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MINI-REVIEW

Immobilized redox mediators for electrochemical NAD(P)$^+$ regeneration

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Abstract The applicability of dissolved redox mediators for NAD(P)$^+$ regeneration has been demonstrated several times. Nevertheless, the use of mediators in solutions for sensor applications is not a very convenient strategy since the analysis is not reagentless and long stabilization times occur. The most important drawbacks of dissolved mediators in biocatalytic applications are interferences during product purification, limited reusability of the mediators, and their cost-intensive elimination from wastewater. Therefore, the use of immobilized mediators has both economic and ecological advantages. This work critically reviews the current state-of-art of immobilized redox mediators for electrochemical NAD(P)$^+$ regeneration. Various surface modification techniques, such as adsorption polymerization and covalent linkage, as well as the corresponding NAD(P)$^+$ regeneration rates and the operational stability of the immobilized mediator films, will be discussed. By comparison with other existing regeneration systems, the technical potential and future perspectives of biocatalytic redox reactions based on electrochemically fed immobilized mediators will be assessed.

Keywords Cofactor · Immobilized mediator · NADH oxidation · Electropolymerization

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Introduction

Nicotinamide-dependent enzymes, in particular alcohol dehydrogenases (ADH), are of increasing interest for the synthesis of fine and bulk chemicals as well as for sensor applications. In vivo, reduced and oxidized cofactors are supplied from cellular metabolism. In vitro, regeneration of nicotinamide cofactors is a crucial step for an economic use of the enzymes (Chenault and Whitesides 1987; Hollmann and Schmid 2004; van der Donk and Zhao 2003; Wichmann and Vasic-Racki 2005; Zhao and van der Donk 2003). From an economic aspect, the cofactors cannot be used in stoichiometric amounts in the process (Chenault and Whitesides 1987; Hollmann et al. 2006). Although the costs for the oxidized cofactors NAD$^+$ and NADP$^+$ are more than fivefold lower than the cost for their reduced form, NADH and NADPH, they are still too expensive to be used in stoichiometric amounts. Furthermore, ADH-catalyzed oxidations often suffer from severe product inhibition and also by the reduced nicotinamide cofactor (NAD(P)H). Thus, for technical enzymatic reactions such as the lactonization of 1,4- and 1,5-diols (Gargiulo et al. 2011), the oxidation of cyclohexanol (Hilt et al. 1997), the oxidation of primary and secondary alcohols (Kroutil et al. 2004a; Lavandera et al. 2008), the resolution of 1-phenyl-1,2-ethanediol (Liese et al. 1996), and methionine oxidation (Findrik et al. 2008), the use of an efficient NAD(P)$^+$ regeneration is required. For sensor applications, the oxidation of NAD(P)H can be used to detect and quantify substrates of different dehydrogenases (e.g., glucose, ethanol, lactate, and glycerol) via the electrochemical current generated by anodic NAD(P)H oxidation. For both application areas, an efficient electrochemical method can help to overcome economic hurdles for the technical use of nicotinamide-dependent enzymes. Mechanistically, the oxidation of NADH can be described by a sequence of two single electron transfers (SET) and one proton transfer (electrochemical–chemical–

electrochemical (ECE) mechanism; Fig. 1) (Blankespoor and Miller 1984; Radoi and Compagnone 2009).

The easiest way to regenerate $NAD(P)^+$ would be the direct oxidation of the cofactor at a bare electrode. Although the reversible potential of NADH oxidation is −0.32 V vs. a normal hydrogen electrode (NHE) (Chenault and Whitesides 1987), both the anodic oxidation of NAD(P)H and the cathodic reduction of $NAD(P)^+$ are chemically irreversible and need high overpotentials (Moiroux and Elving 1980). These overpotentials lead to the oxidation of other electroactive species in the solution, which generates a current that interferes with the analysis (Prieto-Simón and Fàbregas 2004). In biocatalytic applications, formation of unwanted by-products or the oxidation of media components can occur. Furthermore, NAD^+ can adsorb on the electrode surface and hinder the direct electron transfer. Thus, systems based on the direct oxidation of NADH lack stability and sensitivity. A convenient way to decrease the high overpotentials and avoid surface fouling is to use redox mediators. Due to the mainly chemical oxidation of NADH, they lower the working potential to the formal potential of the mediator. The following scheme shows the mechanism of the mediated electrochemical regeneration of the oxidized nicotinamide adenine dinucleotide. The first step is the oxidation of the mediator at a suitable potential (Eq. 1). k_s is the electron transfer rate between the mediator and the electrode. The reduced NAD(P)H will be oxidized by the oxidized mediator (Eq. 2, k_{obs} is a second-order rate constant). The reduced mediator can then be re-oxidized at the electrode. At the cathode, the reduction of oxygen occurs (Eq. 3). The oxidized $NAD(P)^+$ can be used in an enzymatic reaction (Eq. 4). The overall reaction is shown in Eq. 5. NAD(P)H oxidation using electron transfer mediators follows a Michaelis–Menten mechanism (Lobo et al. 1997).

$$\mathrm{Med_{red}} \xrightarrow{k_s} \mathrm{Med_{ox}} + 2\,e^- + 2\,H^+ \quad (1)$$

$$\mathrm{NAD(P)H} + \mathrm{Med_{ox}} \xrightarrow{k_{obs}} \mathrm{NAD(P)^+} + \mathrm{Med_{red}} \quad (2)$$

$$0.5\,O_2 + 2\,e^- + 2\,H^+ \longrightarrow H_2O \quad (3)$$

$$\mathrm{NAD(P)^+} + \mathrm{substrate} \xrightarrow{\mathrm{enzyme}} \mathrm{NAD(P)H} + \mathrm{product} \quad (4)$$

$$\mathrm{substrate} + 0.5\,O_2 \longrightarrow \mathrm{product} + H_2O \quad (5)$$

Mediated oxidation of NADH can be accomplished with a large number of compounds such as metal complexes (Hilt et al. 1997; Hilt and Steckhan 1993), quinones (Ciszewski and Milczarek 2000; Ghanem et al. 2009; Pariente et al. 1996; Prieto-Simón et al. 2007), fluorenones (Mano and Kuhn 1999b; Mano et al. 2001; Munteanu et al. 2002), phenazines (Arechederra et al. 2011; Curulli et al. 1997; Lu et al. 2010), phenoxazine (Lobo et al. 1996), and phenothiazines (Lobo et al. 1996). Furthermore, conducting polymers (Bartlett et al. 1997; Toh et al. 2003) and self-assembled monolayers (SAM; Barzegar et al. 2009; Ju et al. 2002) can be used as catalysts. Generally, two-electron proton acceptor type mediators exhibit higher reaction rates than single electron mediators for NAD(P)H oxidation and yield enzymatically active $NAD(P)^+$ as the reaction product (Santos et al. 2002a).

Most of the redox mediators are also dyes, which are widely used in the textile, rubber, paper, plastic, and cosmetic industry. Some of the chemical substances can be used as both dyes or redox mediators, e.g., methylene green, methylene blue, or Meldola's Blue. Besides the potential hazardous effects, very low concentrations of these pollutants negatively affect the aesthetic value of water streams, also reducing the amount of light entering the water with an adverse effect on photosynthesizing organisms (Novotný et al. 2011). Conventional wastewater treatment has low removal efficiency for reactive and other anionic soluble dyes (Saiful Azhar et al. 2005). Furthermore, some of the substances, as well as their precursors and degradation products, are carcinogenic and/or mutagenic in nature (Manu and Chaudhari 2002). Therefore, retention of the mediators in the reaction system is strongly desired. The retention can be achieved by mediator immobilization at the conducting surface. This review is focused on the applicability of immobilized mediators for the oxidation of reduced NAD(P)H. For more information about the electrochemical basics and mechanistic investigations, please read the following reviews: Gorton and Domínguez (2002), Katakis and Domínguez (1997), Kumar and Chen (2008), and Radoi and Compagnone (2009).

Techniques for immobilizing redox mediators and their application

The first publication of a mediator-modified electrode for NADH oxidization describes the immobilization of two

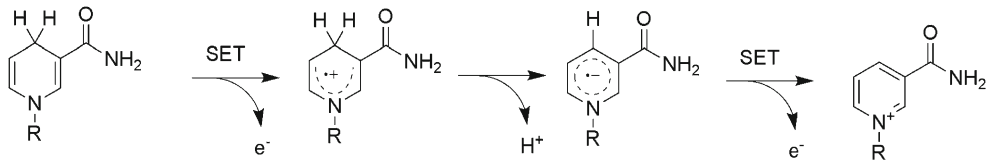
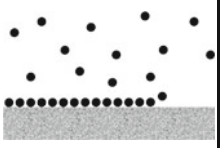
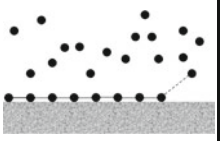
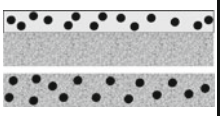
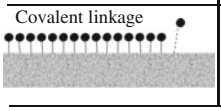


Fig. 1 The ECE redox mechanism of NAD(P)H oxidation (Blankespoor and Miller 1984; Radoi and Compagnone 2009)

primary amines, dopamine and 3,4-dihydroxybenzylamine, forming a monolayer on the surface of activated glassy carbon (GC) electrodes (Tse and Kuwana 1978). In the meantime, numerous ways for immobilization have been explored in order to provide more stable and environmentally friendly approaches (Vasilescu et al. 2003). Mediator immobilization has been performed by adsorption, covalent attachment, polymer modification, or inclusion of the mediator in the electrode material. While adsorption and inclusion in the electrode material are the simplest procedures, more elaborate approaches have been developed in order to obtain mediator layers with enhanced operational stability. To this end, mediators have been electrochemically polymerized at the electrode surface, covalently attached, or physically entrapped in polymers that were enclosed in the electrode material. Another technique is the covalent linkage of the mediator, for example, to a monolayer deposited onto the electrode (Schlereth et al. 1994). Table 1 shows examples of mediators immobilized by different techniques.

Table 1 Examples for mediator immobilization by distinct techniques

Technique	Mediator
Adsorption 	Nile blue (Malinauskas et al. 2000; Ni et al. 1990; Persson and Gorton 1990; Qi-Jin and Shao-Jun 1996), toluidine blue (Malinauskas et al. 2000; Persson and Gorton 1990; Qi-Jin and Shao-Jun 1996), Meldola's Blue (Persson and Gorton 1990; Prieto-Simón and Fàbregas 2004; Qi-Jin and Shao-Jun 1996), dichlorphenolindophenol (Prieto-Simón and Fàbregas 2004), *o*-phenylenediamine (Prieto-Simón and Fàbregas 2004), 3,4-dihydroxybenzaldehyde (Prieto-Simón and Fàbregas 2004), methylene blue (Qi-Jin and Shao-Jun 1996), brilliant cresyl blue (Persson and Gorton 1990; Qi-Jin and Shao-Jun 1996), methylene green (Qi-Jin and Shao-Jun 1996), neutral red (Persson and Gorton 1990), 2,4,7-trinitro-9-fluorenone (Munteanu et al. 2002)
Polymerization 	Toluidine blue (Cai and Xue 1998; Zhou et al. 1998), methylene green (Zhou et al. 1996), bis-phenothiazin-3-yl methane (Gligor et al. 2009b), catechol (Bai et al. 2010), neutral red (Lu et al. 2010), phenosafranine (Saleh et al. 2011b), azure I (Cai and Xue 1997a), Nile blue (Cai and Xue 1997b), Meldola's Blue (Bala et al. 2004), chlorogenic acid (Zare and Golabi 1999)
Entrapping/incorporation 	Meldola's Blue (Doaga et al. 2009; Gründig et al. 1995; Prieto-Simón and Fàbregas 2004), dichlorophenolindophenol (Prieto-Simón and Fàbregas 2004), *p*-benzoquinone (Prieto-Simón and Fàbregas 2004), *o*-phenylenediamine (Prieto-Simón and Fàbregas 2004), 3,4-dihydroxybenzaldehyde (Prieto-Simón and Fàbregas 2004), toluidine blue (Ramirez Molina et al. 1999)
Covalent linkage 	Toluidine blue (Ju et al. 2002; Schlereth et al. 1994), Nile blue (Saleh et al. 2011a)

Adsorption

The adsorption of electrocatalytically active species onto electrodes presents a simple and useful procedure, leading to modified electrodes suitable for many electrocatalytical applications in aqueous solutions (Malinauskas et al. 2000). In general, adsorption of mediators can be performed by immersing the electrode into a mediator solution. Mediators have been immobilized directly on the electrode (Catalin Popescu et al. 1999; Gligor et al. 2009a; Jaegfeldt et al. 1981; Persson and Gorton 1990) or on different supporting materials prior to mixing with the electrode material. As support materials, titanium phosphate (Kubota et al. 2000), zirconium phosphates (Ladiu et al. 2007; Santiago et al. 2006), modified silica gel (de Lucca et al. 2002; Santos et al. 2002b, 2003), and carbon nanotubes (Antiochia et al. 2005; Huang et al. 2007) have been used. Direct adsorption of the mediator cannot be used in technical applications due to a limited stability of the immobilized layers. The limited stability of the layer can be improved by introducing functional groups into the molecule, allowing chemisorption onto the electrode surface (Mano and Kuhn 1999b). Furthermore, the lifetime and performance of the catalyst can be improved by using bivalent cations with substituted nitro-fluorenones (Mano and Kuhn 1999a). The introduction of a carboxy group into the mediator molecule leads to an additional strengthening of the interaction between NADH and the catalyst. The photoelectrocatalytic oxidation of NADH at a graphite electrode adsorbed with polyphenothiazine formaldehyde has also been shown (Gligor et al. 2009a). When the surface of the modified electrode was irradiated with a halogen lamp, a significant increase in the electrocatalytic current was observed. Different mediators have been adsorbed on silica modified with niobium oxide (de Lucca et al. 2002; Santos et al. 2002b, 2003). The electrochemical behavior of methylene green adsorbed on a silica surface modified with niobium

oxide has been investigated, using modified carbon paste electrodes (de Lucca et al. 2002). The electrode showed high stability, which was attributed to the presence of niobium oxide that prevents leaching of the mediator from the electrode surface. Varying the pH between 4 and 8 did not affect the stability or the formal potential of the mediator system. Furthermore, the electrocatalytic oxidation rate of NADH was nearly constant between pH 5 and 8. When immobilizing Meldola's Blue on silica gel coated with niobium oxide, the formal potential was again independent of the pH in the same range (Santos et al. 2002b). The second-order rate constants increased with the solution pH. At pH 5 and 8, the heterogeneous electron transfer was 1,316 and 2,167 M^{-1} s^{-1}, respectively. This indicates that the charge transfer becomes easier at higher pH, providing evidence that the thermodynamic driving force is predominant in the pH change in relation to the charge transfer complex formation. Based on this system, an ethanol sensor was constructed (Santos et al. 2003). The operational stability of the sensor system was high. After 300 measurements, the residual activity was almost 95%. Layered α-zirconium phosphate ($Zr(HPO_4)2{\cdot}H_2O$) was demonstrated to be a promising material for the immobilization of mediators (Santiago et al. 2006). α-Zirconium phosphate is known to be a good ion exchanger because it is a strong acid material due to its phosphate groups and presents ion-conducting properties (Munteanu et al. 2001). These characteristics make it very attractive and convenient to employ as a matrix for immobilization of mediators in order to facilitate electron transfer between the enzyme cofactor and the electrode. Several authors have demonstrated the use of α-zirconium phosphate for the construction of NADH biosensors. Mediators that have been immobilized in α-zirconium phosphate are Nile blue (Malinauskas et al. 2000), toluidine blue (Malinauskas et al. 2000), Meldola's Blue (Ladiu et al. 2007; Munteanu et al. 2001), methylene green (Munteanu et al. 2001), 2,4,7-trinitro-9-fluorenone (Munteanu et al. 2002), and 2,6-dichlorophenolindophenol (Dicu et al. 2003). Cyclic voltammetric investigations of Meldola's Blue, methylene green, and riboflavin immobilized on α-zirconium phosphate performed in buffered solutions at different pHs have also been performed (Munteanu et al. 2001). The midpoint potentials for the mediators were virtually independent of the pH. Munteanu et al. measured the pH dependency of 2,4,7-trinitor-9-fluorenone adsorbed on α-zirconium phosphate in three different buffer systems and found the formal potential of the mediator nearly constant in the tested pH range from 1 to 10 (Munteanu et al. 2002). For sensor and biocatalytic applications, a pH-independent formal potential of the mediator is required. For sensor applications, a measurement should be independent of the pH in the solution and sample preparation should be avoided. Furthermore, for a catalytic system, the pH independency is helpful due to different pH optima of the enzymes and limited buffer capacity of the solution. The addition of Ca^{2+} to a buffered solution of 2,6-dichlorophenolindophenol adsorbed on α-zirconium phosphate in the presence of NADH was shown to increase the oxidation current (Dicu et al. 2003). The kinetic parameters obtained for Meldola's Blue adsorbed via zirconium phosphate on a GC paste electrode were adversely influenced by increased surface coverage (Γ) (Ladiu et al. 2007). The decrease of the second-order constant by increasing the Γ values was explained by hindered access of NADH to the immobilized mediator. The increase in the Michaelis–Menten constant (K_m) with the increase in Γ reflects a decreased affinity of Meldola's Blue to NADH. The presence of polyethylenimine (PEI) improved this behavior. After the addition of PEI to the GC paste, an increased surface coverage could be obtained, resulting in improved kinetic parameters. Imines derived from 3,4-dihydroxybenzaldehyde and 4-aminopyridine have been adsorbed onto platinum electrodes through the pyridine nitrogen and used for electrocatalytic oxidation of NADH (Lorenzo et al. 1995).

Electropolymerization of mediators

Mediators can be polymerized on an electrode via cyclic voltammetry (Bai et al. 2010; Cai and Xue 1998; Li et al. 2011; Saleh et al. 2011b) or chronoamperometry (Murthy et al. 1994). Figure 2 shows the mechanism proposed for the polymerization process. Oxidation of the monomer results in a radical structure. The addition of further monomers leads to polymerized mediators.

At electropolymerized electrodes, the potential for NADH oxidation can be reduced by more than 700 mV compared to unmodified electrodes. A stable electroactive

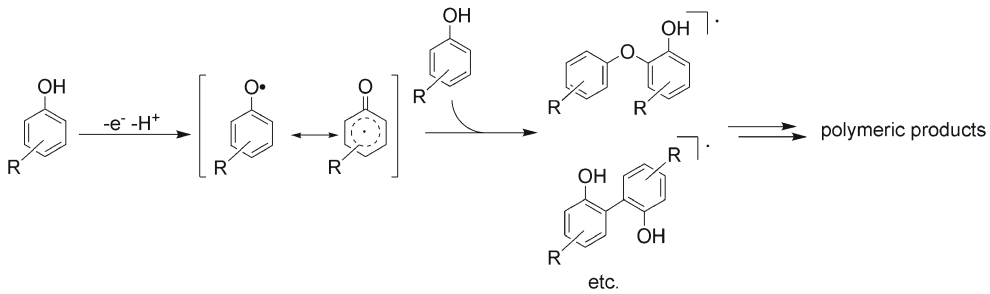


Fig. 2 Proposed mechanism for polymerization process via electrochemistry

film of poly(Nile blue A) has been deposited on the surface of a GC electrode by cyclic voltammetry in an aqueous solution (Cai and Xue 1997b). The modified electrode showed electrocatalytic activity toward NADH oxidation, with an overpotential 660 mV lower than that of the bare electrode. The electrochemical regeneration of NADH/NAD^+ has been studied using poly(phenosafranine) (Saleh et al. 2011b). The modified electrodes reduced the overpotential by 740 mV compared with those at the bare GC electrode. Electropolymerization of poly(methylene green) has been compared with adsorption of the same mediator on electrodes (Zhou et al. 1996). The polymer-modified electrode exhibited a higher stability and better catalytic activity towards NADH oxidation (Zhou et al. 1996). The stability of pyrocatechol violet-modified GC electrodes has been investigated (Golabi et al. 2002). The electrode coverage was followed over time during cyclic voltammetry runs and under storage conditions. Under both conditions, the surface coverage decreases. The surface coverage decreased by approximately 28% after 300 cyclic voltammetric cycles between −0.15 and 0.55 V vs. Ag/AgCl at 100 mV s^{-1}. Under storage conditions, a fast decrease in the surface coverage was observed in the first 10 h, followed by a slower decrease. Higher stability was found for an electropolymerized film of azure I (Cai and Xue 1997a). The stability of the poly(azure I)-modified GC electrode was examined by cyclic voltammetry. Initially, peak currents decreased with continuous scanning. Thereafter, no further changes in height and separation of the cyclic voltammetric peaks were observed during 6 h of repetitive scans (more than 500 cycles). Furthermore, there was no loss of redox activity after storing the modified electrode in a buffer solution for 1 week. The decrease of the surface coverage of a poly(bis-phenothiazin-3-yl methane)-modified electrode has been estimated to be as low as 2×10^{-16} mol cm^{-2} s^{-1} (Gligor et al. 2009b). Saleh et al. have compared two different electrode materials modified with poly(phenosafranine) for the oxidation of NADH (Saleh et al. 2011b). It was shown that a modified basal-plane pyrolytic graphite electrode was a better electrocatalyst compared to a modified GC electrode. This was explained by defects such as steps and edge sites, on the basal-plane pyrolytic graphite electrode surface facilitating an oriented adsorption of poly(phenosafranine) onto the electrode surface. This orientated adsorption improved electron transfer rates between the electrode and the mediator layer as well as between the mediator layer and NADH. Carbon paste electrodes coated with poly(*o*-phenylenediamine) and poly(*o*-aminophenol) conducting films have been prepared by potential scan electropolymerization in aqueous solutions (Lobo et al. 1996). Both polymer films exhibit an effect on the electrocatalytic NAD(P)H oxidation. The poly(*o*-phenylenediamine) coating of the electrode reduced the overpotential by more than 400 mV compared to the bare electrode. The rate of NADH and NADPH oxidation at the poly(*o*-phenylenediamine)-coated electrode at pH 7 was 7.3×10^6 and 4.9×10^6 nA M^{-1}, respectively. At pH 5, the current slopes were 4.6×10^7 nA M^{-1} for the oxidation of NADH and 5.1×10^7 nA M^{-1} for the oxidation of NADPH. These similar values show that poly(*o*-phenylenediamine)-modified electrodes can be used for the oxidation of both nicotinamide adenine dinucleotides. The photoelectrocatalytic oxidation of NADH has been investigated at a GC electrode modified with poly (neutral red) and poly(toluidine blue) (Dilgin et al. 2007, 2011). The modified electrodes showed electrocatalytic as well as photo-electrocatalytic activity towards NADH. When irradiated, the electrode current increased.

Dehydrogenases entrapped in a Nafion® film were deposited on top of a poly(methylene blue)-modified electrode to construct biosensors for glucose, lactate, malate, and ethanol (Karyakin et al. 1994). It was shown that the polymerized mediator can be used to oxidize NADH in a comparable way to soluble methylene blue. The long-term stability of this system was investigated in an automatic flow setup allowing to change between NADH solution and buffer. The stationary plateau current was taken to evaluate the course of the electrocatalytic NADH oxidation property of the electrode system. The authors demonstrated that the electrode can be used for more than 7 days and 500 NADH detections. For the first 2 days, the current is more or less constant. Afterwards, the current decreases with 7 nA day^{-1} (beginning: about 50 nA). A similar approach was used to build an ethanol sensor on the basis of electropolymerized Meldola's Blue on screen printed graphite electrodes (Bala et al. 2004). An ADH was immobilized through cross-linking, by depositing a mixture of the enzyme, bovine serum albumin (BSA) and glutaraldehyde on the surface of electrodes modified with poly(Meldola's Blue). It was shown that this biosensor had good operational stability and sensitivity.

A further method to produce an enzyme electrode is the direct immobilization of the enzyme during the electropolymerization step. This was demonstrated by performing cyclic potential sweep in a buffered solution of toluidine blue and glucose dehydrogenase (Zhou et al. 1998). Karyakin et al. demonstrated that electropolymerized methylene blue had excellent ability and high operation stability for the electrocatalytic oxidation of NADH (Karyakin et al. 1994). Their deposition of a Nafion® membrane with entrapped dehydrogenases onto the poly (methylene blue) layer of the electrode constituted a new amperiometric biosensor for different substrates.

Entrapping/incorporation

Mediators can be immobilized in polymer films or Nafion® as well as in the electrode material itself (Doaga et al. 2009;

Gründig et al. 1995; Huang et al. 2007; Ramirez Molina et al. 1999). The redox mediator Meldola's Blue has been entrapped into the non-conductive poly-1,2-diaminobenzene and the conductive poly-3,4-ethylenedioxythiophene (Doaga et al. 2009). Both polymer films were positively tested for electrocatalytic oxidation of NADH using chronoamperometry at a working potential of 0.1 V vs. Ag/AgCl. The two-electron redox mediator thionine has been incorporated into a carbon nanotube/Nafion® composite film (Huang et al. 2007). The incorporation was promoted by an ion-exchange process between thionine and Nafion® as well as adsorption of thionine on the carbon nanotubes. The films exhibited high and stable electrocatalytic activity towards NADH oxidation with a sensitivity of 4.7 mA M^{-1} and a significant decrease in the required overpotential (working potential −0.297 V vs. NHE). When the mediators Meldola's Blue, dichlorophenolindophenol, *p*-benzoquinone, *o*-phenylenediamine, and 3,4-dihydroxybenzaldehyd were entrapped into an epoxy–graphite electrode, mediator leakage was determined (Prieto-Simón and Fàbregas 2004). The polymer matrix was not able to retain the small and water-soluble mediator inside the matrix. Due to the mediator leakage into the measurement solution, the electrochemical signal was unstable. Gründig et al. immobilized different mediators (e.g., *N*-methyl-phenazinium, 1-methoxy-*N*-methyl-phenazinium, and Medola's Blue) as Reinecke salts in graphite–epoxy composites (Gründig et al. 1995). A sensor based on this composite electrode retained about 50% of the initial sensitivity after 9,500 measurements of NADH in a buffered system. Toluidine blue and glutaraldehyde have been added to well-mixed BSA and graphite powder resulting in a layer with a high sensitivity towards NADH and a high surface coverage of the mediator (Ramirez Molina et al. 1999). The mediators *p*-methylaminophenolsulfate and 3,4-dihydroxybenzaldehyde were mixed with a carbon nanotube paste (Antiochia et al. 2005). *p*-Methylaminophenolsulfate-modified electrode did not have a significant electrocatalytic activity for electro-oxidation of NADH. In contrast, the 3,4-dihydroxybenzaldehyde-modified electrode shows electrocatalytic activity towards NADH. Nevertheless, the oxidation rate was quite low (2.0×10^{3} mol^{-1} L s^{-1}) compared to other systems.

Covalent linkage

Covalent linkage to the electrode surface is a further method to immobilize mediators on an electrode. A toluidine blue O (TBO)-terminated SAM was immobilized on a gold electrode (Ju et al. 2002). In a first step, monolayers of cystamine or 3-mercaptopropionic acid were chemisorbed onto the gold surfaces. Afterwards, toluidine blue was attached through its aromatic amino group in position 3 to the carboxylic groups covering the electrode surface. The TBO terminated SAMs yielded a surface coverage of $(2.5\pm0.5)\times10^{-10}$ mol cm^{-2} and $(6.8\pm0.1)\times10^{-11}$ mol cm^{-2}, respectively, and both monolayers showed good electrocatalytic activities. After covalent attachment of toluidine blue on a 3-mercaptopropionic acid monolayer, Schlereth et al. (1994) have shown that NADH can be oxidized at −297 mV vs. NHE. In another study, the electrochemical oxidation of NADH and its model compound, *N*-benzyl-1,4-dihydronicotinamide, was investigated at a gold electrode modified with SAM of cystamine, mercaptopropionic acid, and mercaptoethanol. Using this modified electrode, an obvious clear decrease in the overpotential could be observed (Retna Raj and Ohsaka 2001). Alpat and Telefoncu immobilized TBO by covalent attachment to a cellulose acetate-modified GC electrode. First, an activated matrix of cellulose acetate was attached to the electrode. In a second step, TBO was covalently attached via an ester bond to cellulose acetate. Further covering of the surface by an ADH and glutaraldehyde/BSA made this electrode applicable as a biosensor for ethanol determination (Alpat and Telefoncu 2010).

Further methods

Doumèche and Blum (2010) investigated the grafting of toluidine blue on screen-printed electrodes. The diazonium salt of the mediator was grafted onto the electrode surface by cyclic voltammetry or by applying a constant potential. At an applied potential of −0.047 V vs. NHE, a significant increase in current was observed after the addition of NADH to the solution. In another study, a poly(3,4-ethylenedioxythiophene) (PEDOT) electrode modified by electrodeposition of 4-phenyl azo aniline (4-PAA) was explored. The electrodeposition occurred via cyclic voltammetric procedure and rendered an electrode with good electrocatalytic activity towards NADH oxidation. Another technique to lower the overpotential for NADH oxidation is the oxidative pretreatment of the anode (Anne et al. 1999; Biade et al. 1992; Fassouane et al. 1990; Obón et al. 1997), which results in the formation of quinoid structures and adsorbed activated oxygen species (Hollmann and Schmid 2004). The 4-PAA/PEDOT/GC electrode had an overpotential for NADH oxidation about 400 mV lower than the bare electrode (Balamurugan and Chen 2008). Toh et al. showed that the addition of Ca^{2+} ions increases the electrocatalytic response towards NADH at poly(aniline)-poly(vinylsulfonate) and poly(aniline)-poly(styrenesulfonate)-modified electrodes 12 and 25 times, respectively (Toh et al. 2003). The effect of bivalent metal ions on the electrocatalysis has also been shown by Milczarek. Lignosulfonic acid and partially desulfonated lignosulfonic acid were oxidatively deposited on an activated GC electrode. Electrocatalytic oxidation of NAD(P)H was studied in buffers having pH of 5.0, 7.5, and 8.5 in the absence and presence of Mg^{2+}.

The determined kinetic constants revealed that the electrocatalysis was strongly affected by the pH, the sulfonation degree, and the presence of the bivalent metal ion (Milczarek 2009).

Poly-2,6-pyridinedicarboxylic acid (PDC) has been fabricated by electropolymerizing of 2,6-pyridinedicarboxylic acid with cyclic voltammetry on a GC electrode surface (Cui et al. 2011). Magnetic Fe_3O_4 nanoparticles were treated with aminopropyltriethoxysilane and subsequently dropped on the PDC/GC electrode to form a composite film. This modified electrode showed an enhanced effective surface area and increased electrocatalytic activity towards the oxidation of NAD(P)H. Besides the oxidation of NAD(P)H, mediators have been immobilized for the reduction of oxygen (Ganesan and Ramaraj 2000; Karnicka et al. 2008; Nogala et al. 2006), the detection of hydrogen peroxide (Liu et al. 1997; Siao et al. 2011), or without any application relation (Pauliukaite et al. 2009).

Kinetic parameters of the $NAD(P)^+$ regeneration with immobilized mediators

The surface coverage Γ of different mediators is usually in the range of 10^{-8} and 10^{-9} mol cm^{-2}. The highest surface coverage of 1.5×10^{-8} mol cm^{-2} has been reported for the entrapment of toluidine blue in carbon paste electrodes (Ramirez Molina et al. 1999). The adsorption of Meldola's Blue on silica coated with niobium oxide resulted in a surface coverage of 4.4×10^{-9} mol cm^{-2} (Santos et al. 2002b). Electropolymerization of phenosafranine leads to a film with a surface coverage of 2.7×10^{-9} mol cm^{-2} (Saleh et al. 2011b). These data show that different immobilization techniques and different mediators result in comparable surface coverage. One of the most important parameters is the rate constant for the chemical oxidation of NAD(P)H by the immobilized mediator. For efficient electrocatalysis, a fast reaction is desirable. The rate constant of this reaction depends on the driving force of the reaction, i.e., on the difference of the redox potentials for both redox couples. For a series of phenoxazine derivatives, a linear dependence of logarithm of the second-order rate constant on the potential of a mediator was disclosed (Gorton 1986). Table 2 shows second-order rate constants for the oxidation of NADH catalyzed by different mediators immobilized by various techniques. The rate constants are in the range of 10^4–10^6 $M^{-1}s^{-1}$.

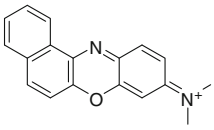
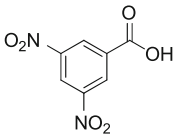
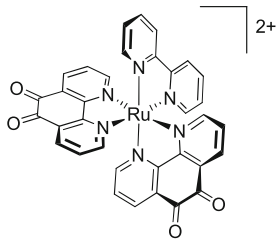
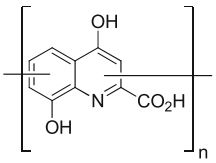
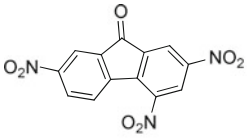
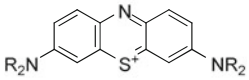
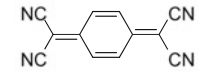
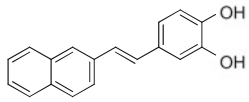
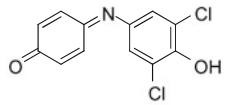
Sensitivity and detection limits in sensor application

A great number of $NAD(P)^+$-dependent enzymes can be used in biochemical analysis. The ability of (immobilized) mediators to react with NAD(P)H results in a high potential for application in these analyses. High sensitivity and a low detection limit are important parameters for sensor applications, and examples are given in Table 3 for different mediators together with their immobilization techniques.

Comparison of processes with dissolved and immobilized mediators

Using mediators in solution is not a very convenient strategy because analysis is not reagentless (Prieto-Simón and Fàbregas 2004). On the other hand, processes with dissolved mediators avoid the need to immobilize the mediator which means that the mediator will keep its original electrochemical properties. In addition, free mediators confer better sensitivities as the mediator can work under optimal conditions (high concentration and free mobility). However, there are economical and ecological advantages using immobilized mediators such as reusability and the avoidance of fouling effects. Prieto-Simón and Fàbregas (2004) compared different mediators, dissolved or immobilized via electropolymerization, entrapment or adsorption, according to their usage for electrochemical NADH oxidation. It was demonstrated that the addition of mediators directly to the analytical media allows the determination of NADH with high sensitivity and at the same time produces very little fouling of the electrode surface. However, this method has crucial disadvantages, e.g., the need to add the mediator in the analytical media and long stabilization times. Therefore, several immobilization strategies have been evaluated (Prieto-Simón and Fàbregas 2004). The first strategy studied was the entrapment of the mediator in an epoxy–graphite composite. This method had some major drawbacks such as leakage of mediator into the solution and low current densities due to the small amount of mediator available on the electrode surface and was concluded to be inappropriate for the construction of robust sensors. Nevertheless, the problem of leakage could be minimized with the use of an appropriate reactant which reduces the solubility of the mediator, for instance, Reinecke salt when entrapping Meldola's Blue (Gründig et al. 1995). The second immobilization strategy studied was the adsorption of the mediator on epoxy–graphite composite electrode surfaces. This approach reduces fouling problems which represents an important improvement compared to the entrapment strategy. However, this approach generates poor sensitivities and low reproducibility (Prieto-Simón and Fàbregas 2004). Finally, the mediator was immobilized by electropolymerization on epoxy–graphite electrode surfaces. Among all these strategies of including a mediator in the amperometric analysis of NADH, the electropolymerization showed the best results regarding the reproducibility of consecutive calibration curves (Prieto-Simón and Fàbregas 2004). Thus, this approach appeared to be the best solution to the problems

Table 2 Second-order rate constants for different mediators immobilized by distinct immobilization techniques

Immobilized mediator	Immobilization technique	Second-order rate constant [M^{-1} s^{-1}]	Applied potential [mV] vs. NHE	Literature
 Meldola´s blue	AD	1.6×10^4	364	Ladiu et al. (2007)
 3,5-dinitrobenzoic acid	CL	3.3×10^4	264	Santhiago et al. (2009)
 bis(1,10-phenanthroline-5,6-dione)-(2,2'-bipyridine)ruthenium(II)	EI	5.7×10^4	276	Santiago et al. (2006)
 poly(xanthurenic acid)	EP	7.7×10^4	414	de Assis dos Santos Silva et al. (2010)
 2,4,7-trinitro-9-flurenone	AD	8.0×10^4	264	Mano and Kuhn (1999b)
	AD	3.2×10^6	564	Munteanu et al. (2002)
 phenothiazin derivates	EP	9.4×10^4	444	Gligor et al. (2009b)
	CL	1.2×10^5	554	Ohtani et al. (1997)
 Tetracyanoquinodimethan	AD	1.5×10^6	624	Surya et al. (1994)
	EP	4.8×10^6	644	Murthy et al. (1994)
 4-[2-(2-Naphthyl)vinyl] catecho	AD	2.0×10^6	429	Jaegfeldt et al. (1981)
 2,6-dichloro henolindo henol	AD	4.6×10^6	794	Dicu et al. (2003)

AD adsorption, *EP* electropolymerization, *EI* entrapment/incorporation, *CL* covalent linkage

Table 3 Sensitivity and detection limit for sensors covered by mediators immobilized by different techniques

Immobilized mediator	Technique	Sensitivity [AM^{-1}]	Detection limit [M]	Literature
Nile blue A	AD	6.5×10^{-4}	1.2×10^{-6}	Zhu et al. (2009)
Meldola's Blue	AD	4.8×10^{-2}	1×10^{-5}	Santos et al. (2002b)
Meldola's Blue	EP, EI, AD	4.0×10^{-9}	3.5×10^{-7}	Mariotti et al. (2006)
Bis-phenothiazin-3-yl methane	EP	1.8×10^{-3}	2×10^{-6}	Gligor et al. (2009b)
catechol	EP	4.6×10^{0}	–	Bai et al. (2010)
o-phenylenediamine	EP	4.6×10^{1}	8.2×10^{-10}	Lobo et al. (1996)
Xanthurenic acid	EP	1.5×10^{-1}	1.0×10^{-7}	de Assis dos Santos Silva et al. (2010)
toluidine blue o	EP	-	5.0×10^{-7}	Cai and Xue (1998)
Meldola's Blue	EI, EP	4.2×10^{0}	2.0×10^{-6}	Vasilescu et al. (2003)
Meldola's Blue	EI	6.1×10^{-3}	5×10^{-5}	Doaga et al. (2009)
thionine	EI	4.7×10^{-3}	2×10^{-6}	Huang et al. (2007)
neutral red	EI	1.2×10^{-2}	1.5×10^{-7}	Lu et al. (2010)
Nile blue A	CL	$2.4 \times 10^{-2}\ cm^{-2}$	1.8×10^{-7}	Saleh et al. (2011a)
o-aminophenol	Electrographed	–	1.5×10^{-7}	Nassef et al. (2006)

AD adsorption, *EP* electropolymerization, *EI* entrapment/incorporation, *CL* covalent linkage

concerning the amperometric detection of NADH. It can be concluded that electropolymerizing the mediator on epoxy–graphite composite electrodes is a valuable strategy in producing chemically modified sensors. This approach generates reproducible surfaces and minimizes the surface fouling of the electrode. It also permits high sensitivities which are vital for the detection of low concentrations of NADH. Nonetheless, this method does not reduce the working potentials significantly which limits the selectivity of these sensors. Vasilescu et al. compared three strategies to develop a NADH detector based on Meldola's Blue (MB) and screen-printed electrodes. They tested (a) the direct mixing of MB in the screen-printing ink, (b) incorporation of MB after precipitation with Reinecke salt (MBRS), and (c) electrodeposition of a MB-derived polymer (poly(MB)) on the surface of graphite electrodes. Cyclic voltammetry studies were performed for these three types of modified electrodes in the absence and the presence of NADH. All of the screen-printed sensors show catalytic ability for NADH oxidation. Poly(MB) sensors provided the largest increase in the anodic current in the presence of

NADH, followed by MB and MBRS sensors. However, if the ratio of the catalytic current to the anodic current in the absence of NADH is to be considered, the sensors with MB showed the most beneficial characteristics (Vasilescu et al. 2003). Interestingly, in another publication, a k_{obs} value was estimated to be 5.7×10^4 $M^{-1}s^{-1}$ using an immobilized ruthenium complex (Santiago et al. 2006), which was much faster than in the solution (2.0×10^3 $M^{-1}s^{-1}$) at comparable experimental conditions (Rivera et al. 1994). Such difference in the catalytic activity occurs since the immobilized mediators are an integral part of the electrode surface. This part of the electrode surface enables electron hopping between mediators inside zirconium phosphate layers, which increases the reaction rate (Santiago et al. 2006).

Immobilized mediators for biofuel cell applications

Immobilized mediators can also be used in biofuel cell applications. Arechederra et al. (2011) have developed a biobattery on the basis of poly(neutral red). Poly(neutral red) was used for both oxidation of NAD(P)H and reduction of $NAD(P)^+$, which is necessary in a rechargeable biobattery environment. For reversibility, the addition of Zn^{2+} was required. Their biobattery had an open circuit potential of 1.019 (±0.051) V vs. NHE, a maximum power density of 0.016 (±0.004) mW cm^{-3}, and a maximum current density of 0.221 (±0.013) mA cm^{-3}.

In another study, Addo et al. immobilized a NAD^+-dependent ADH, an aldehyde dehydrogenase, and a formate dehydrogenase within a tetrabutylammonium bromide-modified Nafion® to oxidize methanol to carbon dioxide (Addo et al. 2010). Electropolymerized methylene green on the surface of an electrode acted as the NADH electrocatalyst. Through this approach, they developed a methanol/air biofuel cell. The biofuel cell had a maximum power density of 261±7.6 μW/cm^3 and current density of 845±35.5 μA/cm^3. This system was characterized according to the oxidation level, temperature, pH, and concentration of fuel and NAD.

Summary and outlook

In general, a useful cofactor regeneration/substitution method has to fulfil several requirements (Adlercreutz 1996). The enzymes, reagents, immobilization matrices, electrodes, and further equipment required should be readily available, inexpensive, easy to manipulate, and stable under the operational conditions. No reagents or by-products of the regeneration/substitution step should interfere with product isolation, and they should be compatible with the rest of the reaction system. In the literature, several enzyme-coupled (Geueke et al. 2003; Riebel et al. 2002, 2003; Seelbach et al. 1996) and substrate-coupled (Kroutil et al. 2004b; Mertens et al. 2003; Schumacher et al. 2006; Stampfer et al. 2002) cofactor regeneration systems are described. There are some disadvantages using an enzyme-coupled regeneration system: costs for a second enzyme and a co-substrate, interferences during product purification caused by secondary products, and reaction conditions have to be optimized for both enzyme reactions. The substrate-coupled approach provides disadvantages such as thermodynamic limitations, costs for co-substrate, reduced catalytic efficiency caused by reaction of the enzyme with co-substrate, and interferences during product purification caused by secondary products. To overcome these drawbacks, an electrochemical mediator-based system can be used. This strategy prevents costs for second enzymes or co-substrates, thermodynamic limitations, and reduced catalytic efficiency. Furthermore, reaction conditions have to be optimized for just one enzyme. Mediators used in these applications can be soluble or immobilized on the electrode material. Although dissolved mediators have benefits (simple utilization, mediator is maintained with its original electrochemical properties), there are also some disadvantages of this concept: it is not reagentless; contact of enzyme with electrode may cause denaturation processes; and the costs for mediator, costs for wastewater treatment, and interferences during product purification caused by secondary products. Immobilized mediators may have the potential to avoid these drawbacks. A suitable immobilized mediator for the oxidation of NAD(P)H should fulfill a number of demands (Gorton and Domínguez 2002): a sustainable reduction of the overvoltage, high regeneration rate (a second-order rate constant of at least 10^6 $M^{-1}s^{-1}$), long-term stability, irreversible immobilization of the mediator, and a high selectivity for NAD(P)H oxidation. It will be one of the most important investigations to identify an "optimal" mediator. Therefore, a systematic comparison of different mediators will be necessary. When mediators are used, a piece of data missing in numerous works is the reaction rate constant for the heterogeneous electron transfer between the mediator and the electrode. As already mentioned, this may be important in many respects. It is also to note that the stability data are determined under different conditions and, in most cases, not well documented in the works described (Katakis and Domínguez 1997).

Compared to other methods of mediator immobilization, electropolymerization provides some advantages. Karyakin and coworkers reported that the long-term operational stability of a poly(methylene blue) film was much higher than that of the adsorbed mediator (Karyakin et al. 1994). Electropolymerization yields modified electrodes with a three-dimensional distribution of mediators. Such electrodes should give a higher catalytic response compared to monolayers, owing to the volume effect

(Cai and Xue 1997b). Electropolymerization is a suitable approach to prepare modified electrodes as it is possible to control film thickness, permeation, and charge transfer characteristics (Siao et al. 2011). Thus, polymer-modified electrodes have many advantages due to the selectivity, sensitivity, and homogeneity in electrochemical deposition, as well as strong adherence to electrode surface and chemical stability of the film.

Recently, the Minteer group has shown that poly(neutral red) can also be used for the oxidation of NADH as well as the reduction of NAD^+ (Arechederra et al. 2011). The reversibility of the catalyst was possible only with the addition of Zn^{2+} for complexation to the redox polymer. The zinc ion complexation with the polymer facilitates electron and proton transfer to/from the substrate and the NAD^+/NADH cofactor. An electrocatalyst that can perform both oxidation and reduction of nicotinamide-based redox reactions with a minimum amount of overpotential is very promising for a wide range of applications. The results presented in this work will promote the development of electrochemical research for biocatalytic applications, biosensors, biofuel cells, and other related bioelectrochemical devices.

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