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Bioelectrochemistry 56 (2002) 117–122

Bioelectrochemistry

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Modified electrodes for NADH oxidation and dehydrogenase-based biosensors

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Received 1 June 2001; received in revised form 5 September 2001; accepted 11 September 2001

Abstract

The direct electrochemical oxidation of β-nicotinamide adenine dinucleotide (NADH) at clean electrodes proceeds through a radical cation intermediate at high overpotentials and is subject to rapid fouling. Consequently, there has been a considerable body of work over the last 20 years looking at ways in which to catalyse the reaction using a wide variety of different types of modified electrode. These studies have resulted in a good knowledge of the essential features required for efficient catalysis. In designing modified electrodes for NADH oxidation, it is not only important to identify suitable redox groups, which can catalyse NADH oxidation and can be attached to the electrode surface; it is also important to ensure facile charge transport between the immobilised redox sites in order to ensure that, in multilayer systems, the whole of the redox film contributes to the catalytic oxidation. One way to achieve this is by the use of electronically conducting polymers such as poly(aniline).

Keywords: NADH oxidation; Dehydrogenase; Biosensor; Charge transport; Conducting polymers

1. Introduction

For simple one-electron outer sphere electron-transfer reactions at electrodes, the choice of metallic electrode material has only a minor effect upon the rate of the electron-transfer reaction (see Ref. [1] for a discussion of the role of the local density of states at the fermi level). In contrast, for reactions which involve multiple electron transfers and the associated formation or breaking of chemical bonds, the choice of electrode material can have a profound effect on the rate of the electrode reaction. For example, for the hydrogen evolution reaction, the rate of the reaction varies by over 10 orders of magnitude depending on the choice of metallic electrode. These large effects arise because the electron transfers proceed sequentially, one electron at a time, and, therefore, the stabilisation of the one-electron intermediate species, $H_{ads}^{\cdot}$ in the case of hydrogen evolution, plays a crucial part in determining the overall reaction rate. The same problem arises in the electrochemistry of many molecules of biological interest, such as β-nicotinamide adenine dinucleotide (NADH), ascorbate, dopamine, etc., where the overall electrode reaction involves the transfer of more than one electron and the associated formation or breaking of chemical bonds (often, proton transfer). Some years ago, it was realised that the deliberate chemical modification of electrode surfaces offered a useful way to catalyse such electrode reactions [2–4]. At a chemically modified electrode, the reactant from solution undergoes oxidation or reduction by *chemical* reaction with a mediator species immobilised at the electrode surface. The resulting reduced or oxidised mediator species is then *electrochemically* reoxidised or reduced, ready to react with further reactants from the solution. The key to the success of this strategy is to find efficient redox mediators together with methods to immobilise them at the electrode surface, which do not limit the catalytic process.

1.1. Modified electrodes for NADH oxidation

The electrochemical oxidation of NADH presents a significant, yet important challenge. NADH is the coenzyme for more than 350 dehydrogenase enzymes and is the terminal electron donor in the mitochondrial electron transport chain [5,6]. Thus, the electrochemical oxidation of NADH to NAD^+ (a 2e $1H^+$ oxidation) provides a route

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1567-5394/02/$ - see front matter
PII: S1567-5394(02)00047-6

to the development of a potentially wide range of amperometric enzyme electrodes for many different substrates. In addition, because a lot is known about NADH and its reactions in homogeneous solution, it provides an ideal model system for testing strategies for the rational design of modified electrodes. A number of reviews of NADH electrochemistry have been published describing the different types of modified electrode that have been studied [7–10].

These modified electrodes for NADH oxidation and their application in dehydrogenase-dependent amperometric enzyme electrodes are examples of what Bard and Faulkner [11] have termed "integrated chemical systems", i.e., assemblies of a number of different chemical components, each specially selected to carry out a particular function, put together in a well-defined way in order to produce a functional structure. In this case, the chemical components comprise the mediator group, some means by which to attach it to the electrode surface, and in the case of the enzyme electrode, the enzyme itself immobilised in some way at the electrode surface. This kind of system can also be used to construct bioelectronic devices as we shall see below. A variety of methods exists for the assembly of the different components at the electrode surface including self-assembly, covalent attachment and electropolymerisation [4,12], together with techniques for the characterisation of the resulting structures.

The direct electrochemical oxidation of NADH at unmodified electrodes only proceeds at high overpotentials and leads to fouling of the electrode surface. This can be understood because the one-electron oxidation product, NADH$^{+\cdot}$, is difficult to form and highly unstable. Studies of the rate of homogeneous oxidation of NADH by a variety of molecules, particularly, the works of Miller et al. [13–18], provide a clear indication of the type of mediator species that one should look to use in a modified electrode for NADH oxidation. Fig. 1 is a linear free energy plot of the rates of oxidation of NADH as a function of the redox potential of the oxidant for a variety of different types of oxidant. From the figure, we can see that one-electron oxidants, such as ferricenium ions, give low rates of oxidation of NADH for high thermodynamic driving forces. In other words, these are a poor choice as mediators for NADH oxidation. The reason for this was demonstrated by Miller et al. [16,17] in their study where they showed that the oxidation of NADH by ferricenium ions proceeds via formation of the unstable NADH$^{+\bullet}$, just as is the case for the electrochemical oxidation of NADH at bare metal electrodes. In contrast, the *ortho*-and *para*-quinones and diaminobenzenes represent a much better choice as mediator species because they give higher reaction rates for lower thermodynamic driving forces. The reason for this is that, for these oxidants, the oxidation of NADH proceeds via either hydride or hydrogen atom transfer followed by electron transfer [14]. Clearly, we should look to mediators of this type as suitable molecules from which to construct modified electrodes for NADH oxidation. Gorton [8] has summarised this requirement by pointing out that good mediators for NADH oxidation should have structures which allow hydride transfer from NADH to the oxidised form of the mediator [9] (Fig. 2).

The rate of reaction of the oxidised mediator with NADH is not the only constraint. It is also essential to arrange for the subsequent efficient reoxidation of the mediator by the electrode. Albery and Hillman [19] pointed out that there were essentially two strategies for the design of modified electrodes. Either one can go for a monolayer modification of the electrode or one could use a multilayer-modified electrode. For the monolayer case, all the mediator molecules are close to the electrode surface and, thus, can be directly oxidised by the electrode. The disadvantage is that the number of mediator molecules is limited, typically around 10^{-10} mol cm^{-2}. For multilayer-modified electrodes, this constraint is removed because more mediator molecules can be incorporated into a layer at the electrode surface. However, there is a price to be paid. Now, if all the mediator molecules are to play a catalytic role, it is essential to ensure rapid charge propagation between the redox species in the film and easy diffusion of the NADH within the film so that the catalytic oxidation of NADH can occur throughout the film. This has not always proved to be easy to achieve [20–22]. The advantage of using the multilayer-modified electrode is that the rate constant for the mediator NADH reaction does not need to be so high in order to sustain the same current density. Albery and Hillman [19] calculated that for a monolayer-modified electrode, the

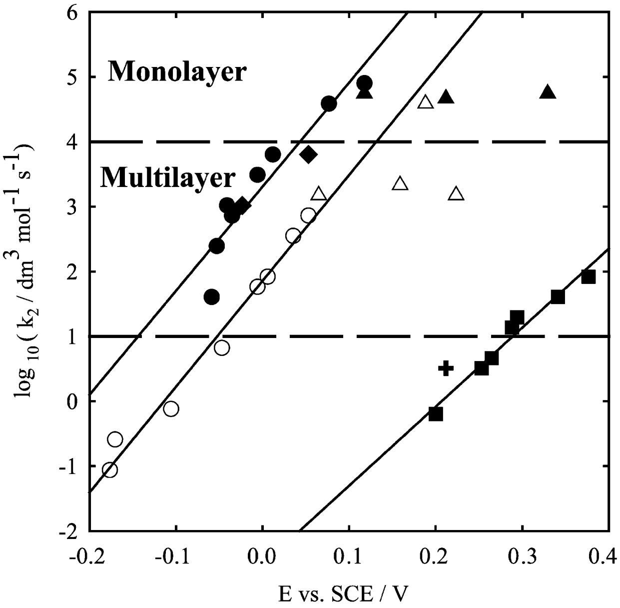

Fig. 1. Linear free energy plot for the rate of oxidation of NADH by a range of possible mediators. ■: Ferricenium ions; +: ferricyanide; ●: *ortho*-quinones; ▲: *ortho*-diaminobenzenes; ○: *para*-quinones; △: *para*-diaminobenzenes; ◆: aminopyrimidines. The figure is based largely on data from the works of Miller et al. [13–18].

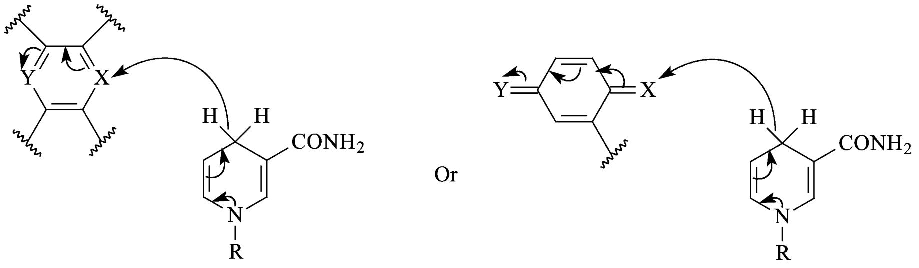

Fig. 2. The general mechanism proposed for efficient oxidation of NADH by a mediator.

second-order rate constant for the catalytic reaction needed to be $>10^4$ dm^3 mol^{-1} s^{-1}, whereas for the multilayer-modified electrode, the corresponding figure was only 10 dm^3 mol^{-1} s^{-1}. In Fig. 1, the horizontal lines indicate these rate constant values. It is clear from the figure that the preferred approach will be the use of a multilayer-modified electrode since this allows a lower overpotential for the electrochemical oxidation and, thus, less interference from other species in solution.

2. Experimental

Experiments were performed using carbon dual microband electrodes (~ 10-μm-wide electrodes, ~ 20 μm gap and ~ 4.5 mm long). Full fabrication details for these electrodes can be found elsewhere [23]. PANI-PVS films were deposited potentiostatically at + 0.9 V vs. SCE across the gap between the two microband electrodes, from a solution containing 21.5% poly(vinylsulfonate) (made from a 25% poly(vinylsulfonate) solution supplied by Aldrich), 1.88 mol dm^{-3} H_2SO_4 and 0.44 mol dm^{-3} aniline (freshly distilled).

The microelectrochemical transistors were first oxidised at + 0.7 V vs. SCE in a conventional three-electrode circuit (with the two microband electrodes connected together) in pH 5 citrate–phosphate solution for 7 min. This procedure turned the device to the "off" state. The device was then transferred to a pH 7 citrate–phosphate buffer solution containing 0.5 M Na_2SO_4 in a thermostated cell in stirred solution at 25 °C. A drain voltage of + 30 mV was applied between the two microband electrodes and NADH (97%, Aldrich) was added to the solution to turn the switch from the "off" to the "on" state. Full details can be found in Ref. [24]. The resulting drain current flowing through the polymer was recorded on a chart recorder (Gould, 60000 series), and the polymer potential was monitored with respect to SCE using a high-impedance pH meter (Corning 145).

For the ethanol experiments, the device in the "off" state was transferred to a 1 mol dm^{-3} Tris–HCl solution containing 30 mmol dm^{-3} NAD$^+$ (98%, Aldrich) and 2 mg ml^{-1} yeast alcohol dehydrogenase (Biozyme). Ethanol (99.86%, BDH) was added to the solution to turn the device from the "off" to the "on" state. The experiments were carried out in a thermostated cell in stirred solution at 35 °C.

3. Results and discussion

An approach that we have been investigating in Southampton is the use of poly(aniline) films as electrodes for the oxidation of NADH. The conducting emeraldine form of poly(aniline) contains *para*-quinonimine groups which fulfill the structural requirements for NADH oxidation, as shown in Fig. 2. In addition, the poly(aniline) films can be deposited by electrochemical polymerisation, making them ideal for use with microelectrodes, microfabricated structures or electrode arrays. The films are also electronically conducting, ensuring rapid propagation of charge throughout the film. The electronic conductivity of the film can be utilised in the construction of bioelectronic devices [25]. One problem, which must be overcome in using poly(aniline), is to ensure that the polymer remains conducting at neutral pH. The conducting emeraldine form of poly(aniline) that we wish to use to oxidise NADH usually deprotonates at about pH 5, forming the insulating emeraldine base [26]. We and others have shown that the pK_a of the emeraldine form can be shifted to pH 7 or 8 by incorporation into the polymer of polymeric counter anions, such as poly(vinylsulfonate) or poly(acrylate), in place of the small anions, such as chloride or bisulfate, which are more usually employed [26–31]. This is because for the small anions, deprotonation of the emeraldine state occurs with loss of both the proton and the anion from the film. In contrast, for the poly(aniline)–poly(anion) composite films, deprotonation can only occur if the loss of the proton is coupled to ingress of another cation into the film because the poly(anion) itself is trapped within the film.

Our experiments have shown that poly(aniline) – poly (anion) composite films are efficient catalysts for the electrochemical oxidation of NADH at potentials around 50 mV vs. SCE and that the reaction occurs through the formation of a complex between the NADH and the polymer, followed by the oxidation of the NADH [28–31]. We have also shown that these films can be used to construct microelectrochemical transistors, or switches, which respond to NADH by measuring the change in the conductivity of the

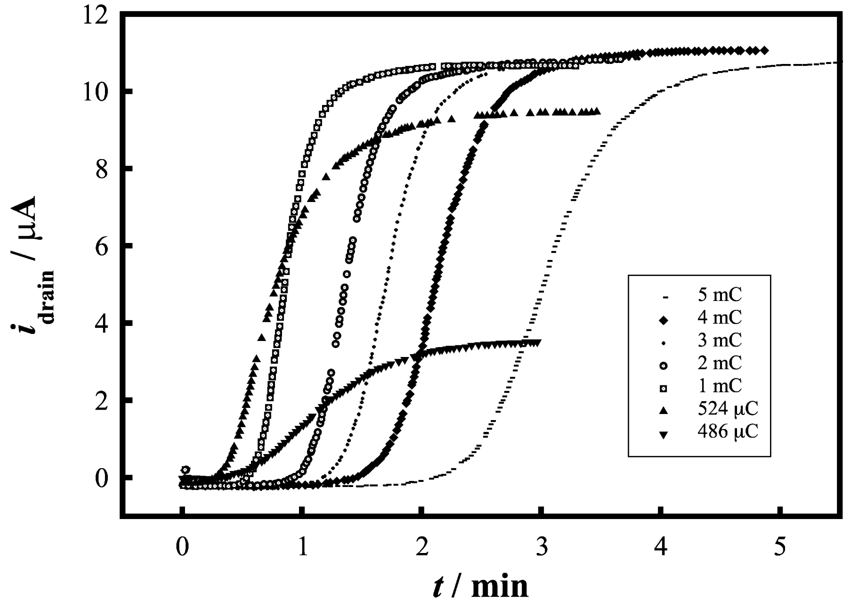

Fig. 3. Responses of a set of poly(aniline)–poly(vinylsulfonate) microelectrochemical transistors to the addition of 4 mM NADH. All measurements were made in 0.1 M phosphate buffer (pH 7) containing 0.5 M Na_2SO_4 at 20 °C. The applied drain voltage was 30 mV. The poly(aniline)–poly(vinylsulfonate) films were deposited across the gaps (~ 20 µm) between pairs of screen-printed microband electrodes (10 µm wide by 4.5 mm long by passing different amount of charge as shown in the inset key).

polymer film when it is reduced by NADH from the insulating, pernigraniline form to the conducting, emeraldine form [24]. Our recent work has concentrated on using these poly(aniline) composite films as electrodes in amperometric enzyme electrodes and in enzyme-based microelectrochemical enzyme transistors. For example, we have investigated the immobilisation of modified forms of lactate dehydrogenase from *Bacillius stearothermophilus* onto poly(aniline)–poly(anion) composite films as part of the next stage in developing integrated chemical systems and bioelectronic devices based on NADH-dependent dehydrogenase enzymes [32–35].

We have also extended our studies of the applications of these films in microelectrochemical enzyme transistors. Fig. 3 shows results for a set of poly(aniline)–poly(vinylsulfonate) composite films deposited across 20-µm gaps between screen-printed carbon microband electrodes. At time zero, before any NADH is added, the films are insulating so that the

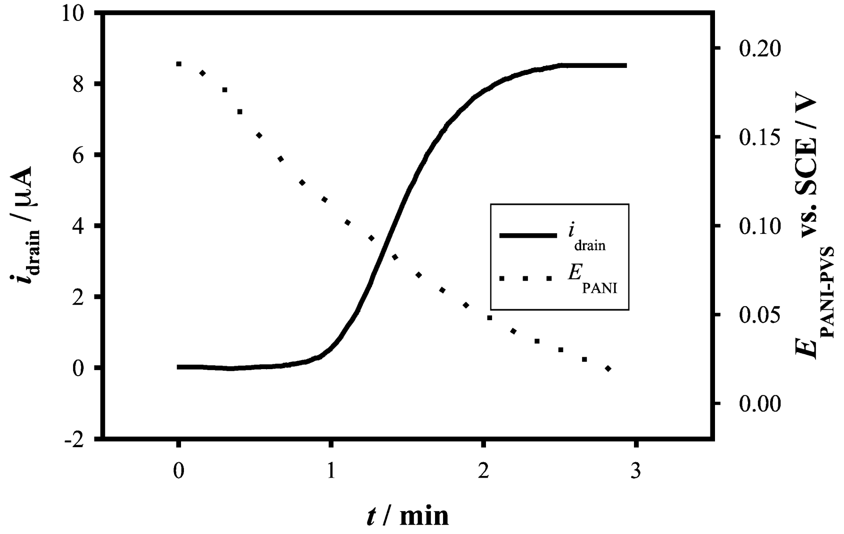

Fig. 4. Response of a poly(aniline)–poly(vinylsulfonate) microelectrochemical transistor to the addition of 0.1 M ethanol in 1 M Tris–HCl buffer (pH 7.1) containing 2 mg ml^{-1} of yeast alcohol dehydrogenase and 30 mM NAD^+ at 35 °C. The applied drain voltage was 30 mV and the polymer film deposition charge was 1 mC. The figure also shows the open circuit potential of the film, $E_{\text{PANI-PVS}}$, measured at the same time as the drain current.

drain current, i_{drain} (i.e., the current flowing through the poly(aniline) film between the two carbon microband electrodes) is very small (< 100 nA corresponding to a device resistance of >3 MΩ). Upon addition of NADH, the poly(aniline) is converted to its conducting form and the drain current increases eventually reaching a constant, plateau value. From the figure, we can clearly see the effect of changing the amount of poly(aniline) deposited, as judged by the charge passed to deposit the film. For thin films, the plateau current increases with film thickness. This is because the conductivity of the poly(aniline) film limits the maximum drain current under these circumstances. For thicker films, the plateau currents are no longer dependent on the amount of poly(aniline) and the device resistance reaches around 3 kΩ, a value determined by the resistance of the carbon microband electrodes rather than the poly(aniline) film itself. We can also see from Fig. 3 that for thicker films, the time taken for the device to switch from its insulating, "off", state to its conducting, "on", state gets longer as the film thickness increases. This is because more NADH must react at the thicker film in order to reduce it to the conducting state than is required for the thinner film. Thus, there is an optimum film thickness in order to obtain a response, which is both large and rapid. For this device, it corresponds to a deposition change of about 1 mC.

Devices of this type can also be made to respond to an enzyme substrate by using the appropriate NADH-dependent dehydrogenase enzyme. Fig. 4 shows an example for the detection of ethanol using yeast alcohol dehydrogenase as the enzyme and a poly(aniline)–poly(vinylsulfonate)-coated microelectrochemical transistor to detect the NADH generated by the enzyme-catalysed reaction. Again, the device switches from "off" to "on", this time, upon the addition of ethanol to the solution. The figure also shows the potential of the poly(aniline) film measured against an SCE reference electrode as the switching occurs. The potential of the film starts off at around 0.18 V where the film is in its insulating form and falls, as the reaction proceeds and the device switches on, to around 0.0 V, thus directly confirming that the measured change in drain current is due to a change in the redox state of the poly(aniline) film and not a local change in pH.

4. Conclusions

Poly(aniline)–poly(anion) composite films can be used for the electrochemical oxidation of NADH at around 50 mV vs. SCE and pH 7. Because the films are electrochemically formed and are electronically conducting in their emeraldine state, these composite films are well suited for use with microelectrodes and arrays of electrodes and can be used to fabricate bioelectrochemical devices such as microelectrochemical transistors. Future directions for research will include the development of methods to immobilise dehydrogenase enzymes on these surfaces to produce integrated devices incorporating the mediator sites, the enzyme and possibly, immobilised derivatives of NADH, with a view to utilising these structures as amperometric biosensors, as bioelectronic devices and in biofuel cells.

Acknowledgements

This work was supported by the US Office of Naval Research.

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