer from the difficulty in depositing uniform and reproducible coatings of the active enzyme on the miniature transducer element. This mini-review highlights the promising prospects of two techniques, electrochemical deposition (ECD) and electrophoretic deposition (EPD), in enzyme immobilization onto miniaturized electrodes and their use as biosensors and biofuel cells. The main differences between ECD and EPD are described and highlighted in the sense to make it clear to the reader that both techniques employ electric fields to deposit enzyme but the conditions from which each process is achieved and hence the mechanisms are quite different. Many aspects dealing with deposition of enzyme under ECD and EPD are considered including surface charge of enzyme, its migration under the applied electric field and its precipitation on the electrode. Still all issues discussed in this mini-review are generic and need to be followed in the future by extensive theoretical and experimental research analysis. Finally, the advantages of ECD and EPD in fabrication of miniature biosensor and biofuel cell electrodes are described and discussed.

Contents

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1. Introduction

Nowadays, the development of high performance and reliable miniaturized enzyme electrodes is an important goal worth pursuing in the field of nano-biotechnology. The reason for this has to do with the fact that miniaturization offers numerous advantages (Dahlin, 2012). First, miniaturized enzyme electrodes are useful in analysis of small sample volumes, hence practical if only small amounts of biological fluids are provided or, waste saving if larger quantities are available. Second, because of their small areas, miniaturized enzyme electrodes can integrate into new technologies like microarray sensor for microfluidic systems. Third, if used in in vivo implantation, miniaturized enzyme electrodes create less damage of the tissue and hence quick healing. Obviously, there are several methods with which enzymes can be deposited onto the transducer element including but not limited to entrapment and crosslinking (Sheldon and van Pelt, 2013). However, an important problem in the application of enzymes for the development of miniaturized electrodes is the difficulty in depositing uniform and reproducible coatings of the active enzyme on the transducer element. To this end, electrochemical (ECD) and electrophoretic deposition (EPD) are techniques, which employ electric fields to produce apparently uniform and reproducible multilayer coatings of enzymes over very small areas. The latter is due to the ability to precisely manipulate the forces exerted on the charged enzymes by the applied external electric field and drive them to deposit on electrodes via electric forces.

ECD and EPD have recently gained increasing interest both in academia and partly in industrial sector because of being versatile and require simple apparatus. Historically, ECD of enzymes is known for several decades that date back to the early 1970s (Wang and Vieth, 1973). By contrast, EPD of enzymes is quite recent (Ammam and Fransaer, 2009). From the fundamental and application standpoints, the basic phenomena involved in ECD and EPD are not very well known and have not yet been the subject of extensive theoretical and experimental research analysis. Hence, further research and development work needs to be done in order to develop a full, quantitative understanding of the fundamental mechanisms of ECD and EPD of enzymes in order to optimize the working parameters for a broader use of especially in the fabrication of miniaturized enzyme electrodes. This mini-review discusses some aspects of enzyme deposition under ECD and EPD and the application of the miniaturized enzyme electrodes in biosensors and biofuel cells.

2. Principles and mechanisms of deposition of enzymes subjected to electric fields

The deposition process of enzymes subjected to external electric fields requires three important steps.

2.1. Step 1: Surface charge of enzymes

When it comes to deposit enzymes on electrodes, the first step that one has to think of is to enhance the surface charge of the dissolved/suspended enzyme in the fluid. It has to be kept in mind that the fluid employed for enzyme dissolution/suspension is usually water. When enzyme molecules make contact with water, the polarity of the solvent water promotes formation of an electrically charged interface (Shaw, 1980). This surface charge arises from essentially the ionization of the functional groups carried out by the aminoacid residues that yields either a positive

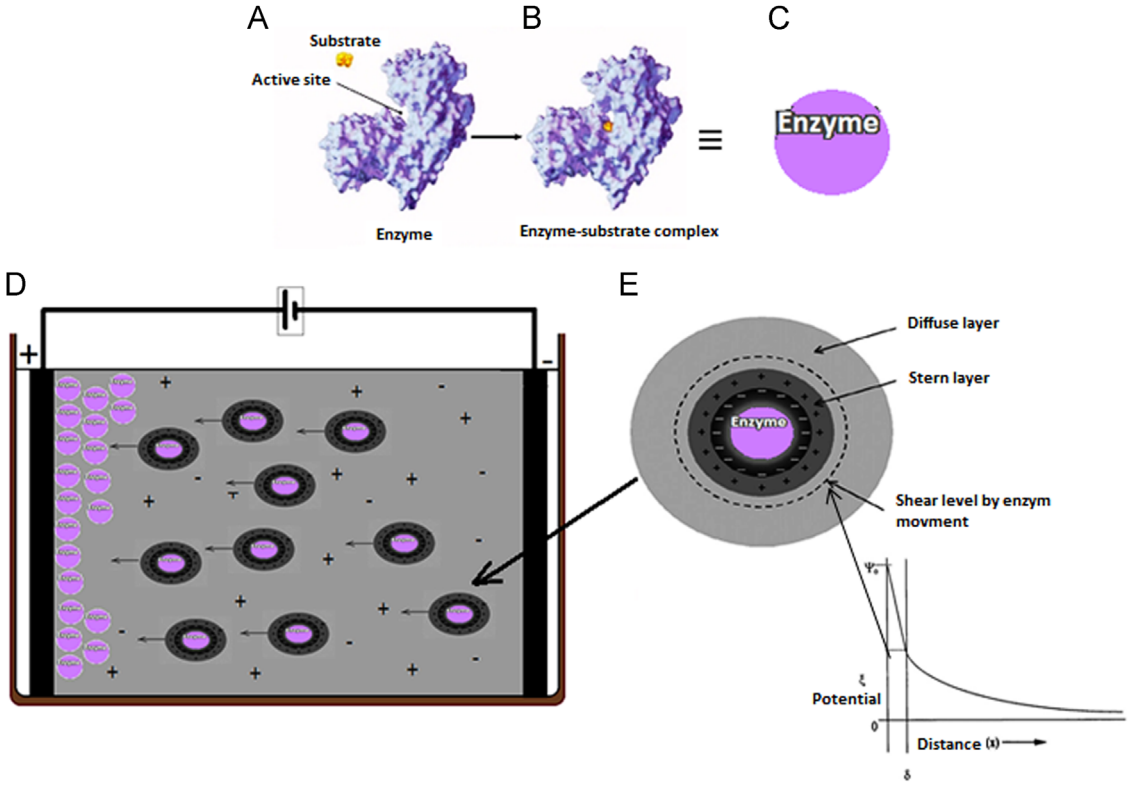

Fig. 1. (A) Structure of the globular protein of an enzyme shown as molecular model. (B) Is (A) binding to substrate (analyte) at the active site of the enzyme. (C) Represents a schematic model of this enzyme that will be used in some of the coming illustrations. (D) Represents scheme showing migration of a negatively charged enzyme towards the anode under an applied electric field. (E) Depiction of the surface charge of a negatively charged enzyme in aqueous media. The enzyme is surrounded by a fixed Stern layer of oppositely charged ions (in this case, positive ions) and outside this layer is a diffuse layer with ions of different polarities. The potential at the shear level in the diffuse layer is known as the zeta potential (ζ).

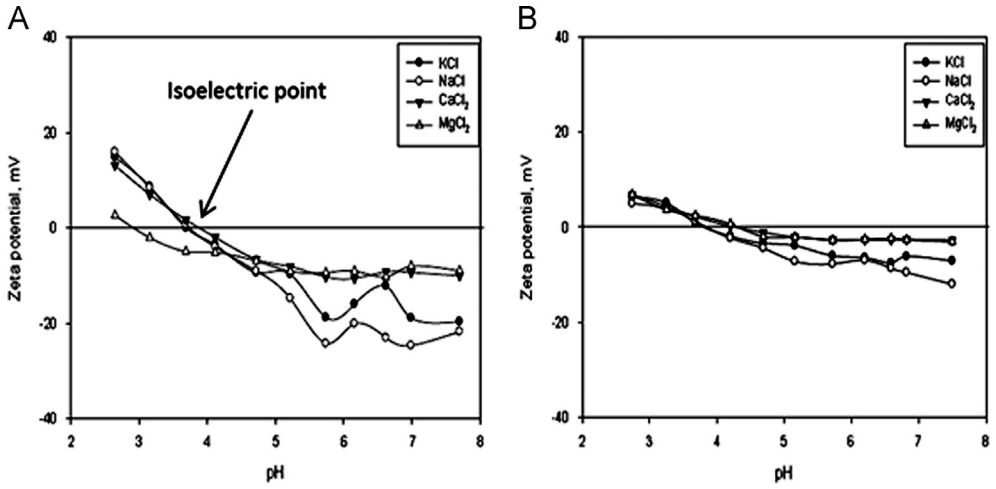

Fig. 2. Zeta potential of amylase enzyme as a function of pH and ionic strength of the electrolyte of 0.001 M (A) and 0.1 M (B). Image taken from Salgın et al. (2012) with permission of Int. J. Electrochem. Sci.

or a negative charge. For electroneutrality, when an enzyme is dissolved/suspended in water, a cloud of ions will surround the charged enzyme (Fig. 1E). Ions of opposite charge (counterions) will be attracted towards the surface of the charged enzyme to form what is known as Stern layer, while ions of similar charge will be pushed away from that surface to form what is known as diffuse double layer (Stern, 1924). The latter yields a net electrical charge on one side of the interface and a charge of opposite sign on the other side of the interface, forming what is called the electrical double layer of the enzyme. The enzyme surface at the share level between Stern and the diffuse layer is characterized by a potential called zeta potential (ζ, expressed in millivolts) (Lyklema, 1977; Velev and Bhatt, 2006). Fig. 2 illustrates a typical example on how ζ varies with pH for amylase enzyme dissolved in two media of low and high ionic strengths (Salgın et al., 2012). In acidic pH, the net charge of enzymes is usually positive, whereas in alkaline pH, the charge is negative (Salgın et al., 2012; Schultza et al., 2008). At a certain pH, enzymes will have no net charge, called isoionic point, which is between pH 3–4 for amylase, depending on nature of the electrolyte (Fig. 2). At neutral pH, enzymes are usually negatively charged (Salgın et al., 2012; Schultza et al., 2008). In general, elevated values of ζ are required for enzymes to avoid coagulation, which in turn facilitate their migration upon application of an electric field. However, as illustrated in Fig. 2, higher ζ are obtained in alkaline pHs at which enzyme activities may be altered and decreased. To keep maximum enzyme activities, it is thus recommended to operate around neutral pH, despite of having moderate values of ζ.

The ionic strength and nature of the supporting electrolyte are two other key parameters to consider in surface charge of dissolved/suspended enzymes. As illustrated in Fig. 2, very low ionic strength of the supporting electrolyte leads to elevated ζ. In addition, the composition of the supporting electrolyte, in terms of anions and cations affect ζ. In general, ζ of enzymes decreases following more or less the reverse Hofmeister series (Salgın et al., 2012):

For anions: $CO_3^{2-} > SO_4^{2-} > HPO_4^{2-} > OH^- > F^- > HCOO^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_3^- > I^- > ClO_4^- > SCN^-$.
For cations: $Ca^{2+} > Mg^{2+} > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+ > NH_4^+$.

In other words, higher ζ should be obtained by using very low ionic strength electrolytes composed of ions like: NO_3^-, ClO_3^-, I^-, ClO_4^-, SCN^- and their counterparts Rb^+, Cs^+ and NH_4^+. The reason for this has to do with the fact that divalent ions like CO_3^{2-}, SO_4^{2-}, Ca^{2+}, Mg^{2+} are more effective at salting out the enzyme solution/suspension than monovalent ions as ClO_4^-, SCN^-, Cs^+ and NH_4^+ (Baldwin, 1996). As a result, divalent ions induce higher ionic strength solutions/suspensions of enzymes, thus reducing their absolute ζ. On the other hand, it has to be kept in mind that commercialized enzymes usually contain salts for electroneutrality, thus, their dissolution/dispersion, even in ultrapure water gives rise to high conductivity solutions/suspensions, which in turn may decrease their ζ. To this end, if feasible, it is recommended to eliminate salts present in commercialized enzymes by washing them by means of chromatography for example.

The last factor worth considering in order to enhance ζ is via surface charge engineering of enzymes by substitution of some charging aminoacids on the protein surface (Koh et al., 2003; Russell and Fersht, 1987). Although from the synthetic standpoint this method might be complicated to accomplish, if it is well designed and successfully carried out, it may lead to a much-elevated values of ζ.

2.2. Step 2: Migration of enzymes

The second step in deposition process of enzymes has to do with migration of the charged enzyme dissolved/suspended in water under the applied electric field. If we assume a negatively charged enzyme surrounded by Stern and diffuse layers, upon application of an electric field, some of the ions outside the Stern layer can be sheared away and induce a migration of the enzyme towards the electrode of opposite charge (Fig. 1D). One important characteristic of the charged enzyme subjected to an electric field is its electrophoretic mobility ($\boldsymbol{\mu_{eph}}$). This quantity can be defined as the coefficient of proportionality between the electric field strength ($\boldsymbol{E}$) and the enzyme velocity ($\boldsymbol{V_{eph}}$) (Velev and Bhatt, 2006; Oddy and Santiago, 2004).

$$\boldsymbol{\mu_{eph(enzyme)} = V_{eph(enzyme)}/E} \quad (1)$$

In turn, $\boldsymbol{\mu_{eph}}$ increases with surface charge (ζ) and decreases with viscosity of the media (η). The coefficient of correlation between $\boldsymbol{\mu}$ and ζ depends on size of the enzyme. $\boldsymbol{\mu_{eph}}$ can be defined as equals to $2/3\varepsilon\varepsilon_0\eta\zeta$ (ε, ε_0 are the dielectric permittivity of water and vacuum, respectively) if the enzyme has a radius r much smaller than the Debye length of the counterionic atmosphere. For enzymes with higher radius $\boldsymbol{r}$, $\boldsymbol{\mu_{eph}}$ can be defined as equals to $\varepsilon\varepsilon_0\eta\zeta$.

By combining these quantities, it is possible to estimate the migration of a charged enzyme subjected to a uniform direct electric field in terms of its displacement $\boldsymbol{x}$ for a finite time t (Oddy and Santiago, 2004):

$$\boldsymbol{x}_{(\text{enzyme})} = \mu_{eph(\text{enzyme})} \boldsymbol{E}\boldsymbol{t} + \boldsymbol{x}_{0(\text{enzyme})} \quad (2)$$

where the constant $\boldsymbol{x}_{0(\text{enzyme})}$ determines the initial location of the enzyme between the two electrodes.

Hence, migration of a charged enzyme dissolved/suspended in water is affected by three factors: voltage, time and electrophoretic mobility. Strong applied electric fields for longer periods should induce larger migrations. With regard to this, it is worth noting that miniaturized electrodes (microelectrodes) offer a number of advantages over "normal" sized electrodes. The small size of microelectrodes and the short distance between the anode and the cathode increase spatial and temporal resolution when compared to their larger counterparts, thus allows for a short migration time scale of the charged enzymes. On the other hand, substantial electrophoretic mobilities, which are directly related to ζ, yields larger migrations. In sum, strong electric fields, longer deposition times and elevated ζ will theoretically yield substantial migrations or displacement of charged enzymes from bulk of the solution/suspension towards the electrode of opposite charge. Nevertheless, interfering phenomena such as water electrolysis induced by strong applied electric fields, low ζ of enzymes due to the lack of using appropriate charging agents are limiting factors in achieving this goal.

2.3. Step 3: Adhesion of enzymes on electrodes

After migration of the charged enzyme from bulk of the solution/suspension to the electrode of opposite charge, the final step in the deposition process under an applied electric field is the adhesion of enzymes on the electrode (Fig. 1D). For decades, the term "electrodeposition" was used to describe enzyme deposition by means of electric fields. However, it is worth noting that the term "electrodeposition" describes a process that uses electrical field to reduce metal cations from a solution to coat a conductive metal surface with thin films of that particular metal (Bicelli et al., 2008). For example, application of an electric field to a simple electroplating system containing copper sulphate solution induces migration of the positively charged copper cations towards the cathode where they are discharged and deposited as metallic copper ($Cu^{2+} + 2e^- = Cu_{\text{metal}}$). In contrast to metal cations, enzymes do not carry out net charges as metal cations. Therefore, enzymes do not adhere on electrodes via charge neutralization as metal cations do. However, the most possible hypothesis to explain adhesion of enzymes on electrodes is via precipitation. In other words, application of an electric field to a negatively charged enzyme dissolved/suspended in water will induce electrolysis of water at the electrodes. The decrease in pH at the immediate vicinity of the positively charged electrode (anode) drives the enzymes present nearby that surface to neutralization ($\zeta = 0$ as shown in Fig. 2 and Eq. (3)), hence induce their precipitation on the electrode (Im et al., 1995; Matsumoto et al., 2002; Ammam and Fransaer, 2009). In this process, size of the electrode may have a big impact on the deposition yield. For miniaturized electrodes (microelectrodes), the small size creates a low double-layer capacitance, which induces minimum drop current in the electrochemical double layer and thus most of the current will be Faradaic (Prieve et al., 2010). In turn, this induces an effective electric strength in the bulk of the enzyme solution/suspension, yielding substantial migration distances, which in turn leads to deposition of thick coatings of enzymes if compared to normal sized electrodes.

$$\textbf{enzyme}^{\zeta}_{(\textbf{solution})} + \boldsymbol{H}^+ = \textbf{enzyme}^{\textbf{0}}_{(\textbf{precipitate})} \quad (3)$$

3. Deposition of enzymes under electric fields

3.1. Electrochemical deposition (ECD)

ECD or commonly reported under the name of electrodeposition consists of applying a direct current (DC) electric field to an electrochemical cell containing the enzyme solution. It is common that ECD is accomplished from high conductivity solutions (buffers). As a result, low applied potentials (0–2 V vs. reference electrode) are employed to prevent generation of high amounts of water electrolysis and extreme heat that may affect the activity of the immobilized enzyme (Ammam and Fransaer, 2011b). Because low strength electric fields are utilized in ECD combined to the moderate ζ created by the high ionic strength buffer solutions, as a result, the migration of enzymes in ECD is generally insignificant (Eq. (2)). The latter yields deposition of thin enzyme coatings because only enzymes present nearby vicinity of the electrode surface precipitate. Fig. 3 depicts an example of thickness of an enzyme coating produced via ECD (Matsumoto et al., 2002). It can be seen that only few tens of nanometer are produced. The thickness of the enzyme coating can be increased to reach few hundreds of nanometers if a surfactant Triton X-100 is added to the enzyme solution prior to its deposition. Although it is not clear what role did Triton X-100 played in enzyme electrokinetics to yield thicker deposited films and only experiments should shed more light, theoretically Triton X-100 may affect the enzyme in two ways. On the one hand, Triton X-100 is a nonionic surfactant that contains large number of oxygen centers in its chain. This may induce a negative non-formal charge and hence facilitates the drifting of the enzymes towards the positively charged electrode (anode) and deposit. On the other hand, it is well known that a number of Triton X-100 molecules may surround a particle like enzyme to form micelles. Formation of micelles yields particles with larger sizes and improved suspension/solution stability. These conditions boost migration of enzyme-surfactant under the applied electric field to form thicker enzyme coatings on the electrode.

A survey of literature shows that ECD is one of the most methods employed for enzyme immobilization. The reason for this has to do with the fact that ECD requires an electrochemical apparatus that is simple, cost effective and easy to use. In addition, ECD is fully automated because a single system operator is the only direct labor required to achieve the task. As a result, throughout the years, ECD has passed by several developments of the process. For instance, ECD can be employed to drive enzymes alone to deposit on electrodes. But, it can also be modified to drive enzymes with other components including but not limited to: (i) Proteins like collagen and bovine serum albumin (Wang and Vieth, 1973; Johnson et al., 1994). (ii) Nobel metal salts like Pt or Pd to form metal catalysts with the deposited enzymes (Ikariyama et al., 1987; Johnson, 1991; Dong et al., 1993). (iii) Monomers such as pyrrole, phenol, 1,3-diaminobenzene, or their derivatives to form insoluble matrices in which the enzymes are entrapped (Fortier et al., 1990; Rishpon and Gottesfeld, 1991; Malitesta et al., 1990; Bartlett and Cooper, 1993; Curulli and Palleschi, 1997; Zhang et al., 1996; Gao et al., 2002). (iv) Small redox mediator molecules like prussian blue and ferrocene or redox centers attached to monomers to form enzymes-polymer-redox mediators coatings (Fei et al., 2003; Smutok et al., 2006; Polsky et al., 2007; Chiu et al., 2009; Qiu et al., 2009; Ackermann et al., 2010). (v) Non-polymeric matrices like sol–gels (Remirez and

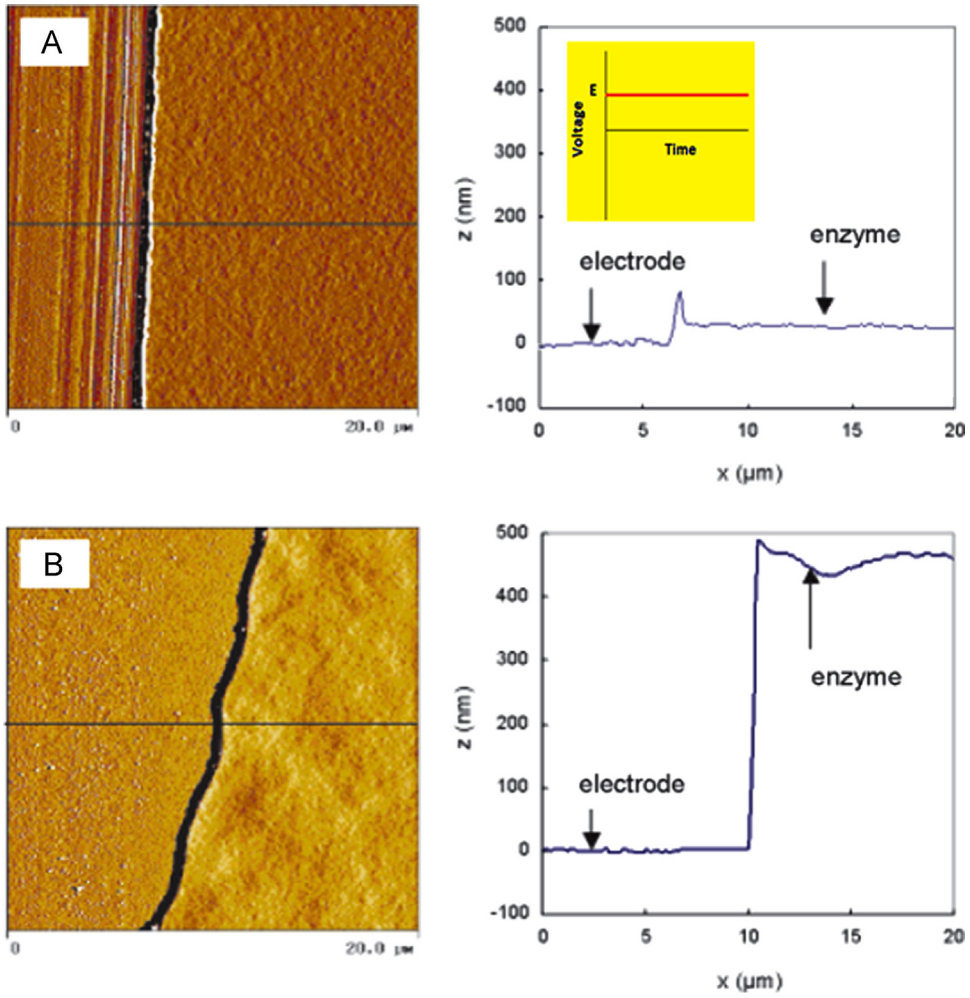

Fig. 3. Atomic Force Microscopy (AFM) images for glucose oxidase coating deposited under ECD at 1.3 V vs. AgCl/Ag for 1 h in a solution containing 10 mg/mL glucose oxidase without the surfactant Triton X-100 (A) and with 0.8 mM Triton X-100 (B). On the left side is shown the estimated thickness of the deposited enzyme coatings in both cases. Image taken from Matsumoto et al. (2002) with permission of ACS publications. In yellow background is shown the DC signal used to deposit the enzyme in both cases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Caruana, 2006), clay minerals (Mignani et al., 2006), and metal–organic films (Lei et al., 2011) utilized to stabilize the immobilized enzymes. (vi) Nano-materials like carbon nanotubes and metal nanoparticles deposited with enzymes (Chiu et al., 2009, Crumbliss et al., 1992; Stonehuerner et al., 1992; Lim et al., 2005; Diaconu et al., 2010; Li et al., 2012). (vii) Combinations between some of these processes (Wang et al., 2005; Kim and Oh, 1996; Chen et al., 2002). In all cases, it has to be kept in mind that the final goal of all these attempts is of course to produce enzyme electrodes with appropriate characteristics in terms of preserved activity, enhanced kinetics and stability to fit with the intended application. In terms of activity, a number of studies have shown that enzyme activities are usually preserved after ECD (Strike et al., 1993; Hoshi et al., 1994). In general, the activity of the enzyme electrodes prepared by ECD depends on thickness of the enzyme films (Im et al., 1995; Johnson, 1991; Almeida et al., 1993). Large thicknesses yield high activities, whereas thin films leads to moderate activities. Large thicknesses can be achieved by using voltages in the oxygen-evolution region, where decrease in pH near the anode drive larger amounts of enzymes to precipitate (Im et al., 1995). In terms of kinetics, several studies reported that enzymes deposited under ECD may or may not keep similar kinetics as dissolved enzymes (Iwuoha et al., 1997; Vidal et al., 1999; Morrin et al., 2003; Eftekhari, 2004). This depends on the microenvironment of the deposited enzyme coating and whether the enzyme is deposited alone or in presence of other components like conducting polymers or redox/electron promoters. Hence, the kinetics may become faster or slower with respect to dissolved enzyme. Finally, in terms of stability, enzyme electrodes fabricated via ECD of enzymes alone or in presence of other proteins or metal catalysts have usually moderate stabilities. The latter can be improved if monomers are electrochemically co-deposited with the enzymes to form enzyme encapsulated within insoluble matrices (Trojanowicz et al., 1995; Pałys et al., 2010).

3.2. *Electrophoretic deposition (EPD)*

The basic difference between ECD and EPD is that the latter is based on enzyme dissolved/suspended in low ionic strength aqueous solvent whereas the former is based on solution of salts (buffers) (Ammam, 2012). In other words, ECD is performed from high conductivity solutions/suspensions (enzyme+electrolyte salts), whereas EPD is conducted from low conductivity aqueous solutions/suspensions (enzyme+water with some charging agent). In turn; this induces high ζ of enzymes in EPD if compared to ECD. Furthermore, whereas ECD needs few volts to deposit enzymes, EPD requires high strength electric fields that may reach several hundreds of volts to move the charged enzymes from bulk of the solution/suspension to the electrode. Both parameters of elevated ζ and high strength electric field yield significant

migration of enzymes under EPD if compared to ECD (Eq. (2)). As a result, more enzymes reach the surface of the electrode and precipitate to form thick-deposited coatings (Ammam and Fransaer, 2009, 2010c). However, it is important to bear in mind that if high strength electric fields are employed in EPD, continuous direct current (DC) can no longer be utilized because it induces heat and extensive evolution of water electrolysis. This causes dramatic changes in temperature and pH near the electrodes, which in turn will decrease the activity of the deposited enzymes (Ammam and Fransaer, 2011b). To overcome the problem, two other categories of electric fields can be employed in EPD of enzymes: pulsed direct current (PDC) and alternating current (AC).

Because PDC contains an OFF time where no or low electric field is applied to the electrodes, it is possible to have the control over the amount of water electrolysis if duration of ON time is short enough to prevent evolution of gases (insert of Fig. 4B). Thus, substantial voltages can be applied for short periods that according to Eq. (2) will lead to higher migrations of the charged enzymes. Therefore, thick enzyme films maybe formed under PDC. As shown in EQCM results of Fig. 4, the deposited mass of glucose oxidase subjected to PDC are several 10-folds higher than those prepared via continuous direct current (CDC). Furthermore, because PDC generates less water electrolysis, it induces minimum shift in pH nearby the electrode surface, leading to preservation of maximum enzymes activities. In this view, Ammam et al. recorded an increase in activity of the deposited enzyme by ~25% when they switched from CDC to PDC under the same conditions (Ammam and Fransaer, 2010c).

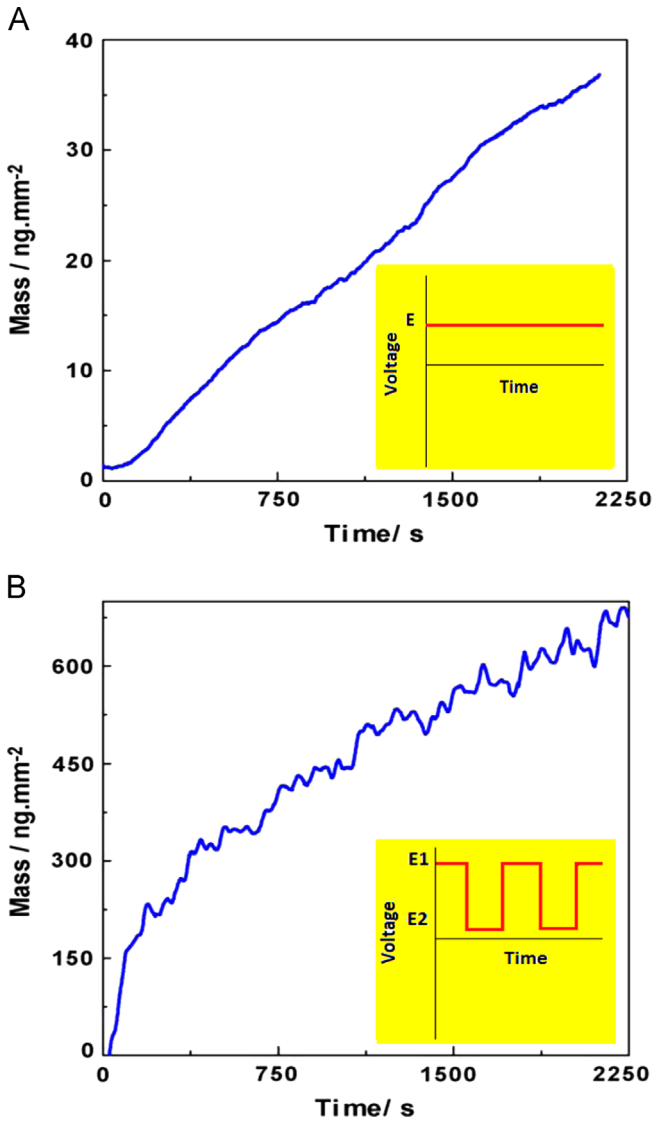

Fig. 4. Electrochemical quartz crystal microbalance (EQCM) mass change during ECD of GOx from (A) phosphate buffer pH 7.4 at 1 V vs. Ag/AgCl and, (B) ultrapure water using square wave potential 4 V vs. Ag/AgCl for 30 s and 0.5 V vs. Ag/AgCl for 10 s. Image taken from Ammam and Fransaer (2010c) with permission of Elsevier. In yellow background is shown the DC and PDC signals used to deposit the enzyme in both cases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The other category of electric fields that is used in EPD of enzymes is based on AC, which if compared to DC contains both positive and negative voltage/current components (Ammam and Fransaer, 2009). It is important to keep in mind that in AC there is no fixed anode or cathode but the polarization of each electrode changes continuously between the positive and negative signs. Thus, if a negatively charged enzyme is subjected to a symmetrical AC signal (inset of Fig. 5A′), it should only oscillate in one location because the migration achieved during the first half cycle when one of the electrodes is positively charged should be neutralized during the second half cycle when the other electrode becomes positively charged. Thus, the net migration of the negatively charged enzyme under symmetrical signals is actually zero. As a result, only thin enzyme coatings can be deposited as shown in Fig. 5A (Ammam and Fransaer, 2009). By contrast, if an asymmetrical AC signal with no net DC component is applied to a negatively charged enzyme (insert of Fig. 5B′), the difference in voltage height and time duration between the two half-cycles of the period, shall push the charged enzyme to migrate significant distance just like in DC fields, if the applied voltage is sufficiently high. The latter can be described by adding a second terms to Eq. (1) with the cubic dependence on the electric field strength to yield Eq. (4) (Dukhin and Dukhin, 2005).

$$V_{eph(\text{enzyme})} = \mu_{eph}E + \mu_{eph,3}E^3 \quad (4)$$

Hence, under asymmetrical AC fields, large amount of enzymes accumulate nearby the electrode surface and because minimum but sufficient electrolysis of water is also induced, the enzymes precipitate to yield thick-deposited coatings (Fig. 5B). In the main time, because AC fields generate minimum water electrolysis and ohmic heat at the electrodes if compared to DC or PDC, thus maximum enzyme activities are preserved after deposition (Ammam and Fransaer, 2009, 2010d, 2010e). Therefore, EPD of enzymes under asymmetric AC fields can successfully be used to produce thick enzyme coatings with higher activities. For this reason, enzymes immobilization under AC is quite investigated despite of being very recent. This can be seen in the numerous enzymes which have been successfully deposited (Ammam and Fransaer, 2009, 2010e, 2012a, 2010b, 2011a, 2012b, 2013). When it comes to deposit enzymes under EPD, one goal is to determine the optimal parameters under which thick and active enzymes coatings are produced. In this respect, Ammam et al. determined five parameters that may affect thickness and activity of the enzyme coatings. The first two parameters deal with voltage and time of which the deposition yield of enzymes is seen to increase quasi-linearly with polarization time and applied voltage then reaches a saturation plateau. At high-applied voltage-time, extensive electrolysis of water may take place, which in turn will play the role not in precipitating more enzymes on the electrode but washing part of it. The third parameter has to do with the frequency of which the deposition rate is recorded to increase up to a certain frequency value then levels off or decreases. If the frequency is changing faster, the charged enzymes will no longer follow the AC

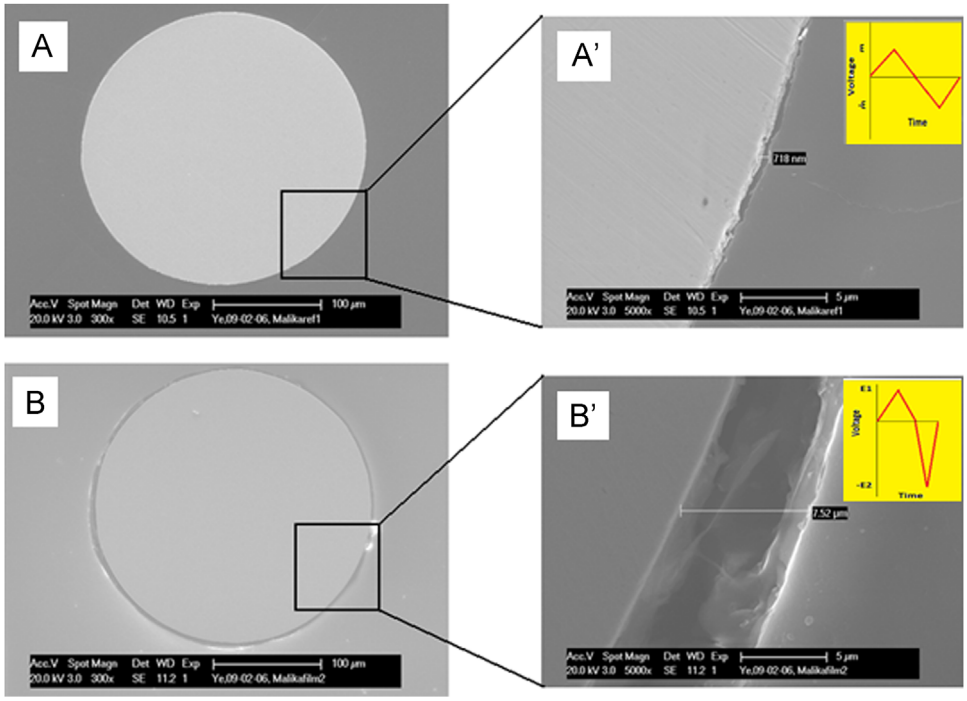

Fig. 5. Scanning electron microscopy (SEM) images showing a cross section of a platinum wire without (A) and with (B) glucose oxidase enzyme modification using symmetrical (A) and asymmetrical (B) AC at 30 Hz, 160 $V_{p\text{-}p}$ for 20 min. (A′) and (B′) are close-ups of the platinum-resin interface. Image taken from Ammam and Fransaer (2009) with permission of Elsevier. In yellow background is shown the symmetrical and asymmetrical triangular signals employed to deposit the enzyme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison between ECD and EPD of enzymes alone. Because additives like nanomaterials, conducting polymers, redox mediators and others may boost both techniques, hence not relevant for comparison.

	ECD	EPD
Representative references	Matsumoto et al. (2002), Chen et al. (2002), Ammam and Fransaer (2010c)	Ammam and Fransaer (2009, 2010c, 2010d)
Equipment	Potentiostat (amperometry, potentiometry or cyclic voltammetry)	Function generator (DC or AC power supplies), amplifier. Potentiostat
Electrolyte	High conductivity solutions (buffers)	Low conductivity solutions (water with some charging agent)
Surface charge of enzymes (ζ)	Moderate	High
Nature of applied electric field	DC	PDC, AC
Applied electric field strength	Low (less than 2 V)	It could very high (up to 200 V AC)
Thickness of deposited enzyme coatings	Thin (monolayers of no more than few hundred of nm)	Thick (multilayers that may be over 10 µm)
Morphology of the deposited enzyme coatings	Smooth	Rough
Activity of the enzyme coatings	Moderate	High
Sensitivities of the enzyme electrodes	Moderate (e.g. for glucose oxidase ~74 nA mM^{-1} mm^{-2})	Large (e.g. for glucose oxidase 430 nA mM^{-1} mm^{-2})

field because of their inertia, hence their net migration is significantly reduced and so the deposited yield. The fourth parameter has to do with the enzyme concentration for which the deposited yield is observed to increase up to a certain value then decreases. Because commercialized enzymes contain salts for electroneutrality, an increase in concentration yields high conductivity solution/suspension of enzymes, which in turn induce evolution of elevated rates of water electrolysis. Finally, shape of the applied AC signal affects also the deposited yield. For instance, triangular asymmetric waves result in higher deposition rates if compared to rectangular and sine waves. In sum, elevated voltages and deposition times that are associated with low water electrolysis, intermediate frequencies and enzyme concentrations under an applied asymmetrical AC triangular wave should yield thick and active enzyme coatings. Table 1 gives a brief comparison between both ECD and EPD techniques.

4. Application of enzyme electrodes by ECD and EPD

4.1. As biosensor electrodes

An enzyme-based biosensor is an analytical device that is employed to determine and measure a particular analyte. It is composed of an immobilized layer of enzymes deposited on a transducer element (electrode). If the properties and activity of the enzyme is preserved after deposition, it will react and recognize the analyte. The molecular recognition between enzyme-analyte induces a signal that defines the biosensor characteristics (Fig. 6A). When it comes to enzyme deposition, the final goal is to produce a biosensor with appropriate characteristics in terms of substantial sensitivity towards the analyte, low response to interferences, extended linear range, short response time and stability (Mehrvar and Abdi, 2004; Wilson and Ammam, 2007). In this respect, ECD

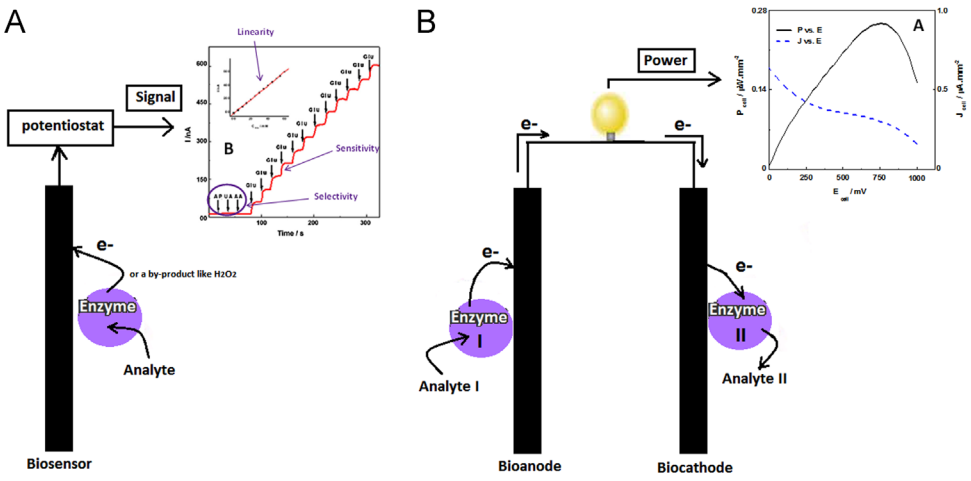

Fig. 6. Scheme representing enzyme electrodes functioning as a biosensor (A) and as an enzyme biofuel cell (B).

and EPD are versatile techniques, meaning that a variety of parameters such as applied voltage, frequency, deposition time, nature of the applied electric signal, enzyme concentration can be controlled to yield enzyme films with desired characteristics and thus better responses under the optimized conditions. Furthermore, because under ECD and EPD, enzymes are driven via electric forces, thus they can be manipulated to coat miniaturized electrodes that have complex shapes with uniform enzyme films. As a result, using ECD and EPD, a number of biosensors with improved sensitivities have been fabricated to detect analytes like glucose, lactose, glutamate, cholesterol, H_2O_2 and others (Dong et al., 1993; Chen et al., 2002; Chiu et al., 2009; Mignani et al., 2006; Lei et al., 2011; Ammam and Fransaer, 2010d, 2010e, 2010b, 2011a). In terms of selectivity towards the interferences, ECD and EPD of enzymes without other components is usually not sufficient to prevent interferences from passing through the enzyme coating to reach the transducer element and induce a signal. To overcome the problem, several ways have been considered. One avenue is to deposit very thick and dense enzyme coatings on electrodes via EPD. The large thickness will prevent part of the interferences from travelling through the enzyme coating (Ammam and Fransaer, 2009, 2010d, 2011a). Another efficient means to exclude interferences is by co-depositing enzymes with polymers, sol–gels and other materials to yield enzymes entrapped in matrices (Chen et al., 2002; Smutok et al., 2006; Iwuoha et al., 1997; Ciriello et al., 2000; Guerrieri et al., 2009). The morphology of the matrices and their low porosity prevent interferences with high molecular weights from travelling through the enzyme-matrix coating to reach the transducer. Because polymers or sol–gels have lower solubilities in aqueous media, the matrix is also beneficial in extending the lifetime and stability. Hence, the biosensors may function for long periods (Chen et al., 2002; Pałys et al., 2010; Qiu et al., 2009; Li et al., 2012). Another advantage of the matrix is that it can also play an important role in boosting the linearity of the biosensor's response, especially if it is applied as an outer layer to regulate the diffusion of the analyte to the enzyme coating. This allows measurement of elevated concentrations of analytes. On the other hand, if compared to coatings containing only enzymes, the presence of the matrix may affect the response time of the biosensor (Lyklema, 1977; Chen et al., 2002). This is quite understandable because the matrix slows down diffusion of the analyte towards the enzyme coating. Since the final goal of the biosensor technology is the measurement of analytes in real samples and real time, a number of biosensors fabricated via ECD and EPD are found dependable for determination of analytes in real samples such as cultivated plants, serum, milk, blood and living human hepatoma cells (Diaconu et al., 2010; Vidal et al., 1999; Ammam and Fransaer, 2010d, 2010e; Rui et al., 2010). The latter is promising if we consider the challenging aspects of biosensors in real samples.

4.2. As biofuel cell anodes and cathodes

Compared to an enzyme biosensor where only one electrode is required in the device construction, an enzyme biofuel cell requires two enzyme electrodes (Fig. 6B): one to act as a bioanode (electrons generator) and another to act as a biocathode (electrons receiver). When the two enzyme electrodes are connected together in presence of the respective analytes (or fuels) like, glucose at the bioanode and O_2 at the biocathode, the electrochemical reactions induced from oxidation/reduction of the analytes lead to an electron flow through the external circuit. This flow is driven by an electromotive force that is raised from the difference in potential between the two enzyme electrodes (Palmore et al., 1998; Katz et al., 1999; Cooney et al., 2008; Meredith and Minteer, 2012; Mano et al., 2003). Compared to a biosensor where electron communication between the deposited enzyme and the electrode may not be necessary (for first and third-generation biosensors) because an induced by-product like H_2O_2 from the immobilized enzyme-analyte reaction or O_2 consumption by the immobilized enzyme suffice to induce a signal that will define the characteristics of the biosensor. By contrast, electron communication between the enzyme and the electrode is necessary in enzyme biofuel cells to induce an electron flow and hence power. The reason for this has to do with enzymes that generally have their redox centers buried inside their complex structures and hence distant from the electrode. To this end, electron mediators/promoters are required to wire the generated/received electrons between deposited enzymes and the electrodes. When it comes to deposit enzymes on electrodes for use as miniaturized biofuel cells, the final goal is to produce enzyme electrodes with enhanced characteristics in terms of high output power and good stability. The power density ($\boldsymbol{P_{cell}}=\boldsymbol{E_{cell}}\times\boldsymbol{J_{cell}}$) is determined by cell voltage ($\boldsymbol{E_{cell}}$) and cell current density ($\boldsymbol{J_{cell}}$). An up to date overview of literature reveals that enzyme electrodes prepared by ECD and EPD have the potential to be used as biofuel cells electrodes. However, if compared to their wide use as biosensors, their application in biofuel cells is still far less. The reason for this is related to the preparation of the enzyme electrodes that require more elaborate procedures to be used as biofuel cells than as biosensors. In other words, when enzymes without electron mediators/promoters are

immobilized on electrodes, they can be useful as first-generation biosensors. However, they cannot be employed as biofuel cells because electron communication between redox centers of the immobilized enzymes and the electrode is not sufficient (Meredith and Minteer, 2012). To this end, because ECD and EPD are versatile techniques, electron mediators/promoters such as conducting polymers, small organic redox mediators and nanomaterials can also be driven by means of electric forces to deposit with the enzymes in one or several steps. In view of this, polymers containing Os-complexes deposited via ECD are found to be excellent in shuttling electrons from electrodes to the deposited enzymes like bilirubin oxidase (Karnicka et al., 2007; Shen et al.; 2013) or laccase (Ackermann et al., 2010) to induce an efficient reduction of O_2 at the biocathode. Other alternatives based on imidazolium ionic liquids, carbon nanotubes and monomers deposited using ECD and EPD are found to promote a better electron-relay between enzyme-electrode to generate high power outputs (Ammam and Fransaer, 2010a, 2012b, 2013, 2012a). The presence of these electron mediators/promoters facilitates the oxidation/reduction of analytes like glucose/O_2 or glucose/O_2–H_2O_2 at bioanode/biocathode. This efficient electron mediation induces shifts in the starting potentials of the electrooxidation/ electroreduction towards earlier values, which in turn improves cell voltages and thus power outputs. As for biosensors, the final goal of miniaturized enzyme biofuel cells fabricated via ECD and EPD is their use to generate energy from real samples and in real time. Examples include providing microelectronic devices like implantable micro-sensors transmitters or artificial organs with sufficient power (Barton et al., 2004; Bullen et al., 2006). To this end, a number of newly constructed enzyme biofuel cells via ECD and EPD are found to be dependable for use in biological fluids like human serum (Ammam and Fransaer, 2012b, 2010a). However, in biological fluids, reductions in power densities are generally observed

Table 2
A brief comparison between the characteristics of biosensor and biofuel cell electrodes constructed by ECD and EPD.

Deposition method	Biosensor electrodes					Biofuel cell electrodes	
	Sensitivity	Selectivity	Linearity	Response time	Stability	Power output	Stability
ECD	• If compared to EPD, the sensitivities are moderate • Optimal sensitivities can be obtained if large amounts of enzymes are deposited on the transducers (i.e., optimal high voltage-time are applied without generating large amounts of electrolysis).	The selectivities are moderate because ECD of enzymes alone do not form dense films that may prevent interferences from travelling through the film. • Co-deposition of polymers or other membranes are required to prevent interferences from reaching the transducer • The membranes can also be applied as inner/outer layers	• The linearity can be extended by co-depositing enzymes with polymers to regulate the diffusion of analytes fluxes reaching the enzyme or by co-depositing the enzymes with redox mediators.	• It depends on the film thickness and its porosity. • If the enzyme film is thick and dense, response time to analyte will be long and if the film is thin, the response time should be short.	• Stabilities are moderate if only enzymes are deposited. • Can be improved by using stabilizers like polymers co-deposited with the enzymes or applied as outer membranes.	• It depends not only on how much enzyme is deposited on the electrode but more importantly on how the generated/ collected electrons are delivered to/from the current collector. • Electron mediator/ promoters such as conducting polymers, small organic redox mediators and nanomaterials are key factors in getting high output power.	• The stability of the enzyme electrodes can be improved by using stabilizers like polymers co-deposited with the enzymes or applied as outer membranes.
EPD	• The sensitivities can be very high if appropriate conditions are employed (i.e., high asymmetric AC voltage, triangular waves, low ionic strength solutions, longer deposition periods without generating high amounts of water electrolysis)	• Because enzyme films fabricated via EPD maybe very thick and dense, thus they can exclude part of the interferences • However, in general, stabilizers like polymers applied as outer or inner membranes are required for enhanced selectivities	• Because enzyme films by EPD are very thick and dense, hence they can self-regulate the diffusion of analyte to the immobilized enzyme, resulting in relatively extended linearities • Can be increased by depositing outer membranes of polymers/sol–gels or by incorporating additional redox mediators with the deposited enzymes	• It depends on the film thickness • Response times maybe long because thick films are generally deposited under EPD	• The more the film is thick and dense, the more the stability increases • However, stabilizers like polymers/ sol–gels applied as outer layers are required to extend lifetime of the biosensors	• Because EPD deposit high amounts of enzymes on electrodes, if redox/ electron promoters are successfully incorporated, it may yield to much higher power outputs if compared to BFCs fabricated via ECD	• Because EPD forms dense and thick enzyme films, the stability is relatively better than films produced via ECD • The stability of the enzyme electrodes by EPD can be boosted using stabilizers like polymers co-deposited with the enzymes or applied as outer membranes
Selected references	Ammam and Fransaer (2009, 2010d, 2011a), Chen et al. (2002), Smutok et al. (2006), Iwuoha et al. (1997), Ciriello et al. (2000), Guerrieri et al. (2009), Pałys et al. (2010), Qiu et al. (2009), Li et al. (2012), Lyklema (1977)					Ammam and Fransaer (2010a, 2012b, 2013, 2012a), Ackermann et al. (2010), Karnicka et al. (2007), Shen et al. (2013)	

if compared to buffers. The latter is related to chemicals/ protein fragments found in biological fluids that after their adsorption on the electrode surface restrict diffusion of analyte molecules to the enzyme coating. In terms of stability, if compared to enzyme electrodes employed as biosensors, biofuel cells generally suffer from lower stabilities. This is caused by water electrolysis and heat emission induced in biofuel cells, which decrease the enzyme activities and result in a quick deterioration. By contrast, a biosensor can be polarized at fixed potentials where electrolysis and heat emission could be minimized or avoided. Table 2 compares the characteristics of the resulting biosensor and biofuel cell electrodes fabricated via ECD and EPD.

5. Summary and conclusions

The reviewed literature revealed that ECD and EPD are promising techniques in deposition of enzymes and manufacturing of biosensors and biofuel cells. Both ECD and EPD employ electric forces to manipulate and drive charged enzymes to deposit on electrodes. Thus, ECD and EPD are suitable to deposit enzymes on electrodes having smaller areas and complex shapes. From the fundamental viewpoint, there are differences between ECD and EPD. ECD is carried out from high conductivity solutions/suspensions containing enzyme with salts. Under these conditions, enzymes gain low values of ζ. Furthermore, because high ionic strength are used, only low electric fields could be utilized to deposit enzymes and prevent massive evolution of water electrolysis that may decrease the enzyme activities. As a result, enzymes migrate meaningless distances to form thin enzyme coatings. By comparison, EPD of enzymes is carried out from low conductivity solutions/suspensions. This enables enzymes to gain substantial ζ. In addition, because low ionic strength solutions are utilized, hence high electric field strengths can be applied without inducing massive water electrolysis. This in turn induces significant migration of enzymes from bulk of solution/ suspension to the electrode of opposite charge, resulting in formation of thick and active enzyme coatings. ECD and EPD techniques have the advantage of being versatile, easy to use, automated and require a simple apparatus. Hence, useful for the manufacturing of enzyme electrodes with higher reproducibilities. Furthermore, if proper fixturing is utilized, numerous electrodes can be prepared simultaneously. As a result, using ECD and EPD, a number of miniaturized biosensors with improved properties in terms of relevant sensitivity and selectivity, extended linear range and stability suitable for use in real samples and real time have been developed. Enzyme electrodes manufactured by ECD and EPD can also be employed as biofuel cells. This can be accomplished by incorporating electron mediators/ promoters like conducting polymers, redox mediators and nanomaterials in order to boost the electron transfer and communication between the immobilized enzymes and the electrodes.

6. Future perspectives

Because of the importance of enzyme biosensors and biofuel cells in today's nano-biotechnology, further quantitative studies of enzyme deposition under ECD and EPD will allow in depth understanding of the principles and mechanisms of the deposition processes. This can be done by using the state of the arts of various physicochemical techniques and their combinations. On the other hand, since parameters like surface charge and particle size are very important in successful enzyme deposition, much attention should be paid for optimizing the preparation conditions of enzymes such as employing appropriate charging agents to enhance ζ of enzymes in order to enhance their migrations under the applied electric fields. This may seem at first exploratory, but if well targeted using synthetic methods, it may lead to elevated ζ of enzymes. The latter should be combined with the use of appropriate electrolytes (anions and cations) as well as low ionic strength electrolytes for dissolution/ suspension of enzymes in order to allow higher strength electric fields to be employed without inducing high rates of water electrolysis. If all these factors are well controlled, the characteristics of the deposited enzymes films will be improved to fit with the design and fabrication of high quality enzyme coatings suitable for novel generation of future advanced enzyme biosensors and biofuel cells.

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