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Review

Direct Electron Transfer: An Approach for Electrochemical Biosensors with Higher Selectivity and Sensitivity

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A abordagem mais promissora para o desenvolvimento de biossensores eletroquímicos é a busca da comunicação elétrica direta entre as biomoléculas e a superfície do eletrodo. Esta revisão apresenta as principais abordagens, estratégias e avanços no desenvolvimento de biossensores eletroquímicos de terceira geração. Os temas discutidos englobam uma breve descrição sobre os fundamentos do fenômeno de transferência de elétrons e sobre o desenvolvimento de biossensores amperométricos (diferentes tipos e novas técnicas de imobilização orientada de enzimas). Um enfoque especial foi dado para as enzimas e proteínas redox capazes de eletrocatalisar reações *via* transferência direta de elétrons. Também foram apresentadas e discutidas as aplicações analíticas e tendências futuras dos biossensores de terceira geração.

The most promising approach for the development of electrochemical biosensors is to establish a direct electrical communication between the biomolecules and the electrode surface. This review focuses on advances, directions and strategies in the development of third generation electrochemical biosensors. Subjects covered include a brief description of the fundamentals of the electron transfer phenomenon and amperometric biosensor development (different types and new oriented enzyme immobilization techniques). Special attention is given to different redox enzymes and proteins capable of electrocatalyzing reactions *via* direct electron transfer. The analytical applications and future trends for third generation biosensors are also presented and discussed.

Keywords: direct electron transfer, electrochemical biosensors, electrodes, redox enzymes, self-assembled monolayer

1. Introduction

Electron transfer (ET) is ubiquitous in biological and chemical systems. Thus, understanding and controlling this process comprise one of the broadest and most active research areas of science nowadays. Usually, electron transfer occurs in nature in connection with energy transduction. For example, in oxidative phosphorylation, NADH releases electrons to O_2 to form water and a substantial amount of energy, used to make ATP.[1] In chemical systems, mechanisms involving bond fracture or bond formation very often proceed by an electron transfer mechanism. Furthermore, the solid state electronics age depends critically on the control of electron transfer and electron transport in semiconductors, while the nascent area of molecular electronics depends, first and foremost, on controlling electron transfer in designed chemical structures.[1,2] Specifically, in electrochemistry the electron transfer between the analytes and the electrode surface is a fundamental process in amperometric techniques, which is one of the most promising and growing areas of analytical chemistry.[3-5]

One field that offers great potential for electron transfer applications is that comprising redox enzymes or proteins. The theoretical framework of biological electron transfer is increasingly well understood, and several properties, such as redox enzymes and proteins carrying out many key reactions of biological and technological importance, make biological redox centers good systems for exploitation.[2] They are also able to perform very selective reactions.[6-7] The essential underlying process for these reactions is electron transfer. Enzyme or protein mediated electron transfer is a fundamental phenomenon, not only in cellular processes, but also in reactions of biotechnological interest, as summarized in Figure 1.

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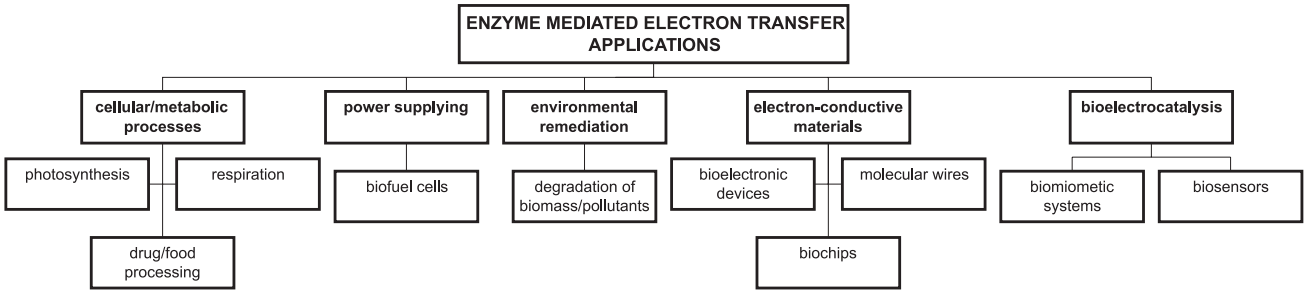

Figure 1. Organizational chart of enzyme mediated electron transfer applications.

Although there is a broad range of applications, this work will focus on bioelectrocatalysis and its applications for the development of highly selective and sensitive electrochemical biosensors.

Much progress has been made over the past ten years in understanding how the protein matrix finely tunes the parameters that are essential to the regulation of biological electron transfer. Marcus' theory of biological electron transfer[8] gained him the 1992 Nobel Prize in Chemistry and fueled many studies that attempted to determine the details of important biological functions.[9-11] Undoubtedly, the protein matrix has a fundamental role in regulating redox functions, even in simple electron-transfer proteins, such as *b*-type and *c*-type cytochromes that contain the same haem iron. A relatively simple parameter, such as the redox potential, varies over a range of 800 mV (from –400 mV to +400 mV for cytochrome c_3 and cytochrome b_{559}, respectively).[2] This broad range highlights the power of the protein matrix in tuning functions. Physical-chemical investigations of direct electron transfer using redox enzyme/protein systems were the focus of intensive investigations during the last two decades.[12-15] This review does not intend to cover exhaustively many involved research areas, but to provide some background regarding the development and analytical application of direct electron transfer-based amperometric biosensors, their current situations and future possibilities, as well as a brief commentary on the main aspects of electrochemical biosensors.

2. Biosensors

The recognition abilities of biological organisms for foreign substances are unparalleled. Scientists have recently developed new chemical analysis tools, known as biosensors, using biochemical molecular recognition from biological organisms or receptors that have been patterned from biological systems. These devices have many favorable analytical characteristics, such as selectivity, sensitivity, portability, speed, low cost and potential for miniaturization.[16,17] Thus, biosensors offer exciting opportunities for numerous decentralized analytical applications and they are quickly becoming useful tools in medicine, food quality control, environmental monitoring and other practical fields.[3,16,17] In principle, biosensors can be tailored to match individual analytical demands for almost any target molecule or compound that interacts selectively with a biological system.[18]

A biosensor is usually defined as a sensing device consisting of a biological recognition element in intimate contact with a suitable transducer, which is able to convert the biological recognition reaction or the biocatalytic process into a measurable signal. Enzymes are the biological components most commonly used in biosensors, while electrochemical transduction is the most popular method, often employing potentiometric or amperometric techniques. In potentiometric devices the analytical information is obtained by converting the biorecognition process into a potential signal, whereas the amperometric types are based on monitoring the current associated with oxidation or reduction of an electroactive species involved in the recognition process.

An amperometric biosensor may be more attractive due to of its high sensitivity and wide linear range.[3] Thus, amperometric enzymatic electrodes hold a leading position among the presently available biosensor systems. These devices combine the selectivity of the enzyme for the recognition of a given target analyte with the direct transduction of the rate of the biocatalytic reaction into a current signal, allowing a rapid, simple and direct determination of various compounds.[3]

3. Amperometric Biosensor Generations

The electronic coupling between redox enzymes and electrodes for the construction of amperometric biosensors can be based on the electroactivity of the enzyme substrate or product (first generation biosensors); utilization of redox

mediators, either free in solution or immobilized with the biomolecule (second generation biosensors), or direct electron transfer (DET) between the redox-active biomolecule and the electrode surface (third generation biosensors). Figure 2 shows a schematic representation of these different approaches in the development of amperometric biosensors.

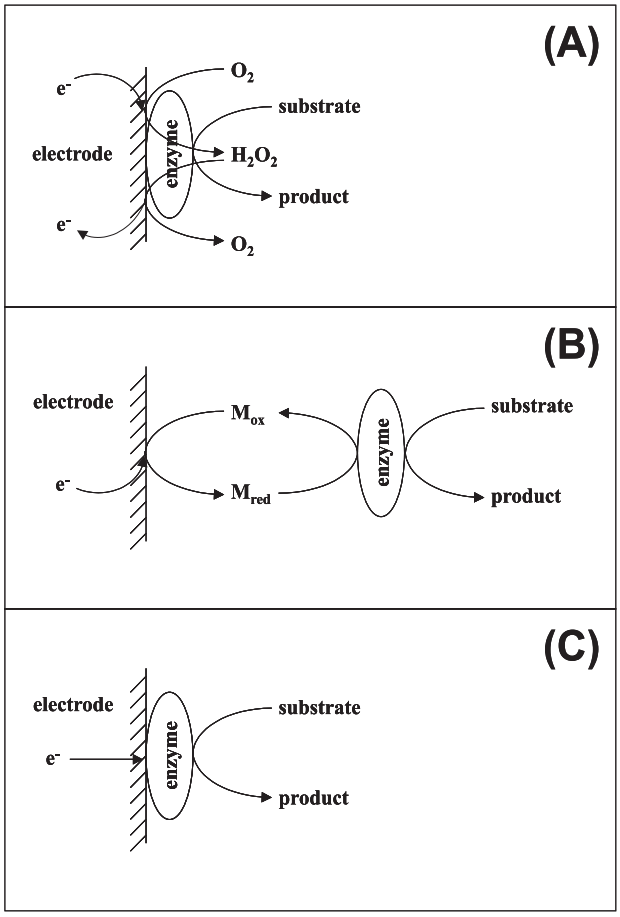

Figure 2. Schematic representation of different biosensor generations.

The drawbacks with first generation biosensors, such as too high applied potentials, put the focus on the use of mediators, which are small redox active molecules (e.g., ferrocene derivates, ferrocyanide, conducting organic salts and quinones) that could react with the active site of the enzyme and with the electrode surface, shuttling the electrons between the enzyme and the electrode. The use of mediators became possible in decreasing the applied potential for many redox enzyme-based biosensors. Unfortunately, the redox mediators used in conjunction with redox enzymes facilitate not only the electron transfer between electrode and enzyme but also various interfering reactions.[14]

In the third generation biosensors the electron transfer is associated with, or occurs during, the catalytic transformation of the substrate to the product. The redox enzyme acts as an electrocatalyst, facilitating the electron transfer between the electrode and the substrate molecule involving no mediator in this process.[13] Thus, this kind of biosensor usually offers better selectivity, because they are able to operate in a potential range closer to the redox potential of the enzyme itself, becoming less exposed to interfering reactions. The higher integration between the biomolecule and the electrode surface can also improve the sensitivity of this kind of biosensor. Recently, a lot of studies have been carried out on the development of electron transferring interfaces between redox enzymes and electrodes to apply them as high-performance amperometric biosensors.[19-28] Another attractive feature of the systems based on direct electron transfer is the presumable simplicity of construction of the enzyme based amperometric devices.

4. Enzyme Orientation and Immobilization

One of the major obstacles to be overcome in the construction of third generation biosensors is how to optimize the electron transfer between the enzyme and the electrode. The best electron transfer mechanism in an amperometric biosensor is direct electrochemical recycling of the prosthetic group of the enzyme at the electrode surface involving an electron tunneling mechanism.[14,15] According to Marcus' theory,[8] the kinetics of electron transfer between two redox species is determined by the driving force (e.g., the potential difference), the reorganizational energy (which qualitatively reflects the structural rigidity of the redox species) and the distance between the two redox centers.

Unfortunately, the distance between the prosthetic group and the electrode surface is often rather long for direct electron transfer, due to shielding by the protein shell, and electron transfer *via* a tunneling mechanism is therefore rarely encountered.[14] Thus, the main aim in the design of optimized amperometric biosensors is to provide fast electron transfer processes based on electrode architectures with predefined electron transfer pathways interconnecting the redox site within the enzyme and the electrode surface. In this way, an optimally designed electrode configuration has to ensure that the electron transfer distance between an immobilized redox biomolecule and a suitable electrode surface is made as short as possible. Moreover, the immobilized biomolecule must have an appropriate orientation, which also should facilitate communication between the active center of the biomolecule and the electrode surface (Figure 3).

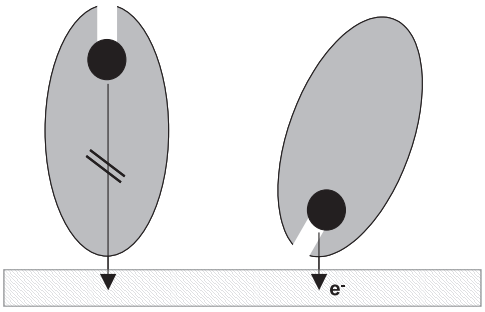

Figure 3. Effect of immobilized enzyme orientation on direct electron transfer.

Thus, the performance of electron transfer depends strongly on the immobilization procedure. Depending on the nature of the support, and the properties and the stability of the biomolecule, several methods can be used for immobilizing the enzyme onto the electrode, including physical adsorption, entrapment behind a dialysis membrane or within a polymeric film, covalent coupling through a cross-linking agent, or incorporation within the bulk of a carbon composite matrix.[18] Usually these methods lead to the formation of a randomly oriented layer, either on the surface of an electrode or in the cavities formed due to the porosity of the matrix. Specifically, for physical adsorption the protein might denature because of multiple contacts and interactions with the surface; binding of ligands might be affected; and unspecific multilayers might prevent substrate accessibility, leading to electrode fouling and unfavorable conditions for electron transfer. In the same way, using incorporation or inclusion in polyelectrolytes and conducting polymers, the enzymes are trapped in these materials that are directly adsorbed or linked to the surface. These immobilization procedures also result in a non-oriented multilayer film; a graphical representation of these immobilization methods is given in Figure 4 (A and B).[2]

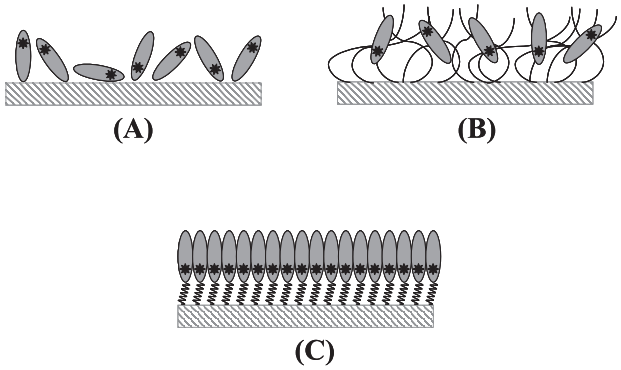

Figure 4. Representation of some methods used to achieve enzyme immobilization. (A) physical adsorption; (B) cross linking or inclusion in polyelectrolytes/conducting polymers; (C) oriented attachment to self-assembled monolayers.

One approach to optimize direct electron transfer is the design of suitable surfaces for the anisotropic and oriented immobilization of enzymes. Chemical modification of the electrode surface increases the stability of the enzyme/protein and introduces the possibility of controlling the orientation, density and environment of the immobilized species.[29] An especially attractive approach involves the formation of self-assembled monolayers (SAM).[27,30-34] These are monomolecular layers that exhibit high organization and are spontaneously formed as a consequence of immersing a solid surface into a solution consisting of amphifunctional molecules. While the adsorption is a result of the affinity of the head group to the surface, the driving force for organization originates from hydrophobic van der Waals interactions of the alkyl chains attached to the head group. The advantages that self-assembled monolayers offer for direct electron transfer are unquestionable. Their high organization and homogeneity, combined with their molecular dimensions, make them very attractive for surfaces tailored with desired properties. Moreover, since the chemical parameters of the monolayer components, such as the length of the hydrophobic chain, can be easily and gradually changed, the properties of the final systems are fully controlable.[32] Thus, self-assembled monolayer modified surfaces can be used as a base for the design of new sensor architectures with high control of orientation and electron transfer facility (Figure 4C).

5. Analytical Applications

Although third generation biosensors present favorable characteristics, only a few groups of enzymes or proteins were found to be capable of interacting directly with an electrode while catalyzing the corresponding enzymatic reaction. Depending on the practical significance of the substrates of these enzymatic reactions, electroanalytical applications of bioelectrocatalysis began to appear in the late eighties.[13]

The first reports on DET with a redox active protein were published in 1977, when Eddows and Hill[35] and Yeh and Kuwana,[36] independently, showed that cytochrome *c* on gold or tin-doped indium oxide electrodes, respectively, showed virtually reversible electrochemistry as revealed by cyclic voltammetry. In 1979 it was found that laccase[37] and peroxidase[38] modified carbon electrodes could promote DET. Later publications on this topic have reported use of other heme containing peroxidases, for which the electrode works as an electron donor to oxidize peroxidase.[39] Third generation biosensors are today still hardly reported, even though the number of examples is

increasing each year. A brief compilation of current ongoing research in this field is presented, focused on peroxidase, laccase, multi-cofactor enzyme and heme containing protein.

5.1. Peroxidases

Peroxidases are defined as oxidoreductases that catalyze the oxidation of organic and inorganic substrates by hydrogen peroxide or organic peroxides. Most peroxidases are heme proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. They have molar masses ranging from 30000 to 150000 (from 251 to 726 residues) and are divided into mammalian and plant peroxidases. The group of mammalian peroxidases includes myeloperoxidase, lactoperoxidase, thyroid peroxidase and prostaglandin H synthetase. The family of plant peroxidase consists of yeast cytochrome *c* peroxidase, plant ascorbate peroxidases, fungal peroxidases and other classical plant peroxidases.[40]

Amperometric peroxidase-modified electrodes have been developed for the detection of hydrogen peroxide, organic hydroperoxides, phenols and aromatic amines. These molecules are substrates, activators, or inhibitors of the reactions catalyzed by peroxidases. Under appropriate conditions and electrode design, these analytes can be selectively monitored in samples of interest.[41]

The peroxidase catalytic cycle occurs through a multistep reaction that involves, first, the reaction of the active site with hydrogen peroxide. This step involves rapid oxygen transfer from a peroxide to the ferric state of the enzyme, formally a two-electron oxidation, to form an unstable intermediate, compound I (cpd I), with the consequent reduction of peroxide to water. In this latter state of the protein, compound I contains an oxyferryl center, with the iron in the ferryl state ($Fe^{IV} = O$), and an organic cation radical which can be an oxidizable amino acid, as in cytochrome *c* peroxidase, or a porphyrin π cation radical, as in horseradish peroxidase. Then, compound I oxidizes a substrate (SH) to give a substrate radical and compound II (cpd II), where the organic cation radical is reduced by a second substrate molecule, regenerating the iron (III) state:[42,43]

$$[\text{heme } (Fe^{III})] + H_2O_2 \longrightarrow [\text{heme } (O{=}Fe^{IV})\text{-}R^{+\bullet}]_{(\text{cpd I})} + H_2O \quad (1)$$

$$[\text{heme } (O{=}Fe^{IV})\text{-}R^{+\bullet}]_{(\text{cpd I})} + SH \longrightarrow [\text{heme } (O{=}Fe^{IV})]_{(\text{cpd II})} + S^{\bullet} \quad (2)$$

$$[\text{heme } (O{=}Fe^{IV})]_{(\text{cpd II})} + SH \longrightarrow [\text{heme } (Fe^{III})] + S^{\bullet} + H_2O \quad (3)$$

When an electrode substitutes the electron donor substrates in a common peroxide reaction cycle, the process is denominated as direct electron transfer. Enzymes immobilized on an electrode can be oxidized by hydrogen peroxide (equation 1) and then reduced by electrons provided by an electrode (equation 4).

$$[\text{heme } (O{=}Fe^{IV})\text{-}R^{+\bullet}]_{(\text{cpd I})} + 2e^- + H^+ \longrightarrow [\text{heme } (Fe^{III})] + H_2O \quad (4)$$

When an electron donor (S) is present in a peroxidase-electrode system, the direct and mediated processes can occur simultaneously, with the reduction of oxidized donor $S^{\bullet}$ by an electrode (equation 5).[14]

$$S^{\bullet} + e^- + H^+ \longrightarrow SH \quad (5)$$

Reactions 1, 2, 3 and 5 are known as mediated electron transfer. In an amperometric sensor, both these approaches result in a reduction current, which may be correlated to the concentration of peroxide in solution. Thus, a simple electrode for peroxide detection can be developed with a layer of peroxidases immobilized onto the electrode surface. Due to the higher currents observed at peroxidase-modified carboneous electrodes, their performance has been extensively studied.[41] Besides horseradish peroxidase and cytochrome *c* modified electrodes, the bioelectrocatalytic reduction of H_2O_2 has also been observed at electrodes modified with lactoperoxidase,[44-46] peroxidase from *Arthromyces ramosus*,[47] chloroperoxidase from *Caldariomyces fumago*,[48] soybean and tobacco peroxidases.[23,33,41,49]

Although there are a great variety of peroxidases, horseradish peroxidase is undoubtedly the most employed in the development of amperometric biosensors. Thus, special attention will be given to this kind of peroxidase.

Horseradish peroxidase (HRP) belongs to the superfamily of heme-containing plant peroxidases and it is the most commonly used enzyme for practical analytical applications, mainly because it retains its activity over a broad range of pH and temperature.

In HRP-based amperometric sensors, the current, in the presence of H_2O_2, is due to electrochemical reduction of cpd-I and cpd-II. This conclusion is supported by the fact that the reduction of peroxide starts at a potential close to the formal potentials of compound-I/II and compound-II/HRP(Fe^{3+}), which were determined to be in the range of 0.63-0.69 V vs SCE at pH 7.[50,51] During direct electron transfer, electrons act as the second substrate for an enzymatic reaction, resulting in a shift of the electrode potential, with the measured current being proportional to the H_2O_2 concentration.[52] However, electrochemical

reduction of cpd-I and cpd-II is assumed to be kinetically slow on the majority of electrode materials. The rate constant of this process increases at lower electrode potentials, and has been estimated to be equal to 0.66 s^{-1} for HRP on spectroscopic graphite at 0 V *vs*. SCE.[53] This is probably due to the insulating properties of the protein shell, which increases the distance between the active heme site and the electrode surface. To overcome this slow heterogeneous electron transfer of HRP, mediators are frequently used to construct HRP modified electrodes.[41] However, as mentioned before, redox mediators used in conjunction with redox enzymes are not selective. Thus, the direct electron transfer of HRP on the electrode surface provides a biosensor with superior selectivity, because it operates in a potential window closer to the redox potential of the enzyme itself. One approach to improve the communication between the active center of the enzyme and the electrode, to get a better direct electron transfer, is focused on the development of oriented binding techniques for HRP.[54]

Since the first observations of direct electrochemical reduction of HRP,[38] there has been increased interest in the development of an amperometric mediatorless sensor for hydrogen peroxide, based on direct electron transfer between the electrode and adsorbed or covalently bound peroxidases, mainly because hydrogen peroxide is an important analyte in a variety of fields including industry, environment protection, and clinical control. For example, the detection of H_2O_2 at the sub μmol L^{-1} concentration range is very important because these peroxide levels can damage mammalian cells.[41,55-57]

Table 1 shows some recently published (1997-2002) DET peroxidase-based biosensors and their applications in hydrogen peroxide detection.

Direct electron transfer between peroxidase and the electrode surface can be used not only for H_2O_2 detection, but also for other metabolites, especially combined with other oxidase enzymes.[12,47,58,59] HRP is also commonly used as an enzyme-label in the development of immunoassays. Immunosensors based on direct electron transfer show great potential to be applied in clinical analysis.[22]

5.2. Laccase

The phenomenon of direct electron transfer in enzymes was first described for a laccase.[37] Laccases are cuproteins belonging to the small group of enzymes called blue copper proteins or blue copper oxidases. The others members of this group are ascorbate oxidase and ceruloplasmin. More than 60 types of laccase were isolated from different sources, such as insects, plants, fungi and bacteria. Although the copper center is similar for all laccase enzymes, differences in thermodynamic and kinetic properties are observed, depending on the source

Table 1. DET peroxidase-based biosensors

Peroxidases	Electrode	Techniques	Reference
HRP	SAM-modified gold	CV and impedance spectroscopy	60
HRP	pyrolytic graphite	CV	61
HRP	graphite	EQCM	62
recombinant HRP	graphite	Amp	63
HRP	SAM-modified gold	CV and Amp	21
HRP	gold	spectroelectrochemical	64
HRP	boron-doped polycrystalline diamond	Amp	65
HRP	pyrolytic graphite	CV	66
recombinant HRP	gold	CV	67
tobacco, peanut, sweet potato peroxidases	graphite RDE	Amp	23
tobacco peroxidase	graphite	Amp	49
HRP	modified glassy carbon	CV	68
HRP	pyrolytic graphite	CV and SWV	69
HRP	modified pyrolytic graphite	CV and SWV	70
tobacco peroxidase	SAM-modified gold	CV and Amp	33
HRP	CP and ormosil modified sol-gel glass	CV and Amp	71
recombinant HRP	gold RDE	Amp	72
recombinant HRP	gold	EQCM	24
native and recombinant HRP	screen-printed graphite electrodes	Amp	73
HRP	graphite	Amp	74
HRP	SAM-modified gold	CV and Amp	27

SAM = self-assembled monolayers; CP = carbon paste; CV = cyclic voltammetry; Amp = amperometry; RDE = rotating disk electrode; SWV = square wave voltammetry, EQCM = electrochemical quartz crystal microbalance.

of the enzyme.[75-78] In general, the laccases exhibit four neighboring copper atoms, which are distributed among different binding sites. They are classified into three types: copper types 1, 2 and 3, which are differentiated by specific characteristics that allow them to play an important role in the catalytic mechanism of the enzyme. According to Call and Mucke,[79] copper types 1 and 2 are involved in electron capture and transfer, while copper types 2 and 3 are involved in binding with oxygen.

Laccase is a phenol oxidase that catalyzes the oxidation of several inorganic and organic compounds (particularly phenols) with the concomitant reduction of oxygen to water.[37,80] Oxygen electroreduction in neutral or weakly acidic solutions on carbonaceous materials is known to proceed at high overvoltage. It was observed, however, that laccase in minor quantities (10^{-9} mol L^{-1}) strongly shifts the potential towards more positive values and accelerates oxygen electroreduction. In addition, these effects do not depend on the nature of the electrode material. An efficient bioelectrocatalysis of O_2 reduction by adsorbed fungal laccase immobilized on pyrolytic graphite, glassy carbon, and carbon black electrodes was first described by Tarasevich and co-workers.[37]

It was demonstrated that the oxygen is reduced at a carbon electrode with immobilized laccase according to a direct (mediatorless) mechanism, where oxygen is reduced to water in a four-electron mechanism:

$$O_2 + 4H^+ + 4e^- \xrightarrow{\text{immobilized laccase}} 2H_2O \quad (6)$$

This direct electron transfer mechanism was used as the basis for the creation of efficient biocatalytic oxygen reduction electrodes. The laccase bioelectrocatalytic properties were experimentally investigated in detail using galvanostatic and potentiodynamic techniques.[81,82]

Yaropolov and Ghindilis[83] investigated the electrochemical transformation of the copper-containing laccase prosthetic group. It was demonstrated that the redox potential of the laccase prosthetic group is about 0.4 V more negative than the zero-current potential of oxygen electroreduction catalyzed by laccase. Thus, the laccase prosthetic group cannot be simply considered as a redox mediator entrapped in the protein structure of the enzyme, with the electron transfer from the electrode to the substrate occurring through it. This indicates that the role of the protein globule of the enzyme is essential for its electrocatalytic activity.[13,83]

A comparative study of the redox transformations between laccase of different sources (*Coriolus hirsitus* and *Rhus vernicifera*) and other copper containing oxidases (tyrosinase, ascorbate oxidase and ceruloplasmin), which have very close substrate specificities and high similarities of the prosthetic group structure, was performed by Ghindilis *et al.*[84] and Yaropolov *et al.*.[85] It was observed that both ceruloplasmin and tyrosinase showed electrochemical redox reactions in anaerobic conditions. However they did not have bioelectrocatalytic properties, while laccase from both sources possesses a significant ability to catalyze oxygen electroreduction. Therefore it was proposed that redox transformation of the prosthetic groups of the enzymes is not sufficient to obtain electrocatalysis. An important parameter, which defines the possibility of bioelectrocatalysis with redox enzymes, is the kinetic mechanism of their catalytic action in homogeneous reactions. The major difference between these enzymes is in their mechanism of action. While laccase catalyzes according to a "ping-pong" or sequencial mechanism,[86] ceruloplasmin and ascorbate oxidase act by formation of a ternary donor-enzyme-acceptor complex. In the case of a mechanism of catalysis with the formation of a ternary complex, rigorous conditions for the structure of this complex are required and the limiting step of oxygen reduction is different from that observed for the laccase enzyme.[84,85]

Table 2 shows some results based on laccase enzymes immobilized on different solid electrodes catalyzing O_2 electroreduction by the direct electron transfer mechanism. It can be observed that the number of papers is not very large, when compared with those using peroxidase enzymes.

5.3. Multi-cofactor enzymes

Multi-cofactor enzymes usually consist of more than one subunit integrating different cofactors, such as pyrroloquinoline quinone (PQQ) (or flavin adenine dinucleotide (FAD)) and heme, linked by an electron transfer pathway (Figure 5). These enzymes provide a unique possibility of studying both heterogeneous and internal electron transfer reactions. Studies of the electrochemistry of multi-cofactor redox enzymes substantially help to understand the biological function of these enzymes as well as biological electron transfer.

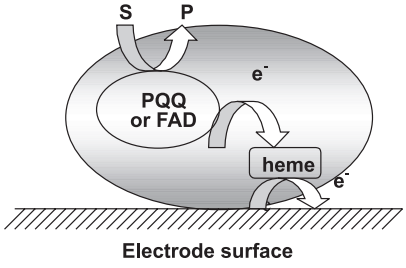

Figure 5. Schematic picture of multi-factor enzymes and the electron transfer pathway.

Table 2. DET for other redox enzymes and protein-based biosensors

Enzyme	Co-factor	Substrate	Electrode process	Reference
Laccase	Cu	O_2	reduction	37,80,85,87
Hydrogenase	Fe-S	H_2	oxidation	88,89
		H^+	reduction	
Superoxide dismutase	Cu-Zn	$O_2^{\bullet}$	reduction	28
Multicofactor enzymes:				
p-cresolmethylhydrolase	FAD-heme	p-cresol	oxidation	98
flavocytochrome c_{552}	FAD-heme	sulfide	oxidation	100
cellobiose dehydrogenase	FAD-heme	cellubiose	oxidation	39,102,103
D-frutose dehydrogenase	PQQ-heme	D-frutose	oxidation	90-93
alcohol dehydrogenase	PQQ-heme	alcohol	oxidation	94-97
fumarate reductase	FAD-Fe-S	fumarate	reduction	98,100
gluconate dehydrogenase	FAD-heme-Fe-S	gluconate	oxidation	104,105
Heme proteins:				
myoglobin	heme	H_2O_2	reduction	110,116
hemoglobin	heme	H_2O_2	reduction	115,117-119
cytochrome c	heme	H_2O_2	reduction	109-114
microperoxidase	heme	H_2O_2	reduction	30,120-122
hemin	heme	H_2O_2	reduction	123

Some of these multi-cofactor enzymes possess the ability to electrocatalyze reactions by a direct electron transfer mechanism. For example, PQQ and heme-containing enzymes (such as D-frutose dehydrogenase[90-93] and alcohol dehydrogenase,[94-97] FAD (or flavin mononucleotide (FMN)) and heme-containing enzymes (such as p-cresol methylhydroxylase,[98] fumarate reductase,[99,100] flavocytochrome c_{552},[101] and cellobiose dehydrogenase[39,102,103]), and trifunctional enzymes (containing a FAD-heme-Fe-S cluster, such as D-gluconate dehydrogenase[104,105]), were shown to display a direct electron transfer mechanism when immobilized on various electrode materials (Table 2).

The mechanism of bioelectrocatalysis in electrodes modified with multicofactor PQQ (or FAD) – heme containing enzymes suggests that the catalytic transformation of the substrate occurs first on the PQQ or FAD site, then the electrons are transferred through the protein molecule to the heme redox site and further to the electrode, where the enzyme is reoxidized through a direct electron transfer,[104,105] as shown schematically in Figure 5. The heme group, in these multi-cofactor enzymes, can be considered as a mediator of internal electron transfer from the specific catalytic site to the electrode.[14] Similar reaction schemes have been suggested for various quino- or flavo-enzymes.[97,101,102,104-106]

5.4. Heme containing proteins

As discussed before, the distance between the active site of the biocatalyst and the electrode surface is of crucial importance for the possibility of direct electron transfer between the immobilized enzyme and the electrode. The isolating properties of the protein shell for large enzymes makes direct electron transfer possible only if the active site of the redox enzyme is properly orientated. An approach to minimize the distance between the active site of the biocatalyst, to get better direct electron transfer, is to use enzyme fragments, such as the heme proteins, instead of the whole peroxidase.

Nature supplies a whole variety of different compounds containing a heme group (an iron porphyrin ring) as the active site, often located close to the outer surface of the protein shell. They all differ in their molar masses, the orientation and fixation of the heme group inside the three-dimensional structure of the protein, and their biological function. In common, they all show extended "peroxidase" activity, catalyzing the reduction of H_2O_2 to water in a two-electron transfer process.[107]

Among the heme proteins, cytochrome *c* was the first redox protein to be studied electrochemically and it has remained one of the most popular in electrochemical studies and applications.[108] Cytochrome *c* is a heme-containing redox active protein in electron transfer pathways, e.g., in the respiratory chain of mitochondria. The electrochemical properties of cytochrome *c* were extensively studied on gold, silver, platinum, carbon and metal oxide electrodes.[109-114] Myoglobin, hemoglobin[110,115-119] and fragments of cytochrome *c*, such as microperoxidase MP11 and haemin, are other examples of heme proteins

which present direct electron transfer properties, and can be used for the development of amperometric biosensors, as shown in Table 2.

Lötzbeyer *et al.*[122] studied the process of direct electron transfer between heme proteins with different sizes immobilized on a gold electrode modified with a self assembled monolayer, aiming to find an "ideal enzyme" for the construction of an amperometric biosensor based on direct electron transfer. They compared the electrocatalytic properties in H_2O_2 reduction of different heme proteins, like horseradish peroxidase, cytochrome *c* and its fragments, microperoxidase MP 11 and haemin. The highest electrocatalytic efficiency with the immobilized biocatalysts in a monolayer was observed for the smallest peroxidase compounds, e.g., microperoxidase MP-11 and haemin. With these results the authors concluded that smaller molecules allow a higher surface concentration, the substrate is be able to reach the active site much easier when it is not hidden by the protein shell and, finally, the direct electron transfer process should have a greater probability to occur with smaller enzymes, independent of their orientation towards the electrode surface.

These studies based on heme proteins showed the possibility for the construction of amperometric sensors, without the necessity of whole enzyme immobilization using only the active site. Obviously, there are some problems with this approach, especially the loss in selectivity, that require more intensive studies in order to lead to reliable sensors.

5.5. Biomimetic systems

Mimicking biological components is an attractive alternative in the search to construct robust biosensors with high sensitivity. The use of biomimetic chemistry and artificial enzymes, that try to imitate natural enzymes with the same efficiency and selectivity, can be applied to the construction of new amperometric biosensors with high sensitivity. These systems are designed with the objective to promote an increase in the electron transfer reaction between the electrode-active site-substrate system and, thus, to increase the sensitivity of the system. In these devices, a redox substance can be immobilized on the electrode surface and act as an active center that catalyzes a determined substrate reaction in the same way as the specific enzyme does.[124] This concept was recently described[125-128] and remains a large field still to be exploited.

Many different configurations have been proposed for the design of these artificial bioactive molecules. Research has been carried out involving simple modification of natural cofactors up to syntheses of compounds that can act as enzymatic models. In the first case, the cofactor or coenzymes that are normally soluble in aqueous solution are modified in order to create a more hydrophobic environment, and facilitate their interaction with the substrate molecule. Some examples of these cofactors are the hemin ring,[129,130] NADH,[130,131] flavins[130,132] and vitamins B_1, B_6 and B_{12}.[130,133] The enzymes can also be simulated by making use of supramolecular chemistry. Biocatalysts based on this type of structure could execute the same reaction processes as the enzymes, without strictly following the same path as they do, with high selectivity and under conditions that denature most enzymes. Among these macrocyclic compounds, the most used are cyclodextrins,[130,134-136] micelles,[130,137] bilayer membranes[130] and cyclophanes.[130,138] Some of these structures contain a transition metal mimicking the active center of the metalloenzymes.

In some cases, a simpler molecule can substitute the enzyme structure. Hasebe *et al.*[127] developed a system to mimic a copper containing enzyme, L-ascorbate oxidase, based on a poly L-histidine-copper complex, as an alternative biocatalyst for the construction of an amperometric biosensor for ascorbate. Another approach was described by Sotomayor *et al.*,[124] who developed an amperometric biosensor for determination of phenolic compounds, mimicking the chemistry of dopamine β-monooxygenase (DbM), a copper containing enzyme, by simply mixing copper phthalocyanine and histidine in a carbon paste.

Recent studies revealed that some proteins (such as myoglobin, hemoglobin and cytochrome *c*) undergo certain functional conversions through noncovalent interactions with lipid membranes. For example, cytocrome *c* was functionally converted to a N-dimethylase-like enzyme *via* supramolecular formation with phosphate lipid membrane.[139,140] Fan *et al.*[115] recently studied the electrochemical properties of hemoglobin entrapped in a SP Sephadex membrane. They observed that the peroxidase activity of hemoglobin is enhanced when it is entrapped in the membrane, providing a good mimetic system for the peroxidase enzyme.

Artificial redox enzymes can also be designed for biosensor purposes by a combination of a specific domain (FAD, PQQ, etc) responsible for the oxidation of a specific substrate with a heme-containing domain working as an electron transfer mediator. The construction of these artificial redox enzymes is a challenging task, since it requires specific knowledge of the electron transfer process in the protein molecules, which, in several cases, are still under investigation.

Some work has been done in this direction, principally using the protein cytochrome *c*, due to its property of exchanging electrons with a whole range of redox enzymes. Different classes of redox enzymes can efficiently communicate electronically with cytochrome *c*. Enzymes such as trinuclear copper containing oxidases (such as laccase, ascorbate oxidase and ceruloplasmin) and similar binuclear oxidases (e.g. tyrosinase) efficiently oxidize cytochrome *c*, mimicking its role with cytochrome oxidase in the mitochondrion respiratory chain.[141] In this case, four equivalents of cytochrome *c* are necessary to complete one turnover cycle with the copper containing enzyme. Cytochrome *c* can also accept electrons from a number of bifunctional redox enzymes containing heme and either a flavin (FMN or FAD) or a PQQ co-factor. Cellobiose dehydrogenase (CDH) and lactate dehydrogenase are examples of flavo-hemoenzymes which, in their reduced states, can transfer electrons to oxidized cytochrome *c*.[142,143] The coupling of cytochrome *c* with the redox enzymes is, in some cases, made by simply mixing them into a lipid modified carbon paste.[142] A more sophisticated technique based on a thiol modified gold electrode has already been used.[141,144]

Table 3 shows some different biomimetic systems designed for the construction of biosensors, which have been described in the literature. It can be observed that some of them present a linear response in the range of μmol L^{-1}, clearly indicating that a more efficient electrical contact between the catalyst (artificial enzyme) and the electrode surface is obtained when using these systems. Moreover, the K_M values obtained indicate a considerable affinity between the catalytic species and the substrate.

The future of biosensor development will probably be based on these artificially designed enzymes, mimicking naturally occurring ones having known electron transfer pathways. However, for this purpose some drawbacks must be addressed. In spite of the significant increase in sensitivity found in DET-promoted biomimetic systems, generally they present lower selectivity than is observed with the original biological systems. Thus, efforts have been directed toward the manipulation of molecular architecture at the electrode surface in order to improve the selectivity of the biomimetic molecules.

Conclusions and Future Trends

Biosensor technology is an open field for innovative approaches to analysis. Research to elucidate and modulate electron transfer mechanisms, direct or mediated, is indispensable for the success of biosensors as a future technology. Moreover, they are also important for understanding the fundamental principles of biological recognition and communication between enzyme and transducer in amperometric biosensors.

The mechanism of direct electron transfer in biosensors is not well understood as yet. There are a wide range of questions to be clarified: *i*) which parameters (structural or kinetic) determine the possibility that a redox enzyme catalyzes a given electrode reaction; *ii*) what is the role of the enzyme prosthetic group in the electrocatalysis and in the electron transfer process; *iii*) what is the influence of the nature of the electrode material and the structure of the electrode surface; and *iv*) what is the relationship between the catalytic mechanism of the enzyme action and the manifestation of direct electron transfer ability. On-going fundamental studies on the synthesis of new bio-electrocatalysts by chemical modification of enzymes, by

Table 3. Different biomimetic systems designed for the construction of biosensors

Enzyme	Biomimetic system	Substrate	Linear range	K_M	Ref.
ascorbate oxidase	poly L-histidine copper complex	ascorbic acid	3-300 μmol l^{-1}	1.3 mmol l^{-1}	127
dopamine β-monooxigenase	copper phtalocyanine and histidine (carbon paste)	dopamine	40-290 μmol l^{-1}	1.1 mmol l^{-1}	124
peroxidase	Prussiam blue	H_2O_2	0.1-100 μmol l^{-1}	-	145-147
	hemoglobin entrapped in SP Sephadex membrane	H_2O_2	5-160 μmol l^{-1}	1.9 mmol l^{-1}	115
hypoxanthine oxidase	Ru and Pb oxide	hypoxanthine	0.1-1μmol l^{-1}	1.2 mmol l^{-1}	126,148
dehalogenases	cobalt tetraphenyl porphyrine	organohalides	5-200 μmol l^{-1}	-	149
tyrosinase	Cu(dipy)	dopamine	40-600 μmol l^{-1}	-	150

gene and protein engineering techniques, and on the development of new, highly efficient electrocatalysts with nonprotein structures should have a tremendous impact on the scope of biosensor applications.

Although these are fundamental challenges, DET-based biosensors have a great potential for the development of new approaches and devices in order to solve many analytical problems. The main advantage obtained with third generation biosensors is the development of simple analytical devices (reagentless sensors) with high sensitivity (due to a closer integration between the recognizer element, which is the signal generator, and the transducer) and, especially, high selectivity (due to the specific interactions between the biomolecules and their substrates). Having all these characteristics in a single sensing element is the goal of most analytical chemists.

Although direct electron transfer between enzymes and electrodes is a very promising approach to obtain this kind of high performance sensor, there are some limitations to be overcome. The first of them is the restricted number of enzymes and proteins that show the phenomenon of DET on electrode surfaces. As described in this brief review, only a few oxidases have this important property. Thus, the scope of third generation biosensors is still limited, and the majority of these sensors have been developed to detect hydrogen peroxide. The attempts to employ multi-cofactor enzymes, heme proteins and biomimetic molecules have contributed efficiently to increase the range of detectable compounds. These approaches usually lead to more sensitive sensors, however there are decreases in selectivity.

Another way to increase the scope of DET-based biosensor systems is the design of molecular sensing systems, which can couple enzymes that show DET ability with a variety of biosensing molecules. For example, a peroxidase-based molecular transducer coupled with glucose oxidase forms a high performance glucose-sensing device, where the oxidation of glucose by molecular oxygen catalyzed by glucose oxidase results in formation of hydrogen peroxide. The peroxidase-based transducer transforms the chemical signal (proportional to hydrogen peroxide concentration) into an electrical signal[13] (Figure 6). Thus, coupling glucose oxidase with a hydrogen peroxide molecular transducer permits mediatorless detection of glucose. Peroxidase, in this case, plays the role of an electrocatalyst for hydrogen peroxide reduction. The detection of a number of different analytes can be achieved by coupling different enzymes with DET ability with those capable of catalyzing the oxidation (or reduction) of specific analytes. The following oxidases have been coupled with amperometric peroxidase

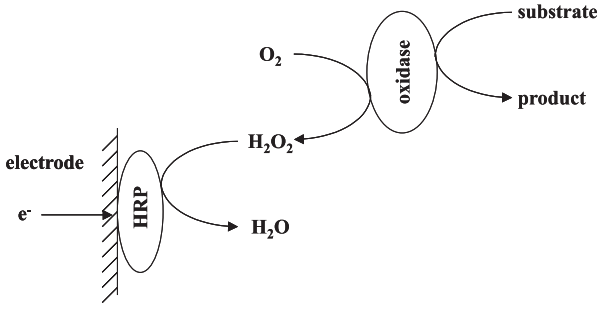

Figure 6. Schematics of coupling of two enzymes.

electrodes for detection of appropriate substrates: glucose oxidase, choline oxidase, cholesterol oxidase, D-amino and L-amino acid oxidase, alcohol oxidase, uricase, lactate oxidase, xanthine oxidase, bilirubin oxidase, glutamate oxidase, putrescine oxidase and polyamine oxidase.

The widespread use of third generation biosensors is also limited by the kinetics of electron transfer between the biomolecules and the electrode surface, which is generally slower than the mediated one. New kinds of electronic communications, biomolecule immobilization, molecular architecture and sensor design have been the targets of many studies in order to improve these kinetics and many exciting developments are expected in the near future.

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