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Bioelectrochemistry 64 (2004) 157–163

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Short communication

A comparison between the use of a redox mediator in solution and of surface modified electrodes in the electrocatalytic oxidation of nicotinamide adenine dinucleotide

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Received 2 October 2003; received in revised form 14 January 2004; accepted 16 January 2004
Available online 20 March 2004

Abstract

Cyclic voltammetry was successfully applied to study the oxidation of nicotinamide adenine dinucleotide (NADH) both in homogeneous and heterogeneous phase. The first case was realized with a solution containing *p*-methylamino-phenolsulphate (MAP) as redox mediator and the diaphorase (DI) from *Clostridium kluveri* as enzyme while the second one by using both a glassy carbon (GC) and a carbon nanotube paste (CNTP) electrode modified with electrodeposited films derived from 3,4-dihydroxybenzaldehyde (3,4-DHB). Such systems were successively coupled with glucose dehydrogenase (GDH) reaction to realize the redox chain present in glucose biosensors. A critical comparison of the two systems was also reported.

Keywords: NADH oxidation; Cyclic voltammetry; *p*-methylamino-phenolsulphate; 3,4-Dihydroxybenzaldehyde; Modified electrode; Carbon nanotube paste electrode

1. Introduction

The electrochemical oxidation of β-nicotinamide adenine dinucleotide (NADH) at low overpotentials is of great importance in biosensor applications because of its role of coenzyme for over 300 dehydrogenase enzymes. However, at solid electrodes the reaction takes place at a very high overpotential, larger than 0.6 V (vs. Ag/AgCl) [1,2]. Oxidation of NADH at lower overpotentials can be successfully achieved by using a redox mediator which can be present in solution or confined at the electrode surface. In the former case, the electron transfer between NADH and the oxidized mediator is quite slow [3] and the diaphorase reaction is usually utilized to enhance the reaction rate [4–11]. On the contrary, in the second case no diaphorase reaction is needed as the kinetics at the modified electrode is much faster. Among the redox mediators used for this purpose thionine [12], phenazine [13] and phenoxazine [14–16] derivatives, *o*-quinones [17,18] and nitrofluorenone compounds [19–22] are most frequently employed simply adsorbed onto carbon electrodes or as electrodeposited films [23–25].

In this work, we study the NADH oxidation by using a bare glassy carbon electrode with the MAP/DI system in solution and both a glassy carbon and a carbon nanotube paste electrode modified with an electrodeposited film derived from 3,4-dihydroxybenzaldehyde (3,4-DHB). The effects of the different kinetics on amperometric biosensor applications are also discussed.

2. Experimental

2.1. Materials

β-NADH, NAD^+, *p*-methylamino-phenolsulphate (MAP) and D-glucose, diaphorase from *Clostridium kluveri* (DI, E.C. 1.8.1.4.) and glucose dehydrogenase from *Bacillus megaterium* (GDH, E.C. 1.1.1.47) were purchased from Sigma (St. Louis, MO, USA). MAP solutions were carefully protected from light. The enzyme solutions were used as received. 3,4-DHB (97% purity), single-wall carbon nanotubes Carbolex (purity 50–70%) were purchased from

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doi:10.1016/j.bioelechem.2004.01.002

Aldrich (Steinheim, Germany). Mineral oil was obtained from Fluka (Buchs, Switzerland).

All other chemicals were from Carlo Erba (Milan, Italy).

All solutions were prepared with high purity water produced by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Cyclic voltammetric measurements were performed using an Autolab computer-controlled potentiostat (Eco Chemie, Utrecht, Netherlands). All experiments were carried out using a 10 ml conventional three-electrode cell. A glassy carbon (GC) and a carbon nanotube paste (CNTP) electrode (both 3 mm diameter) were used as working electrodes, a platinum disk and a Ag/AgCl/KCl(sat.) as the counter and the reference electrode, respectively. All potentials are referred to the Ag/AgCl/KCl(sat). electrode. All measurements were carried out at 22.0 ± 0.2 °C using a thermostatic bath and the supporting electrolyte was a 0.1 M phosphate buffer at pH = 7.0. For measurements with diaphorase, the solution was carefully deaerated before use and maintained under a nitrogen flow during the voltammetric experiment.

2.3. Electrode pretreatment and modification

Before each experiment, GC electrodes were polished with 0.003 μm alumina powder, rinsed with water and sonicated in a water bath for 10 min. CNTP electrodes were prepared by hand-mixing carbon nanotubes and mineral oil with a 60/40 (w/w)% ratio [26]. The electrode modification was carried out by placing the electrodes in a solution containing 1.0 mM 3,4-DHB in 0.1 M phosphate buffer pH = 7.0 at a potential of +0.3 V for 1 min [27–32]. The films so electrodeposited were found to be quite stable with time.

3. Results and discussion

Fig. 1A shows cyclic voltammograms of a solution containing MAP before and after the addition of NADH and of NADH plus diaphorase. The direct oxidation of NADH in the potential range investigated is negligible. The cyclic voltammogram of MAP exhibits a reversible electrochemical behaviour with a peak separation of about 30 mV, typical of a two-electron transfer mediator (curve a). The addition of NADH to the MAP solution causes an increase in the anodic current (curve b). This fact indicates that a direct reaction between NADH and MAP occurs producing a small regeneration of the reduced mediator. A much larger increase of the anodic peak is shown upon addition of the diaphorase to the solution containing MAP and NADH (curve c), thus indicating that the diaphorase effectively accelerates the NADH oxidation. In fact, the

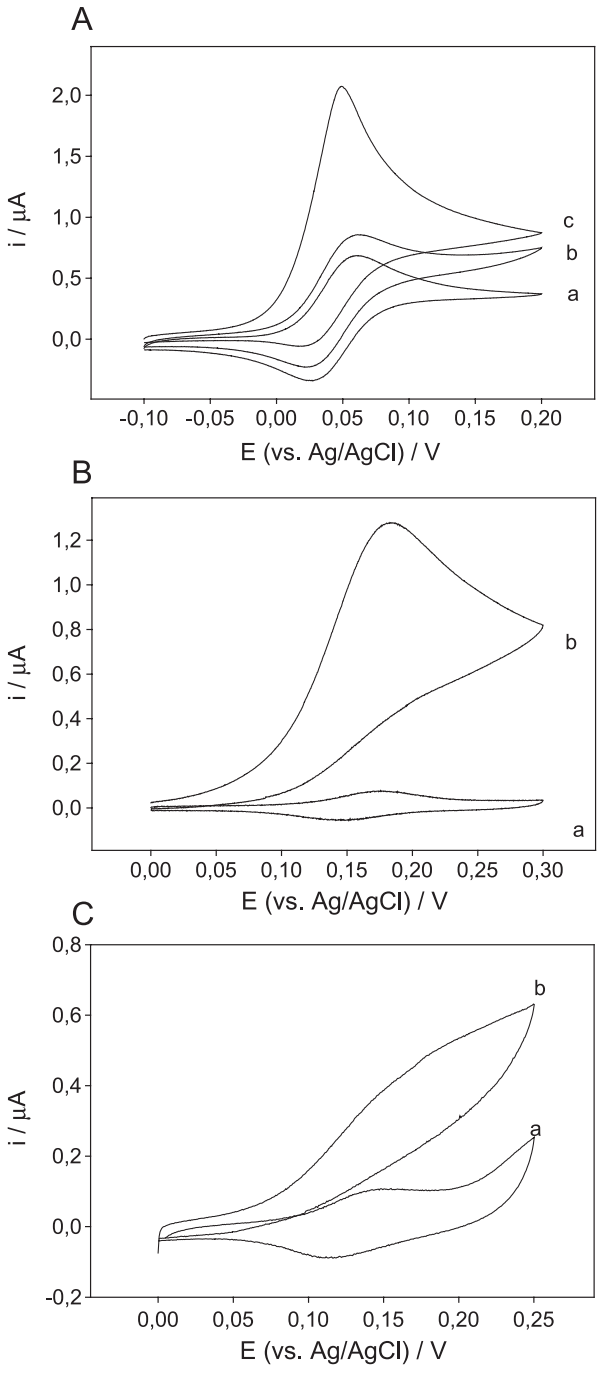

Fig. 1. (A) Cyclic voltammograms of a solution containing: 5×10^{-5} M MAP (a); (a) + 5×10^{-4} M NADH (b); (b) + 5×10^{-6} M DI (c). (B) Cyclic voltammograms for a GC electrode modified with a film derived from 3,4-DHB ($\Gamma^{\circ} = 2.6 \times 10^{-10}$ mol cm^{-2}) in the absence (a) and in the presence (b) of 5×10^{-4} M NADH. (C) Cyclic voltammograms for a CNTP electrode modified with a film derived from 3,4-DHB ($\Gamma^{\circ} = 1.8 \times 10^{-10}$ mol cm^{-2}) in the absence (a) and in the presence (b) of 5×10^{-4} M NADH. Experimental conditions: $v = 2$ mV s^{-1}; GC and CNTP electrode diameters = 3 mm; 0.1 M phosphate buffer pH = 7.0. Curves of A are recorded under anaerobic conditions.

rate constant value of the reaction between NADH and MAP in the presence of diaphorase is of four order of magnitude larger than that obtained in the absence of the

enzyme, as reported in our previous work [11]. The catalytic anodic current depends on the NADH concentration when it is at low concentrations and becomes independent at higher concentrations with resulting sigmoidal voltammetric curves indicating the achievement of the steady-state conditions on the mediator. Therefore, in biosensor applications the NADH concentration which is produced by a preceding reaction catalyzed by a proper dehydrogenase has to be maintained in a suitable ratio [9,11] compared to the mediator concentration in order to guarantee the proportionality between the current signal and the analyte concentration.

Fig. 1B and C shows cyclic voltammograms of a glassy carbon and a carbon nanotube electrode, respectively, both modified with an electrodeposited film of 3,4-DHB in the absence (curves a) and in the presence (curve b) of NADH. As seen the modified electrodes exhibit a quite reversible behaviour (curves a). After addition of NADH, typical electrocatalytic responses are observed (curves b). The voltammograms show an anodic peak current largely en-

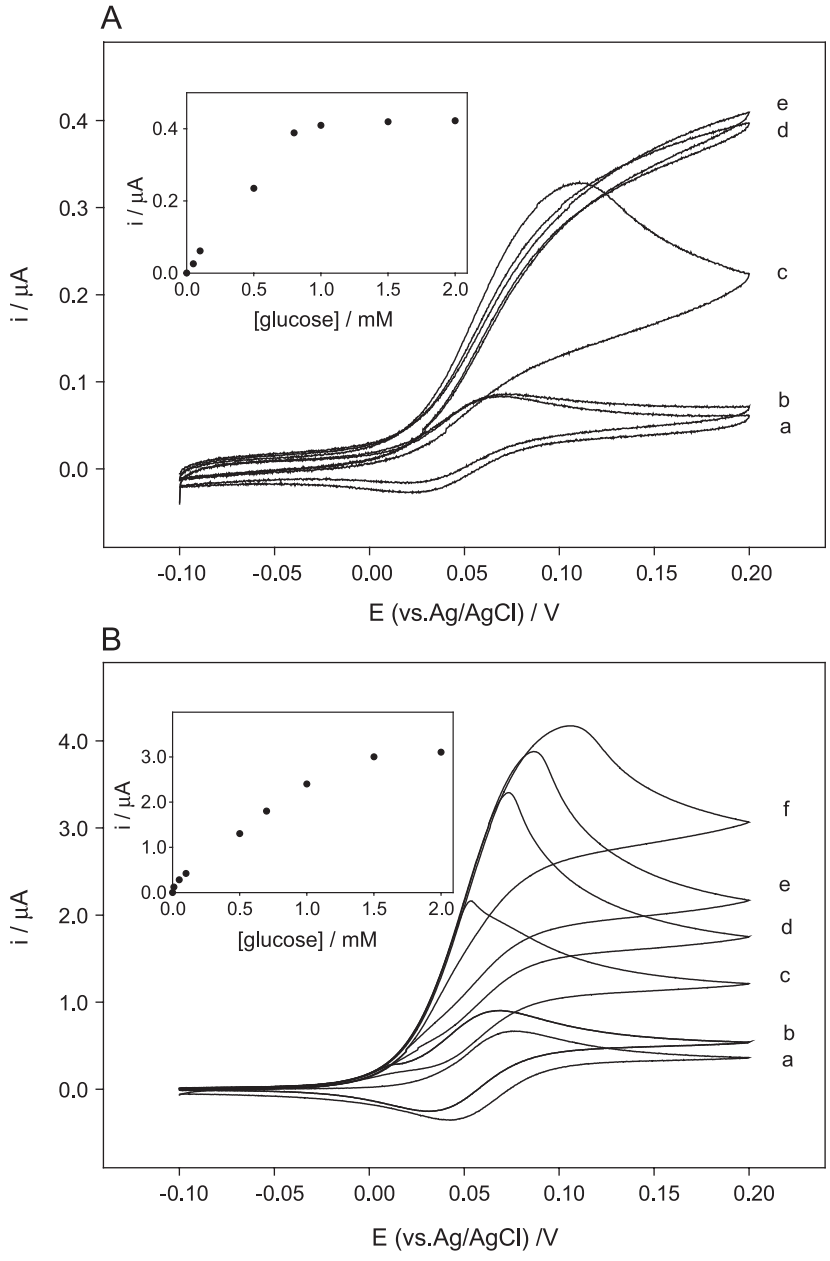

Fig. 2. (A) Experimental cyclic voltammograms of a solution containing 5×10^{-6} M MAP + 5×10^{-6} M DI + 1×10^{-3} M NAD^+ 3×10^{-7} M GDH (a); (a) + 1×10^{-4} M D-glucose (b); (a) + 5×10^{-4} M D-glucose (c); (a) + 8×10^{-4} M D-glucose (d); (a) + 1×10^{-3} M D-glucose (e). (B) As in A but with 5×10^{-5} M MAP. Curve (f) is as curve (a) plus 2×10^{-3} M D-glucose. The insets show the calibration curves of glucose obtained from the relative voltammetric curves. Current values are taken at $E = 0.2$ V. Experimental conditions: $v = 2$ mV s^{-1}; GC electrode diameter = 3 mm; 0.1 M phosphate buffer pH = 7.0; anaerobic conditions.

hanced with no cathodic peak in the reverse sweep with the GC electrode and a lower sigmoidal curve with the CNTP electrode. The heterogeneous rate constants k_1 for the chemical reaction between NADH and the surface-confined mediator were calculated by cyclic voltammetry utilizing the working curve reported by Andrieux and Saveant which reports $i_p/nFSc^\circ_A D_A^{1/2}(n\mathrm{Fv}/RT)^{1/2}$ vs. $\log[lk_1\Gamma^\circ/D_A^{1/2}(nFv/RT)^{1/2}]$ where c°_A is the bulk substrate concentration, l is the number of monolayers in the film and Γ° is the coverage of the film [33]. k_1 values of 1.4×10^3 and 6.3×10^2 M^{-1} s^{-1} were determined for the GC and the CNTP electrode, respectively. The surface coverages of the mediator Γ° were obtained by integration of the voltammetric wave assuming $n = 2$ and resulted to be 2.6×10^{-10} mol cm^{-2} for the GC electrode and 1.8×10^{-10} mol cm^{-2} for the CNTP electrode (values both consistent with $l = 1$). The k_1 value for the GC electrode is in very good agreement with that obtained with rotated disk electrode voltammetry by Pariente et al. [30,31].

Comparing the NADH oxidation peaks obtained with the two experimental approaches, i.e. homogeneous and heterogeneous ones, it is easy to observe that the peak current value in the second case with GC electrode (curve b, Fig. 1B) is 1.6 times larger than that obtained with MAP in the absence of diaphorase (curve b, Fig. 1A) at the same NADH concentration and that the ratio of the peak current obtained with and without NADH (curves b and a, respectively, in Fig. 1A and B) is 15 times larger at the modified electrode than at the bare one. As for the peak potential, at the modified electrode the peak current is recorded at about 0.1 V more positive potential than for the system with MAP in solution but it remains at a sufficiently low potential to avoid most interferences. As for the second heterogeneous system studied, i.e. the CNTP modified electrode, the catalytic current at the same NADH concentration is much lower than that recorded at the GC modified electrode (curve b in Fig. 1C) [34]. The decrease of k_1 on passing from GC to CNTP electrode is in qualitative agreement with the change of curve b in shape and height. The peak shaped curve still indicates a mass transfer control while the S-shaped curve points out the achievement of nearly steady-state conditions.

In Figs. 2A,B and 3, the homogeneous and heterogeneous systems were coupled to the glucose dehydrogenase reaction to realize the redox chain present in glucose biosensors. The NAD^+ reduction by the enzymatic reaction catalyzed by the proper dehydrogenase leads to the formation of NADH which can be recycled in a catalytic electrochemical reaction.

Fig. 2A and B show the voltammetric curves relative to the MAP/DI system with different mediator concentrations. It is possible to observe that a glucose concentration of 8×10^{-4} M is already enough to reach steady-state conditions on the mediator when a MAP concentration of 5×10^{-6} M is employed (curve d in Fig. 2A) while even a glucose concentration of 2×10^{-3} M is not enough to obtain the sigmoidal curve when a 10 times larger MAP concentration is employed (curve f in Fig. 2B). This is due to the fact that in the experimental conditions adopted in Fig. 2A the NADH concentration generated by the GDH reaction is sufficiently large to assure its constancy in the vicinity of the electrode surface. As a consequence the

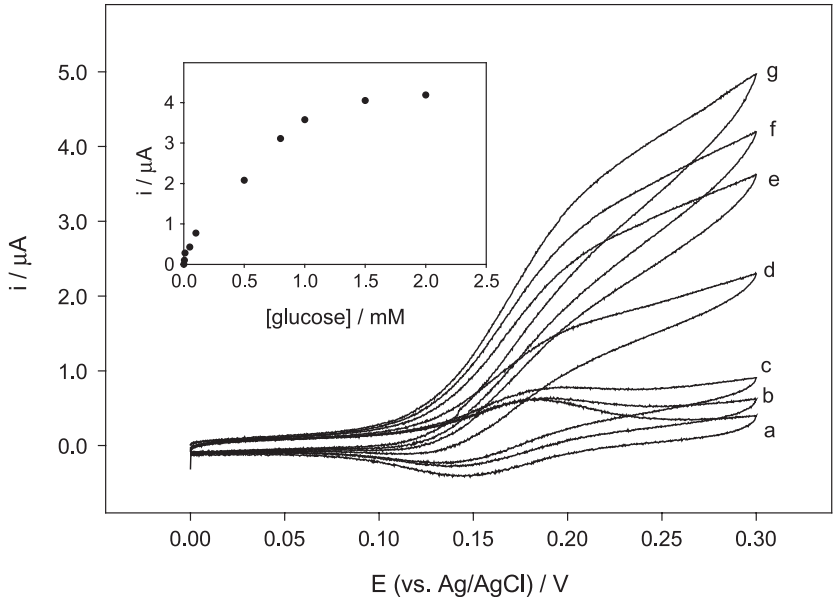

Fig. 3. Experimental cyclic voltammograms for a GC electrode modified with a film derived from 3,4-DHB ($\Gamma^\circ = 2.6 \times 10^{-10}$ mol cm^{-2}) in the presence of 1×10^{-3} M NAD^+ 3×10^{-7} M GDH and the following increasing concentrations of D-glucose: 1×10^{-6} M (a); 5×10^{-5} M (b); 1×10^{-4} M (c); 5×10^{-4} M (d); 8×10^{-4} M (e); 1×10^{-3} M (f); 2×10^{-3} M (g). The inset shows the calibration curve of glucose obtained from the voltammetric curves. Current values are taken at $E = 0.25$ V. Experimental conditions: $v = 2$ mV s^{-1}; GC electrode diameter = 3 mm; 0.1 M phosphate buffer pH = 7.0.

catalytic current becomes independent on the NADH concentration at relatively low glucose concentrations and the dynamic linear interval of the relative glucose biosensor is quite limited. This statement is proved by the fact that the same generated NADH concentration does not produce steady-state currents when a larger mediator concentration is utilized (Fig. 2B). The determining parameter is the ratio between the concentration of NADH coming from NAD^+ via dehydrogenase catalysis and the concentration of the mediator so that for each mediator there is a critical value of the ratio [NAD^+]/[mediator]. Following this argument, two different experimental conditions can be expected when the glucose concentration is larger than NAD^+ concentration: (i) the NADH generated is so large to overcome the critical ratio: a steady-state current is obtained and it does not change with further glucose additions; (ii) the critical ratio is not reached: the catalytic current is peak shaped with a tail due to the depletion of NADH. Further additions of glucose promote a second homogeneous catalytic reaction between electrochemically regenerated NAD^+ and glucose which can annihilate the NADH depletion making the anodic current steady-state in character. Fig. 4 checks the existence of the catalytic regeneration of NADH. The presence of an excess of glucose changes curve a into b at the same overall NAD^+/NADH concentration. As a consequence a possible way to extend the dynamic range of the biosensor could be the use of a larger mediator concentration. Actually, for the two cases investigated the detection limits and the linear dynamic ranges resulted to be 5×10^{-5}, 1×10^{-4}–8×10^{-4} and 1×10^{-5}, 5×10^{-5}–1.0×10^{-3} M, respectively (insets in Fig. 2A and B).

A further remark on the use of the MAP/DI system comes from the circumstance that diaphorase was found to be able to use also molecular oxygen as electron acceptor [35]. This means that some of the NADH generated by the GDH reaction can be oxidized via a mediator independent pathway thus lowering the sensitivity of the biosensor. As a consequence a biosensor based on the MAP/DI/NADH cycle should work in anaerobic conditions.

Fig. 3 shows the voltammetric curves obtained with a GC electrode modified with a film derived from 3,4-DHB. At the beginning, when the glucose concentration is quite low a series of diffusion-limited increasing waves is obtained (curves a–c). Further glucose additions generated steady-state responses both in defect (curves d–f) and in excess (curve g) of glucose in respect to NAD^+ concentration. This unexpected result can be ascribed to the contribute of catalytic regeneration of NADH due to the presence of an excess of glucose. These considerations can explain why a glucose biosensor realized with a 3,4-DHB film shows a larger linear dynamic interval (1×10^{-5}–1×10^{-3} M) compared to the biosensors realized with the MAP/DI system (insets in Figs. 2A,B and 3). The relative detection limit was found to be 5×10^{-6} M, quite lower than the other two values previously reported. On the contrary, the voltammetric curves realized with the modified CNTP electrode did not show any significant increase in the catalytic current with increasing glucose concentration because of the too low kinetics between NADH and the surface confined mediator (curves not shown).

On the basis of the above reported experimental results and of theoretical considerations utilizing the well-known

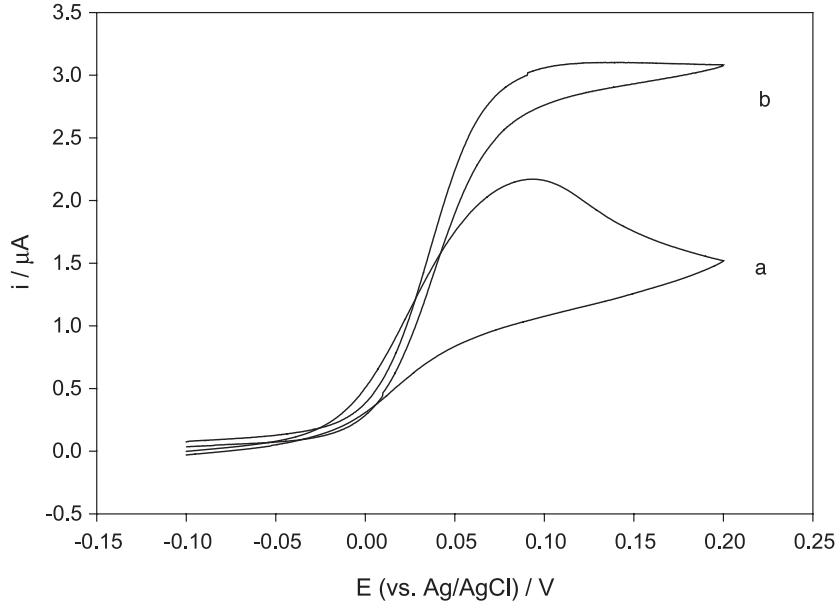

Fig. 4. Experimental cyclic voltammograms of a solution containing 5×10^{-5} M MAP + 5×10^{-6} M DI + 1×10^{-3} M NADH (a) and 5×10^{-5} M MAP + 5×10^{-6} M DI + 5×10^{-4} M NAD^+ 3×10^{-7} M GDH + 5×10^{-2} M D-glucose after the addition of 5×10^{-4} M NADH (b). Experimental conditions as in Fig. 2.

equations for the catalytic currents relative to homogeneous and heterogeneous systems it is worthwhile to enlighten that two different experimental situations are possible for both systems studied. In the homogeneous system, i.e. with the MAP/DI system, the maximum obtainable current value depends on the mediator concentration. Two experimental cases are possible depending on the [NAD^+]/[mediator] ratio as above reported. When this ratio is large the catalytic current assume steady-state characteristics at relatively low amounts of glucose as a NADH concentration suitable to achieve the steady-state conditions is already produced. The current becomes independent on the NADH generated concentration according to the following equation: $I_{lim} = nFS(D_M k c^\circ_{ENZ})^{1/2} c^\circ_M$ where D_M and c°_M are the diffusion coefficient and the bulk concentration of the mediator, respectively, k is the second order rate constant between enzyme and mediator and c°_{ENZ} is the bulk concentration of the enzyme [10]. For this reason, the biosensor cannot detect any further glucose addition. When the [NAD^+]/[mediator] ratio is quite low, the steady-state conditions can be reached only after the addition of large amounts of glucose because of the catalytic regeneration of NADH. The steady-state conditions are not reached because the NADH generated is really in excess, but as a consequence of a balance between consumption and regeneration of NADH. Of course this is possible until the intrinsic kinetic limit of the GDH reaction is not overcome. In the heterogeneous system, i.e. with the 3,4-DHB electrodeposited film, the NAD^+ concentration is now the parameter which can determine the maximum obtainable current value. Also in this case two experimental situations are possible. When the NAD^+ concentration is sufficiently large the steady-state conditions are reached but the catalytic current is still dependent on the NADH bulk concentration (c°_{NADH}) according to the following equation: $I_{lim} = k_1 l \Gamma^\circ F S c^\circ_{NADH}$ [33]. The catalytic current continues to increase as long as NADH is bulky generated from glucose via the GDH reaction (curves a–f in Fig. 3). When the glucose concentration is larger than the NAD^+ one, glucose is still detectable owing to the NADH catalytic regeneration, likely in the MAP/DI system when a small [NAD^+]/[mediator] ratio is employed (curve f in Fig. 2B).Therefore, electrodeposited film biosensors, free from rate-determining steps between diaphorase and mediator, can in principle detect the analyte concentration also after the achievement of steady-state conditions provided that the coenzyme can be still generated.

4. Conclusions

The present study illustrates the advantages of the use of a GC electrode modified with an electrodeposited film derived from 3,4-DHB compared to a bare GC electrode with MAP plus diaphorase in solution in terms of higher sensitivity, lower detection limit, larger linear dynamic range, less waste of mediator and no oxygen interference. A CNTP electrode analogously modified with 3,4-DHB has also been studied but it does not exhibit any significant improvement owing to a lower kinetics between NADH and the confined mediator.

The analysis of our experimental data together with theoretical considerations based on the catalytic limiting current equations for homogeneous and heterogeneous systems allows to elucidate the different possible operative conditions whose knowledge is of great importance for a better understanding of biosensor behaviour.

Acknowledgements

The financial support of the National Council of research (CNR) and the Ministry of University and Scientific Research (MURST) is acknowledged.

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