
Dimitrios V. Stergiou, Mamas I. Prodromidis, Panayotis G. Veltsistas, Nikolaos P. Evmiridis

University of Ioannina, Department of Chemistry, 45110-Ioannina, Greece
*e-mail: panavelt@cc.uoi.gr

Received: April 15, 2003
Final version: July 17, 2003

Abstract
The preparation and the electrochemical study of Disperse Blue 1-chemically modified electrodes (DB1-CME), as well as their efficiency for the electrocatalytic oxidation of NADH is described. The proposed mediator was immobilized by physical adsorption onto graphite electrodes. The electrochemical behavior of DB1-CME was studied with cyclic voltammetry. The electrochemical redox reaction of DB1 was found to be reversible, revealing two well-shaped pair of peaks with formal potentials 152 and 42 mV, respectively, (vs. Ag/AgCl/3M KCl) at pH 6.5. The current $I_p$ has a linear relationship with the scan rate up to 800 mV s$^{-1}$, which is indicative for a fast electron transfer kinetics. The dissociation constants of the immobilized DB1 redox couple were calculated $pK_1 = 4$ and $pK_2 = 5$. The electrochemical rate constants of the immobilized DB1 were calculated $k_1 = 18$ s$^{-1}$ and $k_2 = 23$ s$^{-1}$ ($T=2.36$ mmol cm$^{-2}$). The modified electrodes were mounted in a flow injection manifold, poised at +150 mV (vs. Ag/AgCl/3M KCl) and a catalytic current due to the oxidation of NADH was measured. The reproducibility was 1.4% RSD ($n = 11$ for 30 $\mu$M NADH) The behavior of the sensor towards different reducing compounds was investigated. The sensor exhibited good operational and storage stability.

Keywords: Disperse Blue 1-modified graphite electrode, Cyclic voltammetry, Electrocatalysis of NADH, Flow injection analysis

1. Introduction

The design and characterization of new electrochemical sensors continues to be an extremely attractive and prolific area of research in analytical chemistry [1]. CMEs are progressively finding a growing interest in electrocatalysis of some electrochemical redox processes [2, 3]. The great demand for NADH selective determination with NAD$^+$-dependent dehydrogenase-based biosensors, in clinical, food and environmental chemistry, has resulted to extensive research studies. The development and the electrochemical characterization of the numerous mediator-based electrodes have been extensively reviewed [4, 5].

Various procedures have been reported for the chemical modification of carbon electrodes, with several redox materials. A number of kinetic limitations and chemical interference associated with the oxidation of NADH, such as high activation overpotentials, fouling by free radicals etc, initiated extended investigations on acquisition of more efficient redox mediators, i.e., quinones, catechols, redox dyes, metal complexes and organic conducting salts [6, 7]; organic dyes have been extensively used as mediators for the development of electrochemical sensors and biosensors [8–11]. However, in most cases, the CMEs were found to be of limited success in analytical application for NADH determinations, in multielement real samples.

The present work reports the development of a 1,4,5,8-tetra-aminoanthraquinone-CME, commercially available with the trade name of Disperse Blue 1 [12]. To the best of our knowledge this is the first article dealing with the electrochemical behavior and the potency of this specific mediator towards the electrocatalytic oxidation of NADH. In addition, i) the tested compound is practically insoluble in water and commercially available in rational prices, ii) the reported sensor proved to be highly selective towards NADH, over a wide range of reducing species, which are possible to co-exist in various real samples. This remarkably good selectivity makes this sensor highly promising for further applications in complex matrices and iii) the proposed mediator was found to have a high affinity towards NADH, as revealed from the high values of the apparent electrochemical rate constants, $k_1 = 18$ s$^{-1}$ and $k_2 = 23$ s$^{-1}$, compared with 4 – 8 s$^{-1}$ for the majority of the most popular dyes, e.g., Meldola Blue, Nile Blue. The immobilization of the compound was achieved by physical adsorption onto the graphite electrodes and the electrochemical behavior of it was studied by cyclic voltammetry. CMEs were mounted in a flow through amperometric cell and their applicability as sensors towards the electrocatalytic oxidation of NADH was also tested successfully.
2. Experimental

2.1. Reagents

Disperse Blue 1 (C_{14}H_{12}O_{2}N_{4}, Product No 21,564-3), a dark blue-colored organic dye, was purchased from Aldrich (Gillingham, UK) and was used without further purification. The dye content in the commercial product is approximately 30% and the molecular shape of the active compound is shown in Scheme 1. Recovery of the active dye by extracting the commercial product with acetone and filtering through Whatman 42, gave clear, transparent solutions, which tested on the graphite electrodes, revealed identical electrochemical behavior to the commercial product itself. Consequently, we decided to use the material in our experiments without any pretreatment. β-Nicotinamide adenine dinucleotide reduced form disodium salt (C_{21}H_{27}N_{7}O_{14}P_{2}Na_{2}, Product No N-8129) was purchased from Sigma (St. Louis, USA). The NADH solution was prepared daily in St. Louis, USA. The NADH solution was prepared daily in double-distilled water (DDW) was used throughout.

2.2. Apparatus

All electrochemical experiments were conducted with a computer-controlled potentiostat, the Autolab Electrochemical Analyzer (Eco Chemie, The Netherlands). The flow injection experiments were carried out, by using an in-house fully automated flow injection manifold. A detailed description of the FI-manifold and the electrochemical detector was reported earlier [3]. Cyclic voltammetry experiments were performed with a voltammetric cell (VC2, BAS, IN) with a graphite rod (RW0001, 6.5-mm diameter, Ringsdorff-Werke, Germany) as the working electrode, whose cylindrical surface was covered with parafilm, thus ensuring a constant active surface. A Ag/AgCl/3M KCl (BAS) electrode and a Pt wire (BAS) were, also, used as reference and auxiliary electrodes, respectively. The supporting electrolyte was a 0.25 M phosphate buffer in 0.5 M KCl at pH 2 – 8. All experiments were carried out at room temperature.

2.3. Preparation of the modified electrodes

Spectroscopic graphite rods were polished on emery paper of 1000 grit and they were thoroughly washed with DDW. They were dried at 60 °C for 30 min, heated in a muffle furnace at 700 °C for 90 s in order to remove any small particles from the surface area and allowed to cool in a desiccator. To avoid electrical contact between the sides of the electrodes and the solution, thus ensuring a constant active surface, the sides of the working electrodes were insulated with Parafilm before every electrochemical experiment [13]. The immobilization was achieved by immersing the electrodes for 10 min into a 1 mg/mL solution of the compound in ether. We have chosen the dipping-type procedure, instead of the dropping-type deposition, because the second one strongly suffers from the ether fugacity.

2.4. Procedures

The surface coverage \( \Gamma \) of the modifier and the total effective area \( A \) of the working electrode were determined by executing cyclic voltammetry and double step chronocoulometry experiments, respectively [3].

The applied potential for the flow injection measurements was +150 mV (vs. Ag/AgCl/3M KCl). After the potential had been applied to the CME, the background current was allowed to decay to a stable value under continuous flow. The carrier (0.05 M phosphate buffer, pH 6.5) was continuously pumped at a flow rate of 0.5 mL min^{-1}. Aliquots of NADH were injected with a 70 μL injection loop. The peak heights of the current response were plotted against NADH concentrations, in order to establish a quantitative assay of NADH.

3. Results and Discussion

3.1. Electrochemical Behavior of the Adsorbed Disperse Blue 1

The active surface area \( A \) of the graphite electrodes, polished with the specific emery paper (1000 grit) has been calculated as 0.35 ± 0.05 cm^2 [3]. The electrochemical behavior of the immobilized mediator DB1 was studied by performing cyclic voltammetry. As illustrated in Figure 1, the DB1-CMEs, in a 0.25 M phosphate buffer pH 6.5 in 0.5 M KCl, produce two well-shaped pair of peaks with anodic-cathodic waves almost symmetrical. Thus, we are able to calculate the formal potential \( E^\circ \) by taking the mean of the position of the two redox peaks. They were found 152 mV and −42 mV (vs. Ag/AgCl/3M KCl) for the first and second pairs of peaks, respectively. The separation of potential peaks \( \Delta E_p \) moderately compared with the expected for surface-immobilized redox species; they were found: \( \Delta E_{p1} = 12 – 75 \) mV and \( \Delta E_{p2} = 10 – 74 \) mV at scan rates range 100 – 1000 mV s^{-1} with a surface coverage \( \Gamma = 1.42 \) nmol cm^{-2}. If the adsorption procedure follows the Langmuir isotherm, the expected value of \( \Delta E_p \) for a reversible redox compound is theoretically 0 mV and independent on the scan rate. Deviations from the ideal values of \( \Delta E_p \) seem to be usual for redox-modified electro-

Scheme 1. The structural formula of Disperse Blue 1.
des, even at low coverages and sweep rates due to the interactions among the DB1 molecules, and the redox centers with the support. Broader and flatter peaks can also be attributed to the influence of heterogeneity of the working electrode [14].

The surface coverage is a key-parameter for the electrocatalytic behavior of an adsorbed mediator [15, 16]. Several parameters such as the electron-transfer kinetics between the adsorbed mediator and the underlying graphite phase, the stability of the sensor and the kind of limitations of the produced current (diffusion or kinetic limited) are directly or indirectly affected by its value. In order to determine the optimum concentration of the adsorbed mediator, we tried four different concentrations: 3.5/7/10 and 13 mg DB1 in 10 mL ether. Resulted electrodes were compared based on criteria such as the value of $\Delta E_p$, the ratio of anodic to cathodic current, $I_a/I_c$ and the working stability. The latter was evaluated as the percentage of the total charge decrease at anodic and cathodic region after 300 successive scans over the applied potential window – 0.4 to 0.7 V. As can be seen in Table 1, optimum results were obtained, when a 3.5–7 mg/10 mL immobilization solutions were used and these concentrations were used for further experiments. The value of 1.42 nmol cm$^{-2}$, seems to be the upper limit of the surface coverage so that the absorption of the mediator follows the Langmuir isotherm. Higher concentrations provoke interactions between the absorbed molecules or overlapping between the redox centers, resulting thus in voltammetric peak-broadening, indicative of the formation of multilayers [8]. This phenomenon is also responsible for the decrease of the working stability observed for higher surface coverages.

The molecules of the modifier are physically trapped onto the graphite surface and are restrained through weak intramolecular bonds (e.g., van der Waals), thus resulting in a continuous leaking from the multilayer structure [15].

The choice of the solvent proved also to be a crucial parameter regarding the electrochemical behavior of the DB1-CMEs. Even though the DB1-CMEs were tested in different solvents, it was found to have almost identical formal potentials for both couple of peaks, except the case of the 1:1 mixture of acetone/cyclohexanone, where no peaks were observed at all, the produced currents and the working stability were found to be seriously affected (Table 2).

The peak current $I_p$ for an adsorbed compound is given by the following equation:

$$I_p = \text{constant} \times \text{current efficiency} \times \text{surface area}$$

Table 1. Electrochemical characteristics of immobilized DB1 at different surface coverages.

<table>
<thead>
<tr>
<th>$\Gamma$ (nmol cm$^{-2}$)</th>
<th>$\Delta E_p$ [a] (mV)</th>
<th>$\Delta E_{pc}$ [a] (mV)</th>
<th>$(I_a/I_c)_1$ [a]</th>
<th>$(I_a/I_c)_2$ [a]</th>
<th>% $Q_a$ [b]</th>
<th>% $Q_c$ [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.97</td>
<td>12</td>
<td>10</td>
<td>0.99</td>
<td>1.09</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>1.42</td>
<td>15</td>
<td>12</td>
<td>1.01</td>
<td>1.05</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>1.92</td>
<td>20</td>
<td>15</td>
<td>1.00</td>
<td>1.10</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>2.36</td>
<td>22</td>
<td>17</td>
<td>1.00</td>
<td>1.03</td>
<td>12</td>
<td>18</td>
</tr>
</tbody>
</table>

[a] Scan rate 50 mV s$^{-1}$. [b] Percentage of the total anodic or cathodic charge decrease, after 300 successive scans, pH 6.5, scan rate 200 mV s$^{-1}$.

Table 2. Solvent selection for the immobilization solution.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>1$^{st}$ peak [a] (µA)</th>
<th>2$^{nd}$ peak [a] (µA)</th>
<th>3$^{rd}$ peak [a] (µA)</th>
<th>4$^{th}$ peak [a] (µA)</th>
<th>% $Q_a$ [b]</th>
<th>% $Q_c$ [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether</td>
<td>–115</td>
<td>–166</td>
<td>176</td>
<td>90</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Acetone</td>
<td>–122</td>
<td>–158</td>
<td>161</td>
<td>113</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Chloroform</td>
<td>–89</td>
<td>–129</td>
<td>133</td>
<td>67</td>
<td>70</td>
<td>44</td>
</tr>
<tr>
<td>Ethanol</td>
<td>–94</td>
<td>–136</td>
<td>140</td>
<td>66</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td>Acetone/Triethylamine 95:5</td>
<td>–195</td>
<td>–267</td>
<td>265</td>
<td>181</td>
<td>29</td>
<td>22</td>
</tr>
<tr>
<td>Acetone/Triethanolamine 95:5</td>
<td>–182</td>
<td>–261</td>
<td>267</td>
<td>164</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>Acetone/Cyclohexanone 1:1</td>
<td>–18</td>
<td>–62</td>
<td>77</td>
<td>17</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Acetone/Tetrahydrofuran 1:1</td>
<td>–38</td>
<td>–42</td>
<td>41</td>
<td>35</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Acetone/Acetonitrile 1:1</td>
<td>–193</td>
<td>–257</td>
<td>263</td>
<td>181</td>
<td>51</td>
<td>41</td>
</tr>
</tbody>
</table>

[a] Current height of the 20$^{th}$ scan. [b] Percentage of the total anodic or cathodic charge decrease, after 300 successive scans with surface coverage 1.42 nmol cm$^{-2}$, pH 6.5 and scan rate 200 mV s$^{-1}$.

Electroanalysis 2004, 16, No.11 © 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
\[ I_p = (n^2 F^2 / 4RT) \Gamma A v \]

where \( A \) is the surface area, \( \Gamma \) the surface coverage and \( v \) the sweep rate. The \( n \)-value can be calculated from the above equation – provided that the other quantities are known – only if the Langmuir model is followed.

A plot of the peak current (\( I_p \)) versus the scan rate (\( v \)), reveals a linear relationship up to 800 mV s\(^{-1}\) (Figure 2A), at a surface coverage of 1.42 nmol cm\(^{-2}\), as it has been theoretically predicted for the adsorbed species. The experimental data so far confirm a model in which the redox species are adsorbed on the electrode surface, following a kinetic-limited process. It is important to note that at a higher surface coverage, the linearity is restricted to 400 mV s\(^{-1}\) (data not shown). In the contrary, above 1000 mV s\(^{-1}\) the relationship becomes linear only when \( I_p \) is plotted versus the \( (v^{1/2}) \), thus revealing a mass transport limited process (Figure 2B). This is specifically valid in a regime of high surface coverage, high sweep rates or low buffer capacities, where local pH changes could occur, resulting in increases of \( \Delta E_p \). Under such high sweep rates the diffusional step predominates over the whole procedure. The DB1's redox reaction involves proton mobility (see below) that results in increasing dependence on the diffusional sweep rate even at low surface coverages [6].

From the slope of the linear part in Figure 2A we can also calculate the number of the electrons which participate in the overall redox reaction, according to the aforementioned equation. The results are 1.08 and 1.26 for the first and second pair of peaks, respectively, which are very close to the theoretical value of 1.

The apparent electrochemical (self-exchange) rate constant \( k^' \) (Scheme 2) was calculated from Tafel diagrams according to the method described by Laviron [17]. Anodic, \( \alpha_a \) and cathodic, \( \alpha_c \) transfer coefficients and \( k^' \) were calculated from the slopes and intercepts of plots \( E_p \) versus \( \log(v) \), for high sweep rates. Figure 3 illustrates the plots used for evaluating \( \alpha_a \) and \( k^' \) from the anodic as well as \( \alpha_c \) and \( k^' \) from the cathodic partial reaction for each redox wave, at the surface coverage of 2.36 nmol cm\(^{-2}\). The slope of the linear segment is equal to \(-2.303 RT/\alpha_n F\) for the cathodic and \(2.303 RT/\alpha_p F\) for the anodic peak. The sum of transfer coefficients \( \alpha_a \) and \( \alpha_c \) is close to its normal value of 1. The values of \( k^' \) were calculated 18 s\(^{-1}\) and 23 s\(^{-1}\) for the first and second pair of peaks respectively, by applying the equation \( k^' = 2.303 \alpha_n Fv / RT \), using a value of \( \alpha = 0.5 \) [16]. These values indicate fast kinetics for both oxidative species and explain the good behavior of the mediator at scan rates.

Fig. 2. Plot of the peak current with A) the scan rate and B) with the square root of scan rate. Phosphate buffer 0.25 M in 0.5 M KCl, pH 6.5. Surface coverage: 1.42 nmol cm\(^{-2}\).

Fig. 3. Plot of \( E_p \) vs. \( \log(v) \). Phosphate buffer 0.25 M in 0.5 M KCl, pH 6.5. Surface coverage: 2.36 nmol cm\(^{-2}\).
as high as 3000 mV s\(^{-1}\). The apparent electrochemical rate constants \(k\) of commonly used mediators ranged from 4 to 20 s\(^{-1}\) [3, 16].

The pH dependence of the electrochemical behavior of the adsorbed mediator was studied within the pH range 2.0 to 8.0 by using cyclic voltammetry. Figure 4 shows the pH dependence of \(E^{\ddagger}\). The \(E^{\ddagger}\) value was taken at scan rate 100 mV s\(^{-1}\), where the diffusion effects, which would adversely distort the peak currents, become negligible.

Variation of \(E^{\ddagger}\), calculated as the average value of the formal potentials of the two pair of peaks, respectively, \(E^{\ddagger} = (E^{\ddagger\uparrow} + E^{\ddagger\downarrow})/2\), has been also reported [22].

The dissociation constants of the immobilized DB1 molecules were found \(pK_1 = 4\) and \(pK_2 = 5\), from the intersection of the three straight lines with slopes \(-20\) mV (pH unit\(^{-1}\)) in the acidic region, \(-206\) mV (pH unit\(^{-1}\)) in the pH region \(4 \pm 5\) and \(-57\) mV (pH unit\(^{-1}\)) in the pH range \(5 \pm 7\). The changes in the slope are due to the existence of different DB1 molecules in each pH region respectively. According to the aforementioned, the redox mechanism shows to be practically independent on pH changes in the range \(2 \pm 4\). From pH 4 to 7, based on the different slope values of the straight lines, we can assume a mechanism which involves \(7H^+\) and \(2H^+\); respectively, but up to now the mechanism is under further and more detailed investigation.

The electrochemical behavior of the immobilized compound at pH \(>5\) approximates to the expected one, that is, reduction of the two quinonoid-groups to hydroxy-groups through a \(2H^+/2e^-\) mechanism. On the other hand, the electrochemical behavior of the adsorbed mediator within pH range \(2 \pm 5\) cannot be explained reasonably. Interference arising from the unavoidable coexistence of impurities may be a reasonable explanation of this behavior. These impurities originate: a) from the possible polymerization of DB1 especially in pH region \(4 \pm 5\) and b) from the high content of dye stabilizer.

3.2. Electrocatalytic Oxidation of NADH

The coupling efficiency between the immobilized mediator and NADH was investigated by performing cycling voltammetry. The experiments were carried out in 0.25 M phosphate buffer solution in 0.5 M KCl which contained 3 mM NADH. As illustrated in Figure 5, curve (a) shows the cyclic voltammogram of the DB1-CME (without) and curve (b), (with NADH). Curve (c) is the cyclic voltammogram of NADH taken with an unmodified graphite electrode for comparison. According to earlier reports [3, 6, 18–21] the oxidation of NADH appears at potentials higher than +350 mV. In this case, the oxidation of NADH on the DB1-CMEs gives a plateau from \(-50\) to \(+180\) mV (curve b), almost identical with the formal potential of the adsorbed mediator (curve a). The presence of the reduced cofactor results to the amplification of the anodic current, whereas the cathodic current is decreased accordingly. This effect can be explained by taking into consideration the diffusion of NADH on the electrode surface and the reduction of the oxidized form of the mediator, which is then re-oxidized electrochemically during the sweep, according to the reaction outlined in Scheme 2. As the reductive form of the mediator is regenerated from the reaction of its oxidative form with NADH, there is significant increase in the anodic current, which is actually the measuring signal of the proposed sensing strategy and this phenomenon is well-known as inductive electrochemical catalysis.

3.3. Interference

Interference from various reductive species, coexisting in real samples, was investigated by applying the method of mixed solutions in the presence of 0.1 mM NADH. Interferents were added at concentrations much higher than

![Fig. 4. Formal potentials \(E^{\ddagger}\) for the adsorbed DB1 vs. pH. Phosphate buffer 0.25 M in 0.5 M KCl. Scan rate: 100 mV s\(^{-1}\). Surface coverage: 1.92 nmol cm\(^{-2}\).](image)

![Fig. 5. CVs illustrating the electrocatalytic oxidation of NADH mediated by adsorbed Disperse Blue 1: a) DB1-CME in buffer solution, b) same electrode in buffer containing 3 mM NADH, c) unmodified graphite electrode in buffer solution containing 3 mM NADH. Buffer: 0.25 M phosphate in 0.5 M KCl, pH 6.5. Scan rate: 50 mV s\(^{-1}\).](image)
those normally present in the real samples. The relative responses due to paracetamol, dopamine, glutathione, cysteine, homocysteine (6 mM) and uric acid, thiamine-HCl, tocopherol succinate, riboflavin, Na₂S₂O₈ and NaNO₂ (4 mM) were found 99, 102, 98, 101, 99, 103, 95, 104, 96, 105 and 96%, respectively, all compared to the 100% response, shown from the reference solution of 0.1 mM NADH. Only ascorbic acid (6 mM) exhibits significant positive interference (190%). This problem is acute for the majority of CME’s and could be either minimized by suitable alkaline pretreatment of the samples or eliminated by using ascorbate oxidase [23].

3.4. Flow-through Experiments

The applicability of the DB1-modified electrode as an NADH sensor was investigated by flow injection analysis (FIA). Parameters such as working pH and flow rates of the streams were optimized. The different analytical conditions were implemented with a standard solution of 0.1 mM NADH. Optimum pH value in terms of sensitivity, that is peak height, was found 6.5 (data not shown).

Several flow rates in the range of 0.2–0.70 mL min⁻¹ were tested. An overall flow rate of 0.5 mL min⁻¹ gave fairly high peak intensities and satisfactory sampling throughput (25 samples h⁻¹). A sample volume of 70 μL was selected for performing the experiments, which combines small peak-broadening along with high sensitivity.

Good linearity was achieved within the concentration range 0.002 to 0.1 mM NADH (Figure 6), with a detection limit (signal/noise ratio = 3) 0.002 mM NADH. The correlation coefficient was calculated \( r = 0.9998 \), with a relative standard deviation 1.4% RSD \((n = 11)\) for a standard solution of 30 μM NADH.

The inset in Figure 6 reveals the operational stability of the CME-DB1 sensor, which was obtained by performing successive injections of 30 μM NADH. The sensor shows a continuous decrease of its original activity for the first 15–20 runs, which can be attributed to the successive removal from the electrode surface of any weakly immobilized DB1 molecules. After this stage, which is not recommended for analytical applications, the stability of the sensor is satisfactory and the system can be successfully used to measure more than 50 samples of NADH without intermediate recalibrations. For larger sets of samples, periodical recalibration between runs is strongly suggested, since a 5–7% loss of its activity is observed for every set of 40–50 samples.

The sensors, also, displayed good storage stability if stored dry at + 4 °C when not in use. They retained almost 90% of their initial activity after 2 weeks of storage.

4. References


