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Mediator-modified electrodes for catalytic NADH oxidation: high rate constants at interesting overpotentials

F.D. Munteanu[a], N. Mano[b], A. Kuhn[b], L. Gorton[a,*]

[a]Department of Analytical Chemistry, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
[b]Laboratoire d'Analyse Chimique par Reconnaissance Moléculaire, Ecole Nationale Supérieure de Chimie et de Physique de Bordeaux, 16 avenue Pey Berland, 33607 Pessac, France

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Abstract

Carbon paste electrodes were modified with a nitrofluorenone derivative, 2,4,7-trinitro-9-fluorenone, adsorbed on zirconium phosphate (ZP). After electrochemical reduction of the fluorenone derivative, it turns into a very efficient mediator for electrocatalytic NADH oxidation, with a formal potential of about +250 mV vs. Ag/AgCl. The electrochemistry and the electrocatalytic properties of the mediator were investigated with cyclic voltammetry and rotating disk electrode methodology. The second order rate constant with NADH was evaluated and found to be higher than 10^6 M^{-1} s^{-1}, thus approaching true diffusion controlled currents for NADH oxidation. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: NADH oxidation; Fluorenone; Mediator; Carbon paste electrode; Cyclic voltammetry; Rate constant

1. Introduction

Electrocatalytic reactions of NADH and NADPH at mediator-modified electrodes have been the subject of many studies ever since the first paper published on this topic in 1978 [1–4]. The main reason for these studies has been to be able to very rapidly electrocatalytically oxidise NADH at a low overvoltage but also to ensure that enzymatically active NAD^+ is the product. Various redox mediators commonly used in bioelectrochemistry have been investigated as possible electrocatalysts, but only the 2-electron–proton type acceptors have revealed sufficiently high second order rate constants (k_{obs}) at low overvoltage and at the same time guarantee the production of enzymatically active NAD^+ [4–6]. Possible candidates from virtually all known quinoic groups of redox mediators have been investigated with varying degree of success in terms of lowering the overpotential, reaching high values of k_{obs}, and yielding long-term stability on the electrode surface [2–4]. One of the major drawbacks with these type of mediators (2-electron–proton type acceptors) is the variation of their $E^{\circ\prime}$-value with pH, from which it follows that a change in pH might affect the reaction rate with NADH. This is a common observation with most mediators used that k_{obs} is largely pH dependent [6–11]. One approach to reduce the effect of pH on the response to NADH when the chemically modified electrode is used for analytical purposes would be to reach very high values of k_{obs}, so that the reaction becomes diffusion controlled instead of kinetically controlled. Even though k_{obs} may then still be a function of pH, it remains sufficiently high so that the reaction is still diffusion controlled. Another approach would be to try to find a way to immobilise the mediator onto an electrode surface making its redox reaction on the electrode surface unaffected by the pH of the contacting solution. It is the object of this presentation to show that there should be a way to meet both strategies, i.e., to reach values of k_{obs} higher than 10^6 M^{-1} s^{-1} at an applied potential lower than +300 mV vs. Ag/AgCl, with an immobilised 2-electron–proton-acceptor mediator that has an $E^{\circ\prime}$ independent on pH.

2. Experimental

2.1. Synthesis of zirconium phosphate

Zirconium phosphate (ZP) was synthesised according to a previously reported method [12]. A solution of 1 M

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[*] Corresponding author. Tel.: +46-46-222-7582; fax: +46-46-222-4544.
E-mail address: Lo.Gorton@analykem.lu.se (L. Gorton).

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phosphoric acid (Merck, Darmstadt, Germany) was slowly added onto 500 ml of zirconium chloride (Fluka, Buchs, Switzerland) prepared in 0.1 M HCl (Merck), until a slight excess of phosphoric acid. After that 1.0 M NaOH (Merck) was added until the pH of the solution become higher than 3 [13]. The gelatinous precipitate formed was washed with deionised water (Milli-Q system, Millipore, Milford, MA, USA) 10 times and dried in a thermostated oven for 4 h at 80 °C. The obtained material was ground with a pestle, until the particles size diameter became smaller than 0.05 mm, drizzled in a sieve.

2.2. Immobilisation of the organic dyes

Aqueous solutions of 2,4,7-trinitro-9-fluorenone (Aldrich, structure shown in Fig. 1) with a concentration of 0.001% (m/v) were used for immobilisation. The procedure used to immobilise the mediator was: to 50 ml of dye solution were added 50 mg of ZP and the mixture was shaken for various times up to 1 h. The resulting solid was filtered, washed 10 times with deionised water, and then dried under thermostated conditions at 50 °C for 30 min.

2.3. Preparation of modified carbon paste electrodes

The carbon paste electrodes were prepared by thoroughly mixing 50 mg of graphite (Fluka) and 50 mg of the modified ZP with 25 μl of paraffin oil (Fluka) in an agate mortar. The pastes were put into a cavity of an in-house made Teflon holder using pyrolytic graphite in the bottom for electric contact and then screwed onto a rotating disk electrode device (Tacussel, Villeurbanne, France). The final geometrical area was equal to 0.14 cm^2.

2.4. Electrochemical measurements

All electrochemical measurements were carried out using a typical three electrode electrochemical cell. The modified carbon paste was used as the working electrode, a platinum ring as the counter electrode and an Ag | AgCl as reference electrode. An electrochemical analyser (BAS 100 W, Bioanalytical Systems, West Lafayette, IN, USA) was used connected to a PC microcomputer for potential control and data acquisition for all electrochemical experiments. For some experiments, a Tacussel rotating disk electrode was used. As supporting electrolyte in the electrochemical cell, a 0.1-M KCl solution or a buffer of 0.1 M Tris or 0.1 M HEPES (Sigma) was used. The pH of the solution was adjusted to the required value by adding HCl or KOH (Merck). All solutions were deoxygenated through bubbling nitrogen for 20 min before measurements.

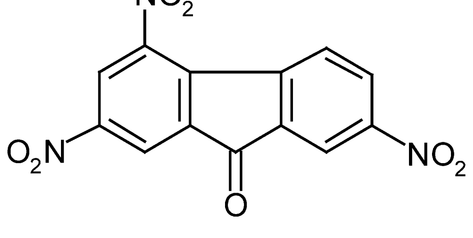

Fig. 1. Structural formula of 2,4,7-trinitro-9-fluorenone.

3. Results and discussion

Among the possible 2-electron–proton type mediators, *ortho*-quinones, phenoxazines, and phenothiazines are the most frequently studied for NADH oxidation [2–4]. Previous observations with carbon paste electrodes modified with these types of 2-electron–proton mediators adsorbed onto titanium or zirconium phosphate (ZP) have revealed some interesting peculiarities such as (i) the $E^{\circ\prime}$ of the mediator becomes virtually independent of the pH of the contacting solution between pH 1 and 9 and (ii) the $E^{\circ\prime}$ becomes much more positive compared with its value at pH 7 in solution or when adsorbed onto carbon [13–19]. As a result of the anodic shift of the $E^{\circ\prime}$, mediators with low reaction rates with NADH in solution become highly active for electrocatalytic NADH oxidation. However, k_{obs} did not reach sufficiently high values to allow a truly diffusion controlled current [20]. Recently, a new type of mediators was studied based on arylnitro derivatives ($ArNO_2$) revealing promising properties in terms of $E^{\circ\prime}$ and k_{obs} [21–24]. The arylnitro compounds as such have no electrocatalytic activity for NADH oxidation. However, when electrochemically reduced the nitro-group is transferred into a hydroxylamino functionality:

$$ArNO_2 + 4e^- + 4H^+ \rightarrow ArNHOH + H_2O \qquad (1)$$

This hydroxylamine can be reversibly oxidised to the corresponding nitroso compound:

$$ArNHOH \leftrightarrow ArNO + 2e^- + 2H^+ \qquad (2)$$

and the ArNO type mediators seem to very efficiently oxidise NADH when adsorbed onto glassy carbon with very promising properties in terms of $E^{\circ\prime}$ and k_{obs} at neutral pH in the order between 10^3 and 10^4 M^{-1} s^{-1} [22–24]. The present study is therefore rationalised as when adsorbed on zirconium phosphate a positive shift of the $E^{\circ\prime}$ is expected and also a much increased k_{obs} value.

Fig. 2 shows a cyclic voltammogram (CV) registered in 0.1 M Tris-buffer at pH 7 for a carbon paste electrode modified with 2,4,7-trinitro-9-fluorenone adsorbed on ZP after being electrochemically reduced (reaction (1)) in a first CV (scan between +450 and −800 mV vs. Ag | AgCl). What is clearly seen is a CV typical of an immobilised redox compound. The $E^{\circ\prime}$ of the adsorbed compound was evaluated by taking the mean value of the anodic and cathodic peak potentials [25,26] and it was found to be +250 mV vs.

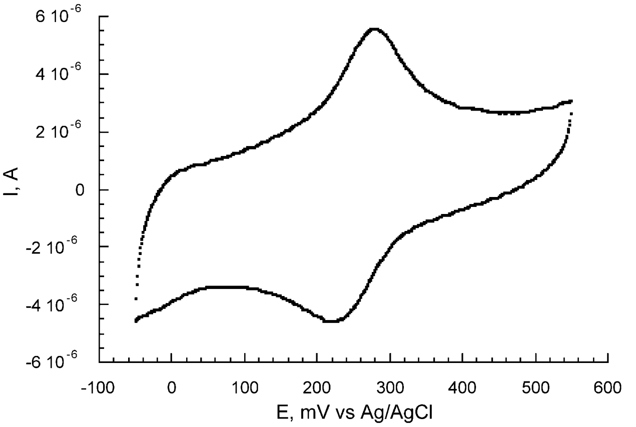

Fig. 2. Cyclic voltammogram obtained for carbon paste modified with 2,4,7-trinitro-9-fluorenone adsorbed on ZP and after being electrochemically reduced. Scan rate 20 mV s^{-1} in 0.1 mol l^{-1} Tris buffer at pH 7.0.

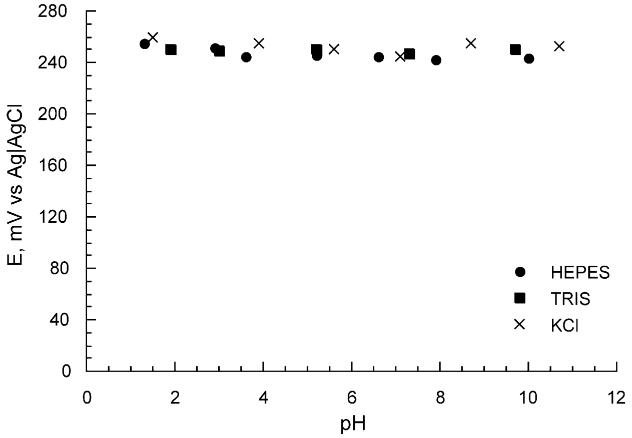

Fig. 3. Variation of the formal potential, $E^{\circ\prime}$, of electrochemically reduced 2,4,7-trinitro-9-fluorenone adsorbed on ZP with pH and buffer constituents.

Ag | AgCl. When compared with the previously published $E^{\circ\prime}$-value of this compound adsorbed on glassy carbon at this pH (− 35 mV vs. Ag | AgCl) [22–24], it is clear that the $E^{\circ\prime}$-value has shifted by almost 300 mV in a positive direction. There is no direct evidence that the irreversible reduction of the nitrogroups leads again to the NO/NHOH couple like on glassy carbon [22–24], and it might be that the immobilisation in the quite acidic ZP matrix changes the electrochemistry of the mediator. However, other possible reactions like reduction into an amino group would not give rise to such a reversible redox behaviour and at the present state we suppose that the catalytically active functionality is again the NO/NHOH couple. The observed anodic shift is in accordance with previous data for a whole series of compounds adsorbed on ZP and TP ranging from flavins, phenazines, phenothiazine, phenoxazines, and viologens [13–20]. When changing the pH of the supporting Tris-buffer for values between 1 and 10, very close to identical $E^{\circ\prime}$-values were registered for the immobilised fluorenone compound (see Fig. 3). This is also in accordance with results obtained for the other redox mediators mentioned above [13–19]. Exchanging Tris for HEPES buffer or KCl solutions with various pHs resulted in very similar $E^{\circ\prime}$-values with a tendency for slightly more positive values in KCl solutions (Fig. 3).

The electrocatalytic activity for NADH oxidation at the fluorenone-ZP-carbon paste (ZPF) electrode was investigated initially with CV. As can be seen in Fig. 4, in the presence of NADH the oxidation peak is drastically increased and the reduction peak decreased (actually it vanished) indicating a very efficient electrocatalytic effect. Catalysis is a result of that NADH diffuses to the electrode surface where it reduces the oxidised mediator (ZPF_{ox}) to form NAD^+ and the reduced form of the mediator (ZPF_{red}) [27,28]:

$$NADH + ZPF_{ox} \overset{k_{obs}}{\rightarrow} NAD^+ + ZPF_{red} \qquad (3)$$

where k_{obs} is the second order rate constant. The reaction is not stochiometrically complete, as it is not clear whether the proton is transferred from NADH to the mediator or to the contacting solution/support material in this case. Reaction (3) is in turn followed by electrochemical reoxidation of ZPF_{red}.

$$ZPF_{red} \overset{k_s}{\rightarrow} ZPF_{ox} + 2e^- + xH^+ \qquad (4)$$

where k_s is the electron transfer rate between the immobilised mediator and the electrode.

In order to obtain kinetic and mechanistic information about the electrocatalytic reactions between NADH and ZPF at pH 7, measurements following the electrocatalytic oxidation of NADH at ZPF-modified carbon paste electrodes were performed at different NADH concentrations using the RDE technique. The choice of pH 7 is rationalised with that most previous values of k_{obs} were reported for pH 7 [4] and

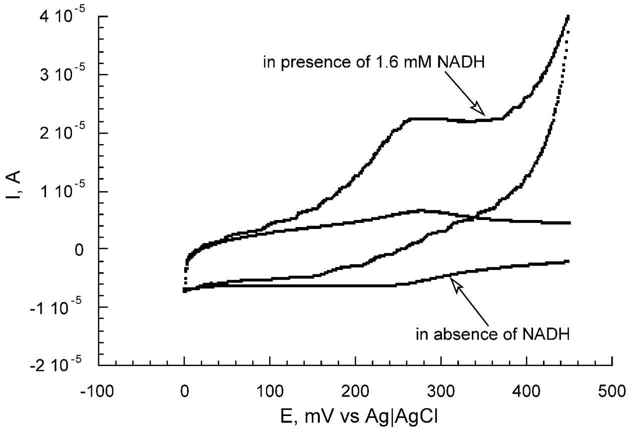

Fig. 4. Cyclic voltammograms obtained for carbon paste modified with electrochemically reduced 2,4,7-trinitro-9-fluorenone adsorbed on ZP in the absence and presence of NADH. Scan rate 20 mV s^{-1} in 0.1 mol l^{-1} Tris buffer at pH 7.0.

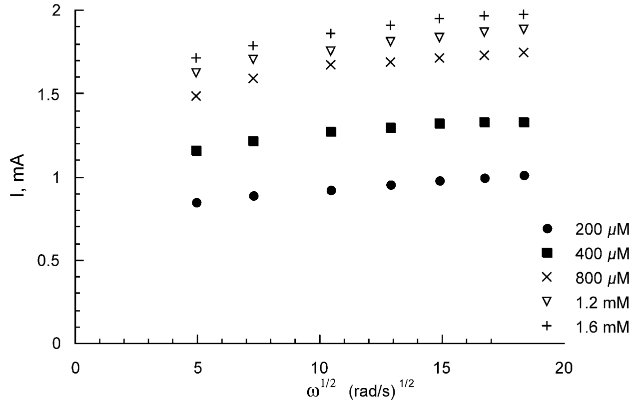

Fig. 5. Levich plot for the steady state electrocatalytic response for a carbon paste RDE modified with electrochemically reduced 2,4,7-trinitro-9-fluorenone adsorbed on ZP at different [NADH]* in 0.1 mol l^{-1} Tris buffer at pH 7.0. Apparent surface coverage 1.5×10^{-10} mol cm^2.

it is also the pH where both NADH and NAD^+ show highest stability when dissolved in aqueous solution [29]. For the investigations on the kinetics between NADH and the immobilised fluorenone, "an apparent surface coverage", Γ, of fluorenone of less than 10^{-9} mol cm^{-2} was chosen [28]. In this report we have deliberately omitted the notation "surface coverage" and rather chosen to use "apparent surface coverage" as for carbon paste electrodes bulk modified with monomeric mediators, in contrast to solid electrodes, it is uncertain how deep into the electrode material the electrolyte can penetrate and thus the electroactivity of the modifier. For higher values of Γ it is anticipated that more than a monolayer of mediator is present [28] and it is from previous results expected that not all mediator molecules would then be easily accessible for NADH and would thus jeopardise a true evaluation of the kinetic constants. Additionally, in all cases, in order to have a global electrode process controlled by the chemical reaction between NADH and the mediator, the applied potential was set 100 mV more positive than the value of the $E^{\circ\prime}$ of ZPF to set the value of k_s high enough to ensure that reaction (4) will not be the rate limiting step [3,15,28].

Fig. 5 shows the Levich plots obtained for the ZPF-modified carbon paste for five different NADH concentrations (from 0.2 to 1.6 mM) at pH 7. The oxidation current of NADH at the modified electrode can be limited by the mass-transfer of NADH to the electrode surface and/or by the kinetics of reaction (3). For the rotating disk electrode, the mass-transfer limited current ($I_{\rm lim}$) depends on the angular velocity, ω, and the bulk concentration of NADH, [NADH]*, according to the Levich equation [26]:

$$I_{\rm lim} = 0.620nFA[\mathrm{NADH}]^* D^{2/3} v^{-1/6} \omega^{1/2} \quad (5)$$

where n denotes the number of electrons, F the Faraday constant, v the kinematic viscosity, and D the diffusion coefficient, respectively. For calculations the values of D and v were 2.4×10^{-6} cm^2 s^{-1} [30] and 0.01 cm^2 s^{-1} [26], respectively.

For a mediated, catalysed reaction, the kinetically limited current ($I_{\rm kin}$) is generally given by the following expression:

$$I_{\rm kin} = nFAk_{\rm obs}\Gamma^*[\mathrm{NADH}]^* \quad (6A)$$

where Γ^* is the total coverage of the fluorenone. For the special case of a rotating disk electrode with the restrictions given, Eq. (6A) reverts to:

$$I_{\rm kin} = \frac{nFAk_{\rm obs}\Gamma^*[\mathrm{NADH}]^* D^{2/3}\omega^{1/2}}{D^{2/3}\omega^{1/2} + 1.61v^{1/6}k_{\rm obs}\Gamma^*}. \quad (6B)$$

As is obvious from Fig. 5, there are some kinetic restrictions in the overall reaction. As the applied potential is set at +100 mV more positive than the $E^{\circ\prime}$-value of immobilised fluorenone the reoxidation reaction of reduced ZPF (reaction (4)) cannot be rate limiting, but rather reaction (3). An electrode consisting of plain ZP-modified carbon paste did not reveal any catalytic current at the same applied potential. Replotting the data in Koutecky–Levich co-ordinates yields perfectly linear plots as revealed in Fig. 6. The general expression for the overall current, I, is given by:

$$I^{-1} = I_{\rm lim}^{-1} + I_{\rm kin}^{-1}. \quad (7A)$$

The Koutecky–Levich equation for the catalytic reaction occurring at mediator-modified electrodes is then with the restrictions given (inversion of Eq. (6B) [31,32]):

$$\frac{1}{I} = \frac{1}{nFAk_{\rm obs}\Gamma^*[\mathrm{NADH}]^*} + \frac{1}{0.620nFAv^{-1/6}D^{2/3}[\mathrm{NADH}]^*}\,\frac{1}{\omega^{1/2}}. \quad (7B)$$

From the slope, the number of electrons (n) participating in the reaction can be evaluated and knowing that then from the intercept $k_{\rm obs}$ (once the Γ^* is known and evaluated by integrating the area under the CV waves) can also be evaluated. For this case, the calculated value of the number of electrons was close to 2 (Table 1). A second way to

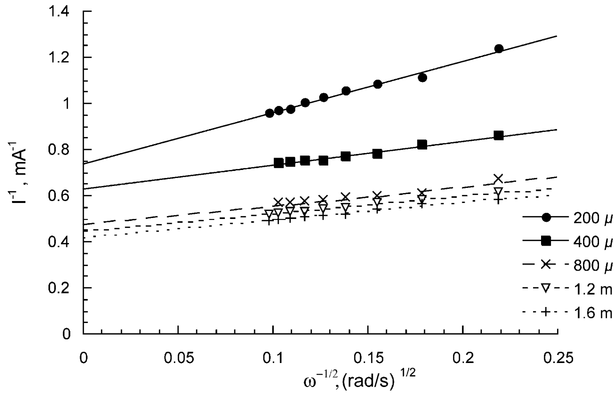

Fig. 6. Koutecky–Levich plots of the same experimental data shown in Fig. 5.

Table 1
Electrochemical and kinetic parameters of electrochemically reduced 2,4,7-nitro-9-fluorenone adsorbed on ZP for NADH electrooxidation (experimental conditions: 0.1 M Tris buffer pH 7.0)

Parameter	2,4,7-Trinitro-9-fluorenone
$E^{\circ\prime}$ (mV)	+250
Γ (mol/cm^2)	1.50×10^{-10}
K_M (mM)	0.20
k_{obs} (M^{-1} s^{-1})	3.20×10^6
k_{+2} (s^{-1})	635
n	2.30

evaluate n is to plot the slope of the Koutecky–Levich plots vs. [NADH]*, then expected to yield a straight line, from which n was also evaluated close to 2. The values of k_{obs} were, however, strongly dependent on [NADH]* for the ZPF-modified carbon paste as revealed in Fig. 7, and this is in accordance with previous results shown for virtually all mediator-modified electrodes used for catalytic NADH oxidation [2–4,8] and has been explained by that the actual reaction between NADH and the mediator occurs through forming a charge transfer (CT) complex before yielding the reaction products. Reaction (3) needs, thus, to be rewritten as:

$$\mathrm{NADH} + \mathrm{ZPMed}_{ox} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} \overset{\overset{k_{obs}}{\longrightarrow}}{\mathrm{CT}-\mathrm{complex}} \overset{k_{+2}}{\leftrightarrow} \mathrm{NAD}^+ + \mathrm{ZPMed}_{red}. \quad (8)$$

This type of reaction mechanism is closely related to the Michaelis–Menten kinetics, and the Michaelis–Menten constant (K_M) can be defined as:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}}. \quad (9)$$

The heterogeneous second order reaction rate, k_{obs}, for any concentration of NADH can thus be expressed as [8,9]:

$$k_{obs} = \frac{k_{+2}}{K_M + [\mathrm{NADH}]^*}. \quad (10)$$

Substitution into Eq. (7B) gives the following version of the Koutecky–Levich equation:

$$\frac{1}{I} = \frac{1}{nFA\left[\frac{k_{+2}}{K_M + [\mathrm{NADH}]^*}\right]\Gamma^*[\mathrm{NADH}]^*} + \frac{1}{0.620nFA\upsilon^{-1/6}D^{2/3}[\mathrm{NADH}]^*}\,\frac{1}{\omega^{1/2}}. \quad (11)$$

Inverting Eq. (10) yields an expression:

$$\frac{1}{k_{obs}} = \frac{K_M}{k_{+2}} + \frac{[\mathrm{NADH}]^*}{k_{+2}} \quad (12)$$

Plots of $1/k_{obs}$ vs. [NADH]* yielded in all cases linear relationships (see Fig. 7) strongly supporting the belief that a CT-complex is formed between the mediator and NADH. From the slope of these graphs values of k_{+2} can be calculated, and by extrapolation to zero NADH concentrations ([NADH] = 0*), i.e., from the intercept, values of K_M can be evaluated. As shown in Table 1, k_{obs} was found to be 3.20×10^6 M^{-1} s^{-1}, k_{+2} 635 s^{-1}, and K_M 0.2 mM.

The previously highest reliable values of k_{obs} were reported for thionine covalently bound to self-assembled monolayer of 3,3'-dithiobis(succinimidylpropionate) on Au ($k_{obs} = 1.2\ 10^5$ M^{-1} s^{-1}) with an $E^{\circ\prime}$ of +240 mV vs. Ag | AgCl (pH 7.0) [33] and for electropolymerised 1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane on glassy carbon ($k_{obs} = 3.6\ 10^5$ M^{-1} s^{-1}) with an $E^{\circ\prime}$ of +135 mV vs. Ag | AgCl (pH 7.4) [34]. As was indicated previously [4,6,8] there seems to be a linear correlation between log k_{obs} vs $E^{\circ\prime}$ (or rather $\Delta E^{\circ\prime}$). Actually, there seem to be two linear relationships between published log k_{obs} and $E^{\circ\prime}$ [4], one with a higher slope and the other with a lower slope. For thionine covalently bound to self-assembled monolayer of 3,3′-dithiobis(succinimidylpropionate) on Au, log k_{obs} falls on the line with the lower slope [4], whereas for both electropolymerised 1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane on glassy carbon and the present case of electrochemically reduced 2,4,7-trinitro-9-fluorenone with an $E^{\circ\prime}$ of +250 mV, the log k_{obs} falls closer on the line with the higher slope.

In comparison with previously published values for other mediator-electrode systems, one can conclude that the here measured k_{obs} value is among the highest ones reported so far [4]. This opens up promising possibilities for practical applications where such high rate constants are indispensable in order to work under mass transfer limited conditions. This could lead to biosensors with low detection limit, a short response time and virtually no dependence on the pH of the sample. However, even though the value of k_{obs} is

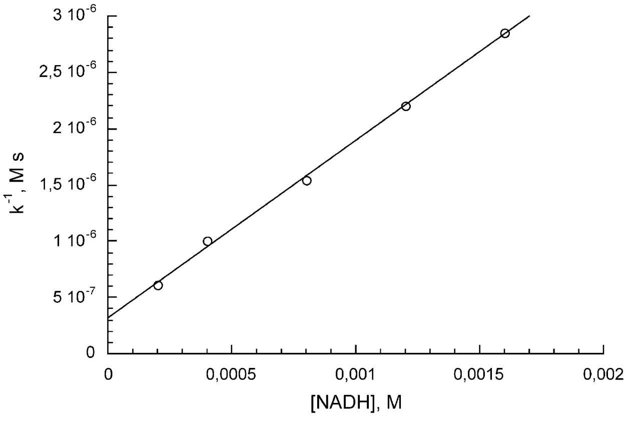

Fig. 7. Variation of $1/k_{obs}$ with [NADH]* for carbon paste modified with electrochemically reduced 2,4,7-trinitro-9-fluorenone adsorbed on ZP. 0.1 mol l^{-1} Tris buffer at pH 7.0. Apparent surface coverage 1.5×10^{-10} mol cm^2.

very high, the value of K_M is rather low (0.2 mM), which means that at these electrodes one would expect linear calibrations characteristics for NADH only for concentrations below $0.1K_M$. This sets a limit to the practical value of this electrode and therefore further investigations are necessary to find alternative mediators that when immobilised onto zirconium phosphate (or similar equivalent materials) have similar k_{obs} but higher K_M (and lower k_{+2}) values.

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