

Small electron transfer proteins as mediators in enzymatic electrochemical biosensors

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Abstract

Electrochemical mediators transfer redox equivalents between the active sites of enzymes and electrodes and, in this way, trigger bioelectrocatalytic redox processes. This has been very useful in the development of the so-called second generation biosensors, where they are able to transduce the catalytic event into an electrical signal. Among other pre-requisites, redox mediators must be readily oxidized/reduced at the electrode surface and easily interact with the biorecognition component. Small chemical compounds (e.g. ferrocene derivatives, ruthenium or osmium complexes and viologens) are frequently used for this purpose, but lately, small redox proteins (e.g. horse heart cytochrome c) have also played the role of redox partners in biosensing applications. In general, the docking between two complementary proteins introduces a second level of selectivity to the biosensor and enlarges the list of compounds targeted for analysis. Moreover, electrochemical interferences are frequently minimized owing to the small overpotentials achieved. This paper aims to provide an overview of enzyme biosensors that are mediated by electron transfer proteins. The article begins with a few considerations on mediated electrochemistry in biosensing

systems and proceeds with a detailed description of relevant works concerning the cooperative use of redox enzymes and biological electron donors/acceptors.

Keywords: electrochemical biosensors, redox partner, electron transfer protein, mediated electrochemistry

Introduction:

Electron transfer in electrochemical systems

The electron transfer (ET) between redox centers of proteins is central in biological processes like the respiratory chain, photosynthesis, metabolic pathways (e.g. glycolysis or the citric acid cycle) and even in the regulation of gene expression, where so-called redox switches are involved [1]. Over the past decades electrochemistry has surrendered important information on the mechanisms, kinetics and thermodynamics of ET reactions in biological systems [2]. Besides achieving a better understanding of redox reactions, the study of the electrochemistry of proteins and enzymes provides the basis for the fabrication of electrochemical biosensors, i.e. integrated analytical devices that combine a biorecognition element displaying catalytic activity (enzymes or whole/fragment cells containing enzymes), with an amperometric or voltammetric transducing system [3,4].

Although some enzymes (typically heme and blue copper proteins) exhibit direct electron transfer (DET) on electrode surfaces with about one hundred examples reported in the literature, this is not the common trend [5,6]. Additionally, it is not often that they also display catalytic activity in DET conditions. One of the main reasons is the intrinsic isolating nature of the polypeptide chains that wrap around the redox centers in many protein structures. If cofactors are shielded by the apoprotein, it is very hard to achieve electrochemical communication with electrodes. Modification of electrode surfaces can help protein interaction with transducers, thus facilitating DET and/or electrocatalysis. The modification of electrode interfaces usually consists in conferring a suitable overall charge or specific functional groups that favor the protein's attachment or orientation on the electrode surface (e.g. with bipyridil, poly-L-lysine or self-assembled monolayers (SAMs)) [7-9]. Nevertheless, when looking into enzyme electrocatalysis a common approach is the use of redox mediators, i.e. small electroactive molecules that can easily interact with the protein's redox centers and consequently exchange electrons between electrodes and the biocatalyst. In this way, mediators can provide the means to follow and measure enzymatic reactions by electrochemical methods [4,10].

Mediated electron transfer

As aforementioned, the electrical contacting of oxidoreductases that lack DET with electrodes can be established by means of a reversible redox couple, one of the forms of which serves as a co-substrate to the enzyme, as depicted in Fig. 1A [11,12]. In mediated electrochemistry (MET), and following an EC' mechanism, the consumption of the redox mediator, i.e. the co-substrate, due to the catalytic reaction is detected as a current amplification (Fig. 1B). Such current increase is directly related to the amount of substrate being processed [6,13].

Fig. 1

Unlike DET, the use of redox mediators takes the control over the protein reaction center away from the electrode [14]. On the one hand, this can be detrimental to the selectivity of detection, since the mediator can react with other species present, and in the case of biosensors, can require a more complex manufacturing process, additional reagents and sophisticated immobilization methods. On the other hand, redox mediation can overcome the frequently sluggish electron communication of enzyme redox centers with electrodes, thus increasing ET rates [4,14].

The mediated transfer of redox equivalents is the working principle of second generation amperometric biosensors (Table 1 briefly compares the three biosensor generations). Mediators are characterized by having a high heterogeneous ET rate that does not compromise electrochemical reversibility and, at the same time, a homogeneous fast electron exchange with the enzyme. Both oxidized and reduced forms should be stable and unreactive with oxygen; also, the reaction should not depend on pH. Furthermore, when selecting a redox mediator for biosensor applications it is important to consider toxicity, biocompatibility, ease of immobilization and, very importantly the redox potentials. Small operating voltages are preferred, allowing appropriate enzyme reaction transduction, while avoiding side reactions. In other words, a mediator should shift redox potentials from the extreme values necessary to detect target analytes, to values around zero, at which fewer interfering species are reduced or oxidized [11,14-16].

Table 1

Over the years, much work has focused on identifying efficient mediators to assure enzyme wiring to electrodes. These are generally organic compounds like viologens, phenazines, quinones, tetrathiafulvalene, tetracyanoquinodimethane or metal complexes such as osmium, ruthenium, ferrocene and derivatives [4,17,18]. However, it is not uncommon to find monohemic cytochromes (cyt) and other small proteins as a source or a sink of electrons in mediated electrochemical systems (Fig. 2). In fact, aside the use of physiological electron donors/acceptors to mimic more faithfully charge transfer processes that occur *in vivo* and thereby study intermolecular ET and enzyme kinetics [19], the *enzyme/mediator protein* coupling can be also exploited in bioelectroanalytical applications, and this will be the subject matter of the present review.

Fig. 2

At this point, it should be mentioned that the use of small ET proteins as redox mediators in biosensors is not limited to naturally inherent redox pairs. In fact, this very interesting ET route has been scarcely exploited up to now, since the physiological partner of the enzyme must be previously identified and isolated. Alternatively, the reaction between versatile electroactive proteins that are not natural redox partners (e.g. horse heart cytochrome c) has also been explored for biosensing purposes, as will be discussed below. In such a case, protein pairing is largely empirical.

Protein redox mediators in biosensors

Protein electron acceptors (or donors) bring some advantages into biosensing. They display high affinity and turnover rates with oxidizing (or reducing) enzymes, specifically if the physiological redox partner is being used [16,27]. Unlike artificial electron shuttles, which are frequently general catalysts and generate unspecific responses, the redox mediation with natural electron shuttles is based on the selective interaction between two complementary proteins, frequently adding a second selectivity element to the biosensor [28]. Also, for *in vivo* applications ET proteins should be considered instead of artificial redox

mediators since the first are less likely to be harmful to biological systems [29,30].

In the following sections we report some of the biosensor proposals which combine enzymes with protein redox mediators. The examples are arranged by mediator protein with subsections detailing the enzyme in use and the electrode design. A selected number of cyclic voltammograms from the works referenced herein are shown in Fig. 3, depicting the mediator redox waves and the catalytic currents following substrate additions.

Fig. 3

Many published studies focus on the interaction of mediator proteins and biocatalysts, but disregard the catalytic activity of the bioelectrodes. This survey was only directed towards works with an analytical perspective and/or aiming at future biosensor applications. Actually, this approach could be considered a recent trend in the R&D of enzyme based biosensing devices, since most of the selected publications report to the last fifteen years.

It must be stressed that, regardless of the proteins couple, the electrode architecture is a very important aspect, since it should guarantee both catalytic turnover and vectorial ET from (or to) the redox enzyme. In particular, the effective immobilization of the small protein mediators and the higher molecular weight enzymes without impairing ET, can be a challenging task. While some approaches make use of simple methods such as entrapment with dialysis membranes [16,34], others integrate the biosensors components with cross-linking agents or polymeric layers [2,33,35-37]. A common trend is also the use of thiol-modified gold electrodes onto which small cytochromes can be attached and display facile DET [37-40]. Nevertheless, some of the reviewed works do not constitute integrated biosensors, because at least one of the components of the mediated system (mediator protein or enzyme) is not immobilized on the electrode [27,29,40-43]. Table 2 summarizes information on the bioelectrode proposals described below, including both protein and non-protein components, as well as the electrode design.

Table 2

Protein mediators and biosensors:

Cytochrome c

Small cytochromes, such as horse heart cytochrome c are by far the most used protein mediators. Cytochrome c is a small globular protein (12 kDa) which encompasses a c-type heme. It is involved in the charge transport of the respiratory chain (Fig. 2A). Because it is a basic protein, it interacts well at negatively charged surfaces (e.g. immobilizing matrices, electrode surfaces or redox partner enzymes) through its positively charged lysine residues [36]. This cytochrome can display a well-defined quasi-reversible electrochemistry on electrode surfaces, directly on highly oxygenated carbon surfaces, such as polished edge pyrolytic graphite, or on promoter covered surfaces, such as thiol-modified gold electrodes [39,58,59]. Additionally, good electrochemical responses can be obtained with some polymeric matrices, like Langmuir-Blodgett thin films [2,36].

Cyt c is the physiological redox partner of many redox enzymes, for instance, cytochrome c peroxidase, sulfite oxidase and lactate dehydrogenase. Furthermore, electron mediation by cytochrome c has been reported with several other enzymes: laccase, ascorbate oxidase [47,60,61], NADPH cytochrome P450 reductase [62], bilirubin oxidase [29,38], xanthine oxidase [46], among others. The role of cyt c on such electrodes falls on the electron shuttling between proteins and electrodes. As earlier mentioned, it is important for cyt c to display a fast ET rate with both partners: heterogeneous with the solid electrode and homogenous with the enzymes. This can be favored by electrode modifications which allow optimal orientation but at the same time grant some mobility to the redox molecule, so that it can successfully accomplish molecular docking [43]. Still, cyt c has also displayed effective electron transfer functions when simply entrapped within films where no specific orientation is expected [2,48,51].

Lactate oxidoreductase / Flavocytochrome b₂

Flavocytochrome b₂ is a lactate oxidoreductase that can be found in yeast mitochondria. It catalyzes the oxidation of L-lactate to pyruvate allowing organism growth on lactate. It is a tetrameric enzyme containing a flavin

dehydrogenase domain housing a flavin mononucleotide (FMN), and a heme domain comprising a b₂-type heme. Cytochrome c is the physiological electron acceptor for the enzyme [63,64].

An electrochemical study showing mediated ET between cyt c (horse heart) and flavocytochrome b₂ (yeast) in solution was presented by Cass et al. [41]. Cyt c displayed quasi-reversible electrochemistry in bis(4-pyridyl) disulfide gold modified electrodes. In the presence of flavocytochrome b₂ and upon addition of *L-lactate* an increase in catalytic current was observed, consistent with the regeneration of the electrochemical mediating cyt c [41].

Liu et al. demonstrated the direct electrochemistry of a flavodehydrogenase domain of flavocytochrome b₂ engineered for L-mandelate dehydrogenase activity [2]. Electrocatalysis of the substrate *L-mandelic acid* could only be achieved using mediator species. A ferrocene derivative was utilized as well as cyt c, with which higher catalytic currents were observed. Both the cyt c and the enzyme were sequentially deposited and air dried on pyrolytic graphite electrodes (PGEs) followed by the deposition of a poly-L-lysine layer. It was shown that the protein mediator could transfer redox equivalents with both the enzyme and the electrode, thus creating the basis for the development of an amperometric biosensor [2].

Cytochrome c peroxidase

Another cyt c based biosensor was proposed by De Wael et al. [35]. In this work a gelatin hydrogel was used for the co-entrapment of horse heart cyt c and *Saccharomyces cerevisiae* cytochrome c peroxidase (CCP), thereby delivering a *hydrogen peroxide* biosensor. CCPs are heme containing monomeric proteins involved in hydrogen peroxide detoxification [65]. Cyt c is the natural redox partner for the enzyme's catalytic reduction of hydrogen peroxide to water. Gold electrodes were first modified with a SAM of 6-mercaptohexanol (MH) after which a mixture of hydrogel and proteins was deposited on top of the electrode surfaces. The immobilization of cyt c was aided by the fact that the hydrogel had an overall negative charge to counter its positive charge yet, it remained mobile in the hydrogel. The cyt c/CCP system had a low overpotential operating range, thus presenting itself as a quite selective system for peroxide detection. The biosensor

had a fast response to H_2O_2 within a linear range of 0 to 0.3 mM and a detection limit of 0.01 mM [35].

Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular protein produced in fungi. This monomeric protein is composed of two domains: one carries a flavin adenine dinucleotide (FAD) cofactor (the catalytic center) and the other has a cyt b-type heme. CDH catalyzes the oxidation of cellobiose and other oligosaccharides using several electron acceptors, including cyt c [66,67].

Fridman et al. have showed that the couple cellobiose dehydrogenase (*Phanerochaete crysosporium*) and cyt c (horse heart) could be used to measure cellobiose [43]. The latter was adsorbed onto an 11-mercapto-1-undecanoic acid (MUA) modified gold electrode and functioned as charge carrier between CDH (in solution) and the transducer surface. Authors have proposed that the FAD center in CDH oxidizes substrate molecules and transfers electrons to oxidized cyt c through its heme b domain; the reduced cyt c is then regenerated on the electrode. Although small catalytic currents could be observed in the presence of cellobiose owing to a direct electrochemical response of CDH, when adding cyt c to the electrolyte a clear catalytic redox wave was displayed, attesting the effective vectorial ET from cellobiose via CDH and cyt c to the electrode [43].

Sarauli et al. have used cellobiose dehydrogenases from two different fungal sources, *Trametes villosa* and *Corynascus thermophiles*, in a study comparing the direct and the cyt c mediated electrochemistry of CDH and cellobiose catalysis [40]. The two enzymes were free in solution, whereas cyt c (horse heart) was adsorbed on gold electrode surfaces modified with a SAM of MUA and 11-mercapto-1-undecanol (MU). Following the addition of the enzyme's substrate - cellobiose - the catalytic currents observed at high pH (6-7) were shown to be cyt c mediated. However, at more acidic pH (4-5) the dominating ET pathway for the bioelectrode response was established via the heme b of the enzyme, consistent with the direct electrochemistry of CDH. At pH 6-7, the interaction of the two protein domains decreased due to the electrostatic repulsion of deprotonated amino acid residues on the surface of both domains and therefore a cyt c MET was necessary to report the catalysis of cellobiose [40].

More recently, CDH (*Trametes villosa*) and cyt c (horse heart) could be co-immobilized in the presence of silica nanoparticles in a supramolecular layered architecture used for *lactose* measurement [44]. For sensor preparation, gold wire electrodes were first modified with MUA and MU onto which cyt c was adsorbed. These electrodes were then incubated alternately with a mix of the proteins (cyt c and CDH) and SiNPs. The bioelectrode assemblies had a defined nanostructure, due to the presence of the nanoparticles, since they constituted the scaffold support for the two protein arrangement. Protein co-entrapment was favored by the electrostatic interactions between CDH (pI 4.3) and cyt c (pI 10). Lactose dependent catalytic currents were followed by cyclic voltammetry and varied linearly with concentrations from 0.01 to 1 mM [44].

Bilirubin oxidase

In another cytochrome c mediated scheme proposed by Dronov et al., this protein was employed as electron shuttle for *Myrothecium verrucaria* bilirubin oxidase (BOD) [29]. This multicopper oxidase has four copper centers in a monomeric structure. It is involved in heme metabolism - it catalyzes the oxidation of bilirubin to biliverdin with concomitant reduction of dioxygen to water [68]. BOD displayed DET on thiol-modified gold-electrodes at low pH values, though not at neutral and physiological pH. Horse heart cyt c could be used as a redox mediator, both in homogeneous solution and immobilized on mixed SAMs of MUA and MU, enabling a fast kinetics for the BOD catalytic reduction of *oxygen* at neutral pH [29].

In a different approach, a number of protein multilayer designs combining cyt c and different enzymes, including BOD, have been reported [38,45-47,49,50]. The bioelectrodes were built using a layer-by-layer self-assembly method. This allowed regulating the amounts of enzyme and protein redox mediator to achieve optimal catalytic activity. The electrostatic interaction between alternating layers of materials containing complementary charged groups was the basis for the protein entrapment. In particular, poly(aniline sulfonic acid) (PASA) was used as a positively charged matrix for cyt c immobilization, which works as ET system. Besides being adsorbed on the polyelectrolyte layers of PASA, the

electrochemistry of the redox mediator protein was facilitated by the surface modification of gold electrodes with a MUA monolayer and complemented with an additional cyt c film (the first layer of cyt c was shown to facilitate further protein adsorption) [69].

The voltammetric characterization of a BOD/cyt c multilayered electrode showed a quasi-reversible electrochemical signal of the small cytochrome. In the presence of *oxygen*, an increased cathodic current was observed as a result of the enzymatic reduction of oxygen to water. The catalytic current had a linear dependence on the oxygen present in solution [38]. The same research group has later showed that the BOD/cyt c multilayered biosensor could be formed with different forms of cytochrome c, such as human cyt c and some of its mutant variants. Interestingly, the amount of cyt c immobilized and the activity of the sensor varied significantly with each of the cyt c forms. Human wild-type cyt c produced the highest catalytic currents for oxygen reduction [45].

Xanthine oxidase

Dronov and co-workers proposed a mediated biosensor based on cyt c and xanthine oxidase (XOD) for *hypoxanthine* determination [46]. Xanthine oxidases take part in purine metabolism where they catalyze the oxidation of hypoxanthine and xanthine to uric acid. The high molecular weight protein (300 kDa) comprises FAD cofactors, iron-sulfur clusters and molybdenum centers (the catalytic site) [70].

In a work reported by Dronov et al., cyt c (horse heart) was immobilized on a polyanionic electrolyte, PASA, while XOD (cow milk) was adsorbed on the polycationic poly(ethylenimin) [46]. Upon addition of hypoxanthine into the electrochemical cell, an oxidation current could be measured at 150 mV *vs* Ag/AgCl. This means that cyt c was responsible for the charge transfer from XOD to the electrode surface, thus translating a hypoxanthine oxidation into a detectable amperometric current. The electrode's response was found to change linearly with hypoxanthine concentration over the range 0.25 to 5 μM , with a sensitivity of $0.3 \text{ A}\cdot\text{M}^{-1}\cdot\text{cm}^{-2}$. The sensor had a low working potential less prone to interferences and side reactions. An additional polyelectrolyte membrane (poly(allylamine), PAA) and thermal treatments improved electrode stability, which retained 60% of its initial activity after 5 days [46].

Laccase

The same strategy was used to develop an *oxygen* sensor using the system horse heart cyt c and *Trametes versicolor* laccase [47]. Fungal laccases are multicopper oxidases thought to be related with morphogenesis, fungal plant-pathogen/host interaction, stress resistance and lignin degradation. The enzyme converts oxygen to water in a four electron reduction reaction accompanied by the oxidation of a broad range of substrates, typically phenolic compounds, such as p-dihydroxy phenol [71]. In spite of not being its physiological electron donor, cyt c was able to reduce the enzyme, as earlier seen by Sakurai et al. [61]. Later, laccase could be immobilized by electrostatic interactions with the PASA-cyt c multilayered film. In air saturated solutions the electrode showed an increase in the catalytic cathodic currents consistent with the sequential reduction of cyt c, laccase and oxygen. Although electrodes prepared with only PASA and laccase also displayed catalytic current for oxygen reduction, in the presence of cyt c, the electrocatalytic signal was considerably amplified. The biosensor exhibited a linear response to oxygen and a pH optimum at 4.5 [47].

Sulfite oxidase

Sulfite oxidase is a dimeric protein which contains, per subunit, a molybdenum cofactor where the catalytic reaction takes place, and a heme b₅, as an electron acceptor center. Sulfite oxidases are involved in sulfur metabolism and can be found in animals, plants and bacteria. The enzyme catalyzes the oxidation of sulfite to sulfate, the final step in the oxidative degradation of the sulfur-containing amino acids cysteine and methionine. It is also involved in the detoxification of sulfite and sulfur dioxide. The enzyme's physiological redox partner is cyt c, as it re-oxidizes the heme cofactor generated during the catalytic cycle [72].

Coury et al. reported an electrochemical study in solution where they have showed that sulfite oxidase from chicken liver can deliver electrons to its redox partner cyt c, even though from a different source (horse heart). This enabled the electrical wiring of the enzyme to a pyrolytic graphite electrode (in the presence of *sulfite*, anodic catalytic currents could be measured) [42].

A decade later, a sulfite biosensor based on the same pair of proteins was described by Abass et al. [48] who mixed sulfite oxidase and cyt c within a carbon ink deposited on screen-printed electrodes (CSPE). Following *sulfite* addition, the enzyme oxidizes it to sulfate in the molybdenum site, electrons are transferred to the heme b₅ center and then to the reduced cyt c, which, in its turn, is re-oxidized by the electrode, generating an anodic current - the analytical signal. In this way, the catalytic sulfite oxidation current could be easily transduced on the biosensor. The sensor was characterized by amperometric measurements at 0.3 V *vs* Ag/AgCl. The response was slow (2-3 min to reach steady state current), but the catalytic current was linear with sulfite between 0.04 and 5.9 mM; the detection limit was 4 ppm. Regarding stability, the biosensor was able to maintain its initial response within a 45 day period. The sensors were shown to be useful for sulfite quantification in water samples, although only recovery tests were performed, because the sulfite concentration in the tested samples was below the detection limit. Except for sulfite related anions (e.g. bisulfite), the current response was not affected by the most common environmental interferents. Thus, authors claimed an enhanced selectivity and sensitivity for sulfite determination by using this mediator [48].

A few years later, the same authors proposed a *sulfur dioxide* sensor using once again the couple sulfite oxidase/cyt c [51]. The amperometric biosensor was similarly constructed, using screen-printing technology. The measurements were based on the principle that sulfur dioxide gas was dissolved in the electrolyte and converted to sulfite ions. These ions then drove the electrocatalytic reaction of sulfite oxidase, with cyt c playing the role of electron acceptor. The sensors were tested at a working potential of 0.3 V *vs* Ag/AgCl. For the optimized configuration the response current was linear from SO₂ concentrations of 4 to 50 ppm, the detection limit was 4 ppm and the response time *t*_{90%} was 110 s. Regarding the electrode stability, biosensors stored over 3 months in the fridge were shown to retain their initial activity. Moreover, the sensor showed great promise for continuous monitoring, with no decay in response over a period of 24h [51].

More recently, Dronov and colleagues developed a bioelectrode with a combination of horse heart cyt c and sulfite oxidase from a different source (human) [31]. A mixture of both proteins was co-adsorbed on gold electrodes previously modified with an alkanethiol promoter layer (MUA and MU) and cyt c. The bioelectrode displayed catalytic currents for the oxidation of *sulfite*; cyt c was the species responsible for charge transfer between the sulfite oxidase and the electrode (Fig. 3A). The bioelectrode could detect sulfite within a 20 μM - 2 mM concentration range, with a K_M^{app} of 310 μM [31].

Spricigo et al. reported a polyelectrolyte multilayered system, based on sulfite oxidase and cyt c [50]. The bioelectrode preparation reproduced the same protocol described for BOD, XOD, and laccase bioelectrodes, mentioned previously [38,46,47]. Sulfite oxidase (expressed in *E. coli*) and cytochrome c (horse heart) were adsorbed by alternated incubation with PASA on gold working electrodes which had been firstly modified with MUA and MU and a monolayer of cyt c. The latter could retain mobility within the network, guarantying a simultaneous interaction with the electrode and sulfite oxidase, as proved by the catalytic activity exhibited in the presence of *sulfite*. The anodic current was dependent on sulfite concentration up to 70 μM and the K_M^{app} was estimated to be 1 μM . Curiously, however, a better response was obtained when the two proteins were mixed and adsorbed together, instead of a sequential multilayered sensor [50].

The same research group later improved on the above configuration by optimizing the number of proteins/PASA layers on the electrode, thereby enhancing the biosensor's response to sulfite. This time, electrochemical measurements were performed at 0.1 V vs Ag/AgCl. Upon sulfite addition, a steady state oxidation current could be obtained in 90 s. The detection limit was 1 μM of sulfite and, as the other analytical parameters of the biosensor, such value could be improved with the number of cyt c/ sulfite oxidase layers. In a 17 layered electrode, the response to sulfite was linear within the range 1 - 60 μM , with a sensitivity of 2.19 $\text{mA}\cdot\text{M}^{-1}$. The apparent K_M was estimated to be 77 μM . Sensor response decreased to 50% after one week storage. After additional protein protection provided by extra PASA and poly allylamine hydrochloride layers and a heat treatment step, at 40 °C the sensors were shown to maintained their initial response for three days, with a subsequent 20% reduction after 5 days of storage at 4 °C. The biosensor

was tested with wine samples; it offered closed to 100% recovery rates, in spiked white wine samples, while the sulfite in the more interferent containing red wine samples was harder to quantify, due to unspecific responses [49].

Microperoxidase-11

Co(II)-protoporphyrin IX reconstituted myoglobin

Microperoxidase-11 (MP-11) is an 11 amino acid heme polypeptide that consists of the microenvironment of cyt c's active site. This polypeptide was used as redox mediator for a Co(II)-protoporphyrin IX reconstituted myoglobin which catalyzed *acetylene dicarboxylic acid* reduction [52]. Co(II)-porphyrins act as catalysts for the hydrogenation of acetylenes, presumably by the intermediate formation of a cobalt hydride species [33]. A monolayer of MP-11 was assembled on MUA SAMs on gold electrodes. The electrodes were then treated with the Co(II) myoglobin and cross-linked with glutaraldehyde. Upon the addition of acetylene dicarboxylic acid, an electrocatalytic cathodic current was observed, indicative of the electrocatalyzed hydrogenation of acetylene dicarboxylic acid to maleic acid. The base layer of MP-11 worked as the electron mediator establishing the electrical contact between the Co(II)-Mb and the underlying electrode. The device presented a linear response to acetylene dicarboxylic acid up to 80 mM and a Michaelis-Menten constant (K_M^{app}) of 90 mM [52].

Nitrate reductase

Microperoxidase-11 mediated nitrate reduction of the cytochrome dependent nitrate reductase from *Escherichia coli* (*E. coli*) was shown for the first time in a study by Narvaez and co-workers [32]. Nitrate reductases catalyze the conversion of nitrate to nitrite in the first step of the nitrate respiration pathway [73]. The proteins have a molybdenum ion as the active site where nitrate reduction occurs [33].

MP-11 was attached on gold electrodes, in the presence 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC), by coupling the protein's carboxylic functions to the amine groups of the cysteamine modified electrodes. The cyclic voltammograms of the bioelectrode showed a pair of well-defined

redox waves from the one electron reduction of MP-11 heme group. In the presence of nitrate reductase and its substrate in solution, enhanced cathodic currents could be measured (Fig. 3B). These were indicative of MP-11 mediation of the catalytic cycle of nitrate reductase. The current showed *nitrate* dependence up to a concentration of ca. 5 mM reaching saturation after that, according to a Michaelis-Menten kinetic behavior ($K_M^{app} = 2.4$ mM) [32].

Later, in a follow up approach, the nitrate reductase was integrated on a MP-11 EDC modified gold electrode, and further incubated with glutaraldehyde, thus creating a more stable configuration to be used as a *nitrate* biosensor. The linear response to nitrate was extended up to ca. 10 mM, accompanied by a K_M^{app} increase to 6.2 mM [53].

Cholesterol oxidase

A *cholesterol* biosensor was prepared on gold electrodes using cholesterol oxidase (*Pseudomonas* sp.) [54]. Signal transduction was attained with microperoxidase-11 as the electron transfer mediator. Cholesterol oxidase is a 56 kDa protein involved in the biosynthesis of bile acids. It contains a FAD cofactor in the active site where cholesterol conversion into cholest-4-en-3-one takes place [74].

The biosensor was prepared on gold surfaces with SAMs of 3-mercaptopropionic acid (MPA) and 3-thiopropanol (TP) which were incubated with MP-11 and the condensing agent EDC. MP-11 was in this way covalently immobilized on the electrode. The enzyme was subsequently immobilized as a nanothin film using layer-by-layer adsorption. The thiol/MP-11 electrodes were therefore sequentially dipped in enzyme and anionic polymer poly(styrenesulfonate) solutions allowing the self-assembly of COx multilayers. The current response of the biosensor to the addition of cholesterol was measured by amperometry at 0 V vs Ag/AgCl. It presented a linear current response within the concentration range 0.2 – 3 mM [54].

Synthetic hemoprotein

A *de novo* synthesized protein with reconstituted heme groups has been utilized as a mediator to wire enzymes to electrodes [33]. The chemically engineered

hemoprotein was designed with specific functional units which allow *i*) the integration of groups analogous to the heme native sites and *ii*) the interaction with reagents that facilitate its immobilization on electrode surfaces. The protein was formed by a four-bundle of antiparallel α -helix elements onto which two Fe^{III} -protoporphyrin IX complexes could be reconstituted (two of the helices included histidine units). For the biosensor construction, gold electrodes were first modified with a cysteamine monolayer followed by coupling of the bifunctional reagent N-succinimidyl-3-maleimidopropionate. A hemoprotein monolayer was then attached to the electrode via covalent linkage of its thiol groups to the maleimide-functionalized gold electrode. Cyclic voltammograms revealed a single reversible pair of peaks with a formal potential of *ca.* -0.35 V vs saturated calomel electrode (SCE), that consists of the two overlapping redox reactions of the heme groups from the synthetic hemoprotein [33].

Nitrate reductase

The *de novo* synthetic hemoprotein was used in integrated bioelectrocatalytic electrodes for *nitrate* reduction [33,55]. The immobilized engineered protein could form an affinity complex with nitrate reductase from *E. coli*, which was stabilized by glutaraldehyde, thus yielding an integrated, electrically contacted enzyme film. This protein assembly could be formed due to the electrostatic interaction between the negatively charged enzyme (pI 4.2) and the positively charged synthetic protein, at pH 7. The resulting bioelectrodes displayed an increased cathodic current upon nitrate addition (Fig. 3C), consistent with the synthetic hemoprotein mediated electrocatalytic reduction of nitrate by nitrate reductase. A linear response could be obtained up to *ca.* 50 mM of substrate, with a K_M^{app} of 42 mM [33,55].

Co(II)-protoporphyrin-reconstituted myoglobin

In the same work, Willner and co-workers have also coupled the bifunctional $\text{Fe}(\text{III})$ -protoporphyrin *de novo* synthesized protein with another semisynthetic protein, Co(II)-protoporphyrin-reconstituted myoglobin [33]. The resulting bioelectrode was shown to electrocatalyze the hydrogenation of *acetylene dicarboxylic acid* to maleic acid. After the reconstitution of the apomyoglobin with Co(II)-protoporphyrin IX, it was interacted with the *de novo* hemoprotein

monolayer-electrode and then cross-linked via glutaraldehyde, as described for the nitrate reductase sensor. The electrode's response to acetylene dicarboxylic acid followed a Michaelis-Menten kinetics with a K_M^{app} of 80 mM [33].

Cytochrome *b*₅₆₂

The cytochrome *b*₅₆₂ (cyt *b*₅₆₂) from *Escherichia coli* is a 12 kDa protein that can be found in the periplasm (Fig. 2B). Although no physiological partners have been identified so far for this small hemic protein, it is likely involved in intermolecular ET. As opposed to other small cytochromes, such as cyt *c*, cyt *b*₅₆₂ does not possess highly charged regions to facilitate its electrostatic docking with redox partners [56].

Glucose oxidase and pyrroloquinoline quinone glucose dehydrogenase

Okuda et al. made use of cyt *b*₅₆₂ as an electron carrier in a couple of *glucose* biosensor systems. Two different enzymes were tested: glucose oxidase (GOx) from *Aspergillus niger* and pyrroloquinoline quinone glucose dehydrogenase (PQQ-GDH) from *Acinetobacter calcoaceticus* [30]. These enzymes are involved in glucose oxidation, catalyzing the conversion of β -D-glucose into D-glucono-1,5-lactone. GOx is a dimeric protein with one bound FAD cofactor per monomer. GOx utilizes oxygen as the electron acceptor of the catalytic reaction with concomitant hydrogen peroxide production [75]. The PQQ-GDH from *Acinetobacter calcoaceticus* is a soluble protein that can be found in the bacteria's periplasmic space. It is composed of two identical 50 kDa subunits that contain a pyrroloquinoline quinone (PQQ) as prosthetic group. The enzyme converts mono and disaccharides into lactones. Contrary to GOx, it does not require oxygen as electron acceptor; it can donate electrons to small cytochromes, such as cyt *b*₅₆₂ [56,76].

The bioelectrodes were prepared by mixing the cytochrome and each enzyme in carbon paste. In the presence of glucose, the cyclic voltammograms of these electrode preparations exhibited an increase in current correlated with the glucose concentration (Fig. 3D). Hence, both enzymes could transfer electrons from their redox centers through cyt *b*₅₆₂. Measurements were performed at potentials close

to cyt b₅₆₂ reduction potential, i.e. 0.189 V vs standard hydrogen electrode (SHE). The detection limits of the biosensors were 0.1 mM and 0.8 mM, while the quantification ranges spanned for 2 mM and 20 mM, for PQQ-GDH and GOx, respectively. The sensitivity was higher with the PQQ-GDH based sensor with 43.2 $\mu\text{A} \cdot \text{M}^{-1} \cdot \text{cm}^{-2}$ versus 12.2 $\mu\text{A} \cdot \text{M}^{-1} \cdot \text{cm}^{-2}$ for the GOx biosensor. Because cyt b₅₆₂ was not the natural redox partner of both enzymes, a high ratio cyt b₅₆₂ to enzyme (100 to 1) was used to ensure efficient electrical wiring. Nevertheless, this cytochrome displayed great versatility in achieving ET with both enzymes, showing its potential application in oxidoreductase based sensors free of synthetic mediators [30].

In a previous work also published by Okuda and collaborators, cyt b₅₆₂ (and cyt c) was shown to improve the catalytic efficiency of *glucose* sensors based on PQQ-GDH and artificial redox mediators (potassium ferricyanide or 1-methoxy-5-methylphenazinium methylsulfate (PMS)) [56]. The authors proposed that the protein mediator interacted with the enzyme and sequentially transferred the received electrons to the artificial electron shuttle. All proteins (cytochromes and PQQ-GDH) were mixed in carbon paste electrodes and fixed with glutaraldehyde. The amperometric response of the biosensors to glucose was registered by monitoring the oxidation of the artificial mediators (in solution) at 0.4 V and 0.1 V vs Ag/AgCl, for ferricyanide and PMS, respectively. While the electrochemical response was in fact mediated by the chemical electron carriers, which could exclude this work from the context of this review, it was made evident that the inclusion of the cytochromes greatly benefitted the bioelectrode's response to glucose; to be precise, a *ca.* 30 fold increase in catalytic currents was observed in their presence, as compared to a biosensor response based simply on PQQ-GDH and the artificial mediators [56].

Cytochrome c₅₅₀

Cytochrome c₅₅₀ from *Paracoccus denitricans* is a small 15 kDa monohemic c-type cytochrome involved in charge transfer (Fig. 2C). It has been indicated as the physiological electron acceptor for quinoxinohemoprotein amine dehydrogenase (QH-AmDH), mediating the ET from the enzyme to the respiratory chain. QH-

AmDH catalyzes the oxidative deamination of amines (e.g. n-butylamine, methylamine, histamine) to aldehyde and ammonia, allowing organism growth using amines as the sole source of carbon and energy. The enzyme has two heme c groups and a quinone as cofactors in its heterotrimeric structure [77].

Quinohemoprotein amine dehydrogenase

Yamamoto's et al. have designed a *histamine* biosensor using QH-AmDH and cyt c_{550} from *Paracoccus denitricans* [16]. The cytochrome replays its physiological role mediating the ET between the transducer interface and the enzyme. The two proteins were co-immobilized by means of a dialysis membrane adjusted on the transducing surface. Gold electrodes were treated with bis(4-pyridyl)disulfide in order to attain reversible electrochemistry of the cyt c_{550} . A clear redox wave was observed in the cathodic and anodic scans attributed to a reversible one electron exchange (Fig. 3E). The formal potential for cyt c_{550} was estimated at 44.5 mV vs Ag/AgCl. A comparison study on the biosensor's kinetic parameters was performed with the cyt and several artificial mediators (ferricyanide, quinones and phenylenediamines). The physiological mediator proved to be more efficient for enzymatic bioelectrocatalysis both in terms of kinetic parameters, redox potential and heterogeneous electron transfer kinetics. The favorable electrostatic interaction between the two proteins was pointed out as taking part in the superior behavior of this mediated electrochemical system. The amperometric sensor (tested at 0.120 V vs Ag/AgCl) could be used for two weeks within a range of 500 nM to 1.5 mM of histamine [16].

Cytochrome c_{551}

cd₁ nitrite reductase

cd₁ nitrite reductase (cd₁NiR) takes part in the denitrification pathway; it reduces nitrite to nitric oxide, being an important endogenous source of this molecule in bacteria. The enzyme can be found in the periplasm as a soluble dimer composed of two 60 kDa subunits. Each subunit contains a d₁- type heme group, where the reduction of nitrite occurs, and a c-type heme involved in ET (accepts reducing

equivalents from small cytochromes and copper proteins and transfers them to the catalytic center) [73].

Loujou et al. have shown the mediated *nitrite* reduction activity of *Pseudomonas aeruginosa* cd₁NiR through its putative redox partner cytochrome c₅₅₁ [34]. Cyt c₅₅₁ is an ET protein which contains one heme group in its small 9 kDa structure (Fig. 2D) [23]. Contrary to cyt c, it is acidic, with a 4.7 isoelectric point (pI); nevertheless, it was able to exhibit a quasi-reversible electrochemical behavior on PGEs (Fig. 3F). This was attributed to favorable interactions of the hydrophobic patch near cyt c₅₅₁ heme group with the PGE, and to a ring of positive lysine side chains also located near the heme cleft. The formal potential of the redox protein was 305 mV *vs* normal hydrogen electrode (NHE) [34]. The electron donor protein and cd₁NiR were secured on the PGEs with a dialysis membrane. With the addition of nitrite, the cd₁NiR mediated response was dependent on nitrite concentration in agreement to a Michaelis-Menten profile ($K_M^{app} = 20 \mu\text{M}$). Interestingly, no electrocatalytic activity was detected when artificial electron shuttles (ferrocene, benzoquinone and ferricyanide) were employed [34].

Cytochrome c₅₅₂

cd₁ nitrite reductase

In a more advanced approach, a *nitrite* biosensor based on cd₁NiR from *Marinobacter hydrocarbonoclasticus* and its physiological redox partner cyt c₅₅₂ was proposed by our own research group [28]. Cytochrome c₅₅₂, a homodimeric 20 kDa protein with one c-type heme per monomer, was indicated as the most probable physiological electron donor for the enzyme in *M. hydrocarbonoclasticus* (Fig. 2E) [73,78]. This small cytochrome (pI 6.8) exhibits a quasi-reversible electrochemistry on 4,4'-dithiodipyridine modified gold electrodes (formal potential 265 mV *vs* NHE) and carbon paste screen-printed electrodes (254 mV *vs* NHE) [28,78].

The cd₁NiR and cyt c₅₅₂ were co-entrapped within a photopolymerisable polyvinyl alcohol (PVA) derivative matrix. The biosensor was developed on disposable CSPEs onto which a mixture of the proteins and the polymer was deposited. Cyclic voltammograms of the bioelectrode showed the quasi-reversible

electrochemistry of the cytochrome in the absence of nitrite, and intense catalytic currents in its presence (Fig. 3G). Besides enabling intermolecular ET to cd_1NiR , cyt c_{552} allows settling the work potential at -0.1 V (vs Ag/AgCl), minimizing interference issues. The linear range was determined to be $10\text{--}200\text{ }\mu\text{M}$, with a detection limit of $7\text{ }\mu\text{M}$. The sensitivity, considering the amount of immobilized cyt c_{552} , was $2.49 \pm 0.08\text{ A.mol}^{-1}.\text{cm}^2.\mu\text{M}^{-1}$ [28].

Pseudoazurine

Pseudoazurine is a small (14 kDa) bacterial blue copper protein that acts as electron donor for several enzymes (e.g. enzymes in the denitrification pathway) (Fig. 2F) [79]. Pseudoazurine possesses a type-1 copper center close to the protein surface, which facilitates ET with its redox partners [80].

Copper nitrite reductase

Prospecting a future application in a *nitrite* biosensor device, the interaction between the copper containing nitrite reductase (CuNiR) and pseudoazurine from *Alcaligenes faecalis* S-6, both in the soluble form, were studied by Astier et al. [27].

Copper nitrite reductase can be found in the bacteria periplasm where it catalyzes the reduction of nitrite to nitric oxide. It is involved in nitrate respiration as one of the steps of nitrate reduction to dinitrogen in the denitrification pathway. This enzyme is composed by three identical subunits each containing two copper centers: a type-1 copper center, involved in ET, and a type-2 copper center, as the catalytic site. Among other small proteins (such as cyt c_{552}), CuNiR can use pseudoazurine as its electron donor partner [73].

According to the proposal of Astier and colleagues, the reaction between pseudoazurine and CuNiR can be electrochemically transduced on gold electrodes modified with cysteine-containing hexapeptides, which create a biocompatible surface for the interaction of the small ET protein [27]. In fact, cyclic voltammograms of pseudoazurine displayed a pair of redox waves consistent with a quasi-reversible electrochemical process and, in the presence of nitrite, the

enzyme's turnover could be measured (Fig. 3H). The authors also tested an inorganic mediator, ruthenium hexamine, though a more limited linear range of 1-100 μM (compared to 0-1500 μM with the physiological electron donor) was attained [27].

The same set of physiological redox partners was immobilized by Tepper on SAM-modified gold electrodes using specific DNA tethers [57]. The electrodes were first immersed in the anchor DNA solution, followed by deposition of a 1-mercaptohexanol SAM. Pseudoazurine and nitrite reductase were modified with complementary DNA tags which allowed them to hybridize with the gold anchored SH-DNA strands. Such electrode architecture enabled a tight association of the two proteins with the transducer interface but without compromising charge transfer and catalytic turnover. However, this bioelectrode preparation was not characterized from an analytical perspective [57].

Final Remarks and Conclusions:

Mediated biosensors based on the physiological partners of redox enzymes can simulate the ET cascades typical of biological processes. The efficiency of charge propagation of these natural electron transport systems is a very attractive feature for biosensors development, as proved by the considerable number of publications in this area. Mediators used in this class of biosensors are almost exclusively small hemic proteins, such as horse heart cytochrome c and bacterial cytochromes (cf. Table 2). The reasons behind this trend are quite obvious; on the one hand, these biomolecules display facile (quasi-reversible) electrochemistry on electrode surfaces. On the other hand, they can shuttle electrons easily, not only to their natural oxidoreductase couples but also to other non-physiologically related enzymes.

The main advantages and drawbacks of protein mediated biosensors are summarized in Table 3.

Table 3

An important advantage from the joint use of an enzyme and its redox partner is the efficient electrochemical wiring of biocatalysts that otherwise would not be able to interact with electrode surfaces, sometimes even using small electroactive synthetic mediators [34]. This opens up the range of possibilities for enzymes that can be used in biosensors, thereby expanding the list of target analytes.

Other advantages in using natural electron transfer proteins in biosensors are clearly illustrated by the works in which they are directly compared with artificial mediators - the biosensor's performance is superior with the first [2,16,27]. In the particular case of the glucose biosensors based on PQQ-GDH, when protein mediators were introduced in the electron relay between the enzyme and the chemical mediator [substrate–enzyme–mediator (cyt b_{562} -ferricyanide/PMS)–electrode] the sensor response improved significantly [56]. Curiously, a superior analytical performance in the presence of electron shuttle protein was also attained in CDH and laccase based bioelectrodes in which the mediated response was compared to their DET based bioelectrocatalysis [43,47].

It is worth emphasizing that protein mediators operate by intermolecular electron exchange in protein–protein complexes [81]. This has several implications on the electrode design. In particular, it is important for the mediator protein to have some mobility within the sensor biofilm. The simplest situations are the bioelectrodes that do not constitute integrated biosensors because proteins are in solution, or the immobilization is achieved with dialysis membranes. In these cases the enzymes and mediator proteins are expected to interact freely [16,27,34,40-43]. In fully integrated biosensors, the mobility and coupling between the biomolecules is highly influenced by the type of immobilization method. Several proposals take advantage of the interactions between the redox couples, just relying on the formation of a complex between the primarily immobilized protein mediator and the redox enzyme [31,52,53]. In other cases, the addition of cross-linking agents, such as glutaraldehyde, is necessary; this helps stabilizing and integrating the components on the bioelectrode and leads to improved analytical parameters [33,52,53,55,56]. Entrapment into polymeric matrices is quite common as well; the proteins can be simply deposited on the electrode in a mix with the polymer or incorporated on multilayered systems. Frequently, the polymer matrices counterbalance the overall charge of the protein molecules (enzyme, mediator protein or both) thereby improving the

immobilization efficiency [35,38,45-47,49,50]. However, the most common method to immobilize proteins is through SAMs (Table 2). In fact, the great majority of bioelectrodes are constituted by a primary layer of thiolated SAM onto which the protein electron shuttle is attached [29,31,38,40,43-47,49,50,52]. The choice of SAMs is normally related with the overall charge of the protein mediators. In general, negatively charged MUA or polar MH are used for positively charged mediator proteins (cyt c and pseudoazurine) and positively charged cysteamine or polar MH are selected for more acidic mediators (MP-11 and cyt c₅₅₀) (Table 2).

As often mentioned throughout the text, both interfacial and intermolecular ET involves a suitable orientation between redox partners. In other words, the redox proteins should have favorable orientations towards both the electrode and the enzyme, in order to guarantee effective electron shuttling for the protein electrochemistry and the coupled enzyme catalysis [1]. Most proposals have some degree of molecular organization. Structured biofilms may be built through a layer-by-layer method, frequently using thiol-modified gold electrodes, onto which a first layer of cyt c is adsorbed, followed by the remaining components [38,44-47,49,50]. In this way, besides allowing attachment of the redox mediator protein, the underlying SAMs can optimize its orientation on the electrode interface. On the other hand, good results are also attained in less organized biosensor constructions based on mixed carbon pastes or polymeric layers [2,28,30,49,50,56]. Perhaps non-structured biofilms are less constraining to the interacting proteins.

Another important advantage comes from the usually low potentials required to drive the electrochemical reactions. This should contribute to an increased selectivity and sensitivity, especially when compared to biosensors that rely on detection of electroactive co-substrates (e.g. O₂ or artificial mediators) or products (e.g. H₂O₂) [61]. With the exception of microperoxidase-11 and synthetic hemoprotein, the majority of protein electron carriers operate in a potential window close to 0 mV *vs* NHE (± 200 mV) (cf. Table 2). As a consequence, some bioelectrodes are reported to be less prone to interferences [46,51], even allowing detection of hydrogen peroxide at around -200 mV *vs* NHE [35]. Nonetheless, despite beneficial, the lowered working potentials do not deliver interference free biosensors, as has been pointed out for sulfite biosensors [48,49].

Some of the examples discussed in this review article indicate that the affinity of the mediator protein to the enzyme is very important for the biosensor's response [30,45,56], with the physiological redox couples generally offering better electrode performances. When the two proteins are not natural redox partners, high protein mediator/enzyme ratios may have to be used to compensate minor responses [30]. Consequently, identification and purification of the natural electron donors/acceptors of the enzymes is highly recommended.

Before concluding, it is worth mentioning that, due to the elimination of the frequently hazardous chemical mediators, protein mediators based biosensors can be viewed as a step forward in the development of more environmentally friendly devices.

From the overall analysis of the works detailed in the present review, we may anticipate a growing and widespread interest on the use of small electron transfer proteins as mediators in electrochemical biosensors. Although a few challenges need to be overcome, they are generally common to the biosensor field.

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FIGURE CAPTIONS

Fig. 1 Schematic representations of the working principle of protein mediated biosensors. A) The enzymatic oxidation (or reduction) of the substrate is linked to the electrochemical oxidation (or reduction) of the mediator; the red arrow indicates the direction of the electron flow in biosensors based on oxidase enzymes and the gray arrow on reductase enzymes. B) Cyclic voltammograms of a redox mediator/oxidoreductase electrode (reversible electrochemical process), recorded (a) in the absence and (b) in the presence of the enzyme's substrate.

Fig. 2 Three-dimensional structures of redox protein mediators used in biosensors. A) horse heart cyt c (1HRC.pdb) [20]; B) *Escherichia coli* cyt b₅₆₂ (1QPU.pdb) [21]; C) *Paracoccus denitrificans* cyt c₅₅₀ (155C.pdb) [22]; D) *Pseudomonas aeruginosa* cyt c₅₅₁ (351C.pdb) [23]; E) *Marinobacter hydrocarbonoclasticus* cyt c₅₅₂ (1CNO.pdb) [24]; F) *Alcaligenes faecalis* pseudoazurine (1PAZ.pdb) [25]. Protein atomic coordinates were obtained from the Brookhaven Protein Data Bank. Structures were prepared with the UCSF Chimera package [26].

Fig. 3 Bioelectrocatalysis mediated by redox proteins. A) Cytochrome c on MUA/MU modified gold co-adsorbed with sulfite oxidase ((a) - without sulfite, (b)–(f) with 60 µM to 1 mM sulfite) [31]; B) Microperoxidase-11 on a MUA modified gold ((a) alone, (b) with nitrate reductase, (c) with nitrate reductase and nitrate) [32]; C) Synthetic hemoprotein and nitrate reductase on cysteamine modified gold ((a) without nitrate (b)–(e) with 12 to 68 mM nitrate; Inset: calibration curve for amperometric measurements at -0.48 V vs SCE) [33]; D) Cytochrome b₅₆₂ and GOx incorporated on carbon paste (immobilized: thin line - GOx, dotted line - GOx and bovine serum albumin, dashed line - GOx and cyt b₅₆₂ thick line - GOx and cyt b₅₆₂ and glucose) [30]; E) Cytochrome c₅₅₀ (membrane bis(4-pyridyl)disulfide modified gold with: solid line - cyt c₅₅₀, dotted line - cyt c₅₅₀, QH-AmDH and n-butylamine) [16]; F) Cytochrome c₅₅₁ and cd₁ nitrite reductase on membrane PGE in the presence of 5 mM nitrite ((a) without enzyme, (b) 0.25 µM enzyme, (c) 1.5 µM enzyme) [34]; G) Cytochrome c₅₅₂ and cd₁NiR immobilized in PVA on carbon paste screen-printed electrodes (solid line - without nitrite, dashed line - with 10 mM nitrite) [28], H) Pseudoazurine on hexapeptide modified gold ((a) alone, (b) with CuNiR, (c) with CuNiR and 0.5 mM nitrite) [27]. Images reprinted with permission from the respective reference.

TABLES

Table 1 Generations of enzyme based amperometric/voltammetric biosensors. MET – mediated electron transfer, DET – direct electron transfer

Generation	ET mode	Electroactive species		Examples
1 st	MET	natural	co-substrates	O ₂
			products	H ₂ O ₂
2 nd	MET	artificial		ferrocene, viologens
		natural ET molecules		cytochrome c
3 rd	DET	enzyme redox cofactors		laccase, hemoglobin, peroxidases, nitrite reductase

Table 2 Characteristics of the biocomponents and electrode composition of biosensors based on protein mediators and enzymes. Biomolecule sources can be found in the text. (N.D. not determined, * pIs are theoretical values predicted from sequence analysis according to databases and tools from the DOE Joint Genome Institute and ExPASy Proteomics Server, ¹ approximate formal potentials were extrapolated from cyclic voltammograms, [#] engineered protein).

Mediator protein (cofactor)	MW (kDa)	pI	Enzyme catalyst (cofactors)	MW (kDa)	pI	Biosensor analyte	e ⁻ flow	Natural electron couple	Biosensor design	Mediator E ⁰ in biosensor	References
Cytochrome c (heme c)	12	10	Lactate oxidoreductase (FMN, heme b ₂)	249	8.59*	Lactate	O	yes	Au/disulfide SAM/cyt c and enzyme in solution	250 mV vs NHE	[41]
						L-mandelic acid [#]	O	yes	PGE/cyt c + enzyme + poly-L-lysine	250 mV vs SCE	[2]
			CCP (heme c)	40	5.95*	Hydrogen peroxide	R	yes	Au/MH SAM/cyt c + CCP + collagen hydrogel	18 mV vs SCE	[35]
			CDH (FAD, heme b)	90	4.2	Cellobiose	O	no	Au/MUA SAM/cyt c/CDH in solution	60 mV vs Ag/AgCl (pH5)	[43]
				98/87	4.4/4.1				Au/MUA-MU SAM/cyt c/CDH in solution	0 mV vs Ag/AgCl (pH6-7)	[40]
			BOD (Cu centers)	90	4.3	Lactose	O	no	Au/MUA-MU SAM/cyt c/(cyt c + CDH/SiNPs) _n	50 ¹ mV vs Ag/AgCl	[44]
				64	5.16*	Dioxygen	R	no	Au/MUA-MU SAM/cyt c/BOD in solution	40 ¹ mV vs Ag/AgCl	[29]
			XOD (FAD, Fe-S clusters, Mo center)	300	7.76*	Hypoxanthine	O	no	Au/MUA SAM/cyt c/(cyt c/XOD/PASA/poly(ethylenimin)) _n	-13 mV vs Ag/AgCl	[38]
									Au/MUA SAM/cyt c/(cyt c/BOD/PASA) _n	-12 mV vs Ag/AgCl	[45]
			Laccase (Cu centers)	68	ca. 3.8	Dioxygen	R	no	Au/MUA SAM/cyt c/(cyt c/laccase/PASA) _n	N.D.	[47]
			Sulfite oxidase (Mo center, heme b ₅)	110	5.5	Sulfite	O	yes	PGE/cyt c and enzyme in solution	150 ¹ mV vs Ag/AgCl	[42]
									CSPE/SO + cyt c + carbon ink	N.D.	[48]
Microperoxidase-11 (heme c)	1.9	6.70*	Reconstituted myoglobin (Co(II)-protoporphyrin)			Acetylene carboxylic acid	R	no	Au/ MUA-MU SAM/cyt c/enzyme + cyt c	-25 ¹ mV vs Ag/AgCl	[31]
									Au/MUA SAM/cyt c/(cyt c + enzyme + PASA) _n	N.D.	[49,50]
			Nitrate reductase (Mo centers, Fe-S clusters)	200	6.05*	Nitrate	R	no	CSPE/SO + cyt c + carbon ink	-125 ¹ mV vs Ag/AgCl	[51]
									Au/MUA SAM/MP-11/enzyme & glutaraldehyde	-420 ¹ mV vs SCE	[52]
			Cholesterol oxidase (FAD)	56	5.98*	Cholesterol	O	no	Au/cysteamine SAM/MP-11 & EDC/enzyme in solution Au/cysteamine SAM/MP-11 & EDC/enzyme & glutaraldehyde Au/MPA-TP SAM/MP-11 & EDC/(enzyme/poly(styrenesulfonate)) _n	-400 mV vs SCE -390 mV vs Ag/AgCl	[32] [53] [54]

Synthetic hemoprotein (Fe(III)-protoporphyrin X)			Nitrate reductase (Mo centers, Fe-S clusters)	200	6.05*	Nitrate	R	no	Au/cysteamine SAM/synt hemoprotein & N-succinimidy-3-malimidopropionate/enzyme & glutaraldehyde	-350 mV <i>vs</i> SCE	[33,55]
			Reconstituted myoglobin (Co(II)-protoporphyrin)			Acetylene carboxylic acid	R	no			[33]
Cytochrome b ₅₆₂ (heme b)	12	6.12*	GOx (FAD)	160	4.2	Glucose	O	no	Carbon paste + cyt b ₅₆₂ + GOx	189 mV <i>vs</i> SHE	[30]
									Carbon paste + cyt b ₅₆₂ + GOx & glutaraldehyde, artificial mediators in solution		[56]
			PQQ-GDH (PQQ)	100	8.93*	Glucose	O	no	Carbon paste + cyt b ₅₆₂ + PQQ-GDH		[30]
Cytochrome c ₅₅₀ (heme c)	15	4.85*	QH-AmDH (quinone, heme c)	60	4.90*	Histamine	R	yes	Au/disulfide SAM/ cyt c ₅₅₀ + QH-AmDH/dialysis membrane	44.5 mV <i>vs</i> Ag/AgCl	[16]
Cytochrome c ₅₅₁ (heme c)	9	4.7	cd ₁ nitrite reductase (heme c, heme d ₁)	120	7.75*	Nitrite	O	yes	PGE/cyt c ₅₅₀ + cd ₁ NiR/dialysis membrane	305 mV <i>vs</i> NHE	[34]
Cytochrome c ₅₅₂ (heme c)	20	6.8	cd ₁ nitrite reductase (heme c, heme d ₁)	120	5.05	Nitrite	O	yes	CSPE/polyvinyl alcohol + cd ₁ NiR + cyt c ₅₅₂	254 mV <i>vs</i> NHE	[28]
Pseudoazurine (Cu center)	14	7.8	Copper nitrite reductase (Cu centers)	111	4.5	Nitrite	O	yes	Au/ cysteine-thiolated hexapeptides/CuNiR and pseudoazurine in solution	40 ⁱ mV <i>vs</i> SCE	[27]
									Au/SH-DNA anchors/ MH SAM/DNA tagged pseudoazurine and CuNiR	275 mV <i>vs</i> SHE	[57]

Table 3 Advantages and challenges of biosensors based on protein mediators

<i>Pros</i>	<i>Cons</i>
<ul style="list-style-type: none">• Mimic the efficient charge transfer processes that occur <i>in vivo</i>• Natural electron transfer proteins have high turnover rates with partner enzymes• Specific interaction between natural partner proteins may improve sensor selectivity and sensitivity in some cases• Not limited to physiological redox couples• Protein mediators usually have low redox potentials (ca. 0 V <i>vs</i> NHE) enabling electrochemical reactions at low driving forces• Biocompatible and nontoxic - environmentally friendly mediators	<ul style="list-style-type: none">• Effective immobilization while retaining enzymatic catalysis may be difficult• Intermolecular electron exchange within sensor films requires some protein mobility• Optimization of electrode interfaces to improve protein favorable orientations• Complex immobilization methods for small size mediator proteins and higher molecular weight enzymes• Enzyme natural electron donors/acceptors must be identified• Purification of a second biomolecule rises the costs• No direct control over the enzymatic reaction by the electrode