

## Review



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# Direct enzymatic bioelectrocatalysis: differentiating between myth and reality

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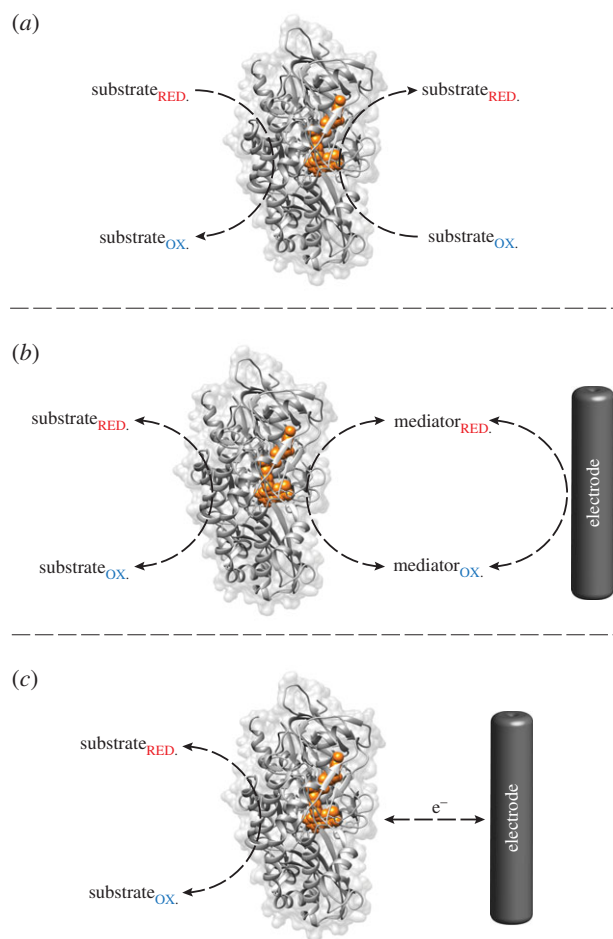
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Enzymatic bioelectrocatalysis is being increasingly exploited to better understand oxidoreductase enzymes, to develop minimalistic yet specific bio-sensor platforms, and to develop alternative energy conversion devices and bioelectrosynthetic devices for the production of energy and/or important chemical commodities. In some cases, these enzymes are able to electronically communicate with an appropriately designed electrode surface without the requirement of an electron mediator to shuttle electrons between the enzyme and electrode. This phenomenon has been termed direct electron transfer or direct bioelectrocatalysis. While many thorough studies have extensively investigated this fascinating feat, it is sometimes difficult to differentiate desirable enzymatic bioelectrocatalysis from electrocatalysis deriving from inactivated enzyme that may have also released its catalytic cofactor. This article will review direct bioelectrocatalysis of several oxidoreductases, with an emphasis on experiments that provide support for direct bioelectrocatalysis versus denatured enzyme or dissociated cofactor. Finally, this review will conclude with a series of proposed control experiments that could be adopted to discern successful direct electronic communication of an enzyme from its denatured counterpart.

## 1. Introduction

Oxidoreductase enzymes are biocatalytic proteins that catalyse the coupled oxidation and reduction in two substrates; thus, transferring an electron(s) between the two substrates with the involvement of the cofactor of the enzyme. While multiple mechanisms of biocatalysis and the transfer of electrons exist, figure 1*a* illustrates a commonly adopted mechanism by which both enzymatic substrates bind to the protein. In the case of enzymatic bioelectrocatalysis, the second substrate of the enzyme is replaced with an electrode, so that the catalytic oxidation of the first substrate of the enzyme can supply electrons to the electrode (bioelectrocatalytic oxidation). Conversely, the electrode can be used to reduce an enzyme and ultimately facilitate enzymatic reduction in the second substrate (bioelectrocatalytic reduction). Electron transfer (ET) to an enzyme, however, is most commonly not trivial (especially when the redox cofactor is deeply buried within the protein) and a small electron mediator is employed to facilitate ET (figure 1*b*). In some cases, enzymes are able to undergo ET directly with an electrode surface (figure 1*c*) [1].

The ability to electronically contact an oxidoreductase enzyme has resulted in a wealth of research pertaining to enzymatic fuel cells (EFCs), amperometric biosensors and bioelectrosynthetic devices as well as mechanistic studies of oxidoreductase activity such as inhibition [2–7]. EFCs are devices that use the bioelectrocatalytic capabilities of enzymes to oxidize and reduce substrates in the production of electrical energy [2]. The most common EFCs use O<sub>2</sub>-reducing enzymes for the cathodic reaction (producing H<sub>2</sub>O by a 4e<sup>−</sup> reduction), whereas saccharide-, molecular hydrogen (H<sub>2</sub>)- or alcohol-oxidizing enzymes are frequently used for the anodic reaction [8–13]. Saccharide/O<sub>2</sub> EFCs have the added benefit of their potential implantation into living hosts that circulate saccharides (such as glucose) or produce lactate or glucose externally, including mammals and insects [14–19].



**Figure 1.** (a) Simplified mechanism for the oxidation and reduction of two substrates by an oxidoreductase (grey) with its redox cofactor highlighted in orange. (b) MET of bioelectrochemical oxidation or reduction of a substrate by an oxidoreductase. (c) DET of bioelectrochemical oxidation or reduction in a substrate by an oxidoreductase. (Online version in colour.)

The inherent substrate specificity of enzymes coupled with their ability to undergo heterogeneous electronic communication with electrodes has also spurred significant research towards creating biosensors for the detection of physiological glucose [20]. Progressing through several developments of electronic communication strategies with oxidoreductases, glucose is now most commonly sensed by the use of an electron mediator that is covalently grafted onto a polymer support, which is also conveniently used to immobilize the enzyme at the electrode surface [21]. The combination of low potential redox mediators and high-specificity enzymes permits the detection of glucose at potentials where other physiological components are seldom interferents (such as ascorbic acid and acetaminophen/paracetamol).

In contrast with EFCs and biosensors that typically produce electrical energy in the presence of substrate/analyte, bioelectrosynthetic systems have been explored, whereby oxidoreductase enzymes are used to convert common substrates into more valuable chemical commodities by the consumption of electrical energy. Most commonly, CO<sub>2</sub> reduction has been explored with the aim of producing formate or alkanes/alcohols, although NAD<sup>+</sup> reduction to NADH is frequently attempted where NAD-dependent enzymes can support reductive reactions [22–24]; Vincent and co-workers [25] have also demonstrated that the coimmobilization of hydrogenase and NAD<sup>+</sup>-reducing proteins onto

conductive particles can facilitate H<sub>2</sub>-driven NADH production. Recent research has also explored the possibility of reducing dinitrogen (N<sub>2</sub>) to an important chemical commodity, ammonia (NH<sub>3</sub>), while simultaneously producing electrical energy [26].

## 2. Electron transfer mechanisms

### 2.1. Mediated electron transfer

As illustrated in figure 1*b*, enzymes are able to use small electroactive molecules to undergo mediated ET (MET) with an electrode, although some prerequisites are necessary to establish electronic communication. First, the electronic properties of the electron mediator must align with the desired bioelectrocatalytic reaction. In the case of oxidative MET, the reduction potential of the mediator should be more positive than the reduction potential of the redox cofactor of the enzyme to enable spontaneous MET (equation (2.1)). The opposite is true of reductive MET, where the reduction potential of the mediator should be more negative than that of the redox cofactor to enable its reduction and subsequent reduction in the substrate. While the optimal magnitude of potential difference between the mediator and redox cofactor has been reviewed to be somewhere between 50 and 170 mV, the reaction quotient defines the cell potential (as per the Nernst equation, equation (2.2)) and must also be considered [2]. The reaction quotient becomes more important when MET can support pseudo-reversible enzymatic MET, as in the case of some hydrogenases where reductive and oxidative catalytic responses can be observed in the same experiment using the same mediator.

$$\Delta G = -nFE_{\text{emf}} \quad (2.1)$$

and

$$E = E^{\circ'} + \frac{RT}{nF} \ln \frac{[O]}{[R]}, \quad (2.2)$$

where  $G$  is the Gibbs free energy,  $n$  the number of electrons,  $F$  the Faraday constant,  $E_{\text{emf}}$  the potential in terms of electromotive force (between the enzyme's cofactor and electron donor/acceptor),  $E$  the potential of the cell,  $E^{\circ'}$  the formal potential of the species,  $R$  the gas constant,  $T$  the absolute temperature,  $O$  the oxidized species and  $R$  the reduced species.

Once a series of electron mediators with suitable electronic properties has been selected, they must next be screened for their ability to interact with the enzyme in a similar fashion to a substrate; this has greater importance in cases where the redox cofactor is deeply buried within the protein structure. We recently demonstrated that naphthoquinone derivatives were able to undergo efficient MET with one type of glucose oxidizing enzyme, while another species did not exhibit any activity even though the enzymes possess the same redox cofactor where their potentials are expected to have negligible effect on the required overpotential of MET, reemphasizing the importance of cofactor accessibility by the electron mediator [27].

### 2.2. Direct electron transfer

In contrast with MET, some oxidoreductases are able to undergo ET with an electrode surface without the need of an exogenous electron shuttle, where the process is termed

direct ET (DET, as illustrated in figure 1c) [1,28]. Typically, a/ the redox cofactor of the enzyme is proximal to the electrode, so that efficient heterogeneous ET can take place, where the distance of the redox cofactor from the electrode surface should not exceed 20 Å (2 nm) [2,29].

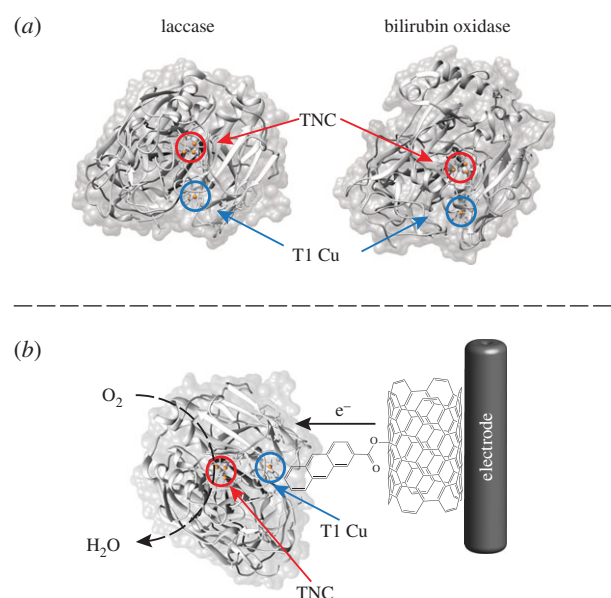
For the purpose of this review and as most commonly found in the relevant literature, ET is considered to be DET in the sense of direct bioelectrocatalysis if the process meets the following criteria: (i) the redox cofactor(s) of the enzyme remain bound or associated with the enzyme during ET, (ii) ET is not mediated (in the electrochemical sense of the word) by a non-diffusive and/or synthetic electroactive species on the enzyme or electrode surface, and (iii) substrate oxidation/reduction can be observed upon its addition to the system. By applying these criteria, nicotinamide adenine dinucleotide-(NAD) dependent enzymes are not considered to undergo DET, as the NAD cofactor is freely diffusing and not bound to the enzyme. Although we will not consider NAD-dependent enzymes within this review (containing only NAD as the cofactor), it is important to note that researchers have immobilized the NAD cofactor on electrode surfaces, thereby installing a pseudo-DET architecture [30]. There are also naturally occurring enzymes and engineered enzymes that employ an electroactive, non-catalytic subdomain within their protein structure to facilitate DET; this inter-domain ET (IET) pathway will be discussed in the following text.

Since its conception in 1978 where DET between a multi-copper oxidase (MCO) (laccase) and a carbon electrode afforded enzymatic  $O_2$  reduction, DET has evolved into an established ET pathway for many oxidoreductases, most commonly (but not limited to) laccase, bilirubin oxidase (BOx), nitrate reductase, hydrogenase and fructose dehydrogenase [31–34].

### 3. Direct bioelectrocatalysis of metalloenzymes

Many metalloenzymes have been studied by DET and direct bioelectrocatalysis at electrode surfaces. Commonly occurring metallocofactor motifs of such metalloenzymes are: haem centres, iron–sulfur clusters ([Fe–S]), iron (Fe) centres, copper (Cu) centres, molybdenum centres (commonly called Moco-factors, Moco) and tungsten, including various iterations and combinations thereof. In many cases, only a single catalytic redox cofactor is found, although others can be found to be involved in internal ET (commonly the case of [4Fe–4S] containing proteins). Frequently, oxidoreductases that contain non-metallocofactors (such as flavin adenine dinucleotide (FAD) and pyrroloquinoline quinone (PQQ)-dependent enzymes) also use metallocofactors to transfer electrons to or from their redox partners, or in our case, electrode surfaces.

MCOs are perhaps the most commonly studied enzymes for direct bioelectrocatalysis, namely laccase and BOx, due to interest in employing them in  $O_2$ -reducing EFCs. Typically, MCO enzymes harbour multiple ‘types’ of Cu centres, termed T1, T2 and T3 (binuclear), where their differences in terminology arise from their coordination numbers, the amino acids to which they are coordinated within enzymes, electron paramagnetic resonance spectroscopic properties and light absorption properties [35–39]. In these MCOs, a T1 site is proximally located and is responsible for single  $e^-$  oxidations of their respective substrates (phenolic substrates, bilirubin and ascorbate) and the T2 Cu is combined with the binuclear T3 Cu in a trinuclear cluster (TNC) where  $O_2$  undergoes a  $4e^-$  reduction to  $H_2O$  once the TNC

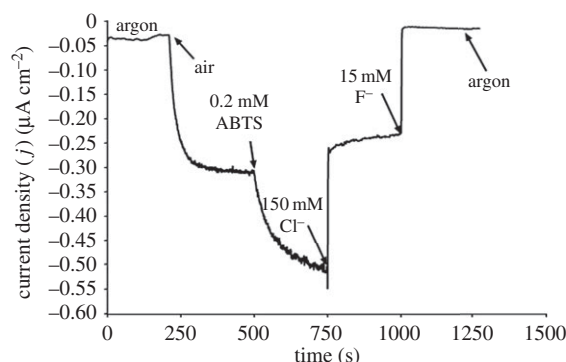


**Figure 2.** (a) Crystal structure of laccase from *T. versicolor* (PDB accession code: 1KYA) and BOx from *M. verrucaria* (PDB accession code: 2XLL). (b) Favourable orientation of laccase towards a modified electrode surface for improved direct bioelectrocatalysis. (Online version in colour.)

has been suitably reduced. Figure 2a presents a crystal structure of two typical MCOs: laccase (from *Trametes versicolor*) and BOx (from *Myrothecium verrucaria*), where the proximity of the T1 Cu centres to a hypothetical electrode surface can be easily envisaged.

These MCOs, specifically laccase and BOx, are renowned for their largely orientationally regulated ability to reduce  $O_2$  to  $H_2O$  by direct bioelectrocatalysis at electrode surfaces. In the case of laccase, substrate mimics have been shown to greatly improve the catalytic currents that can be observed whereby these mimics promote the orientation of the T1 Cu centre (the electron acceptor of laccase) towards the surface of the electrode, thereby minimizing the distance of electron tunnelling to the T1 centre and improving direct bioelectrocatalysis (figure 2b). Typically, polyaromatic hydrocarbons (anthracene, naphthalene and derivatives thereof) are the most commonly employed orientational agents [40–44]. In the case of BOx, efficient DET can be afforded by immobilization onto multi-walled carbon nanotubes, using charged multi-walled carbon nanotubes, immobilized polyaromatic hydrocarbons and even natural substrates (bilirubin and its metabolites) [33,45–49].

Multiple experimental approaches have been adopted to demonstrate that direct bioelectrocatalysis does in fact arise from electronic communication with intact enzymes and not products of their degradation, such as unfolding and/or dissociation of their redox cofactors. Initial experiments usually seek to vary the dissolved  $O_2$  concentration for cyclic voltammetry or steady-state amperometric  $i-t$  analysis, where a direct bioelectrocatalytic current is only observed in the presence of dissolved  $O_2$ . The specific orientation and association of laccase and BOx to their substrates or substrate mimics provides strong evidence that the enzyme must remain partially correctly folded (as a minimum). Knowledge surrounding the inhibition of these enzymes by small halide anions has resulted in studies that provide additional experimental evidence to support the argument that  $O_2$  reduction is facilitated by oriented intact enzyme versus dissociated

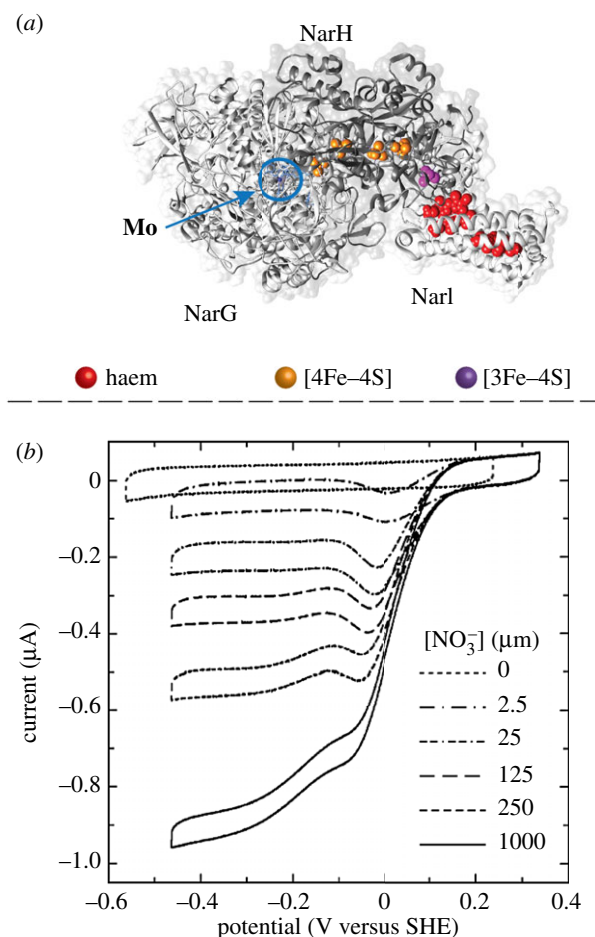


**Figure 3.** Inhibition of laccase by  $\text{Cl}^-$  and  $\text{F}^-$ . Initially, a reductive current is observed for  $\text{O}_2$  reduction by laccase undergoing direct bioelectrocatalysis upon the introduction of air. The addition of ABTS (0.2 mM) as an electron mediator results in an increase in the reductive current, due to the establishment of MET with laccase that was not already undergoing DET. The addition of  $\text{Cl}^-$  (150 mM) results in the loss of MET only, where  $\text{F}^-$  (15 mM) results in a complete loss of MET and DET.  $E_{\text{appl.}} = +0.2$  V versus Ag/AgCl (3 M), pH 4.5 citrate/phosphate buffer. Reproduced from [43] with permission from the Royal Society of Chemistry.

cofactor [40,48,50].  $\text{Cl}^-$  has been shown to act as a competitive inhibitor to some laccases, competing with substrate inhibition at the T1 Cu centre; thus, electrode architectures designed to possess substrate mimics of laccase are able to out-compete  $\text{Cl}^-$  inhibition up to 150 mM  $\text{Cl}^-$ . The addition of  $\text{F}^-$  as an inhibitor results in the complete loss of direct bioelectrocatalysis (figure 3) [40,48]. Additionally, loadings of laccase onto electrodes at quantities greater than an expected monolayer result in some enzyme that is not orientated, and thus does not undergo direct bioelectrocatalysis. The addition of an additional T1 Cu centre electron mediator (such as ABTS) increases the catalytic current observed on the electrode, due to a mixture of MET and DET pathways. The addition of 150 mM  $\text{Cl}^-$  typically quenches this additional MET pathway (due to  $\text{Cl}^-$  competitive inhibition at the T1 Cu centre), providing further evidence for enzymatic orientation [40,48].

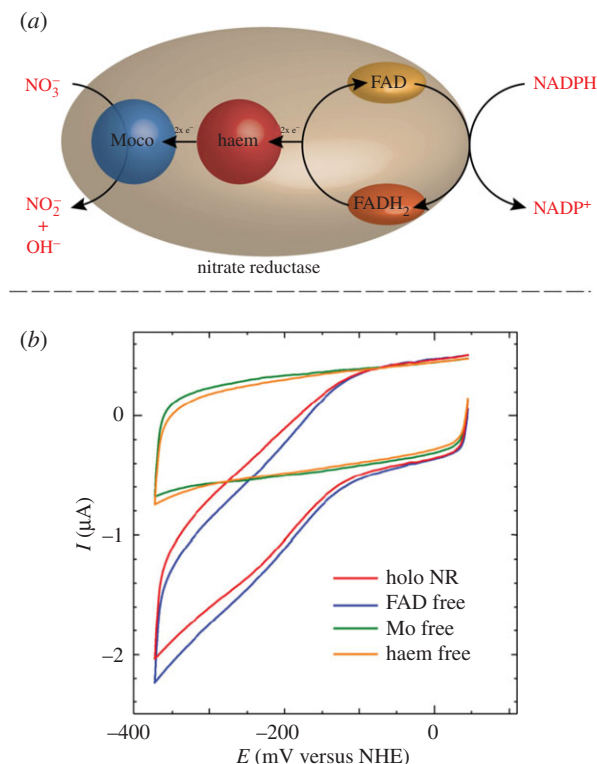
Very recently, Dagys *et al.* [51] reported on an efficient laccase bioelectrode whereby ET via the T1 Cu centre was negated altogether and DET was established between an Au electrode and the TNC of the enzyme, achieving high catalytic current densities of nearly  $1 \text{ mA cm}^{-2}$  (at pH 4). In addition to showing that  $\text{F}^-$  had a markedly less inhibitory effect on their electrode, where  $\text{F}^-$  is largely considered to affect ET between the T1 and TNC Cu centres of MCOs, the authors also turned to the use of surface-enhanced Raman spectroscopy and differential spectroelectrochemistry of the T1 and TNC Cu centres to prove that the enzyme was indeed oriented about its TNC.

For many of the scientific reports detailing direct bioelectrocatalysis of MCOs for  $\text{O}_2$  reduction at electrode surfaces, enzyme orientation and thus the heterogeneous interfacial ET rate ( $k_0$ ) is typically considered to be homogeneous and the catalytic reductive current often appears to reach a plateau; however, this is not always the case. Léger *et al.* [52] explored the case where a dispersion of enzyme orientations (a dispersion of  $k_0$  values) results in a catalytic response that does not always reach a plateau within a reasonable potential window. It is important to consider and account for a range of enzyme orientation may be present on an electrode surface when attempting to extrapolate mechanistic and kinetic details.



**Figure 4.** (a) Crystal structure of NarGHI NR from *E. coli* (PDB accession code: 1Q16). The haem cofactors are shown in red, the [4Fe–4S] clusters are shown in orange, the [3Fe–4S] cluster is shown in magenta and the Moco is shown in blue. (b) Catalytic cyclic voltammogram of NarGHI NR from *E. coli* at a PGE electrode, in the absence or presence of nitrate, where the reductive catalytic current magnitude increases under higher concentrations of nitrate. Experiments were performed at  $30^\circ\text{C}$  pH 7.0 on a rotating electrode. Potentials were recorded versus a saturated calomel electrode (SCE) and are reported versus the standard hydrogen electrode (SHE,  $E_{\text{SHE}} = E_{\text{SCE}} + 241 \text{ mV}$  (at  $25^\circ\text{C}$ )). The IUPAC convention was used to plot current versus potential, where positive potentials and currents are oxidative [48]. Reprinted (adapted) with permission from [53] (copyright © 2004 American Chemical Society). (Online version in colour.)

Other metalloenzymes are known that possess intricate internal ET pathways, where such an enzyme could function as a transmembrane enzyme (where the enzyme must stretch across and function on either side of a membrane) or the enzyme contains a deeply buried catalytic redox cofactor. One of the most fascinating examples of such an internal ET pathway can be found in prokaryotic nitrate reductase (Nar NR), such as that in *Escherichia coli* (NarGHI NR). Evaluation of this NarGHI NR reveals the protein to be organized as a dimer of heterotrimers (figure 4a), harbouring an Mo-dependent cofactor (Moco) in the NarG subunit. Amazingly, this enzyme is able to transfer electrons from its outer haem cofactor (located in the NarI subunit) over a distance of approximately  $75 \text{ \AA}$  (spanning the NarH subunit; by the way of six internal cofactors that facilitate ET:  $1 \times$  haem,  $4 \times$  [4Fe–4S] clusters and  $1 \times$  [3Fe–4S] cluster [54]. Interest in ET of the different variations of this enzyme (both prokaryotic and eukaryotic) has led to multiple studies of its direct



**Figure 5.** (a) Electron flow and catalysis within holo NR from *N. crassa*, as described in [51]. (b) Direct bioelectrocatalytic nitrate reduction by holo NR and mutants lacking the Moco, haem or FAD cofactor. Potentials were recorded versus an Ag/AgCl reference electrode and are reported versus the normal hydrogen electrode (NHE,  $E_{\text{NHE}} = E_{\text{Ag/AgCl}} + 196 \text{ mV}$  (at  $25^\circ\text{C}$ )). The IUPAC convention was used to plot current versus potential, where positive potentials and currents are oxidative. Reprinted from [51] with permission from Elsevier. (Online version in colour.)

bioelectrocatalysis at electrode surfaces [53,55–58]. Figure 4b presents a catalytic cyclic voltammogram for a pyrolytic graphite edge (PGE) electrode modified with NarGHI NR, in the absence and presence of nitrate. In this particular study, the PGE electrode was modified by immersing the electrode into a  $10 \mu\text{M}$  solution of NarGHI NR containing  $200 \mu\text{g ml}^{-1}$  polymyxin, before being rinsed prior to electrochemical testing [53].

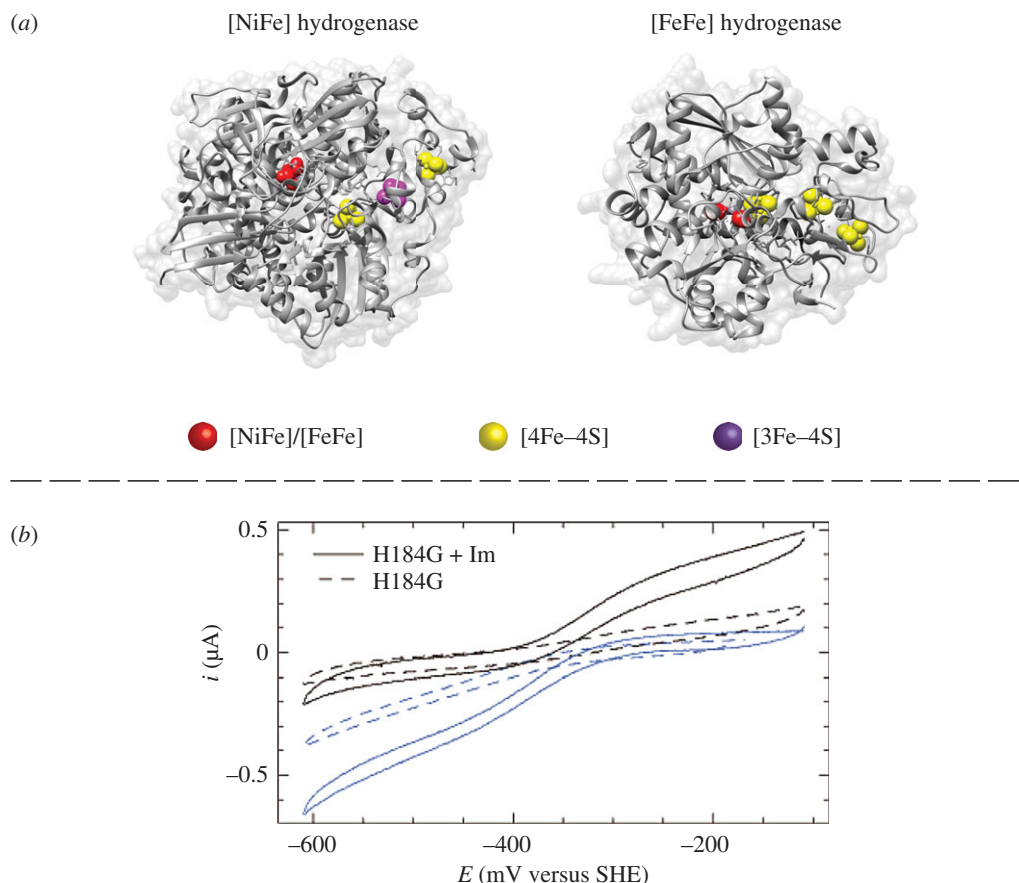
A recent study of eukaryotic NR from *Neurospora crassa* from the Bernhardt group investigated the necessity of the different cofactors of this NR for direct bioelectrocatalysis [58]. This NR contains a Moco active site, along with a haem cofactor and an FAD cofactor; NAD(P)H serves as the physiological electron donor of this NR via the FAD cofactor (figure 5a). Initially, the authors demonstrated that this NR could directly reduce nitrate ( $\text{NO}_3^-$ ) when immobilized at an electrode surface, although they note that DET was not always obtained and various promoters of DET were used (polymyxin and poly(ethylenimine)) to maintain electrical contact. As the orientation of the enzyme at the electrode surface affects its direct bioelectrocatalysis, this is the first piece of evidence to suggest that direct bioelectrocatalysis is in fact due to immobilized holo-enzyme and not from denatured cofactor. The authors also demonstrated that direct bioelectrocatalysis is only observed when mutants containing the Moco are evaluated, and further, that the haem group is necessary for direct bioelectrocatalysis, whereas the FAD cofactor is not (figure 5b). Interestingly, the authors also note that the observed redox properties observed in

the presence of FAD-containing mutants are largely due to dissociated cofactor.

Hydrogenases are another class of FeS cluster-containing enzymes that have been extensively characterized at electrode surfaces. The most common hydrogenases contain a catalytic cofactor (typically [NiFe] or [FeFe]) that is buried within the enzyme structure alongside accompanying FeS clusters that facilitate ET from the surface of the protein (figure 6a) [60–62]. These two types of hydrogenases can usually facilitate the reduction of  $2\text{H}^+$  to  $\text{H}_2$  and the oxidation of  $\text{H}_2$  to  $2\text{H}^+$ , and their electrochemistry has been explored for over 30 years [63–66].

In the case of metalloenzymes that contain metallocofactors and can degrade to form various inorganic catalytic species, it is important to demonstrate that activity is from active enzyme and not from degraded cofactors or denatured proteins. In 2006, Léger and co-workers [59] investigated mutations to the ligation of the distal [4Fe–4S] cluster of hydrogenase, which is presumably the first port-of-call for ET to or from the enzyme at an electrode surface (figure 6a). The authors interestingly note that the  $k_0$  of the enzyme is modified upon replacing the single His ligation of the distal cluster with a Gly, where a diminished direct bioelectrocatalytic signal is observed (figure 6b). Upon the addition of exogenous imidazole to the electrolyte (as a substitute for the deleted His ligating amino acid),  $k_0$  is enhanced along with the apparent direct bioelectrocatalytic currents that are observed at the electrode. Additionally, exogenous imidazole improved the oxidative activity of the Gly mutant; however, no significant increase in oxidative activity was observed for the wild-type enzyme. These findings strongly support the hypothesis that the observed apparent direct bioelectrocatalytic response is observed from holo-enzyme, and not from denature/degraded/dissociated cofactor that is adsorbed to the electrode surface. In 2008, Lojou *et al.* demonstrated that the orientation of hydrogenase ([NiFe] from *Desulfovibrio fructosovorans*) significantly impacted the direct bioelectrocatalysis response observed at an electrode surface. Direct bioelectrocatalytic  $\text{H}_2$ -oxidation and  $2\text{H}^+$ -reduction currents were observed at modified gold electrode surfaces, where a combination of thiol and/or carbon nanotube modifications was employed [64]. As discussed above for the case of MCOs, orientational studies provide strong evidence to suggest that the observed bioelectrocatalytic response is in fact due to holo-enzyme.

When considering oxidoreductases that oxidize/reduce their substrate at their FAD redox cofactor, three enzymes have been deeply studied for their direct bioelectrocatalytic properties: FAD-dependent glucose dehydrogenase (FAD-GDH), cellobiose dehydrogenase (CDH) and fructose dehydrogenase (FDH). FAD-GDH has been extensively investigated as a replacement enzyme to glucose oxidase (discussed below), because it does not employ  $\text{O}_2$  as its natural electron acceptor; thus, MET does not compete with dissolved  $\text{O}_2$  for electrons and peroxide is not produced (from the  $2\text{e}^-$  reduction of  $\text{O}_2$ ) [67]. FAD-GDH has been identified from three different sources to date: Gram-negative bacteria, fungi and insects [68]. Fungal and bacterial FAD-GDH has been studied at electrode surfaces, but only the bacterial FAD-GDH has been shown to undergo DET/direct bioelectrocatalysis [69–71]. Sode and co-workers [70] have performed extensive research into the structure of this bacterial FAD-GDH (further abbreviated to bFAD-GDH in this review),



**Figure 6.** (a) Crystal structures of NiFe hydrogenase (from *Desulfovibrio vulgaris* Miyazaki F, PDB accession code: 1WUK) and FeFe hydrogenase (from *Desulfovibrio desulfuricans*, PDB accession code: 1HFE). The NiFe or FeFe catalytic centres are shown in red, the [4Fe-4S] clusters are shown in yellow and the [3Fe-4S] cluster is shown in magenta. (b) Direct bioelectrocatalytic  $H_2$  oxidation and  $2H^+$  reduction by NiFe hydrogenase (from *D. fructosovorans*) mutants in the absence and presence of exogenous imidazole (Im).  $H_2$  oxidation was performed under a  $H_2$  atmosphere (black lines), whereas  $2H^+$  reduction experiments were performed under an atmosphere of Ar (blue lines, scan rate  $10\text{ mV s}^{-1}$ , 1000 r.p.m. rotation, pH 6,  $40^\circ\text{C}$ ). The IUPAC convention was used to plot current versus potential, where positive potentials and currents are oxidative [60]. Reprinted (adapted) with permission from [59] (copyright © 2004 American Chemical Society). (Online version in colour.)

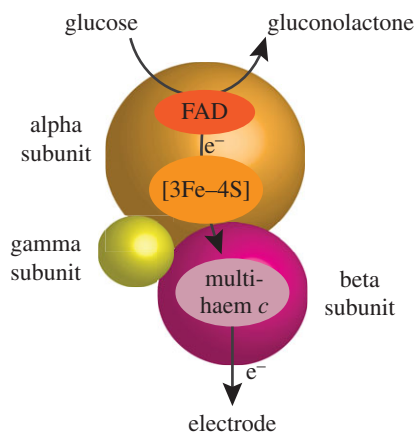
revealing the enzyme to be a heterotrimer comprised of an FAD-containing catalytic subunit, a small chaperone subunit and a multi-haem subunit (responsible for DET). Figure 7 illustrates the structure of bFAD-GDH where glucose is oxidized and electrons are shuttled to the electrode from the FAD cofactor via a [3Fe-4S] cluster and a multi-haem c unit.

Sode and co-workers [69] also performed a series of mutations to the FAD-harboring domain of bFAD-GDH (from *Burkholderia cepacia*) to alter its substrate specificity, where mutants were selected for reduced reactivity with maltose oxidation, and thus more specificity towards glucose oxidation. Upon selecting a suitable mutant by traditional dye-mediated enzymatic activity assays, the direct bioelectrocatalytic activity of bFAD-GDH mutants was evaluated for glucose selectivity, providing strong information to suggest that the observed direct bioelectrocatalytic currents were due to holo-bFAD-GDH and not from dissociated FAD cofactor. In contrast with performing mutations that affect substrate specificity, it is important to investigate the integrity of direct bioelectrocatalysis by evaluating the extent to which an immobilized enzyme can still catalyse a range of natural substrates (i.e. does enzyme immobilization eliminate reactivity towards key substrates).

CDH is an enzyme that oxidizes aldoses and is commonly employed for glucose oxidation [72]. This enzyme contains an FAD-dependent dehydrogenase subunit as well as a haem-

dependent subunit that is connected by a flexible peptide linker (figure 8a). In the literature, the ET mechanism of CDH is commonly referred to as IET because the saccharide substrate is oxidized at the FAD cofactor within the dehydrogenase subunit, from which electrons are transferred to the final electron acceptor of the enzyme via the haem subunit of the enzyme. For the purposes of this review and in the light of the wild-type CDH being comprised of the FAD dehydrogenase domain alongside the haem domain, we consider CDH to undergo direct bioelectrocatalysis. For the last 20 years or so, researchers have investigated the nature of DET between CDH and electrode surfaces, which has been concluded to be largely afforded via the haem domain (figure 8a) [73–77].

In early research of CDH at electrode surfaces, researchers used a protease to cleave the peptide linker of the two subunits (papain) allowing the purification of both components and subsequently their individual electrochemical analysis (figure 8b) [78]. Electrochemical evaluation of both of the individual subunits revealed the prominent redox peaks presented by CDH to originate from the haem subunit; following the addition of cellobiose, an oxidative response is observed that is coupled to the electrochemistry of the haem subunit, implying its role in facilitating DET from the FAD-dependent dehydrogenase domain to the electrode surface. Further, the authors reported that direct bioelectrocatalysis of cellobiose oxidation was only observed in the presence of both the



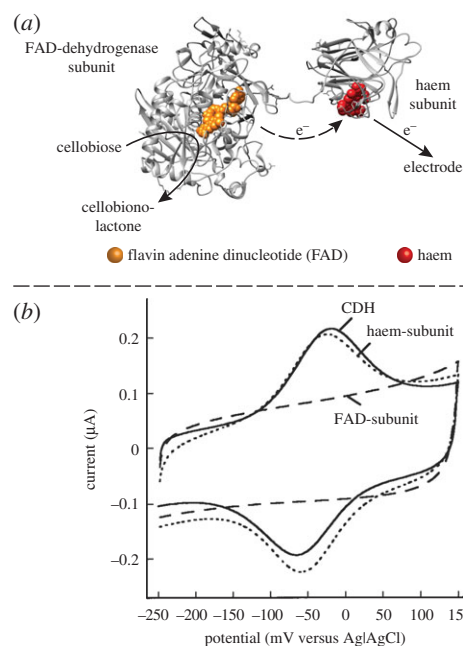
**Figure 7.** Proposed ET pathway of bacterial FAD-dependent glucose dehydrogenase (bFAD-GDH), as described in [64]. (Online version in colour.)

FAD-dependent dehydrogenase domain and the haem domain, providing further evidence that direct bioelectrocatalysis was afforded by holo-CDH and not from dissociated cofactor. Importantly, CDH is not known to produce  $\text{H}_2\text{O}_2$ , which could also be electrooxidized at the electrode surface.

A later study of CDH evaluated the effect of deglycosylation on direct bioelectrocatalysis; CDHs are typically glycosylated by about 9–16%, which is thought to limit the intimacy between CDH and an electrode surface (thereby restricting direct bioelectrocatalysis) [73]. The authors demonstrated that the deglycosylation of CDH resulted in a significant enhancement of the direct bioelectrocatalytic currents obtained for lactose oxidation, providing further evidence to support direct bioelectrocatalysis by holo-CDH and not from dissociated cofactor. The authors importantly note that direct bioelectrocatalysis was not observed for the FAD-dependent dehydrogenase subunit of CDH when separated from the haem subunit.

Researchers have studied the direct bioelectrocatalysis FAD-dependent fructose dehydrogenases from acetic acid bacteria that have a haem-containing subunit. These enzymes allow for internal relay of electrons between FAD and haem in a similar manner to CDH. The enzyme has very high specific activity and therefore has been studied for its ability to provide high current densities for sensor and fuel cell applications. The majority of the work on FAD-dependent fructose dehydrogenase has focused on high surface area electrode structures for decreasing the internal ET distance. Kano and co-workers [79] have shown that Ketjen black provides a high surface area carbon environment for facilitating DET of FAD-dependent fructose dehydrogenase. Cyclic voltammetry in the presence and absence of fructose is consistent with the potential for the haem c communicating with the electrode. Nishizawa and co-workers [80] showed that Ketjen black can be combined with different carbon fibres, cloth, etc. to produce high current density fructose bioanodes ( $\text{mA cm}^{-2}$ ). Nishizawa and co-workers [81] have developed forests of carbon nanotubes for ensuring short ET distances and high current densities. Overall, this enzyme is interesting, because it readily adsorbs to carbon while maintaining high activity and this has allowed for the fabrication of high current density fructose bioanodes.

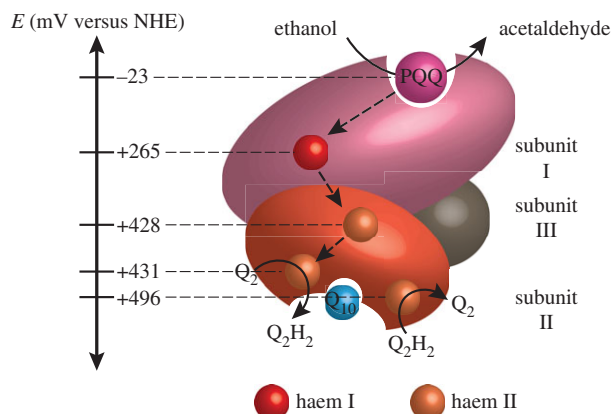
FAD-dependent dehydrogenases are not the only class of dehydrogenases that can contain haem subunits. There are three types of PQQ-dependent dehydrogenases and the



**Figure 8.** (a) Crystal structure of CDH from *N. crassa* (PDB accession code: 4QI7). The FAD cofactor is shown in orange and the haem cofactor is shown in red. Cyclic voltammetry of complete CDH (solid line), isolated FAD dehydrogenase subunit (dashed line) and isolated haem subunit (dotted line) immobilized at a gold electrode (scan rate  $50 \text{ mV s}^{-1}$ , pH 5.1 acetate buffer). The IUPAC convention was used to plot current versus potential, where positive potentials and currents are oxidative. Reprinted from [71] with permission from Elsevier. (Online version in colour.)

type II and type III are quinohaemoproteins containing both the PQQ cofactor and at least one haem. Type II are defined as soluble, periplasmic quinohaemoproteins and type III are membrane-bound quinohaemoproteins [82]. These dehydrogenases are similar to FAD-dependent dehydrogenases in that they do not include a diffusional cofactor and can use the haem-containing subunit as a relay to internally transfer electrons to/from the electrode. Calcium ions are used to bind the PQQ cofactor in the binding site [83] and alternative ions like strontium have been used to bind the PQQ cofactor more tightly and extend the stability of the enzyme on an electrode. Of the PQQ-dependent dehydrogenases, the most common enzymes for bioelectrocatalysis are PQQ-dependent glucose dehydrogenase and alcohol dehydrogenase. It is important to note that there is a commercially available PQQ-dependent glucose dehydrogenase that is a quinoprotein that does not contain a haem, but is similar to glucose oxidase in that the cofactor is too deeply buried for direct ET. Owing to the oxygen independence of PQQ-dependent glucose dehydrogenase, it has been widely studied for mediated bioelectrocatalysis for biosensor applications, but is outside of the scope of this review due to inability for facile direct ET.

PQQ-dependent alcohol (ADH) and aldehyde (ALDH) dehydrogenases from *Gluconobacter* have been studied for bioelectrocatalysis [84–86]. These membrane-bound quinohaemoproteins have been studied for alcohol biosensing and alcohol-based EFCs [86,87]. Although PQQ-ALDH is a quinohaemoprotein and it has been used for studying the effect of orientation of the direct bioelectrocatalysis with multi-subunit proteins [88], PQQ-ADH is the more common enzyme studied electrochemically. PQQ-ADH is a three-subunit



**Figure 9.** ET pathway of PQQ-dependent alcohol dehydrogenase (ADH) highlighting the reduction potentials of its various cofactors at pH 4.5, as described in [78]. (Online version in colour.)

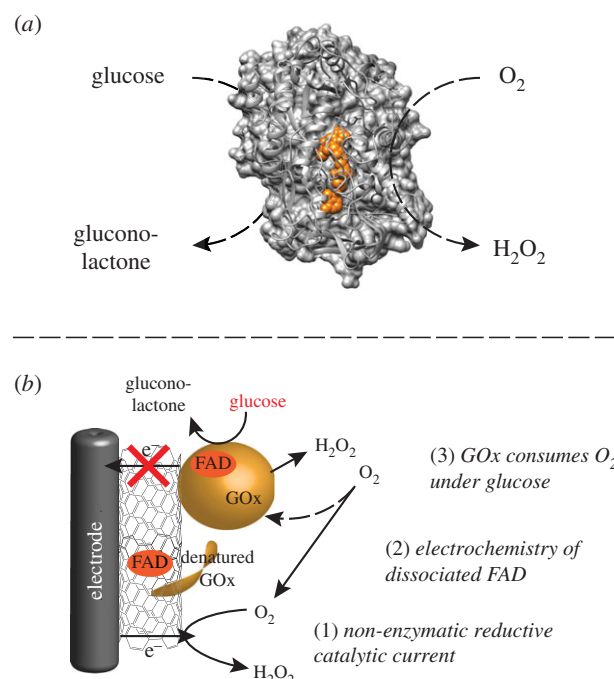
quinohaemoprotein with an active subunit that contains a PQQ cofactor and a single haem c, a structural subunit that contains no cofactors and a third subunit that contains  $3 \times$  haem c centres. There is a substantial loss in potential from the PQQ cofactor to the haems, but the haems provide an internal relay to transfer electrons to the electrode (figure 9) [89]. Many researchers have shown direct bioelectrocatalysis of this enzyme on a variety of electrodes, including: carbon nanotubes, glassy carbon, Toray carbon paper and polyaniline [12,90,91]. As PQQ-ADH has a multi-haem subunit that facilitates rapid internal electron relays, Sode and co-workers [92] addressed the buried cofactor of PQQ-GDH by genetically fusing the multi-haem domain of PQQ-ADH with the type 1 quinoprotein PQQ-GDH. This promoted direct ET and increased current densities.

There are several other quinohaemoproteins in the literature that have been studied for direct bioelectrocatalysis, including: PQQ-dependent aldose dehydrogenase [93], lactate dehydrogenase [94] and pyruvate dehydrogenase [95]. PQQ-dependent glycerol dehydrogenase is similar to PQQ-ADH and PQQ-ALDH, in that it is a promiscuous enzyme and it frequently loses substantial activity upon immobilization on electrode surfaces, but it has been shown to do mediated bioelectrocatalysis with either phenazine methosulfate or ferrocenylphenol [96], but up until now has shown no evidence of direct bioelectrocatalysis.

Many researchers have also investigated direct bioelectrocatalysis of cytochrome P450 and derivatives thereof. P450s are enzymes that catalyse a wide range of reactions, such as alkane hydroxylation, olefin epoxidation and dealkylation reactions, with their most common reactivity being termed 'monooxygenase' where only one O atom from  $O_2$  is transferred to the oxidized substrate (product) [97]. The active site contains an iron(III) protoporphyrin-IX (tetradentate), which is also covalently linked to the protein via a proximal cysteine ligand. Despite the degree of research into direct bioelectrocatalysis of P450s [98], a large number of reports do not provide adequate evidence for DET.

#### 4. Direct bioelectrocatalysis of non-metalloenzymes

The most commonly studied non-metalloenzyme for direct bioelectrocatalysis is glucose oxidase (GOx). Fungal GOx is



**Figure 10.** (a) Crystal structure and turnover of glucose oxidase (GOx) from *Aspergillus niger* (PDB accession code: 1GAL). (b) Commonly observed pseudo-DET response for GOx-modified bioelectrodes. (1) Under aerobic conditions,  $O_2$  is reduced by the supporting electrode. (2) A pair of reversible redox peaks is observed for the FAD cofactor of GOx in the absence and presence of  $O_2$ , although it is frequently due to dissociated cofactor. (3) The addition of glucose results in the consumption of  $O_2$  by some GOx that remains active, which yields a change in the electrochemical reduction of  $O_2$  to  $H_2O_2$  by the supporting electrode. (Online version in colour.)

an FAD-dependent enzyme that couples the  $2e^-$  oxidation of glucose to gluconolactone with the  $2e^-$  reduction of  $O_2$  to  $H_2O_2$  (figure 10a). Owing to the biotechnological desire to quantify physiological glucose concentrations for diabetes care, many researchers have attempted to develop GOx-based electrochemical devices due to the known selectivity of enzyme/substrate pairs. While a large number of devices have successfully employed polymeric materials that have been modified with redox mediators for MET, DET-based glucose strategies are frequently debated. Wooten *et al.* [99] recently evaluated the construction of a typical GOx DET-type bioelectrode, where chitosan was used as an immobilization matrix alongside carbon nanotubes. The authors observed the commonly reported characteristic responses observed for GOx DET systems, such as a reversible couple for the redox cofactor of GOx (FAD) and an amperometric response following the addition of glucose. Finally, the authors came to the conclusion that DET may not actually be taking place and that the observed electrochemistry of the FAD cofactors is most likely due to dissociated cofactor.

The most frequent pseudo-bioelectrocatalytic response observed and used as rationale to support GOx DET is: 'An enzymatic response to glucose is only observed upon the addition of glucose when  $O_2$  is present' (figure 10b). Typically, researchers observe a reductive current around the potential region of the FAD cofactor of GOx, which shifts to 'less-reductive' currents upon the addition of glucose to the electrolyte, resulting in a positive shift in current magnitude. Further, researchers prepare control bioelectrodes with denatured GOx or another protein/enzyme that is inert to

glucose and this 'less-reductive' response is not observed. In this case, it is becoming more commonly agreed upon that the FAD redox response is in fact due to FAD cofactor that has dissociated from GOx and adsorbed to the electrode surface, while a portion of active GOx still remains adsorbed to the electrode surface. The reductive current arises from O<sub>2</sub> reduction by the supporting electrode (typically carbon, commonly modified with carbon nanotubes) and the addition of glucose results in O<sub>2</sub> consumption by GOx and thus less O<sub>2</sub> reduction by the supporting electrode.

It is important to note that the use of the terms 'anaerobic' and 'anoxic' to describe solutions in which dissolved O<sub>2</sub> has been displaced by purging with an inert gas is often an overstatement; trace amounts of O<sub>2</sub> can give rise to pseudo-glucose responses and the actual concentration of dissolved O<sub>2</sub> should be reported, or, work should be conducted in an anoxic chamber and the concentration of atmospheric O<sub>2</sub> provided. Additionally, GOx does not require O<sub>2</sub> to turn-over glucose if a suitable electron mediator is employed (ferrocene, Os complexes, etc.) [100–102].

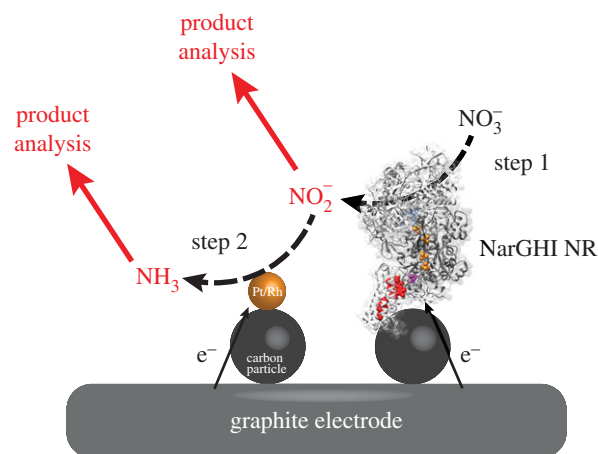
## 5. Control experiments to prove direct bioelectrocatalysis

The above review of direct bioelectrocatalysis of several metalloenzymes presents multiple approaches to confirm direct bioelectrocatalysis by a series of properly designed control experiments. This section is dedicated to summarizing and discussing experiments that can be used to differentiate direct bioelectrocatalysis from responses that arise from dissociated cofactor. It is possible, however, that a small conformational change in the enzyme takes place once the enzyme is immobilized on an electrode surface, but it is presumed that the activity of the enzyme will likely be lower due to resulting changes in the active site. Finally, a single control experiment does not always confirm direct bioelectrocatalysis and a multitude of controls should be used to confirm this ET pathway.

### 5.1. Product analysis

Perhaps the first port-of-call is to determine the production of the expected product of the enzymatic reaction. This does not eliminate the possibility that the reaction takes place by dissociated cofactor, although it does confirm that the reaction of interest is actually taking place. One recent example of suitable product analysis was demonstrated by Duca *et al.* [57], where an NR was co-immobilized with a noble metal catalyst to afford the stepwise reduction of NO<sub>3</sub><sup>−</sup> to ammonia (NH<sub>3</sub>) through its intermediate reduction to nitrite (NO<sub>2</sub><sup>−</sup>). Initially, NO<sub>3</sub><sup>−</sup> reduction to NO<sub>2</sub><sup>−</sup> was confirmed by using the 'Griess assay' to detect NO<sub>2</sub><sup>−</sup>, which was also correlated to the theoretical quantity of NO<sub>2</sub><sup>−</sup> that should be produced by its bioelectrochemical reduction (using Coulomb's law and by calculating the charge passed during the experiment, figure 11). The authors subsequently employed noble metal catalysis to reduce the NO<sub>2</sub><sup>−</sup> produced from the first step to NH<sub>3</sub>, where a different fluorescence reagent (*o*-phthalaldehyde) was used to quantify NH<sub>3</sub>.

Product analysis is especially important when using an enzyme that can perform the reduction in several substrates; nitrogenase, primarily an N<sub>2</sub>-reducing enzyme, can also



**Figure 11.** Duca *et al.* demonstrated the co-immobilization of NarGHI NR and Pt/Rh nanoparticles for the reduction of NO<sub>3</sub><sup>−</sup> to NH<sub>3</sub> on a hybrid electrode, as reported in [50]. The 'Griess' assay was used to detect and quantify NO<sub>2</sub><sup>−</sup> production, whereas *o*-phthalaldehyde was used to detect and quantify NH<sub>3</sub>. (Online version in colour.)

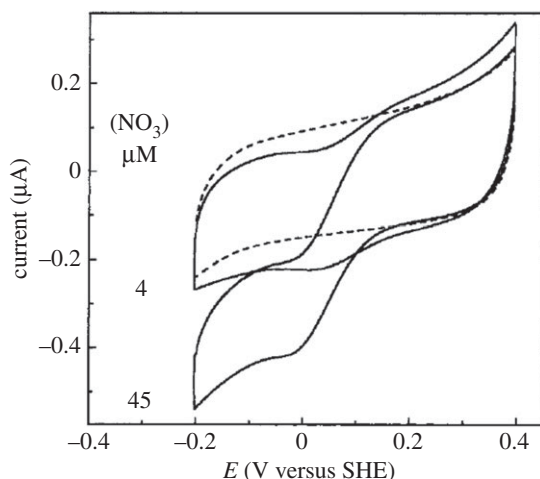
reduce H<sup>+</sup>, carbon dioxide (CO<sub>2</sub>), NO<sub>2</sub><sup>−</sup> and azide (N<sub>3</sub><sup>−</sup>) in addition to other substrates [103]. We recently reported on the immobilized and non-immobilized electrochemistry of nitrogenase where product analysis was performed to confirm the successful production of NH<sub>3</sub> [26,104]. In the absence of other substrates, nitrogenase will revert to 100% H<sup>+</sup> reduction; thus, a catalytic current will always be present when undergoing ET.

### 5.2. Enzyme denaturation and non-catalytic proteins

Often, the tertiary and quaternary structures of oxidoreductases are important to their activity and these enzymes are frequently denatured at elevated temperatures (although thermostable enzymes are being increasingly investigated for improved stabilities). Enzymes can be briefly heated at elevated temperature (80–100°C for 10–30 min) to render them inactive, where their loss of activity can be evaluated following heat treatment by conventional activity assays. Additionally, proteins that are inert to the reaction of interest (such as bovine serum albumin) can be used; this is useful if the tertiary/quaternary structure of the protein is expected to be beneficial (such as in the case of immobilized films). In the event that the enzyme loses activity, it can then be used to verify direct bioelectrocatalysis, or indeed, that cofactor can dissociate from the enzyme and undergo electrocatalysis. We recently demonstrated an MET-type bioelectrode that simultaneously employed two oxidoreductases, where heat-denatured proteins were prepared to confirm the bioelectrochemical activity of each counterpart [105]. An alternative to heat denaturation would be to digest the oxidoreductase with a promiscuous protease (such as trypsin).

### 5.3. Inhibition of enzymatic activity

As highlighted above, direct bioelectrocatalysis by holoenzyme at an electrode surface can also be indirectly determined by initiating turnover of the substrate at the electrode followed by the addition of an inhibitor to enzymatic activity. An example of this was demonstrated by the Butt group in 2001, where NarGH NR (free of its NarI component) was studied at an electrode surface and NO<sub>3</sub><sup>−</sup>



**Figure 12.** Direct bioelectrocatalysis of NarGH NR on a gold electrode. Electrodes were initially analysed in 4  $\mu\text{M}$  of substrate ( $\text{NO}_3^-$ ), which was increased to 45  $\mu\text{M}$  prior to the injection of 100  $\mu\text{M}$  inhibitor ( $\text{N}_3^-$ , dashed line). Scan rate 10  $\text{mV s}^{-1}$ , 3000 r.p.m., pH 6 and 25°C [49]. Reprinted (adapted) with permission from [56] (copyright © 2004 American Chemical Society).

reduction was inhibited by the addition of  $\text{N}_3^-$  (figure 12). As discussed above for laccase and shown in figure 3, Vaz-Dominguez *et al.* demonstrated the inhibition their laccase direct bioelectrocatalytic electrodes by the addition of two different inhibitors ( $\text{Cl}^-$  and  $\text{F}^-$ ). In the case of dissociated metallocofactor, inhibitors such as cyanide ( $\text{CN}^-$ ) should be used with caution as  $\text{CN}^-$  could react with the dissociated metallocofactor and yield a pseudo-inhibitory response.

#### 5.4. Mutations and modifications

Finally, perhaps the most elegant and subtle approach used to confirm possible direct bioelectrocatalysis involves the generation of mutated or modified oxidoreductases that have altered catalytic properties or properties that effect their orientation or immobilization on an electrode surface, of which a few examples were provided above. Such mutations and modifications can include single-point mutations, the cleavage of component subunits or even the deglycosylation of enzymes. If the mutation is expected to alter the catalytic

properties of the enzyme, then evaluation of the apparent steady-state kinetics (i.e. Michaelis–Menten kinetics) may result in a significant change in these properties (i.e. maximum velocity,  $V_{\text{MAX}}$ , or the Michaelis constant,  $K_{\text{M}}$ ).

Mutations can also be made to alter the mechanism of ET, or internal ET, within a protein. As outlined above, an example of this was demonstrated in 2006 by Léger and co-workers [59], who altered the ligation of the distal  $[\text{4Fe–4S}]$  cluster of a hydrogenase. As a result, the interfacial ET rate ( $k_0$ ) of the enzyme at the electrode surface changed and direct bioelectrocatalysis was altered. The introduction of exogenous imidazole (presumably to replace the His residue that was replaced with a Gly residue) resulted in improved direct bioelectrocatalysis (figure 6b). In many cases, however, the generation of mutant enzymes is neither straightforward nor trivial.

Finally, the potential applied to the electrode may indicate whether direct bioelectrocatalysis of active enzyme is taking place, provided that the reduction potential of the enzyme's cofactor has been predetermined and that its reduction potential is not mildly altered upon any small conformational changes that may occur by immobilizing the enzyme.

## 6. Conclusion

This review article describes a number of metalloenzymes that have been shown to undergo internal ET to promote direct bioelectrocatalysis and the properties necessary for facile direct bioelectrocatalysis. However, the review also describes the challenges in the literature with verifying direct bioelectrocatalysis and the need for a variety of control experiments. Although mutations and modifications of the enzymes can be challenging for commercial enzyme systems, denatured controls, inhibition studies and product analysis are the required minimum experiments for evaluating direct bioelectrocatalysis.

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**Competing interests.** We declare we have no competing interests.

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