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TRENDS

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, initiate bioelectrocatalytic redox processes. This has been very useful in the development of the so-called second-generation biosensors, in which they transduce a catalyzed reaction into an electrical signal. Among other pre-requisites, redox mediators must be readily oxidized and/or reduced at the electrode surface and readily 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 been used as redox partners in biosensing applications. In general, docking between two complementary proteins introduces a second level of selectivity to the biosensor and enlarges the list of compounds analyzed. Moreover, electrochemical interferences are frequently minimized owing to the small overpotentials achieved. This paper provides an overview of enzyme biosensors that are mediated by electron-transfer proteins. The paper begins with a brief discussion of mediated electrochemistry in biosensing systems and proceeds with a detailed description of relevant work on the cooperative use of redox enzymes and biological electron donors and/or acceptors.

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Introduction

Electron transfer in electrochemical systems

Electron transfer (ET) between redox centers of proteins is of crucial importance in such biological processes as the respiratory chain, photosynthesis, metabolic pathways (e.g. glycolysis or the citric acid cycle), and even in the regulation of gene expression, in which so-called redox switches are involved [1]. In recent decades electrochemistry has provided important information on the mechanisms, kinetics, and thermodynamics of ET reactions in biological systems [2]. Besides leading to a better understanding of redox reactions, the study of the electrochemistry of proteins and enzymes provides the basis for fabrication of electrochemical biosensors, i.e. integrated analytical devices that combine a biorecognition element with catalytic activity (enzymes, whole cells, or fragments of cells containing enzymes), with an amperometric or voltammetric transducing system [3, 4].

Although direct electron transfer (DET) is observed for some enzymes (typically heme and blue copper proteins) on electrode surfaces, with approximately one hundred examples reported in the literature, this is not the common trend [5, 6]. In addition, it is not often they also have catalytic activity under DET conditions. One of the main reasons is the intrinsic isolating nature of the polypeptide chains wrapped 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 facilitate interaction of proteins with transducers, thus facilitating DET and/or electrocatalysis. This modification of electrode interfaces usually consists in conferring a suitable overall charge or specific functional groups

that promote specific attachment or orientation of the protein on the electrode surface (e.g. with bipyridyl, poly-L-lysine, or self-assembled monolayers (SAMs)) [7–9]. Nevertheless, when studying 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 mentioned above, electrical contact 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 of the enzyme, as depicted in Fig. 1a [11, 12]. In mediated electrochemistry (MET), and following an EC′ mechanism, consumption of the redox mediator, i.e. the co-substrate, because of the catalytic reaction, is detected as a current amplification (Fig. 1b). This current increase is directly related to the amount of substrate being processed [6, 13].

Unlike DET, use of redox mediators takes control over the protein reaction center away from the electrode [14]. On the one hand, this can be detrimental to the selectivity of detection, because the mediator can react with other species present, and, for 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].

Mediated transfer of redox equivalents is the working principle of second-generation amperometric biosensors (the three biosensor generations are briefly compared in Table 1). Mediators are characterized by having a high heterogeneous ET rate that does not compromise electrochemical reversibility and, at the same time, homogeneous, rapid 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. Low operating potentials are preferred, enabling 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 near zero, at which fewer interfering species are reduced or oxidized [11, 14–16].

Over the years, much work has focused on identifying efficient mediators to ensure enzyme wiring to electrodes. These are generally organic compounds, for example viologens, phenazines, quinones, tetrathiafulvalene, and 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, besides to the use of physiological electron donors and/or acceptors to mimic more faithfully the charge-transfer processes that occur in vivo, and thereby study intermolecular ET and enzyme kinetics [19], the *enzyme–mediator protein* coupling can be exploited in bioelectroanalytical applications, which is the topic of the present review.

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 scarcely been exploited, because the physiological partner of the enzyme must be previously identified and isolated. Alternatively, reactions between versatile electroactive proteins that are not natural redox partners (e.g. horse heart cytochrome c) have also been investigated for biosensing purposes, as will be discussed below. In such a case, protein pairing is largely empirical.

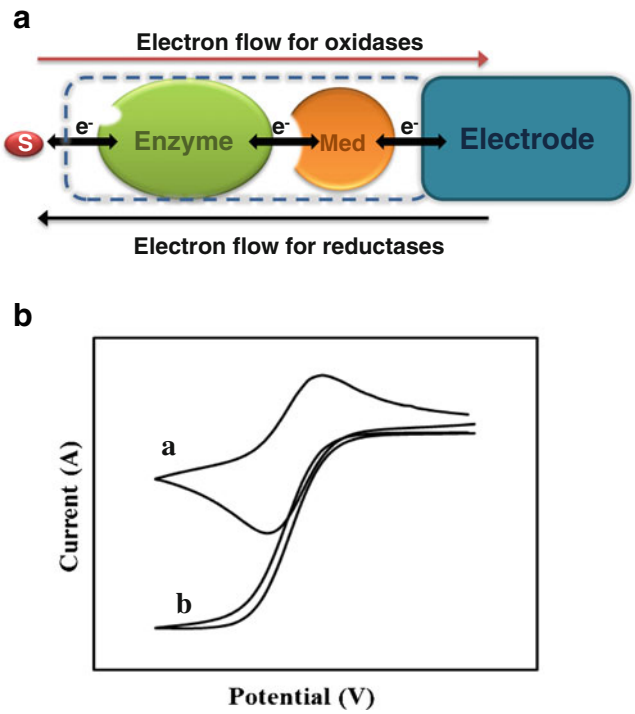


Fig. 1 Schematic representations of the working principle of protein mediated biosensors. (**a**) Enzymatic oxidation (or reduction) of the substrate is linked to the electrochemical oxidation (or reduction) of the mediator. The *red and black arrows* indicate the direction of electron flow in biosensors based on oxidase enzymes and reductase enzymes, respectively. (**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

Table 1 Generations of enzyme based amperometric and voltammetric biosensors

Generation	ET mode	Electroactive species		Examples
1st	MET	Natural	Co-substrates	O_2
			Products	H_2O_2
2nd	MET	Artificial		Ferrocene, viologens
		Natural ET molecules		Cytochrome c
3rd	DET	Enzyme redox cofactors		Laccase, hemoglobin, peroxidases, nitrite reductase

MET, mediated electron transfer; DET, direct electron transfer

Protein redox mediators in biosensors

Protein electron acceptors (or donors) have some advantages in biosensing. They have high affinity for oxidizing (or reducing) enzymes and turnover rates are high, especially if the physiological redox partner is being used [16, 27]. Unlike artificial electron shuttles, which are frequently general catalysts and generate unspecific responses, 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, because the first are less likely to be harmful to biological systems [29, 30].

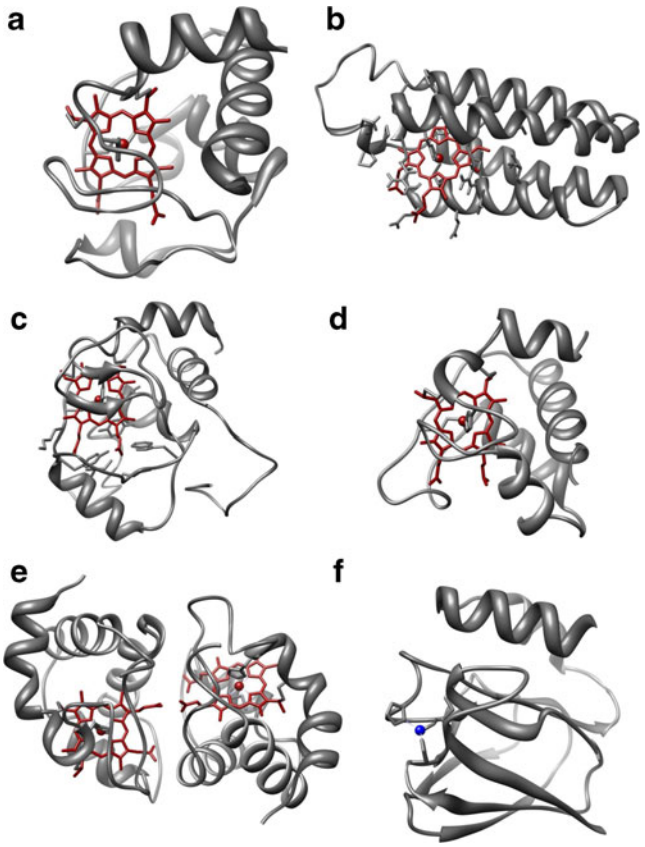


Fig. 2 Three-dimensional structures of redox protein mediators used in biosensors. (**a**) horse heart cyt c (1HRC.pdb) [20]; (**b**) *Escherichia coli* cyt b_{562} (1QPU.pdb) [21]; (**c**) *Paracoccus denitrificans* cyt c_{550} (155C.pdb) [22]; (**d**) *Pseudomonas aeruginosa* cyt c_{551} (351C.pdb) [23]; (**e**) *Marinobacter hydrocarbonoclasticus* cyt c_{552} (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]

In the following sections we report some biosensor designs 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. Cyclic voltammograms selected from the referenced works depicting the mediator redox waves and the catalytic currents after substrate addition, are shown in Fig. 3.

Many published studies focus on the interaction of mediator proteins and biocatalysts, but disregard the catalytic activity of the bioelectrodes. This survey only includes work with an analytical perspective and/or the prospect of future biosensor applications. This approach can be regarded as a recent trend in the R&D of enzyme-based biosensing devices, as most of the papers selected were published in the last fifteen years.

It must be stressed that, irrespective of the proteins couples, electrode structure is a very important aspect, because it should guarantee both catalytic turnover and vectorial ET from (or to) the redox enzyme. In particular, effective immobilization of the small protein mediators and the higher molecular weight enzymes without impairing ET can be a challenging task. Whereas some approaches make use of simple methods, for example entrapment with dialysis membranes [16, 34], others integrate biosensor components with cross-linking agents or polymeric layers [2, 33, 35–37]. A common trend is also the use of thiol-modified gold electrodes, on to which small cytochromes can be attached and for which facile DET is observed [37–40]. Nevertheless, some of the papers cited do not report use of 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]. Information about the bioelectrodes described below, including protein and non-protein components and electrode design, are summarized in Table 2.

Protein mediators and biosensors

Cytochrome c

Small cytochromes, for example horse heart cytochrome c, are, by far, the protein mediators most often used. Cytochrome

c is a small globular protein (12 kDa) which encompasses a c-type heme. It is involved in charge transport in the respiratory chain (Fig. 2a). Because it is a basic protein, it interacts well with negatively charged surfaces (e.g. immobilizing matrices, electrode surfaces, or redox partner enzymes) through its positively charged lysine residues [36]. Well-defined quasi-reversible electrochemistry of this cytochrome can be observed on electrode surfaces, either directly on highly oxygenated carbon surfaces, for example polished edge pyrolytic graphite, or on promoter-coated surfaces, for example thiol-modified gold electrodes [39, 58, 59]. Good electrochemical responses can also be obtained with some polymeric matrices, for example Langmuir–Blodget 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, namely, laccase, ascorbate oxidase [47, 60, 61], NADPH cytochrome P450 reductase [62], bilirubin oxidase [29, 38], xanthine oxidase [46], among others. The function of cyt c on such electrodes is electron shuttling between proteins and electrodes. As previously mentioned, rapid ET between cyt c and both partners, i.e. heterogeneous with the solid electrode and homogenous with the enzymes, is important. This can be favored by electrode modifications which enable optimum orientation but at the same time afford some mobility to the redox molecule, so it can successfully accomplish molecular docking [43]. Electron transfer can, however, still be effective when cyt c is simply entrapped within films in which no specific orientation is expected [2, 48, 51].

Lactate oxidoreductase—flavocytochrome b_2

Flavocytochrome b_2 is a lactate oxidoreductase that can be found in yeast mitochondria. It catalyzes the oxidation of L-lactate to pyruvate, enabling 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 b_2-type heme. Cytochrome c is the physiological electron acceptor for the enzyme [63, 64].

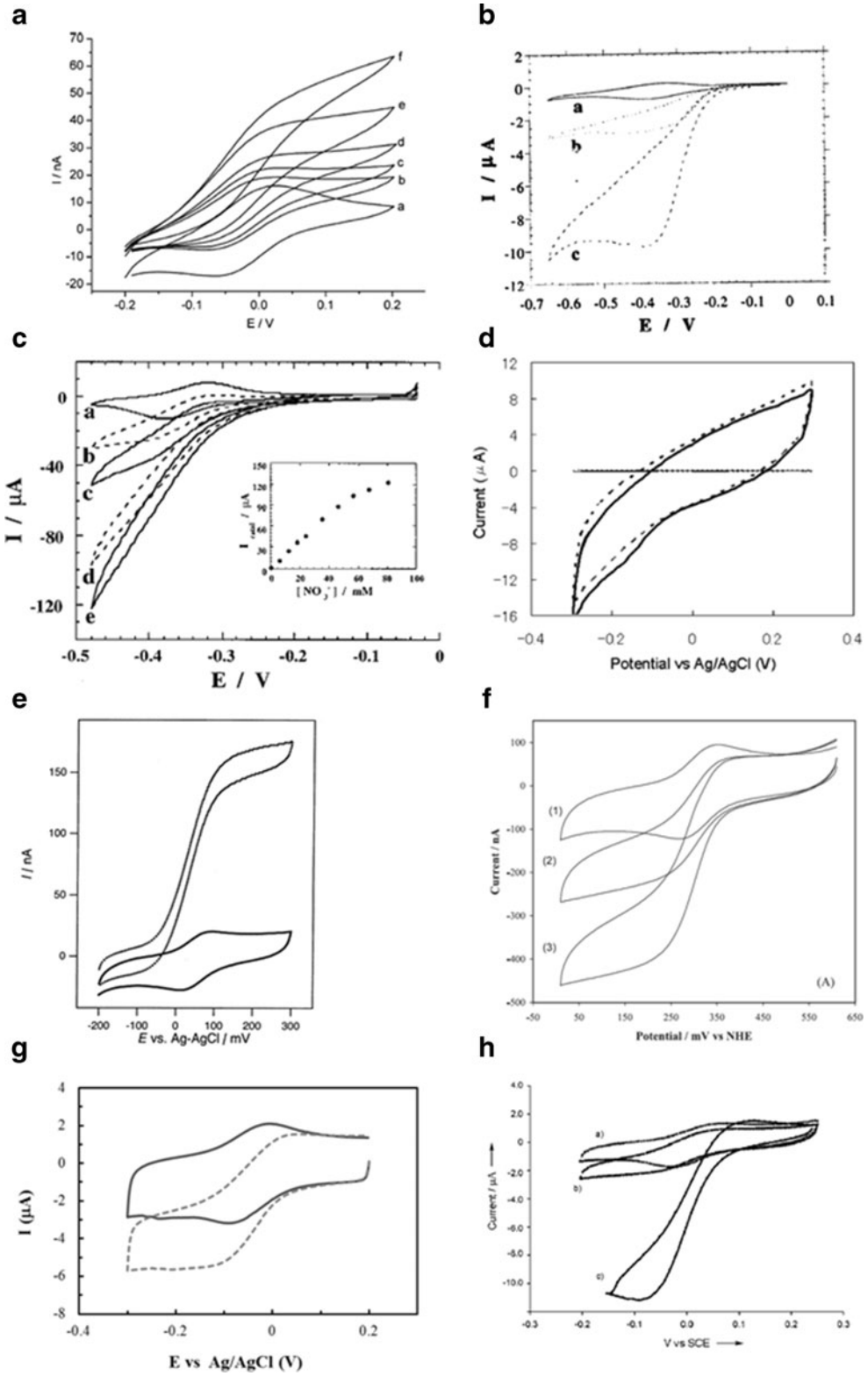
An electrochemical study showing mediated ET between cyt c (horse heart) and flavocytochrome b_2 (yeast) in solution was reported by Cass et al. [41]. Quasi-reversible electrochemistry is observed for cyt c in bis(4-pyridyl) disulfide gold-modified electrodes. In the presence of flavocytochrome b_2 and after 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 b_2 engineered for L-mandelate dehydrogenase activity [2]. Electrocatalysis of the substrate L-mandelic acid could only be achieved by use of mediator species. A ferrocene derivative was used 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].

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 μmolL^{-1} to 1 mmolL^{-1} sulfite [31]. (**b**) Microperoxidase-11 on 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 mmolL^{-1} nitrate; *inset*, calibration curve for amperometric measurements at −0.48 V vs SCE) [33]. (**d**) Cytochrome b_{562} and GOx incorporated in carbon paste (immobilized: *thin line*, GOx; *dotted line*, GOx and bovine serum albumin; *dashed line*, GOx and cyt b_{562}; *thick line*, GOx and cyt b_{562} and glucose) [30]. (**e**) Cytochrome c_{550} (membrane bis(4-pyridyl)disulfide-modified gold with: *solid line*, cyt c_{550}; *dotted line*, cyt c_{550}, QH-AmDH, and *n*-butylamine) [16]. (**f**) Cytochrome c_{551} and cd_1 nitrite reductase on membrane PGE in the presence of 5 mmolL^{-1} nitrite: (*a*) without enzyme, (*b*) 0.25 μmolL^{-1} enzyme, (*c*) 1.5 μmol L^{-1} enzyme [34]. (**g**) Cytochrome c_{552} and cd_1NiR immobilized in PVA on carbon paste screen-printed electrodes (*solid line*, without nitrite; *dashed line*, with 10 mmolL^{-1} nitrite) [28]. (**h**) Pseudoazurin on hexapeptide modified gold: (*a*) alone, (*b*) with CuNiR, (*c*) with CuNiR and 0.5 mmolL^{-1} nitrite [27]. Panel (**a**) Reprinted with permission from [31]. Copyright 2008 American Chemical Society, (**b**) This figure was published in [32], Copyright Elsevier 1997, (**c**) Reprinted with permission from [33]. Copyright 1999 American Chemical Society, (**d**) Reprinted from [30], Copyright 2003, with permission from Elsevier, (**e**) From [16]. Copyright 2001 by Wiley-VCH. Reprinted by permission of John Wiley & Sons, Inc., (**f**) Reprinted from [34], Copyright 2003, with permission from Elsevier, (**h**) From [27]. Copyright 2005 by Wiley-VCH. Reprinted by permission of John Wiley & Sons, Inc.

Cytochrome c peroxidase

Another cyt c-based biosensor (for hydrogen peroxide) was proposed by De Wael et al. [35]. In this work a gelatin hydrogel was used for co-entrapment of horse heart cyt c and *Saccharomyces cerevisae* cytochrome c peroxidase (CCP). 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. Immobilization was aided by the overall negative charge of the hydrogel, which countered the positive charge of the cyt c, yet the cyt c remained mobile in the hydrogel. Because the cyt c–CCP system



had a low overpotential operating range, it enabled quite selective peroxide detection. The biosensor had a fast response to H_2O_2 within a linear range of 0 to 0.3 mmolL^{-1} and a detection limit of 0.01 mmolL^{-1} [35].

Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular protein produced in fungi. This monomeric protein is composed of

Table 2 Characteristics of the biocomponents and electrode composition of biosensors based on protein mediators and enzymes

Mediator protein (cofactor)	MW (kDa)	p*I*	Enzyme catalyst (cofactors)	MW (kDa)	p*I*	Biosensor analyte	e^- flow	Natural electron couple	Biosensor design	Mediator $E^{0'}$ in biosensor	Refs.
Cytochrome c (heme c)	12	10	Lactate oxidoreductase (FMN, heme b_2)	249	8.59[a]	Lactate	O	Yes	Au/disulfide SAM/cyt c and enzyme in solution	250 mV vs NHE	[41]
						L-Mandelic acid[b]	O	Yes	PGE/cyt c + enzyme + poly-L-lysine	250 mV vs SCE	[2]
			CCP (heme c)	40	5.95[a]	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]
				90	4.3	Lactose	O	No	Au/MUA-MU SAM/cyt c/(cyt c + CDH/SiNPs)$_n$	50 mV vs Ag/AgCl[c]	[44]
			BOD (Cu centers)	64	5.16[a]	Dioxygen	R	No	Au/MUA-MU SAM/cyt c/BOD in solution	40 mV vs Ag/AgCl[c]	[29]
									Au/MUA SAM/cyt c/(cyt c/BOD/PASA)$_n$	−13 mV vs Ag/AgCl	[38]
										−12 mV vs Ag/AgCl	[45]
			XOD (FAD, Fe–S clusters, Mo center)	300	7.76[a]	Hypoxanthine	O	No	Au/MUA SAM/cyt c/(cyt c/XOD/PASA/ poly(ethylenimine))$_n$	N.D.	[46]
			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 mV vs Ag/AgCl[c]	[42]
									CSPE/SO + cyt c + carbon ink	N.D.	[48]
									Au/ MUA-MU SAM/cyt c/enzyme + cyt c	−25 mV vs Ag/AgCl[c]	[31]
									Au/MUA SAM/cyt c/(cyt c + enzyme + PASA)$_n$	N.D.	[49, 50]
						Sulfur dioxide	O	Yes	CSPE/SO + cyt c + carbon ink	−125 mV vs Ag/AgCl[c]	[51]
Microperoxidase-11 (heme c)	1.9	6.70[a]	Reconstituted myoglobin (Co(II)-protoporphyrin)			Acetylene carboxylic acid	R	No	Au/MUA SAM/MP-11/enzyme and glutaraldehyde	−420 mV vs SCE[c]	[52]
			Nitrate reductase (Mo centers, Fe–S clusters)	200	6.05[a]	Nitrate	R	No	Au/cysteamine SAM/MP-11 and EDC/enzyme in solution	−400 mV vs SCE	[32]
									Au/cysteamine SAM/MP-11 and EDC/enzyme and glutaraldehyde		[53]
			Cholesterol oxidase (FAD)	56	5.98[a]	Cholesterol	O	No	Au/MPA-TP SAM/MP-11 and EDC/(enzyme/ poly(styrenesulfonate))$_n$	−390 mV vs Ag/AgCl	[54]

Table 2 (continued)

Mediator protein (cofactor)	MW (kDa)	p*I*	Enzyme catalyst (cofactors)	MW (kDa)	p*I*	Biosensor analyte	e^- flow	Natural electron couple	Biosensor design	Mediator $E^{0'}$ in biosensor	Refs.
Synthetic hemoprotein (Fe(III)-protoporphyrin X)			Nitrate reductase (Mo centers, Fe–S clusters)	200	6.05[a]	Nitrate	R	No	Au/cysteamine SAM/synt hemoprotein and *N*-succinimidy-3-malimidopropionate/enzyme and glutaraldehyde	−350 mV vs SCE	[33, 55]
			Reconstituted myoglobin (Co(II)-protoporphyrin)			Acetylene carboxylic acid	R	No			[33]
Cytochrome b_{562} (heme b)	12	6.12[a]	GOx (FAD)	160	4.2	Glucose	O	No	Carbon paste + cyt b_{562} + GOx	189 mV vs SHE	[30]
									Carbon paste + cyt b_{562} + GOx and glutaraldehyde, artificial mediators in solution		[56]
			PQQ-GDH (PQQ)	100	8.93[a]	Glucose	O	No	Carbon paste + cyt b_{562} + PQQ-GDH		[30]
Cytochrome c_{550} (heme c)	15	4.85[a]	QH-AmDH (quinone, heme c)	60	4.90[a]	Histamine	R	Yes	Au/disulfide SAM/ cyt c_{550} + QH-AmDH/dialysis membrane	44.5 mV vs Ag/AgCl	[16]
Cytochrome c_{551} (heme c)	9	4.7	cd_1 nitrite reductase (heme c, heme d_1)	120	7.75[a]	Nitrite	O	Yes	PGE/cyt c_{550} + cd_1NiR/dialysis membrane	305 mV vs 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_1NiR + cyt c_{552}	254 mV vs 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 mV vs SCE[c]	[27]
									Au/SH-DNA anchors/ MH SAM/DNA tagged pseudoazurine and CuNiR	275 mV vs SHE	[57]

[a] These are theoretical p*I* values predicted from sequence analysis according to databases and tools from the DOE Joint Genome Institute and ExPAsy Proteomics Server

[b] Engineered protein

[c] Approximate formal potentials were extrapolated from cyclic voltammograms

Sources of biomolecules are given in the text

N.D., not determined; R, reduction; O, oxidation

two domains, one with a flavin adenine dinucleotide (FAD) cofactor (the catalytic center) and the other with 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. showed that the couple cellobiose dehydrogenase (*Phanerochaete crysosporium*) and cyt c (horse heart) could be used to measure cellobiose [43]. Cyt c was adsorbed on an 11-mercapto-1-undecanoic acid (MUA)-modified gold electrode and functioned as charge carrier between CDH (in solution) and the transducer surface. The authors suggested 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 cyt c was added to the electrolyte a clear catalytic redox wave was observed, indicative of effective vectorial ET from cellobiose via CDH and cyt c to the electrode [43].

Sarauli et al. 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). After 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, interaction of the two protein domains decreased, because of electrostatic repulsion of deprotonated amino acid residues on the surface of both domains and, therefore, cyt c MET was necessary to monitor catalysis of cellobiose [40].

More recently, CDH (*Trametes villosa*) and cyt c (horse heart) have been co-immobilized, in the presence of silica nanoparticles (SiNPs), in a supramolecular layered structure used for *lactose* measurement [44]. For sensor preparation, gold wire electrodes were first modified with MUA and MU, on to which cyt c was then adsorbed. These electrodes were then incubated alternately with a mixture of the proteins (cyt c and CDH) and SiNPs. The bioelectrode assemblies had a defined nanostructure, due to the presence of the nanoparticles, which constituted the support for the two-protein arrangement. Protein co-entrapment was favored by the electrostatic interactions between CDH (isoelectric point (p*I*) 4.3) and cyt c (p*I* 10). Lactose-dependent catalytic currents were followed by cyclic voltammetry and varied linearly with concentrations from 0.01 to 1 mmolL^{-1} [44].

Bilirubin oxidase

In another cytochrome c-mediated design proposed by Dronov et al., this protein was used as an 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 oxidation of bilirubin to biliverdin with concomitant reduction of dioxygen to water [68]. DET was observed for BOD on thiol-modified gold-electrodes at low pH, although not at neutral or 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 rapid kinetics for BOD-catalyzed reduction of oxygen at neutral pH [29].

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

Voltammetric characterization of a BOD–cyt c multilayered electrode revealed 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 was linearly dependent on the oxygen present in solution [38]. The same research group later showed that BOD–cyt c multilayered biosensors 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 substantially 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 determination of hypoxanthine [46]. Xanthine oxidases participate in purine metabolism, catalyzing 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 the work reported by Dronov et al., cyt c (horse heart) was immobilized on a polyanionic electrolyte, PASA, and XOD (cows' milk) was adsorbed on the polycationic poly (ethylenimine) [46]. After addition of hypoxanthine to 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 turning 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 $\mu mol L^{-1}$, with a sensitivity of 0.3 A $mol^{-1} L cm^{-2}$. Because the sensor had a low working potential its susceptibility to interferences and side reactions was also low. An additional polyelectrolyte membrane (poly(allylamine), PAA) and thermal treatment improved electrode stability, thereby enabling the electrode to retain 60 % of its initial activity after five days [46].

Laccase

The same strategy was used to develop an oxygen sensor by use of horse heart cyt c and *Trametes versicolor* laccase [47]. Fungal laccases are multicopper oxidases believed to be involved in 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 oxidation of a broad range of substrates, typically phenolic compounds, for example *p*-dihydroxyphenol [71]. Despite not being its physiological electron donor, cyt c was able to reduce the enzyme, as reported previously by Sakurai et al. [61]. Later, laccase was immobilized by electrostatic interaction with the PASA-cyt c multilayered film. In air-saturated solutions, the increase in the catalytic cathodic current was consistent with sequential reduction of cyt c, laccase, and oxygen. Although a catalytic current for oxygen reduction was also observed for electrodes prepared with only PASA and laccase, in the presence of cyt c the electrocatalytic signal was substantially amplified. The biosensor responded linearly to oxygen and the pH optimum was 4.5 [47].

Sulfite oxidase

Sulfite oxidase is a dimeric protein which contains, per subunit, a molybdenum cofactor, where the catalytic reaction occurs, and a heme b_5, 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 in which they 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 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). After sulfite addition, the enzyme oxidizes it to sulfate in the molybdenum site, electrons are transferred to the heme b_5 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 $mmol L^{-1}$; the detection limit was 4 ppm. With regard to stability, the initial response of the biosensor was maintained for 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 interferences. Thus, the authors claimed enhanced selectivity and sensitivity for sulfite determination by use of this mediator [48].

A few years later, the same authors proposed a sulfur dioxide sensor using, once again, sulfite oxidase and cyt c [51]. The amperometric biosensor was similarly constructed, by the employment of 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 as electron acceptor. The sensors were tested at a working potential of 0.3 V vs Ag/AgCl. For the optimum configuration the response current was linear for SO_2 concentrations from 4 to 50 ppm, the detection limit was 4 ppm, and the response time $t_{90\ \%}$ was 110 s. With regard to electrode stability, biosensors stored over three months in the fridge were shown to retain their initial activity. Moreover, the sensor had good potential for continuous monitoring, with no decay in response over a period of 24 h [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 showed catalytic currents for 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 $\mu mol L^{-1}$ to 2 $mmol L^{-1}$ concentration range, with a Michaelis–Menten constant (K_M^{app}) of 310 $\mu mol L^{-1}$ [31].

Spricigo et al. reported a multilayer polyelectrolyte system based on sulfite oxidase and cyt c [50]. The procedure used for bioelectrode preparation was a reproduction of that described for BOD, XOD, and laccase bioelectrodes, already mentioned [38, 46, 47]. Sulfite oxidase (expressed in *E. coli*) and cytochrome c (horse heart) were adsorbed by alternate incubation with PASA on gold working electrodes which had first been modified with MUA and MU and a monolayer of cyt c. The last of these retained its mobility within the network, guaranteeing simultaneous interaction with the electrode and sulfite oxidase, as proved by the catalytic activity detected in the presence of sulfite. The anodic current was dependent on sulfite concentration up to 70 $\mu mol L^{-1}$ and K_M^{app} was estimated to be 1 $\mu mol L^{-1}$. Curiously, however, a better response was obtained when the two proteins were mixed and adsorbed together, rather than as a sequential multilayer [50].

The same research group later improved on this configuration by optimizing the number of protein–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. After addition of sulfite, a steady state oxidation current could be obtained in 90 s. The detection limit was 1 $\mu mol L^{-1}$ sulfite and, in the same way as the other analytical properties of the biosensor, the value could be improved by increasing the number of cyt c–sulfite oxidase layers. The response of a 17-layer electrode to sulfite was linear within the range 1–60 $\mu mol L^{-1}$, with sensitivity of 2.19 $mA\,mol^{-1}L$. The apparent K_M was estimated to be 77 $\mu mol L^{-1}$. Sensor response decreased to 50 % after storage for one week. After additional protein protection provided by extra PASA and polyallylamine hydrochloride layers and a heat-treatment step at 40 °C the sensors were shown to maintain their initial response for three days, with a subsequent 20 % reduction after storage for 5 days at 4 °C. The biosensor was tested with wine; recovery was close to 100 % for spiked white wine samples whereas the sulfite in red wine, which contained more interferences, was harder to quantify, owing to unspecific responses [49].

Microperoxidase-11

Co(II)-protoporphyrin IX reconstituted myoglobin

Microperoxidase-11 (MP-11) is an 11-amino-acid heme polypeptide that corresponds to the microenvironment of cyt c's active site. This polypeptide has been 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 hydrogenation of acetylenes, presumably via 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. In the presence of acetylene dicarboxylic acid, an electrocatalytic cathodic current was observed, indicative of electrocatalyzed hydrogenation of acetylene dicarboxylic acid to maleic acid. The base layer of MP-11 worked as the electron mediator, establishing electrical contact between the Co(II)-Mb and the underlying electrode. The response of the device to acetylene dicarboxylic acid was linear up to 80 $mmol L^{-1}$ and K_M^{app} was 90 $mmol L^{-1}$ [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 et al. [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 to gold electrodes, in the presence 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC), by coupling the protein's carboxyl functions to the amine groups of the cysteamine-modified electrodes. Cyclic voltammograms showed a pair of well-defined redox waves from the one-electron reduction of the 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 was dependent on nitrate concentration up to ca 5 $mmol L^{-1}$, after which it reached saturation, in accordance with Michaelis–Menten kinetic behavior (K_M^{app}=2.4 $mmol L^{-1}$) [32].

Later, in a follow up approach, the nitrate reductase was integrated on an MP-11 EDC modified gold electrode, and further incubated with glutaraldehyde, thus creating a more stable configuration. The linear response to nitrate was extended to ca 10 $mmol L^{-1}$, accompanied by a K_M^{app} increase to 6.2 $mmol L^{-1}$ [53].

Cholesterol oxidase

A cholesterol biosensor on a gold electrode has been prepared by use of cholesterol oxidase (*Pseudomonas* sp.) [54]. Signal transduction was achieved with microperoxidase-11 as the electron transfer mediator. Cholesterol oxidase (COx) 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 occurs [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 a layer-by-layer adsorption. The thiol–MP-11 electrodes were therefore sequentially dipped in enzyme and anionic polymer poly(styrenesulfonate) solutions, enabling self-assembly of COx multilayers. The current response of the biosensor after addition of cholesterol was measured by amperometry at 0 V vs Ag/AgCl. A linear current response was observed within the concentration range 0.2–3 mmolL^{-1} [54].

Synthetic hemoprotein

A de novo synthesized protein with reconstituted heme groups has been used as a mediator to electron wire enzymes to electrodes [33]. The chemically engineered hemoprotein was designed with specific functional units which enable:

1. integration of groups analogous to the heme native sites; and
2. interaction with reagents that facilitate its immobilization on electrode surfaces.

The protein was formed from a bundle of four antiparallel R-helix elements on to which two Fe^{III}-protoporphyrin IX complexes could be reconstituted (two of the helices included histidine units). For construction of the biosensor, 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 have a single reversible pair of peaks with a formal potential of ca −0.35 V vs the saturated calomel electrode (SCE); these were indicative 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 through the electrostatic interaction between the negatively charged enzyme (p*I* 4.2) and the positively charged synthetic protein, at pH7. The cathodic current of the resulting bioelectrodes increased in the presence of nitrate (Fig. 3c), consistent with the synthetic hemoprotein-mediated electrocatalytic reduction of nitrate by nitrate reductase. A linear response was obtained up to ca 50 mmolL^{-1} substrate, with a K_M^{app} of 42 mmolL^{-1} [33, 55].

Co(II)-protoporphyrin-reconstituted myoglobin

In the same work, Willner et al. also coupled the bifunctional Fe(III)-protoporphyrin de novo synthesized protein with another semisynthetic protein, Co(II)-protoporphyrin-reconstituted myoglobin [33]. The resulting bioelectrode was shown to electrocatalyze hydrogenation of acetylene dicarboxylic acid to maleic acid. After reconstitution of the apomyoglobin with Co(II)-protoporphyrin IX, it interacted with the de novo hemoprotein monolayer electrode and was then cross-linked with glutaraldehyde, as described for the nitrate reductase sensor. The electrode's response to acetylene dicarboxylic acid followed Michaelis–Menten kinetics with a K_M^{app} of 80 mmolL^{-1} [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 yet been identified for this small hemic protein, it is likely to be involved in intermolecular ET. As opposed to other small cytochromes, for example cyt c, cyt b_{562} does not have 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 two *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. In contrast with GOx, it does not require oxygen as electron acceptor; it can donate electrons to small cytochromes, e.g. 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 obtained from these electrode preparations showed an increase in current correlated with 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 the cyt b_{562} reduction potential, i.e. 0.189 V vs the standard hydrogen electrode (SHE). The detection limits of the biosensors were 0.1 mmol L^{-1} and 0.8 mmol L^{-1}, and the quantification ranges reached 2 mmol L^{-1} and 20 mmol L^{-1} for PQQ-GHD and GOx, respectively. Sensitivity was higher with the PQQ-GHD-based sensor, 43.2 μA mol^{-1} L cm^{-2} compared with 12.2 μA mol^{-1} L cm^{-2} for the GOx biosensor. Because cyt b_{562} was not the natural redox partner of both enzymes, a high ratio of cyt b_{562} to enzyme (100:1) was used to ensure efficient electrical wiring. Nevertheless, this cytochrome was extremely versatile, achieving ET with both enzymes and enabling potential application in oxidoreductase-based sensors free from synthetic mediators [30].

In previous work also published by Okuda and collaborators, cyt b_{562} (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 recorded 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. Although the electrochemical response was mediated by the chemical electron carriers, which could exclude this work from the context of this review, it was made evident that inclusion of the cytochromes greatly improved the bioelectrode's response to glucose; to be precise, a ca 30-fold increase in catalytic current was observed in their presence, compared with the response of a biosensor based simply on PQQ-GDH and the artificial mediators [56].

Cytochrome c_{550}

Cytochrome c_{550} from *Paracoccus denitricans* is a small 15-kDa monohemic c-type cytochrome involved in charge transfer (Fig. 2c). It has been reported to be the physiological electron acceptor for quinohemoprotein amine dehydrogenase (QH-AmDH), mediating 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, enabling 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 et al. have designed a histamine biosensor using QH-AmDH and cyt c_{550} from *Paracoccus denitricans* [16]. The cytochrome's physiological function, i.e. mediation of ET between the redox partners is used. The two proteins were co-immobilized by means of a dialysis membrane adjusted to the transducing surface. Gold electrodes were treated with bis(4-pyridyl)disulfide to achieve reversible electrochemistry of the cyt c_{550}. A clear redox wave observed in the cathodic and anodic scans was attributed to a reversible one-electron exchange (Fig. 3e). The formal potential for cyt c_{550} was estimated to be 44.5 mV vs Ag/AgCl. The kinetic parameters of the biosensor were compared with those obtained with different artificial mediators (ferricyanide, quinones, and phenylenediamines). The physiological mediator proved more efficient for enzymatic bioelectrocatalysis, in terms of kinetic data, redox potential, and heterogeneous electron-transfer kinetics. It was pointed out that favorable electrostatic interaction between the two proteins was responsible for 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 nmol L^{-1} to 1.5 mmol L^{-1} histamine [16].

Cytochrome c_{551}

cd_1 nitrite reductase

cd_1 nitrite reductase (cd_1NiR) participates in the denitrification pathway; it reduces nitrite to nitric oxide, and is 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 d_1-type heme group, in which 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* cd_1NiR via its putative redox partner cytochrome c_{551} [34]. Cyt c_{551} is an ET protein which contains one heme group in its small 9-kDa structure (Fig. 2d) [23]. In contrast with cyt c, it is acidic, with a p*I* of 4.7; despite this, its electrochemical behavior on PGEs is quasi-reversible (Fig. 3f). This was attributed to favorable interactions of the hydrophobic patch near the cyt c_{551} 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 the

normal hydrogen electrode (NHE) [34]. The electron-donor protein and cd_1NiR were attached to the PGEs by means of a dialysis membrane. After addition of nitrite, the cd_1NiR-mediated response was dependent on nitrite concentration in agreement with a Michaelis–Menten profile (K_M^{app} = 20 μmol L^{-1}). Interestingly, no electrocatalytic activity was detected when artificial electron shuttles (ferrocene, benzoquinone, and ferricyanide) were used [34].

Cytochrome c_{552}

cd_1 nitrite reductase

In a more advanced approach, a nitrite biosensor based on cd_1NiR from *Marinobacter hydrocarbonoclasticus* and its physiological redox partner cyt c_{552} was proposed by our own research group [28]. Cytochrome c_{552}, a homodimeric 20 kDa protein with one c-type heme per monomer, was reported to be the most probable physiological electron donor for the enzyme in *M. hydrocarbonoclasticus* (Fig. 2e) [73, 78]. The electrochemistry of this small cytochrome (p*I* 6.8) is quasi-reversible on both 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 cd_1NiR and cyt c_{552} were co-entrapped within a photopolymerizable poly(vinyl alcohol) (PVA) derivative matrix. The biosensor was developed on disposable CSPEs, on to which a mixture of the proteins and the polymer was deposited. Cyclic voltammograms demonstrate a quasi-reversible electrochemistry of cyt c_{552} in the absence of nitrite, and intense catalytic currents in its presence (Fig. 3g). Besides transferring electrons to cd_1NiR, cyt c_{552} enables settling of the work potential at −0.1 V (vs Ag/AgCl), minimizing interference. The linear range was determined to be 10–200 μmol L^{-1}, with a detection limit of 7 μmol L^{-1}. The sensitivity, considering the amount of immobilized cyt c_{552}, was 2.49±0.08 A mol^{-1} cm^2 μmol^{-1} L [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 has 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, Astier et al. studied the interaction between the copper-containing nitrite reductase (CuNiR) and pseudoazurine from *Alcaligenes faecalis* S-6, both in the soluble form [27].

Copper nitrite reductase can be found in the periplasm of the bacteria, 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 comprises 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. Aside other small proteins (e.g. cyt c_{552}), CuNiR can use pseudoazurine as its electron donor partner [73].

According to the proposal by Astier and colleagues, reaction between pseudoazurine and CuNiR can be electrochemically transduced on gold electrodes modified with cysteine-containing hexapeptides, which create a biocompatible surface for interaction of the small ET protein [27]. In fact, cyclic voltammograms of pseudoazurine have 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, but the linear range was more limited, 1–100 μmol L^{-1} compared with 0–1500 μmol L^{-1} with the physiological electron donor [27].

The same set of physiological redox partners was immobilized on SAM-modified gold electrodes by use of 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 where modified with complementary DNA tags which enabled them to hybridize with the gold-anchored SH-DNA strands. This electrode architecture enabled tight association of the two proteins with the transducer interface but without compromising charge transfer and catalytic turnover. This bioelectrode was not characterized analytically, however [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 development of biosensors, as proved by the substantial number of publications on this topic. Mediators used in this class of biosensor are almost exclusively small hemic proteins, for example horse heart cytochrome c and bacterial cytochromes (cf. Table 2). The reasons for this trend are quite obvious. On the one hand, the electrochemistry of these biomolecules on electrode surfaces is simple and quasi-reversible. 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 disadvantages of protein-mediated biosensors are summarized in Table 3.

Table 3 Advantages and disadvantages of biosensors based on protein mediators

Advantages	Disadvantages
• Mimic the efficient charge-transfer processes that occur in vivo	• Effective immobilization while retaining enzymatic catalysis may be difficult
• Natural electron-transfer proteins have high turnover rates with partner enzymes	• Intermolecular electron exchange within sensor films requires some protein mobility
• Specific interaction between natural partner proteins may improve sensor selectivity and sensitivity	• Optimization of electrode interfaces to improve favorable protein orientations
• Not limited to physiological redox couples	• Complex immobilization methods for small size mediator proteins and higher-molecular-weight enzymes
• Protein mediators usually have low redox potentials (ca 0 V vs NHE) enabling electrochemical reactions at low driving forces	• Enzyme natural electron donors/acceptors must be identified
• Biocompatible and nontoxic, environmentally friendly mediators	• Purification of a second biomolecule increases the costs
	• No direct control over the enzymatic reaction by the electrode

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

Other advantages of using natural electron-transfer proteins in biosensors are clearly illustrated by work in which they were directly compared with artificial mediators—the performance of the biosensor is superior if natural electron-transfer proteins are used [2, 16, 27]. For the specific example of glucose biosensors based on PQQ-GDH with protein mediators introduced in the electron relay between the enzyme and the chemical mediator (substrate–enzyme–mediator (cyt b_{562}-ferricyanide/PMS)–electrode) sensor response improved significantly [56]. Curiously, a superior analytical performance in the presence of electron shuttle protein was also achieved by use of CDH and laccase-based bioelectrodes in which the mediated response was compared with that of 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 of, and coupling between, the biomolecules is highly affected by the method used for immobilization. Several proposals take advantage of the interactions between the redox couples, relying solely on the formation of a complex between the initially immobilized protein mediator and the redox enzyme [31, 52, 53]. In other cases, addition of cross-linking agents, for example glutaraldehyde, is necessary; this aids stabilization and integration of the components on the bioelectrode and leads to improved analytical performance [33, 52, 53, 55, 56]. Entrapment in polymeric matrices is also quite common; the proteins can simply be deposited on the electrode, as a mixture with the polymer, or incorporated in multilayered systems. Frequently, the polymer matrices counterbalance the overall charge of the protein molecules (enzyme, mediator protein, or both) thereby improving the efficiency of immobilization [35, 38, 45–47, 49, 50]. However, the most common method of immobilization of proteins is through SAMs (Table 2). In fact, for the great majority of bioelectrodes the protein electron shuttle is attached to a primary layer of thiolated SAM [29, 31, 38, 40, 43–47, 49, 50, 52]. The choice of SAM is normally related to 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 c_{550}) (Table 2).

As often mentioned throughout the text, interfacial and intermolecular ET both require suitable orientation of the redox partners. In other words, the redox proteins should be favorably orientated toward both the electrode and the enzyme, to guarantee effective electron shuttling for the protein electrochemistry and the coupled enzyme catalysis [1]. Most proposals include some molecular organization. Structured biofilms may be built by a layer-by-layer method, frequently using thiol-modified gold electrodes, on to which a first layer of cyt c is adsorbed, followed by the remaining components [38, 44–47, 49, 50]. In this way, besides enabling attachment of the redox-mediator protein,

the underlying SAMs can optimize its orientation on the electrode interface. Good results are also achieved by use of less organized biosensor constructions based on mixed carbon pastes or polymeric layers [2, 28, 30, 49, 50, 56]. Perhaps non-structured biofilms result in less constraint of the interacting proteins.

Another important advantage is the low potentials usually required to drive the electrochemical reactions. This should contribute to increased selectivity and sensitivity, especially compared with biosensors that rely on detection of electroactive co-substrates (e.g. O_2 or artificial mediators) or products (e.g. H_2O_2) [61]. With the exception of microperoxidase-11 and synthetic hemoprotein, most protein electron carriers operate at a potential close to 0 mV vs NHE (±200 mV) (cf. Table 2). As a consequence, some bioelectrodes are reported to be less prone to interference [46, 51], even enabling detection of hydrogen peroxide at approximately −200 mV vs NHE [35]. Nonetheless, although beneficial, the reduced working potentials do not result in interference-free biosensors, as has been pointed out for sulfite biosensors [48, 49].

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

Before concluding, it is worth mentioning that, owing to elimination of frequently hazardous chemical mediators, protein mediator-based biosensors can be regarded as a progress in the development of more environmentally friendly devices.

From an overall analysis of the work detailed in this review, we may expect growing and widespread interest on the use of small electron-transfer proteins as mediators in electrochemical biosensors, although a few problems must still be overcome.

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