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# Electrochemical study in both classical cell and microreactors of flavin adenine dinucleotide as a redox mediator for NADH regeneration

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## A B S T R A C T

The electrochemical reduction of flavin adenine dinucleotide (FAD) is studied in a classical electrochemical cell as well as in two types of microreactors: the first one is a one-channel reactor and the other one, a multichannel filter-press reactor. The ultimate goal is to use the reduced form of flavin (FADH<sub>2</sub>), in the presence of formate dehydrogenase (FDH), in order to continuously regenerate the reduced form of nicotinamide adenine dinucleotide (NADH) for chiral syntheses. Various voltammetric and adsorption measurements were carried out for a better understanding of the redox behavior of the FAD as well as its adsorption on gold. Diffusivity and kinetic electrochemical parameters of FAD were determined.

## 1. Introduction

NAD<sup>+</sup>/NADH (nicotinamide adenine dinucleotide, oxidized and reduced forms) is a coenzyme that is involved as a cofactor in several [1–6] dehydrogenation reactions (catalyzed by enzymes such as dehydrogenases). Because of the high cost of the pyridinic cofactor (NADH), as well as for its very interesting reactivity, continuous regeneration represents an important industrial challenge. The literature contains many works concerning this coenzyme regeneration through chemical [7], photochemical [8], enzymatic [9,10], biological or electrochemical [11,12] means.

Fig. 1 shows the reaction scheme for the indirect electrochemical regeneration of NADH, using FAD/FADH<sub>2</sub> as a redox mediator, in the presence of formate dehydrogenase (FDH), as well as its enzymatic reaction, using L-lactate dehydrogenase (LDH), on achiral pyruvate to synthesize L-lactate.

Flavin adenine dinucleotide (FAD/FADH<sub>2</sub>) is an interesting mediator for syntheses [13–15], as compared to other molecules such as methyl viologen, rhodium complexes, etc. The main advantages of this biocompatible molecule are its stability and its activity after several cycles [14]. Moreover, NADH oxida-

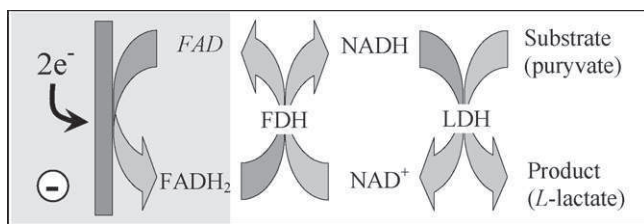
tion through FAD reaction (1) is spontaneous at pH 7.0 [15].



The ultimate objective of this work is to carry out chiral syntheses; the electrochemically reduced form of flavin was used to continuously regenerate the reduced form of nicotinamide adenine dinucleotide, the required cofactor for these enzymatic reactions. The main reason for choosing an indirect method is that the direct electro-regeneration of NADH seems not to be selective [17–19]. In fact, the challenge is to shift the reaction (1) in the reverse direction, i.e., the NADH production (–1); nevertheless, because of the negative value of the Gibbs energy, this reverse reaction is not spontaneous from the thermodynamic point of view [16].

In our previous works [3–6,19], the feasibility of continuous electrogeneration of NADH in a microreactor, using FAD/FADH<sub>2</sub> as a redox mediator, was experimentally demonstrated; in addition, mass balance experiments (and theoretical simulation) were carried out in order to use electrogenerated NADH for the syntheses of chiral L-lactate from achiral pyruvate. The aim of this study is to focus more specifically on the electrochemical behavior of the flavin on a gold electrode in phosphate buffer media in order to get a better understanding of the reversibility/irreversibility of this system, to access these physicochemical parameters and finally, to define the limiting step of its electrochemical reduction.

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**Fig. 1.** Reaction scheme for the indirect enzyme-catalyzed electrochemical regeneration of NADH, using FAD/FADH<sub>2</sub> as redox mediator, in presence of formate dehydrogenase (FDH), as well as its enzymatic reaction, using the L-lactate dehydrogenase (LDH), on the achiral pyruvate to synthesize L-lactate.

## 2. Experimental

The chemicals used, i.e., enzymes, phosphate buffer, flavin adenine dinucleotide disodium salt hydrate (FAD and FADH<sub>2</sub>) and nicotinamide adenine dinucleotide disodium salt (NAD<sup>+</sup> and NADH), were supplied by Acros, Sigma–Aldrich, Merck or Prolabo.

The enzyme L-lactate dehydrogenase (L-LDH) is obtained from rabbit muscle and the formate dehydrogenase (FDH), from yeast *Candida Bodinii*.

A phosphate buffered solution (K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, total concentration: 0.05 mol/L, pH 7.0) was used as supporting electrolyte.

The electrochemical apparatus used was an Autolab PGSTAT 30 and the experiments were performed using various setups:

- Electrochemical kinetic experiments and the determination of physical parameters were performed using a three-electrode system immersed in a 50 cm<sup>3</sup> isothermal cell under inert atmosphere (nitrogen, 1 atm). Electrode potentials were measured with respect to a saturated calomel electrode (SCE) connected to a Luggin capillary located near (3–4 mm) the working electrode and containing the electrolyte in large excess. The working electrode was a wire or a rotating gold disk, carefully polished with abrasive paper (Norton A621 4/0) before each experiment. A platinum counter electrode was fitted into a separate compartment. Preliminary chronopotentiometric measurements were performed using a ferricyanide 5 mM/KCl 0.2 M solution in order to determine the electrochemically active surface of the working electrode (a disk with a geometrical surface area  $S_g$ : 2.9 mm<sup>2</sup>). Diffusivity of ferricyanide was assumed to be  $7.6 \times 10^{-10}$  m<sup>2</sup>/s [19]. Results indicate no dependence of the Sand factor on the current ( $0.8 \times 10^{-5} < (I \cdot \sqrt{\tau}/C_0)_{A_{S0.5}m^3/mol} < 0.9 \times 10^{-5}$ ) and allow determining the electrochemically active surface area of the disk electrode ( $S_{EA} = 5.0$  mm<sup>2</sup>); despite suitable polishing, the ratio  $S_{EA}/S_g$  reaches a value of 1.7.
- Two types of electrochemical microreactors were involved in this study.

The first of these was a previously designed one microfluidic Y-shaped channel [5]. The Y-shaped channel with integrated electrodes was sandwiched between gasket layers of PDMS and the dimensions of the microchannel are as follows: length = 3 cm; height = width = 1000 μm. Both anolyte and catholyte flowed along the same channel. The cathode and anode were made of gold sputtered on polycarbonate blades. Moreover, a wolfram (W) wire immersed in the catholyte inlet compartment was used as a pseudo-reference/comparison electrode; its potential was constant due to the constant composition of the feed. In all experiments, the flow rate  $\phi$  was regulated by a syringe pump with typical values in the range of 0.006–1 mL/min.

The second device is a previously designed multichannel filter-press microreactor [3–4,6]. Two rectangular plates (5 cm × 5 cm × 0.2 cm), made out of gold and platinum, were used

respectively as working and counter electrodes, separated by a Nafion proton exchange membrane (N1135). Each electrode contains 130 semi-cylindrical channels and their specific area reaches 250 cm<sup>-1</sup>. A platinum wire, which opened into the electrolyte inlet channel, was used as a comparison electrode.

In both microreactors, it is difficult to polish the electrodes and to maintain a reproducible state of the surface of the gold working electrode; in order to operate at the same state of the area of the electrode, before performing measurements for curve plots, the solution was flowed within the microreactor for at least five minutes.

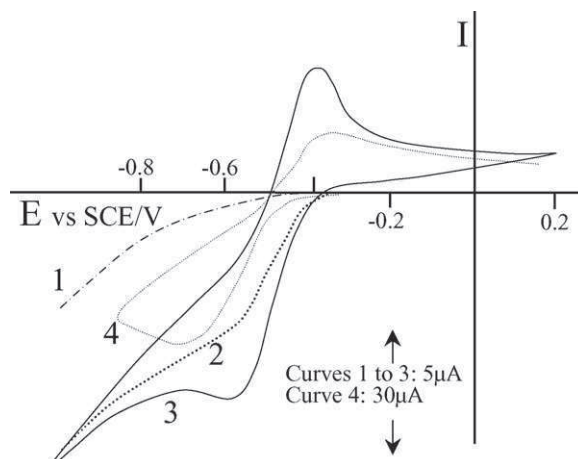
## 3. Results and discussion

### 3.1. Study in a three-electrode classical electrochemical cell

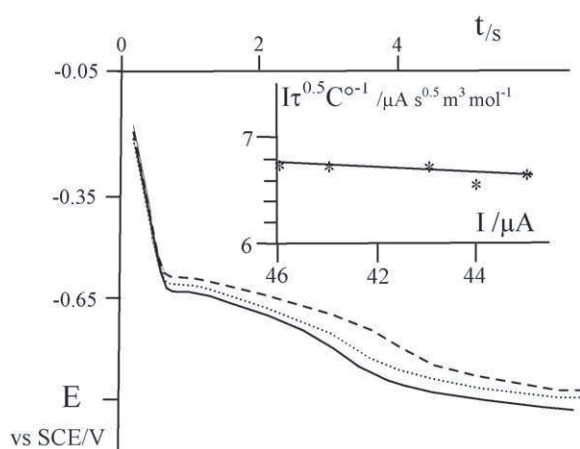
#### 3.1.1. Electrochemical behavior of flavin on a gold wire electrode

Fig. 2 presents current–potential curves obtained on a gold electrode immersed in a flavin solution within a classical electrochemical cell. Curve 2, obtained at steady state conditions (stirring, 5 mV/s), indicates that the FAD reduction occurs at potentials lower than –0.4 V/SCE. Despite a low resolution, the obtained signal seems to contain a diffusion wave (–0.3 to –0.5), the plateau of which does not indicate a constant limiting current. The shape of the curve clearly shows an augmentation of the limiting current in the range of –0.6 to –0.8 V, probably caused by a second electrochemical step. This signal (–0.3 to –0.8 V) was attributed to the flavin bielectronic reduction, in accordance with the results obtained in a thin-layer electrochemical cell by Bergel and Comtat [16].

In addition, flavin adsorption at the electrode may occur. Curve 3, obtained in transient state conditions (no stirring, 100 mV/s), reveals one cathodic peak at –0.6 V/SCE. The curvature of the curve slightly changes in the range of –0.7 to –0.8 V/SCE, just before the reduction of water (approximately –1 V/ref). The reverse scan indicates one anodic peak, the position of which shows a certain irreversible behavior of this electrochemical system ( $\Delta E_{\text{anodic/cathodic peaks}} = 0.17$  V > 0.059 V/n for a reversible system). The comparison, for the anodic and the cathodic signals, of both the magnitude of the current and the amount of charge shows significant differences (~35%), confirming the appreciable irreversibility of this redox system. In addition, for curve 4, obtained



**Fig. 2.**  $I=f(E)$  curves obtained at gold cathode, immediately after immersion in the solution, with flavin adenine dinucleotide solution in phosphate buffer pH 7.0; N<sub>2</sub> 1 atm.; working electrode: gold wire ( $l = 1$  mm;  $d = 0.5$  mm) for curves 1–3, and gold disk ( $d = 2$  mm) for curve 4. Counter electrode: platinum; solution stirred for curves 1 and 2 and no stirred for curves 3 and 4. (1) Residual current, 5 mV/s; (2) FAD 0.08 M, 5 mV/s; (3) FAD 0.008 M, 100 mV/s; (4) FAD 0.01 M, 50 mV/s.



**Fig. 3.** Typical chronopotentiograms obtained on gold disk electrode with a flavin  $10^{-2}$  M solution in phosphate buffer pH 7.0, for various applied currents; counter electrode: platinum. Inset: Sand's parameter against applied current.

with a carefully polished gold disk, the peak-to-peak potential is higher ( $\Delta E_{\text{an./cath. peaks}} = 0.33$  V), indicating a different electrocatalytic behavior. The difference in magnitude between anodic and cathodic current could be caused by:

- a partial or complete adsorption of the different forms of flavin
- a certain irreversibility of the system.

These assumptions are in agreement with literature studies; indeed, many works have been carried out [20,21] on the adsorption of various flavins, especially the flavin mononucleotide (FMN), on electrodes such as carbon, platinum, or mercury. In this work, adsorption of FAD on gold electrodes will be validated in the following paragraphs.

### 3.1.2. Chronopotentiometric measurements for determination of diffusivity of flavin

Chronopotentiometric measurements were carried out on a gold disk electrode ( $S_R/S_g = 1.7$ ) using the Sand equation in order to obtain the diffusivity of flavin in pH 7.0 phosphate buffer. Fig. 3 shows the results obtained with a  $10^{-2}$  M flavin solution for various applied currents; the inset indicates that the Sand parameter remains essentially constant versus applied current; its value ( $6.6 \times 10^{-6} \text{ A s}^{0.5} \text{ m}^3/\text{mol}$ ) allows determining the flavin diffusion coefficient:  $0.61 \times 10^{-10} \text{ m}^2/\text{s}$ .

The same types of experiments were carried out for various concentrations of flavin and the results are presented in Table 1, indicating that the flavin diffusivity decreases with its concentration. Decreasing the concentration by a factor of approximately 1.3 causes an increase in the diffusion coefficient of the same magnitude. Nevertheless, diffusivity seems to become constant for FAD concentrations below 7.5 mM. In addition, FAD diffusivity values are relatively low in comparison with the diffusivities of classical simple molecules (values in the range  $10^{-10}$  to  $10^{-9} \text{ m}^2/\text{s}$ ) because of the relatively high size of the molecule of FAD. Acid/base equilibria between various forms of FAD can cause the evolution of diffusivity with concentration. At the operating pH, the flavin oxidized form is planar, rather than the butterfly form of the reduced

**Table 1**  
Diffusion coefficient dependency with concentration of flavin in phosphate buffer pH 7.0; Results obtained by chronopotentiometry using a gold disk as working electrode (extracted from Fig. 3)  $\text{N}_2$ .

[Flavin]/mM	5.3	7.5	8.6	10.0
$D_{\text{FAD}} \times 10^{+10}/\text{m}^2 \text{ s}^{-1}$	0.82	0.82	0.75	0.62

flavin. Moreover, there are three different forms of flavin: semi, hydro and quinonic. An increase in the concentration shifts the equilibrium and modifies the distribution of the various acidic and basic forms; it is probable that the molecular form interacts by extramolecular hydrogen bonds either with another FAD molecule or with another ionic form of FAD [19]. In addition, interactions could lead to a possible change in the conformation of the flavinic cofactor and consequently, in its solvation degree. Regardless, the size of the resulting entities increases, explaining the decrease of the diffusion coefficient.

### 3.2. Study of the redox behavior of flavin in presence of $\text{NAD}^+$ in microreactors

Previous works carried out on gold [5,6] indicate that, in the cathodic potential range from  $-0.1$  to  $-1$  V of W (or Pt) wire, only the flavinic compound is reducible; the other species required for indirect pyruvate reduction to the chiral L-lactate molecule (formate dehydrogenase, lactate dehydrogenase,  $\text{NAD}^+$ ,  $\text{NADH}$ ) are not electroactive. Solvent reduction takes place at potentials below approximately  $-1$  V/Pt wire. The same previous works demonstrate that the reduction of the flavinic compounds is not an elementary step and that many physicochemical phenomena govern it. Here, a relatively simple electrochemical microreactor was used to study the electrochemical behavior of the flavinic system FAD/ $\text{FADH}_2$  on a gold electrode; cyclic voltammetry was used to examine the influence of the operating parameters (concentration  $C$ , flow rate  $\phi$ , potential scan rate  $r$ , use of enzyme, etc.).

#### 3.2.1. Study using a one-channel microreactor

**3.2.1.1. Flow rate dependence.** The study of the flow dependence on the flavin reduction was carried out using the one-channel microreactor ( $\mu\text{R}$ ) with two gold electrodes. Two different types of experiments were performed: in the absence of the FDH enzyme (Fig. 4A) and in its presence (Fig. 4B). The potential scan rate was  $0.5 \text{ V s}^{-1}$  and the examined flow range was 300 to  $667 \mu\text{L}/\text{min}/\text{channel}$ .

The results show that cathodic curves consist of two peaks (not strongly defined) at approximately  $-0.35$  V and approximately  $-0.45$  V. These potentials are less cathodic than those obtained for the FAD reduction on a gold wire electrode ( $-0.55$  V), showing the catalytic effect of the electrode material (in the  $\mu\text{R}$ , gold is chemically deposited on the polymeric membrane) and the uncertainty generated by the tungsten wire, which was used as a pseudo-reference (comparison) electrode instead of a real reference electrode. The reversal scan towards anodic potentials reveals the presence of a peak at  $-0.1$  V to  $-0.15$  V instead of  $-0.3$  V for the peak obtained on a gold wire electrode (Fig. 2). Moreover, a shoulder was observed for a potential range of approximately  $-0.25$  to  $-0.15$  V.

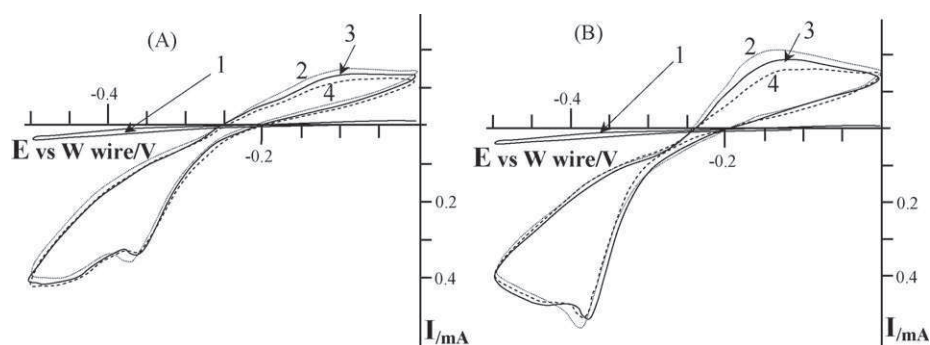
The analysis of the results obtained without the enzyme does not show any influence of the flow ( $\phi$ ) on the magnitude of the cathodic peak at  $-0.35$  V; in addition, the curves show very little influence on the current of the second peak at  $-0.45$  V.

$$\ln I_{\text{peak at } -0.45 \text{ V}} = -7.33 + 0.057 \ln(\nu) \quad R^2 = 0.99; \quad (2)$$

where  $\nu$  is the linear flow velocity (m/s).

Indeed, a linear dependence of the logarithm of the current is observed against the logarithm of the linear flow velocity  $\nu$  of the electrolyte (2), but the exponent in the linear velocity is close to zero.

This relationship can also indicate the dependence of the flow mass transfer coefficient on volumetric flow according to:  $k_{/m s^{-1}} = \text{cnste} \times \phi^{+0.06}$ .



**Fig. 4.** Flow rate dependence of cyclic voltammetry curves obtained in one compartment micro-electrochemical reactor ( $l=3$  cm;  $h=w=1000$   $\mu\text{m}$ ). Two streams in the compartment (anolyte and catholyte); working and auxiliary electrodes: Au, electrodes' area:  $1\text{ mm} \times 30\text{ mm} = 30.10^{-6}$   $\text{m}^2$ , phosphate buffer pH 7.0,  $\text{N}_2$ ,  $[\text{FAD}] = [\text{NAD}^+] = 5$  mM,  $0.5\text{ V/s}$ , (A) no enzyme; (B) FDH enzyme  $0.5\text{ g/L}$ . (1) Residual current,  $667\text{ }\mu\text{L}/\text{min}/\text{channel}$ ; (2)  $333\text{ }\mu\text{L}/\text{min}/\text{channel}$ ; (3)  $500\text{ }\mu\text{L}/\text{min}/\text{channel}$ ; (4)  $667\text{ }\mu\text{L}/\text{min}/\text{channel}$ . The first scan is plotted in the cathodic direction.

For the study of a classical electrochemical system with this microreactor, the exponent in the linear velocity reaches  $0.44$   $\{k=f(\phi^{+0.44})\}$ .

These results allow attributing the first cathodic signal to the reduction of flavin adsorbed forms and the limitation on the magnitude of the current is due to adsorption and not to diffusion; thus, the flows do not have any influence. It is noteworthy that adsorption of the oxidized form of flavin (FAD) was demonstrated by Shinohara's studies [22] on titanium dioxide films, as well as by Gorton and Johansson [20] on a graphite electrode.

Both Shinohara and Gorton proposed relatively high values for heterogeneous electronic transfer constant  $k$  ( $0.25\text{ m/s}$  and  $0.01\text{ cm/s}$ , respectively), indicating a relatively rapid redox system.

On the other hand, adsorption of the reduced form of flavin  $\text{FADH}_2$  on gold electrodes was reported by Wang et al. [21].

The observed shoulder may correspond to the reduction of a FAD intermediate form arising from any acid/base equilibrium, not specifically modified by the stream of the catholyte.

Concerning the second cathodic peak ( $-0.45\text{ V}$ ), the magnitude of the current slightly ( $<5\%$ ) increases with the flow and this low dependence could in part be explained by the low resolution of the global cathodic signal. Indeed, because of the high specific area of the microelectrochemical reactor, the adsorbed quantity of flavin on the electrode is important in comparison with the free flavin in the solution. This allows the ratio of adsorption current to diffusion current to increase and the first peak masks the second one. There is no influence of the volumetric flow on the main adsorption signal and the influence on the second one is masked.

The oxidation signal, obtained during the reversal scan towards anodic potentials (Fig. 4, graph A) shows a shoulder (approximately  $-0.2\text{ V}/\text{ref}$ ), followed by a peak at approximately  $-0.1\text{ V}/\text{W}$  wire.

The influence of the flow on the magnitude of the shoulder (approximately  $-0.2\text{ V}/\text{W}$  wire) is negligible, meaning that this signal corresponds to the oxidation of the adsorbed  $\text{FADH}_2$ .

Conversely, the flow rate has a negative effect on the magnitude of the peak at  $-0.1\text{ V}$  (3).

$$\ln I_{\text{peak}-0.1\text{ V}} = -11.549 - 0.32 \ln(v/\text{m s}^{-1}),$$

$$R^2 = 0.995 \leftrightarrow (k = \text{cnste}' \times \phi^{-0.32}). \quad (3)$$

This signal is attributed to the free  $\text{FADH}_2$  oxidation (as in the study performed on a gold wire; Fig. 2) and its magnitude is lower than the corresponding cathodic one because of the flow; indeed, removing the electrolyte stream of solution containing the reduced form of flavin  $\text{FADH}_2$  causes a decrease of its concentration in the microchannel and reduces the quantity available for oxidation.

Similar results are obtained in presence of the enzyme (Fig. 4–graph B):

- the global shape of the curve is not modified;
- the potential of the anodic signal decreases at  $\sim 50\text{ mV}$  (in comparison with the one obtained without the enzyme), indicating that the flavin ( $\text{FADH}_2$ ) oxidation was facilitated;
- the magnitudes of both cathodic and anodic currents significantly increase ( $\sim 54\%$  at approximately  $-0.35\text{ V}$  and  $45\%$  at approximately  $-0.1\text{ V}$ , respectively), in comparison with the corresponding ones obtained without the enzyme. Enhancement indicates that apparent concentrations of flavin forms increase and confirms that the regeneration of NADH takes place in the microchannel, as this phenomenon was demonstrated in our previous work [5];
- similarly to the results obtained without FDH, the flow dependence on the current is low for the cathodic signals and has a negative effect on the oxidation. Indeed, the analysis of the oxidation peak at  $-0.12\text{ V}$ , attributed to the free  $\text{FADH}_2$  oxidation, shows that the increasing flow rate decreases the magnitude of the current (4):

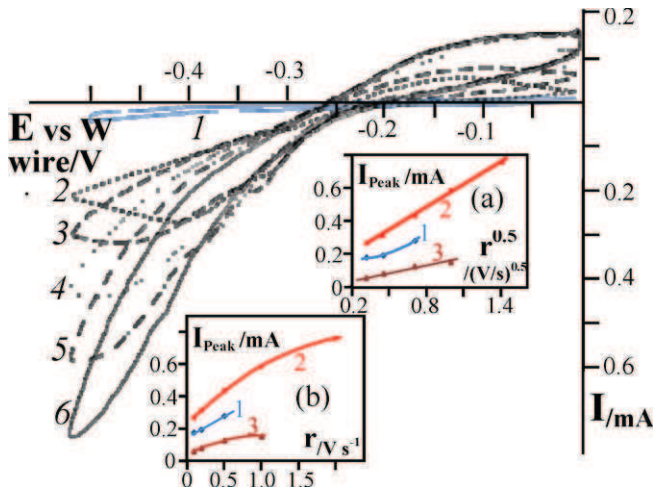
$$\{\ln I_{\text{peak}-0.1\text{ V FDH}} = -12.144 - 0.43 \ln(v/\text{m s}^{-1});$$

$$R^2 = 0.999 \quad (k = \text{cnste}'' \times \phi^{-0.43}) \quad (4)$$

In addition, the presence of FDH causes a more important ( $0.43/0.32 = 1.34$ ) diminution of the magnitude of the current of the peak at  $-0.12\text{ V}$ .

While plotting the curve (1 s), a part of the mixture contained in the working electrode compartment, i.e., the flavinic solution, was renewed because of the flow. For curves 2–4, the residence times of the solution are, respectively, 2.7, 1.8 and 1.35 s, corresponding to 36, 55 and 74% of solution renewed per second. Consequently, a portion of the electrogenerated  $\text{FADH}_2$  (in the cathode) was removed by the flowing solution before being oxidized and leads to the drop of the current; another portion of  $\text{FADH}_2$  may remain adsorbed on the gold surface. These results seem to indicate that the presence of the enzyme allows the reverse reaction 1 to take place and consequently avoids the reoxidation of  $\text{FADH}_2$  by residual oxygen; the redox system  $\text{FAD}/\text{FADH}_2$  appears to become more reversible in the presence of FDH.

**3.2.1.2. Scan rate dependence.** The study of the potential scan rate ( $r$ ) dependence on the flavin reduction was carried out at the same conditions as previously using the same one-channel micro-electrochemical reactor. Two different types of experiments were achieved under flow conditions ( $500\text{ }\mu\text{L}/\text{min}/\text{channel}$ ): without the enzyme FDH (Fig. 5) and with FDH, performing identical experiments (similar shape curves, not presented here). The curves are similar to those indicated in Fig. 4; indeed, the cathodic part exhibits two signals (1:  $-0.35\text{ V}$  and 2:  $-0.45\text{ V}/\text{W}$ ) characterized by a low resolution, which decreases for higher values of the scan rate. The



**Fig. 5.** Scan rate dependence of cyclic voltammetry curves obtained in one-channel micro-electrochemical reactor. Analyte and catholyte flow in the same channel; working and auxiliary electrodes are in Au;  $S = 30 \times 10^{-6} \text{ m}^2$ ;  $500 \mu\text{L}/\text{min}/\text{channel}$ , phosphate buffer pH 7.0;  $\text{N}_2$ ;  $[\text{FAD}] = [\text{NAD}^+] = 5 \text{ mM}$ ; (1) residual current;  $r = 0.1, 0.2, 0.5, 1$  and  $2 \text{ V/s}$  for respectively curves 2–6. Inset: (a) square root of the potential scan rate dependence of the magnitude of the peaks current; (b) potential scan rate dependence of the magnitude of the peaks current; (1) peak at  $-0.35 \text{ V}$ ; (2) peak at  $-0.45 \text{ V}$ ; (3) peak at  $-0.1 \text{ V}$ .

anodic part of the curve contains one peak in the examined potential range.

The analysis of these signals (Fig. 5, inset) confirms previous results even if the resolution of cathodic signals is low. As indicated below, the dependence of the logarithm of the potential scan rate on the peak potential seems to be linear for all signals.

$$E_{1\text{st cathodic peak}}/\text{V} = -0.0353 \ln(r) - 0.3897 \quad R^2 = 0.97 \quad (5)$$

$$E_{2\text{d cathodic peak}}/\text{V} = -0.0343 \ln(r) - 0.4899 \quad R^2 = 0.99 \quad (6)$$

$$E_{1\text{st anodic peak}}/\text{V} = 0.0247 \ln(r) - 0.0641 \quad R^2 = 0.99 \quad (7)$$

Assuming that the classical relationship of cyclic voltammetry (8) for irreversible systems [23]

$$E_{\text{peak}} = E^{\circ'} - \frac{RT}{\alpha nF} \left\{ 0.78 + \ln \frac{\sqrt{D_{\text{FAD}}}}{k^{\circ}} + \ln \left( \sqrt{\frac{\alpha nF}{RT}} \sqrt{r} \right) \right\} \quad (8)$$

could be applied in the case of the microelectrochemical reactor at flow conditions (the validity of this assumption will be examined in a future work), kinetic parameters for FAD reduction can be estimated from the second cathodic signal; results led to  $\alpha = 0.37/n$  and  $k^{\circ} = 1.1 \times 10^{-6} \text{ m/s}$  considering the following values:

- standard potential of FAD at pH 7.0:  $E^{\circ'} = -0.236 \text{ V}$  ([24])
- diffusivity of FAD:  $D_{[\text{FAD}] = 5 \text{ mM}} = 8.2 \times 10^{-10} \text{ m}^2/\text{s}$  [this work].

This estimated value of  $k^{\circ}$  is low in comparison with literature values (Shinohara et al. [22], Gorton and Johansson [20]) and seems to indicate a slow electrochemical system. This value arises from experimental measurements carried out using a pseudo-reference electrode (W wire introduced in the inlet of the catholyte channel); its potential is not rigorously defined by Nernst law because of the absence of the reduced form of flavin in the feed solution.

Relationships (9) and (10) provide the dependence of the magnitude of the current on the potential scan rate (insets a and b); for the three signals (1:  $-0.35 \text{ V}/\text{ref.}$ ; 2:  $-0.45 \text{ V}/\text{ref.}$ ; 3:  $-0.1 \text{ V}/\text{ref.}$ ), the magnitude of the current increases with the square root of the potential scan rate.

For the signal at approximately  $-0.35 \text{ V}$ , despite the insufficient resolution, the magnitude of the current seems to linearly vary

with the potential scan rate, meaning that adsorption is the limiting phenomenon.

For signals at approximately  $-0.45 \text{ V}$  (reduction) and approximately  $-0.1 \text{ V}$  (oxidation), linear dependences (9) and (10) were observed between the magnitude of the current and the square root of the potential scan rate. This evolution is in agreement with a mass transfer limited phenomenon.

$$I_{\text{peak at } \sim -0.45 \text{ V}}(\text{A}) = 0.0005 r^{0.5} + 0.0001 \quad R^2 = 0.998 \quad (9)$$

$$I_{\text{peak at } -0.1 \text{ V}} = 0.0001 r^{0.5} + 10^{-5} \quad R^2 = 0.98 \quad (10)$$

Curves of a similar shape were obtained when operating with FDH enzyme at  $0.5 \text{ g/L}$  (results not shown).

- The magnitudes of the currents of both cathodic and anodic signals are higher than the ones obtained in the absence of the enzyme (for peaks at  $-0.1 \text{ V}$ , the current increases from 40 to 100% when  $r$  increases from 0.2 to  $2 \text{ V/s}$ ).
- The dependence of the magnitude of the current on the square root of the potential scan rate and the dependence of the peak potential on the logarithm of the potential scan rate are similar to those obtained without the enzyme.
- In all experiments, an anodic peak at  $-0.1 \text{ V}$  indicates the presence of the reduced form of flavin ( $\text{FADH}_2$ ), showing that its interfacial concentration is never zero. This means that the rate of the electrochemical reduction of FAD is faster than the rate of the reverse chemical reaction (1) between  $\text{NAD}^+$  and  $\text{FADH}_2$ .

In summary, this section discusses the reduction of FAD in a one-channel micro-device (undivided cell) and the results allow:

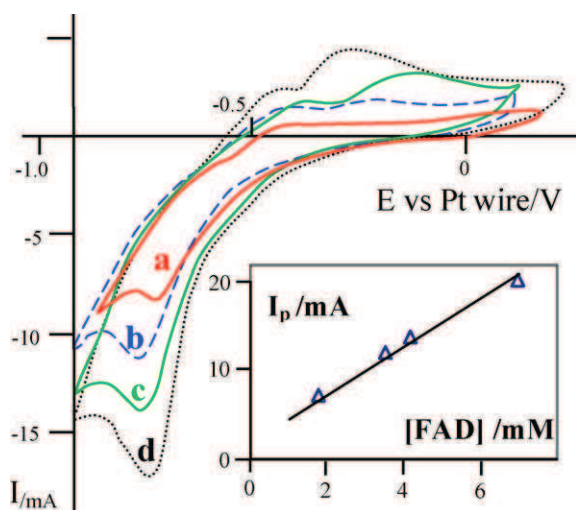
- plotting of useful  $I = f(E)$  curves,
- identifying the limitation of the electrochemical rate,
- obtaining approximate values of the electrokinetic parameters of an electrochemical system, even if the absence of a real reference electrode does not allow obtaining rigorous values,
- showing that adsorption phenomena take place and that the magnitude of the global adsorption and diffusion limited cathodic current does not significantly change by increasing the flow rate because of the high specific area of the microreactor (the adsorption is confirmed by the study of the influence of the scan rate on the magnitude of signals),
- demonstrating that regeneration of NADH takes place and that the rate of the reverse chemical reaction (1) is lower than the rate of the electrochemical reduction of FAD (nevertheless, the magnitude of the current of the anodic signal remains lower than the cathodic one). A partial mixing of the flow of anodic and cathodic streams limits the wide use of this microelectrochemical reactor; the subsequent part of this study deals with a divided filter-press electrochemical microreactor.

### 3.2.2. Study using a multichannel filter-press microreactor

**3.2.2.1. Concentration dependence.** The influence of the flavin concentration is examined in the range of  $1\text{--}7 \text{ mM}$  without flow. The curves (Fig. 6) indicate two cathodic and two anodic signals.

In the cathodic range of  $-0.4$  to  $-0.6 \text{ V}$  over a Pt wire, the curves show a very low resolution signal ( $N^{\circ} 1$ ), similar to the first signal observed in the one-channel microreactor (Fig. 4, approximately  $-0.3\text{--}0.4 \text{ V}$ ). The low resolution does not allow a quantitative analysis of this signal; thus, according to previous results, this shoulder is attributed to the adsorbed flavin reduction and this assumption will be validated in the next sections.

The second cathodic signal ( $N^{\circ} 2$ ), located at approximately  $-0.63 \text{ V}$ , is better defined as a real peak; its magnitude of the current slightly decreases ( $<10\%$ ) after the first scan and remains constant over more than ten successive scans. The inset gives the current (at



**Fig. 6.** Cyclic voltammetry (CV) curves obtained in a multichannel microelectrochemical filter-press reactor with various concentration of flavin; working electrode: Au; auxiliary electrode Pt; No flow; anolyte and catholyte contain phosphate buffer pH 7.0;  $N_2$ : 1 atm;  $r=100$  mV/s; a–d respectively 1.7, 3.5, 4.1 and 7.0 mM. Inset: magnitude of the current (first scan) at  $-0.63$  V against FAD concentration.

$-0.63$  V) dependence against the FAD concentration and the linear evolution translates a mass transfer limitation. The non-zero intercept corresponds to the residual current at  $-0.63$  V.

The reverse anodic scan shows two signals, attributed to the oxidation of the electrogenerated flavin. The increase of the FAD concentration causes the magnitude of the current of both anodic signals to increase, as well as shifting their peak potential to more cathodic values. This means that  $FADH_2$  is sufficiently stable and it is possible to prepare solutions containing a relatively high concentration at the local scale (within the microchannel). Even if the resolution of these peaks seems to increase with the flavin (FAD) concentration, it is insufficient to perform a rigorous analysis of the peak current evolution or to rigorously conclude about the nature of the associated reactions and limitations of the overall oxidation rate. Adsorbed and free  $FADH_2$  may be the reacting species and this assumption will be confirmed in the next section.

**3.2.2.2. Scan and flow rate dependence.** The influence of the potential scan rate on the FAD electrochemical behavior in the microreactor was examined with and without flowing (Fig. 7, graphs (i)–(iii) and graph (0), respectively). The shapes of the curves are similar to the ones obtained in the study of the concentration influence.

The cathodic part of the curves (obtained with and without flowing) clearly indicates a main wave ( $-0.7$  to  $-0.8$  V) attributed to the non-adsorbed FAD reduction; indeed, a linear dependence was observed between the peak current and the square root of the potential scan rate ( $I_{peak/A} = 0.04098 \times r^{0.5}/(V/s)$ ;  $R^2 = 0.999$ ), indicating a mass transfer limitation.

The dependence of the logarithm of the potential scan rate ( $r$ ) on the main cathodic peak potential appears linear, indicating a certain irreversibility for the flavinic cofactor.

$$\{E_{peak/v/Pt\ wire} = -0.798 - 0.061 \times \ln r_{V/s-1}; R^2 = 0.995\} \quad (11)$$

The peak potential shifts 80 mV towards the cathodic values when the potential scan rate increases from 50 to 200 mV/s. Using the same assumptions as for previous experiments and the classical relationship in cyclic voltammetry for irreversible systems [23] allows the estimation of kinetic parameters for the FAD reduction; i.e.,  $\alpha = 0.207/n$  and  $k^0 = 0.25 \times 10^{-6}$  m/s.

The value of the intrinsic constant is similar to the one obtained with the one-channel microreactor ( $k^0 = 1.1 \times 10^{-6}$  m/s) but lower than the literature values (see above discussion of reasons for this effect).

The cathodic part of the curves (obtained with and without flowing) contains a shoulder before the main peak (approximately  $-0.6$  V), which is not seen clearly on the figure (even if present) because of the reduced scale of the graphs.

The anodic curves, obtained during the reverse scan, indicate two signals in the range of  $-0.7$  to  $-0.1$  V on a Pt wire. Increasing the potential scan rate causes an increase of the current magnitudes of both signals. The low resolution (graph (0), without flowing) does not allow obtaining a theoretical evolution of  $I$  against the potential scan rate  $r$ .

The flow dependence was examined over the same potential scan rate range (Fig. 7, graphs (i) to (iii)) and similar curve shapes were obtained. The current magnitude of the main cathodic signal (the reduction of free FAD) increases counter to the flow rate and corresponds to a mass transfer limited process. In addition, the shape of this signal is a peak instead of a diffusion wave (with a limiting current) in spite of the flow of the solution; depletion of concentration in the microchannel causes the magnitude of the current to decrease, even if the flow contributes to renew the solution within the microchannel.

The anodic section of the curves obtained with a flow (graphs (i) to (iii)) presents two very well distinguished peaks. Resolution, position and magnitude of the first peak, located at approximately  $-0.7$  to  $-0.5$  V, do not depend of the volumetric flow rate; instead, the flow strongly modifies the parameters of the second signal located at approximately  $-0.4$  to  $-0.1$  V.

The magnitude of the current of the first peak increases linearly counter to the potential scan rate, according to the following relationship:

$$I_{1/A} = 0.0194r_{V/s-1} \quad R^2 = 0.99 \quad \text{flow rate : } 130 \mu\text{L/min}$$

$$I_{1/A} = 0.0193r_{V/s-1} \quad R^2 = 0.99 \quad \text{flow rate : } 250 \mu\text{L/min}$$

consequently, it is attributed to the bielectronic oxidation of the adsorbed flavin ( $FADH_2$ ).

The second (very wide) signal is located at approximately  $-0.4$  to  $-0.1$  V. Its magnitude increases with  $r$  but decreases with the flow rate; the signal disappears for flows higher than  $200 \mu\text{L/min}$ .

The following relationships were determined for the influence of the potential scan rate on the magnitude of the maximum current:

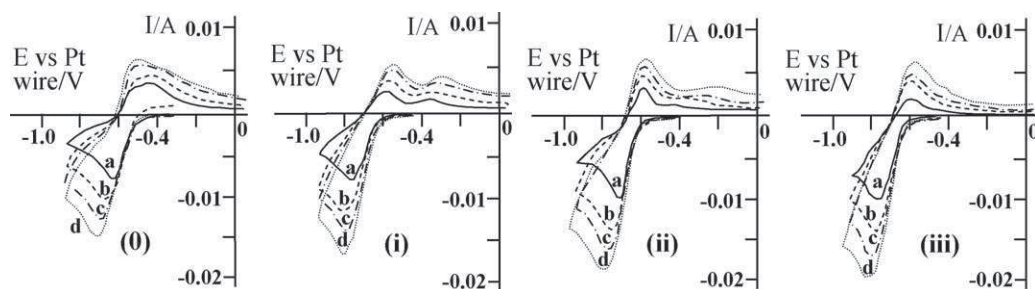
$$I_{1/A} = 0.0108 \times r^{0.5}/(V/s); \quad R^2 = 0.99 \quad \text{flow rate : } 130 \mu\text{L/min.}$$

$$I_{1/A} = 0.0096 \times r^{0.5}/(V/s); \quad R^2 = 0.98 \quad \text{flow rate : } 250 \mu\text{L/min.}$$

Theoretically, for dissolved species, increasing the flow rate enhances both the mass transfer and the magnitude of the current. This is true for the cathodic signal (FAD bielectronic reduction) because the feed solution contains FAD, but not for the anodic signal ( $FADH_2$  oxidation) at approximately  $-0.4$  to  $-0.1$  V; indeed, increasing the flow removes the free (not adsorbed)  $FADH_2$  from the working electrode compartment and contributes to reducing its concentration and consequently, the magnitude of the current.

Nevertheless, the flow strongly influences the position of this signal; indeed, increasing the flow shifts the peak to the anodic potentials, meaning that oxidation becomes more difficult and more irreversible. This evolution may be explained by the presence and the oxidation of flavin intermediate forms ( $FADH$ , for example). Indeed, increasing the flow allows renewing the anodic compartment solution and modifies the acid/base equilibria of the various





**Fig. 7.** Flow rate dependence on cyclic voltammety curves obtained with a 3 mM flavin solution, in a multichannel micro-electrochemical filter-press reactor for various potential scan rates; working electrode: Au; auxiliary electrode Pt; anolyte and catholyte contain phosphate buffer pH 7.0; N<sub>2</sub>: 1 atm; a–d respectively 50, 100, 150 and 200 mV/s. (0): no flow; (i)  $\phi = 130 \mu\text{L}/\text{min}$ ; (ii)  $\phi = 250 \mu\text{L}/\text{min}$ ; (iii)  $\phi = 500 \mu\text{L}/\text{min}$ .

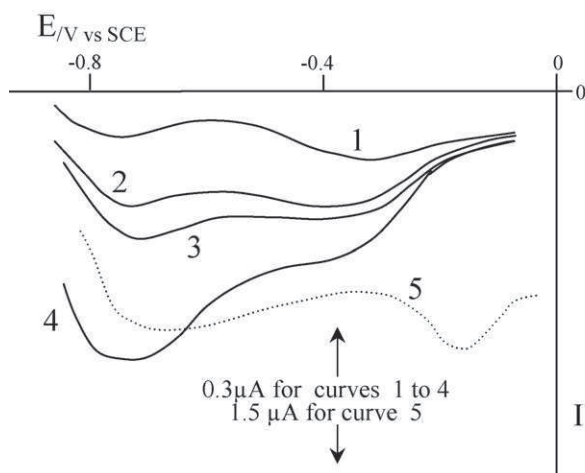
forms of flavin or favors the disproportionation of the FADH intermediate form. The oxidation of intermediate forms takes place at higher potentials and explains the large width of this signal.

These experiments confirm that the reduced form of flavin (FADH<sub>2</sub>) could be adsorbed on gold and that monoelectronic oxidation of intermediate forms can occur. A theoretical simulation of these evolutions is complicated because this type of measurement combines principles of cyclic voltammety in the transient state and statements in a continuous plug flow reactor; this will constitute the goal of a future work on the field.

### 3.3. Study of flavin adsorption on a rotating disk gold cathode

The aim is to quantify the adsorption of the FAD on a gold cathode in phosphate buffer (pH 7.00) and to confirm the previous results. A set of coulometric measurements was carried out using a carefully polished rotating disk electrode gold (diameter of 2.9 mm) as the adsorption support. The electrode was immersed in 7 mM FAD de-aerated solution for various durations (5–60 min), rinsed using distilled water, immersed in a phosphate buffered and de-aerated solution not containing flavin; finally, current–potential curves were plotted in the transient state (Fig. 8).

The analysis of curves 1–4 shows two peaks at  $-0.32 \text{ V}/\text{SCE}$  and  $-0.71 \text{ V}/\text{SCE}$ . The current magnitude of these peaks remains low ( $<1 \mu\text{A}$ ), despite increasing with immersion duration. Curve 5, obtained after a 1-h immersion of the gold electrode in an aerated phosphate buffer solution (no FAD), shows two peaks (approximately  $-0.15 \text{ V}$  and from  $-0.3$  to  $-0.8 \text{ V}$ ), their current magnitudes



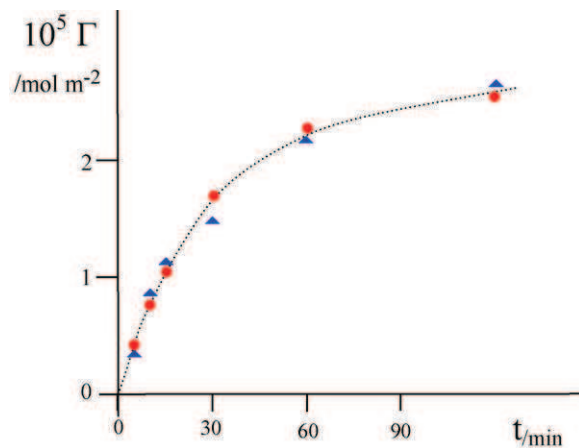
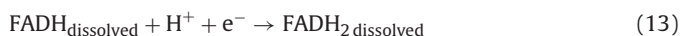
**Fig. 8.** Current–potential curves plotted into a phosphate buffer solution, using a gold cathode previously immersed during various durations within a flavin solution ([FAD] = 7 mM, pH 7.0),  $r = 0.05 \text{ V}/\text{s}$ . Immersion time: 5, 10, 30 and 60 min for respectively curves 1–4. Curve 5: aerated phosphate buffer solution.

are  $\sim 3 \mu\text{A}$ . The signal at approximately  $-0.15 \text{ V}$  (curve 5) was attributed to the first bielectronic reduction of dissolved oxygen ( $\text{O}_2/\text{H}_2\text{O}_2$ ); the very wide signal ( $-0.3$  to  $-0.8 \text{ V}$ , curve 5) may correspond to the second bielectronic reduction of dissolved oxygen ( $\text{H}_2\text{O}_2/\text{H}_2\text{O}$ ).

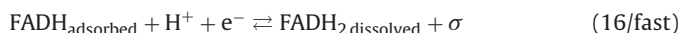
The comparison of the shape and magnitude of curves 1–4 with curve 5 shows significant differences and demonstrates that the signals in the curves 1–4 do not correspond to the dissolved oxygen but rather exclusively to the FAD and more particularly, to the reduction of the adsorbed form; the presence of two peaks could be explained by two successive monoelectronic steps in the flavin reduction because there is no dissolved FAD and consequently no mass transfer limitation.

Assuming that both signals correspond to the reduction of flavin and accounting for two electrons for the overall reduction, the integration of  $I = f(E)$  curve leads to the superficial concentration  $\Gamma$  of flavin on the gold electrode as a function of the immersion time. The electrochemical area of the gold electrode was determined by other (chronopotentiometric) experiments and its value corresponds to  $5 \text{ mm}^2$ . Fig. 9 represents the experimental evolution of  $\Gamma$  as a function of time.

A possible scheme for flavin reduction is:



**Fig. 9.** Superficial concentration of flavin adsorbed on gold disk as a function of immersion time in a flavin (7 mM) solution. Integration of both cathodic signals of  $I = f(E)$  curves allows to determine  $\Gamma$ . Solution stirred during adsorption. (▲) experimental evolution and (●) theoretical evolution.



In these experiments, there is no free FAD; assuming that flavin adsorption requires one site ( $\sigma$ ) on the gold cathode and constitutes the limiting step of the reduction, the overall rate can be written as follow:

$$\frac{d\theta}{dt} = k_a C_{\text{FAD}}(1 - \theta) - k_d \theta \quad (17)$$

where  $\theta$  is the coverage ratio of the gold surface ( $\theta = \Gamma/\Gamma_{\text{max}}$ ),  $\Gamma$ , the superficial concentration of the adsorbed flavin.

$\Gamma_{\text{max}}$ , the superficial concentration of the adsorbed flavin for a gold saturated surface.

The integration of this equation leads to the superficial concentration of flavin adsorbed on the gold cathode as a function of the immersion time:

$$\Gamma = \frac{\Gamma_{\text{max}} k_a C_{\text{FAD}}}{k_a C_{\text{FAD}} + k_d} \times (1 - \exp(-(k_a C_{\text{FAD}} + k_d)t)) \quad (18)$$

In order to estimate the adsorption/desorption rate constants from the experimental data, the previous relation is approximated by a first-order exponential development:  $\Gamma \approx \Gamma_{\text{max}} k_a C_{\text{FAD}} \times t$

Analysis of the experimental data for a duration of less than 15 min leads to the

$$\Gamma_{(\text{mol}/\text{m}^2)} = 9.2 \times 10^{-7} t_{\text{min}} - 6 \times 10^{-7} \quad (19)$$

relationship (19) and allows accessing the adsorption  $k_a$  and desorption  $k_d$  constants, assuming that  $\Gamma_{\text{max}} = 2.71 \times 10^{-5} \text{ mol}/\text{m}^2$  (extrapolation on infinite immersion time, curve  $\Gamma = f(t)$  of Fig. 9).

$$k_a = 4.85 \times 10^{-3} \text{ m}^3/\text{mol}/\text{min}; \quad k_d = 7.4 \times 10^{-4} \text{ min}^{-1}$$

The adsorption equilibrium constant ( $K = k_a/k_d = 6.5 \times 10^3$ ) leads to the adsorption standard free energy  $\Delta G^\circ = 21.7 \text{ kJ}/\text{mol}$ , a relatively low value, indicating a weak adsorption.

The  $\Gamma_{\text{max}}$  value is lower than the one determined by Gorton and Johansson [20] ( $2 \times 10^{-8} \text{ mol}/\text{cm}^2$ ) after an immersion duration of 4 h of a graphite electrode within the flavinic solution. The high electrochemical area of graphite, in comparison with gold, could explain the difference in the values of  $\Gamma_{\text{max}}$ . The authors indicate that the adsorption is not strong and that the adsorbed layer disappears after a simple rinsing of the electrode.

The overall electrochemical behavior observed for the flavin reduction in this work appears to be irreversible, which is in accordance with the work of Wang et al. [21]; the authors used a quartz microbalance to study the flavin adsorption on gold. The voltammograms obtained in acidic media (pH < 4.5) were reversible, in contrast to voltammograms obtained with a higher pH, which appeared to be less reversible. The authors indicate that for pH 1.5 and flavin concentrations lower than  $2 \times 10^{-4} \text{ mol}/\text{L}$ , mass transfer phenomena seem to limit the flavin reduction rate; conversely, for higher concentrations, adsorption becomes limiting.

In summary, the reduction of FAD within a filter-press microreactor takes place mainly in a dissolved and free (not adsorbed) form. The observed shoulder may correspond to the reduction of both forms (an adsorbed and an intermediate) of FAD arising from an acid/base equilibrium.

#### 4. Conclusions

In this work, microreactors were used to study the electrochemical behavior of the flavinic redox system FAD/FADH<sub>2</sub>, as well as to use this mediator in the NADH indirect electro-regeneration. The study leads to a better understanding of the reduction scheme and of the physicochemical phenomena that limit the overall reduction rate of FAD.

Two different setups were used:

- A conventional three-electrode cell to determine the flavin diffusion coefficient in phosphate media for various concentrations. The coefficient value decreases from  $0.8 \times 10^{-10}$  to  $0.6 \times 10^{-10} \text{ m}^2/\text{s}$  with increasing concentration in the range of 7–10 mM; this evolution can be explained by the significant size of the flavin molecule and the equilibrium between various acid/base forms.
- Microreactors (one-channel and filter-press multichannel microreactors) in order to examine the influence of various operating parameters (C, flow, potential scan rate and so on). The reduction and oxidation of flavinic compounds take place in two different ways. Adsorption phenomena seem to limit the first steps of both reduction and oxidation. The second step is diffusion limited for these processes. The peak-to-peak potential reaches values higher than 150 mV, translating a certain irreversible behavior for adsorption or diffusion of the flavinic redox system FAD/FADH<sub>2</sub>. In addition, a low value ( $10^{-6} \text{ m}/\text{s}$ ) is determined for the apparent intrinsic electronic transfer constant  $k^\circ$ .

The flavin adsorption equilibrium requires a relatively long time (>1 h) to be reached and depends strongly on the electrode surface; indeed, the reproducibility of the adsorption signals is difficult to obtain, meaning that adsorption is weak ( $k_a = 4.85 \times 10^{-3} \text{ m}^3/\text{mol}/\text{min}$ ). The adsorbed quantity remains low ( $27 \text{ } \mu\text{mol}/\text{m}^2$ ) in comparison with literature values. This study also allows demonstrating that for preparative electrosyntheses, the adsorption phenomena were not limitative because the low adsorption rate and the reduction rate of flavin are mainly governed by mass transfer phenomena.

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#### References

- [1] R. Devaux-Basseguy, A. Bergel, M. Comtat, *Enzyme Microbial Technol.* 20 (1997) 248.
- [2] H.K. Chenault, E.S. Simon, G.M. Whitesides, *Biotechnol. Genet. Eng. Rev.* 6 (1988) 221.
- [3] T. Tzedakis, C. Kane, A. Launay, French Patent, n(04.12305, 2004), US patent (2006)0108215A1, PCT (2006), N(WO (2006)053962.
- [4] E.R. Choban, P. Waszczuk, T. Tzedakis, P.J.A. Kenis, S.K. Yoon, C. Kane, US Patent No. 10/844,058, 2004.
- [5] S.K. Yoon, E.R. Choban, C. Kane, T. Tzedakis, P.J.A. Kenis, *J. Am. Chem. Soc.* 127 (30) (2005) 10466.
- [6] C. Kane, T. Tzedakis, *AIChE J.* 54 (5) (2008) 1365.
- [7] P.S. Wagenknecht, J.M. Penney, R.T. Hembre, *Organometallics* 22 (2003) 1180.
- [8] K. Nakamura, R. Yamanaka, *Chem. Commun.* (2002) 1782.
- [9] F. Zambianchi, P. Pasta, G. Carrea, N. Colonna, N. Gaggero, J.M. Woodley, *Biotechnol. Bioeng.* 78 (2002) 489.
- [10] B. Orlich, H. Berger, M. Lade, Schomaecker, *Biotechnol. Bioeng.* 70 (2001) 638.
- [11] E. Steckhan, T. Arns, W.R. Heineman, G. Hilt, D. Hoormann, J. Jörissen, L. Kröner, B. Lewall, H. Pütter, *Chemosphere* 43 (2001) 63.
- [12] Y.H. Kim, Y.J. Yoo, *Enzyme Microbial Technol.* 44 (2009) 129.
- [13] S.B. Sobolov, M.D. Leonide, A. Bartosko-Malik, K.I. Voivodov, F. McKinney, J. Kim, A.J. Fry, *J. Org. Chem.* 61 (1996) 2125.
- [14] H. Durliat, M.B. Barrau, M. Comtat, *Bioelectrochem. Bioenerg.* 19 (1988) 413.
- [15] John T. Edsall, H. Gutfreund, *Biothermodynamics*, John Wiley & Sons Inc., 1983, ISBN-10: 0471102571 ISBN-13: 978-0471102571. The Study of Biochemical Processes at Equilibrium. (Monographs in Molecular Biophysics and Biochemistry).
- [16] A. Bergel, M. Comtat, *J. Electroanal. Chem.* 302 (1991) 219.
- [17] H. Jaegfeldt, *Bioelectrochem. Bioenerg.* 8 (1981) 355.
- [18] J. Cantet, A. Bergel, M. Comtat, *Enzyme Microbial Technol.* 18 (1996) 72.
- [19] C. Kane, PhD, Toulouse France, 2005.

- [20] L. Gorton, G. Johansson, *J. Electroanal. Chem.* 113 (1980) 151.
- [21] Y. Wang, G. Zhu, E. Wang, *Anal. Chim.* 338 (1997) 97.
- [22] H. Shinohara, M. Graetzel, N. Vlachopoulos, M. Aizawa, *Bioelectrochem. Bioenerg.* 26 (2) (1991) 307.
- [23] A.J. Bard, L.R. Faulkner, *Electrochemical Methods, Fundamentals and applications*, 2nd edition, John Wiley & Sons Inc., New York, 2001.
- [24] R.F. Anderson, L. Gray, *Berichte der Bunsen-Gesellschaft* 80 (10) (1976) 969.