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K. Délécouls-Servat · A. Bergel · R. Basséguy

Membrane electrochemical reactors (MER) for NADH regeneration in HLADH-catalysed synthesis: comparison of effectiveness

Abstract Two membrane electrochemical reactors (MER) were designed and applied to HLADH-catalysed reduction of cyclohexanone to cyclohexanol. The regeneration of the cofactor NADH was ensured electrochemically, using either methyl viologen or a rhodium complex as electrochemical mediator. A semi-permeable membrane (dialysis or ultra-filtration) was integrated in the filter-press electrochemical reactor to confine the enzyme(s) as close as possible to the electrode surface. When methyl viologen was used, the transformation ratio of cyclohexanone varied from 0 to 65% depending on the internal arrangement of the reactor. Matching the reactor configuration to the reaction system was essential in this case. With the rhodium complex, the ultra-filtration MER was tested in continuous and recycling configurations. The best conditions led to 100% transformation of 0.1 L volume of 0.1 M cyclohexanone after 70 h with the recycling mode. Finally, the performances of the reactors are discussed with respect to different evaluations of the production yields.

Keywords Electrochemistry · Enzyme catalysis · Membranes · Electrochemical reactor · Synthesis

Introduction

Mastering oxidoreductase-catalysed reactions has the potential to open a wealth of new synthesis routes. In this field, NAD-dependent oxidoreductases are of great interest, because they are at the crossroads of synthetic routes to numerous high-value-added compounds: pharmaceuticals, food additives, perfumes, insecticides, pesticides, etc. [1]. Nevertheless, to promote economically efficient processes, the regeneration of the pyridine cofactor (NAD^+ or NADH), which is a very expensive compound, remains a key problem to solve.

Enzyme-catalyzed regeneration of the cofactor

The most widely used method for NADH regeneration consists of associating the synthesis reaction to a second oxidoreductase-catalysed reaction, which ensures NAD^+ reduction into NADH. Several substrate/enzyme couples have proved efficient. Exhaustive reviews of these techniques have already been proposed elsewhere [2, 3]. The formate/formate dehydrogenase (FDH) method seems to give the best results at the moment, and large scale applications have been foreseen [4]. This reaction only produces CO_2 as side-product, which avoids downstream separations:

$$NAD^+ + HCO_2^- \xrightarrow{FDH} NADH + CO_2$$

This system has been successfully used for NADH regeneration in a membrane reactor for L-leucine synthesis (high conversion 90–99.7% maintained during more than 25 d) [5] or D-lactate synthesis (complete reaction in 7 d) [6]. The formate dehydrogenase generally used so far is specific to NAD^+, but a new formate dehydrogenase has been produced [7] that is able to work with $NADP^+$. The main disadvantage of the system is its rather high cost.

The glucose/glucose dehydrogenase system also gave good results for NADH regeneration. In a six-day synthesis of D-lactate, a NADH turnover of 36,000 was reached with no loss of activity [8]. The glucose dehydrogenase from *Bacillus cereus* accepts either NAD^+ or $NADP^+$ [3]. Like FDH, the cost of glucose dehydrogenase is a disadvantage.

Several anaerobic bacteria produce hydrogenases (E.C. 1.12.1.2) that catalyse the direct reduction of NAD^+ by hydrogen. The conversion of 2-oxoglutarate

into glutamate reached 98% after 3 h [9]. However, hydrogenase is not commercially available.

Using ethanol and alcohol dehydrogenase is an attractive process because of the low cost of both compounds. Nevertheless, this system produces acetaldehyde, which inhibits alcohol dehydrogenase. Methods for removing acetaldehyde have been developed such as sweeping with nitrogen [10] or trapping with sodium bisulphite [11]. Wong and Whitesides added aldehyde dehydrogenase to oxidize acetaldehyde into acetate [12]. They obtained thus the complete transformation of 120 mmol pyruvate to L-lactate in 2 d. These authors also examined regeneration methods based on the oxidation of methanol, which needs three enzymes. These methods seem less practical, but they remain attractive thanks to the low cost of the compounds involved.

Electrochemical regeneration

The electrochemical reduction of NAD^+ into NADH should be a regeneration technique that avoids the production of any side-products:

$$NAD^+ + H^+ + 2e^- \rightarrow NADH$$

Nevertheless, this direct reaction is neither rapid nor selective enough to be used for synthesis [13]. Surface-modified electrodes have been proposed to improve the selectivity of the reaction [14]. Adsorption of some hydrogenases on a platinum electrode has also yielded efficient catalysis [15, 16]. In a batch reactor, the hydrogenase-catalysed electrochemical reduction of NAD^+ gave NAD-turnover numbers of 207 h^{-1} when coupled with the L-glutamate dehydrogenase-catalysed transformation of α-ketoglutaric acid into L-glutamic acid [17].

Numerous attempts for NADH regeneration have been made for the synthesis of lactate catalysed by a lactate dehydrogenase, free in solution or immobilised on a soluble suspension. Several solutions have been proposed to create the most effective electrochemical chain to transfer the electrons from the electrode to NAD^+:

- DiCosimo et al. used methyl viologen as a mediator [18]. Lipoamide dehydrogenase was required to catalyse the reaction of the reduced methyl viologen with NAD^+. A 94% transformation has been obtained after 9 d for the production of L-lactate.
- Ruppert et al. replaced methyl viologen by a rhodium complex [$Cp(Me)_5$ Rh(bipy)Cl] [19]. No enzyme is required in this case to catalyse the reaction between the mediator and NAD^+. A transformation ratio of 70% was obtained after 3 h, with an initial pyruvate concentration of 0.02 M corresponding to a D-lactate production rate of 233 μmol/h.
- Covalent co-immobilisation of the electrochemical mediator (N,N'-diaminopropyl-4,4'-dipyridinium-dication) and a viologen-accepting pyridine nucleotide oxidoreductase on a carbon electrode gave 36 nmol/h/cm^2 lactate, with lactate dehydrogenase in solution [20].
- Fry et al. proposed to immobilise methyl viologen and lipoamide dehydrogenase in a Nafion film, whereas lactate dehydrogenase was in solution [21]. They obtained a production of 700 nmol/h/cm^2. Fresh lactate dehydrogenase had to be added every two days, since lactate dehydrogenase from rabbit muscle has a short half-life.

Different techniques have been used to improve the enzyme stability. Maeda and Kajiwara have shown that adding lipoamide stabilises malate dehydrogenase and diaphorase [22]. When cross-linked lactate dehydrogenase crystals were used, the lactate yield was improved 3-fold (2115 nmol/h/cm^2) [23] compared with synthesis using soluble lactate dehydrogenase (700 nmol/h/cm^2) [21].

Membrane electrochemical reactors

In an electrochemical continuously stirred tank reactor, the greatest part of the enzyme is in the bulk and does not participate in the synthesis. Actually, only the enzyme close to the electrode surface is really efficient. In order to use the enzyme optimally, it must be confined near the electrode. In the literature, many methods are proposed such as adsorption, entrapment in a matrix, covalent attachment or a combination [24–26] but the effectiveness of these processes is strongly dependent on the nature of the enzyme. We propose here to confine the enzyme at the electrode surface with a semi-permeable membrane, which is integrated in the electrochemical reactor. This technique should become widely applied, because it does not require any previous enzyme preparation, and the enzyme does not undergo any chemical modification that may affect its catalytic properties.

For classic enzyme-catalysed synthesis, in the absence of any electrochemical step, the membrane technique has been widely used. Two types of reactors can be distinguished according to the membrane nature: dialysis or ultra-filtration. The method called membrane enclosed enzymatic catalysis (MEEC) consists in entrapping enzymes in their soluble form in a dialysis tube. This technique, applied to the reduction of 75 mmol pyruvate, led to 98% transformation after 32 h [27]. Ultra-filtration membranes were more often used to separate the reaction synthesis from the co-factor regeneration. Kula and Wandrey proposed to weigh down the compounds to increase the efficiency of the ultra-filtration membrane retention [28]. This process applied to L-leucine production, coupled with the FDH cofactor regeneration, ran continuously for 48 d with a maximum yield of 99.7% on roughly the 20th day [5]. For the same synthesis, Seelbach and Kragl preferred a nanofiltration membrane [29] and obtained 370 g/l/day of L-leucine. The use of charged filtration membranes

allowed the compounds to be retained by associating size exclusion and electrostatic repulsion effects [30–32]. This bioreactor used for sorbitol production gave 114 g/l/day sorbitol over 836 h [33]. Hydrophobic interactions were also emphasized with micro-filtration membranes: production of l-menthol was thus maintained for 270 h with a space-time yield of 46.1 g/l/day [34].

However, the membrane technique has been scarcely used in association with electrochemical reactors. Nevertheless two types of electrochemical membrane reactor were found in the literature dealing with enzyme-catalysed synthesis. In one type, the electrochemical and membrane reactors are separated. The membrane reactor allows the continuous removal of the product and keeps the enzyme(s) in the working loop [35–37]. In the other type, the electrochemical and enzymatic reactors are in the same device, a so-called ''compact reactor''. A theoretical study showed that the performance should be higher if the electrochemical and enzymatic reactions are conducted as the same process rather than being split in two different reactors [38]. The advantages of a compact reactor should be emphasized. This design allows the enzyme to be confined in a very small volume near the electrode surface whereas separated processes do not. Only a minimal quantity of enzyme is required for maximal transformation. Indeed, the enzyme, confined near the electrode surface, does not circulate in the pumps, the tubes or the storage tank. Consequently, numerous denaturing phenomena such as adsorption on the walls are avoided. Moreover, when the reaction requires nitrogen bubbling to eliminate oxygen, the creation of two phase interface is a strong denaturing factor for enzymes. In a compact reactor, nitrogen bubbling is no longer a problem, because it can be performed in the storage tank where no enzyme is present; the enzyme, confined near the electrode surface, is not in contact with the dispersed gas phase. To our knowledge, the first reactor of this type was proposed by Grimes and Drueckhammer [39]. The working electrode was simply inserted in a dialysis tube and the results were promising. The reaction was complete in 8 d for the reduction of α-ketoacid (pyruvate or butyrate).

The membrane-enclosed electrochemical reactors have been recently improved by integrating a flat dialysis membrane [40] or a flat ultra-filtration membrane [41] into a filter-press electrochemical reactor. These reactors possess the combined advantages of confining the enzymes close to the electrode surface without any modification. They may also benefit from all the experience gained in classic membrane reactors applied to enzyme catalysis. The purpose of this work was to compare the behaviours of the dialysis-membrane and ultra-filtration-membrane electrochemical reactors (D-MER and UF-MER) applied to the HLADH-catalysed reduction of cyclohexanone to cyclohexanol. The electrochemical NADH regeneration was achieved either with methyl viologen or rhodium complex as mediator. The work focused the importance to match the internal design of the reactors to the reaction to be processed.

Materials and methods

Chemicals and materials

The chemicals were purchased from Sigma, Boehringer Mannheim and Fluka. Lipoamide dehydrogenase LipDH (E.C.1.8.1.4) from *Porcine heart* type III was purchased from Sigma. Alcohol dehydrogenase (E.C.1.1.1.1) from *Horse liver* (HLADH) was purchased from Fluka. The rhodium complex, pentamethylcyclopentadienyl-2,2'-bipyridinechloro-rhodium(III), was not commercially available but synthesised by the late Prof. E. Steckhan's group (University of Bonn, Germany).

The working electrode was either a platinum grid (196 mesh.cm^{-2}) from Engelhard-CLAL or a carbon felt (RVG 2000) from Carbone Lorraine (France). The dialysis membrane (cut-off 12–14 kDa) made of regenerated cellulose was supplied by Bioblock Company. The ultra-filtration membranes made of cellulose were purchased from Hoechst High Chem Society (Wiesbaden, Germany). Two cut-off values were selected: 5 kDa and 30 kDa. Before use, the membranes were immerged in the buffer solution for at least 24 h.

Analysis

The study dealt with the transformation of cyclohexanone to cyclohexanol. The activity of HLADH was consequently defined here with respect to cyclohexanol production, instead of cyclohexanol consumption as commonly done. The HLADH unit was thus defined as the quantity required to transform one micromole of cyclohexanone into cyclohexanol per minute. This activity was determined spectrophotometrically by recording NADH consumption at $\lambda = 340$ nm in a solution containing 0.1 M phosphate buffer (pH 6.6), 7 mM cyclohexanone and 0.44 mM NADH. The enzyme HLADH was added last.

The transformation of cyclohexanone to cyclohexanol was followed by gas chromatography (HP 5890 series II), with a HP1 (Methyl Silicone Gum) 10 m×0.53 mm×2.65 μm column. This method allowed simultaneous measurement of the concentrations of substrate and product as a function of time. Cyclohexane was chosen as an internal standard. The temperature gradient started at 40°C, increased to 200°C at 50°C/min, and finally to 210°C at 5°C/min to extract all the compounds from the column. In these conditions, the retention times were 1.7, 8.8 and 9.3 min for cyclohexane, cyclohexanone, and cyclohexanol respectively.

Electrochemical measurements

All electrochemical experiments, were performed with an electrochemical interface 1286 Solartron Schlumberger connected to a computer. The potential of the working electrode was monitored versus a saturated calomel reference electrode (SCE), which was connected to the electrochemical cell or reactor through a Luggin capillary. During cyclic voltammetry the potential of the working electrode varied linearly as a function of time, and the results are reported in the form of current-potential curves. During electrolysis, the potential of the working electrode was maintained at a constant value, and the current was recorded as a function of time.

Reactors and reactions

Reaction system

The membrane electrochemical reactors (MER) were applied to the horse liver alcohol dehydrogenase

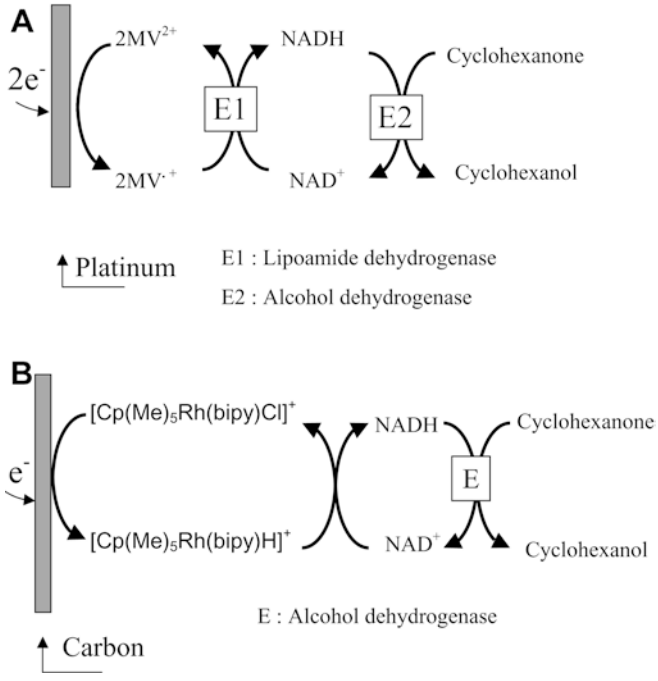

Fig. 1 Scheme of the two NADH regeneration systems with methyl viologen or the rhodium complex

(HLADH)-catalysed synthesis of cyclohexanol from cyclohexanone coupled to the electrochemical regeneration of NADH. Two different electrochemical mediators were used: methyl viologen and a rhodium complex (Figure 1). The electrochemical reaction was either:

$$MV^{2+} + 1e^- \rightarrow MV^{\circ +} \quad (1a)$$

or:

$$Cp(Me)_5Rh(bipy)Cl^+ + H^+ + 2e^- \rightarrow Cp(Me)_5Rh(bipy)H^+ + Cl^- \quad (1b)$$

NADH regeneration with methyl viologen requires the enzyme lipoamide dehydrogenase to catalyse the reaction between $MV^{\circ +}$ and NAD^+:

$$2\ MV^{\circ +} + NAD^+ + H^+ \ \text{LipDH}\ NADH + 2\ MV^{2+} \quad (2a)$$

On the contrary, regeneration with the rhodium complex does not require any enzyme:

$$Cp(Me)_5Rh(bipy)H^+ + NAD^+ + Cl^- \rightarrow Cp(Me)_5Rh(bipy)Cl^+ + NADH \quad (2b)$$

NADH was used in the HLADH-catalysed synthesis:

$$NADH + C_6H_{10}O + H^+ \xrightarrow{\text{HLADH}} C_6H_{12}O + NAD^+ \quad (3)$$

The electrodes were a platinum mesh and a carbon felt for methyl viologen and the rhodium complex, respectively. The different experimental conditions are reported in Table 1.

Description of the membrane electrochemical reactors (MERs)

The MERs shown in Figs. 2 and 3 were based on a filter-press electrochemical reactor made of three pieces of Plexiglas. Both working and auxiliary electrodes were of 15×2 cm^2 surface area. The auxiliary electrode (1) was a platinum grid. The saturated calomel reference electrode (3) was connected to the reactor with a Luggin capillary. When necessary, a Nafion membrane (2) was inserted between the working and the auxiliary compartments to avoid any transfer of reactant or product between the compartments. With or without the Nafion membrane, the solution(s) circulated in loop(s), each including the reactor compartment, a gear pump and a storage tank. Nitrogen was bubbled through the storage tank to maintain anaerobic conditions.

The working electrode (5) of the dialysis membrane electrochemical reactor (D-MER) (Fig. 2) was a platinum grid covered by a dialysis membrane (4), which confined the enzyme in solution close to the electrode. The solution flowed tangentially with respect to the dialysis membrane and the transport of compounds

Table 1 Experimental conditions

Regeneration method		Methyl viologen	Methyl viologen	Rhodium complex	Rhodium complex	Rhodium complex
Electrode material/ geometric area (cm^2)		platinum/30	platinum/30	carbon/18	carbon/30	carbon/30
Process		D-MER in recycling mode	UF-MER in continuous mode	Beaker	UF-MER in continuous mode	UF-MER in recycling mode
Volume (mL)		100	100	8	100	100
Cyclohexanone concentration (mM)		85	100	100	100	100
Mediator concentration (mM)		5	5	1	1	1
Cofactor concentration (mM)		1	1	1	1	1
Enzymeconcentration (U)	LipDH	787 to 1187	590 to 1325	None	None	None
	HLADH	64	60	22	60	73

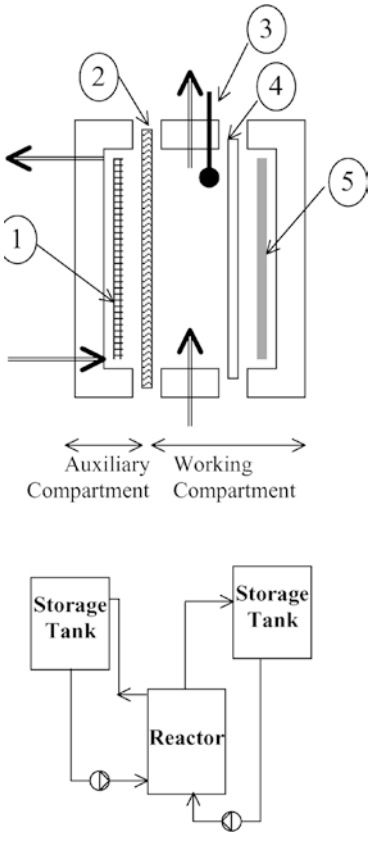

Fig. 2 Scheme of the dialysis membrane electrochemical reactor (D-MER)

through the membrane was driven by the concentration gradients only. The D-MER was always used in recycling mode.

The ultra-filtration membrane electrochemical reactor (UF-MER) (Fig. 3) was equipped with a platinum or a porous carbon felt working electrode (5). The solution circulated through this electrode, then through the membrane (4') until reaching the solution collector (6). In this case, the driving force is the pressure gradient. The UF-MER was used in continuous or recycling configurations. The working loop was open to get the continuous mode. The solution flowed from the storage tank to the working compartment and was collected at the outlet. The pressure gradient obtained by the difference of heights between the storage tank and the reactor was sufficient to ensure the solution flow through the 5 kDa cut-off membrane. The outlet solution was collected at hourly intervals, each sample consequently gave 1-hour-average concentrations. The results were expressed as the 1-hour-average transformation ratio versus the sample number, or equivalently versus time. The recycling mode was obtained with a closed working loop. In this case, the solution had to be pumped around and a 30 kDa cut-off UF-membrane was used. The results were expressed as the transformation ratio in the storage tank versus time.

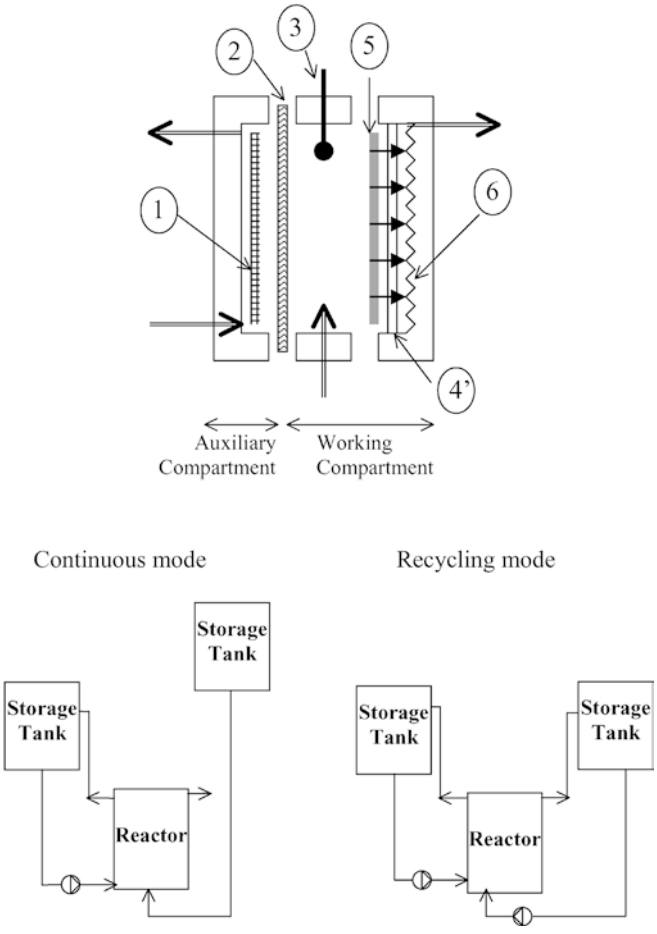

Fig. 3 Scheme of the ultra-filtration membrane electrochemical reactor (UF-MER)

Results

Characterisation of mediators

A preliminary electrochemical study was carried out to determine the potential to be imposed for electrolysis. Current-potential curves were recorded on platinum with 1 mM methyl viologen in a 0.1 M phosphate buffer (Fig. 4). The MV^{2+} reduction wave was observed from −0.6 V/SCE, followed by the solvent reduction at −0.8 V/SCE. Consequently the value of −0.7 V/SCE was chosen for the synthesis performed with methyl viologen and a platinum electrode.

Current-potential curves were recorded on a carbon electrode with 0.5 mM rhodium complex in tris-HCl buffer 0.1 M (Fig. 5). The first wave noted I, at -0.5 V/SCE, was assigned to oxygen reduction, whereas wave II ($E_{peak} = -0.85$ V/SCE) corresponded to the reduction of the rhodium complex. The increase of signal II, which was observed after the addition of NAD^+, indicates a very fast reaction between the reduced rhodium complex and NAD^+. The potential chosen for the synthesis using the rhodium complex on carbon felt was consequently −0.8 V/SCE.

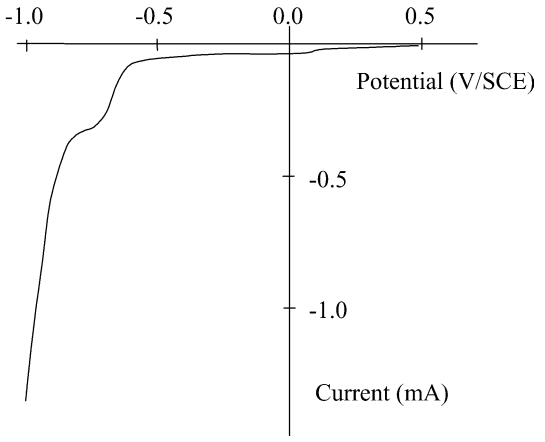

Fig. 4 Current-potential curve for a platinum electrode in a buffer solution containing methyl viologen (1 mM). Scan rate 10 mV/s

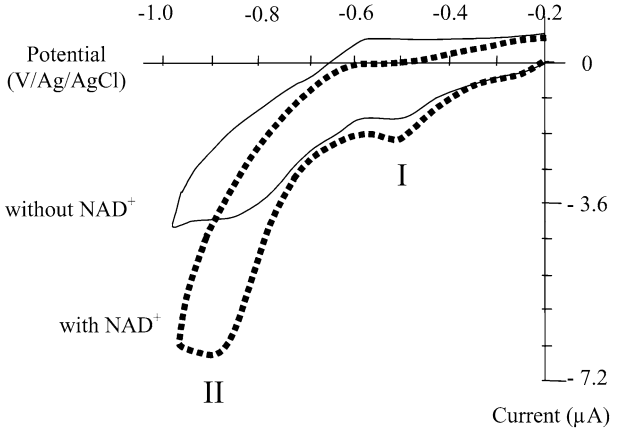

Fig. 5 Current-potential curves for a carbon felt electrode in a buffer solution containing the rhodium complex (0.5 mM) without or with NAD^+ (0.25 mM). Scan rate 25 mV/s

For these two potentials (−0.7 V/SCE and −0.8 V/SCE on platinum and carbon electrode respectively), it was verified that cyclohexanone and cyclohexanol did not react directly on the electrodes.

Regeneration with methyl viologen

The D-MER was equipped with a platinum electrode maintained at –0.70 V/SCE. The solution composition is reported in Table 1. At the beginning of the experiment 787 U LipDH were put in the reaction layer of the reactor. No trace of cyclohexanol was detected after 62 h, in spite of addition of 400 U LipDH after 18 h. Several attempts confirmed this absence of cyclohexanol production.

The UF-MER was then used with the same platinum electrode, in continuous mode, it means with an open loop and only one passage of the solution in the reactor. The initial concentration of cyclohexanone, methyl viologen and NAD^+ are gathered in Table 1. Sixty U HLADH and 590 U LipDH were put in the reaction layer. The potential was imposed after 19 h, to verify that there was no transformation without electrochemical assistance. The results are presented in Fig. 6. After a few hours of electrolysis at –0.7 V/SCE, cyclohexanol was detected, and a constant transformation ratio of 26% was obtained during 7 h (from samples 35 to 42, each sample corresponding to 1 h collection) with only one passage through the reactor at 0.47 mL/h. Successive additions of LipDH (+260 U, +350 U and +125 U) led to successive increases of the transformation yields; after a certain latency period, 45, 65 and up to 72% yields were obtained, respectively. This demonstrates that the LipDH-catalysed reaction between NAD^+ and the reduced methyl viologen is the limiting step. Production yields that were derived from these results are expressed in nmol/h/U (Table 2) so that they can be compared with results from the literature.

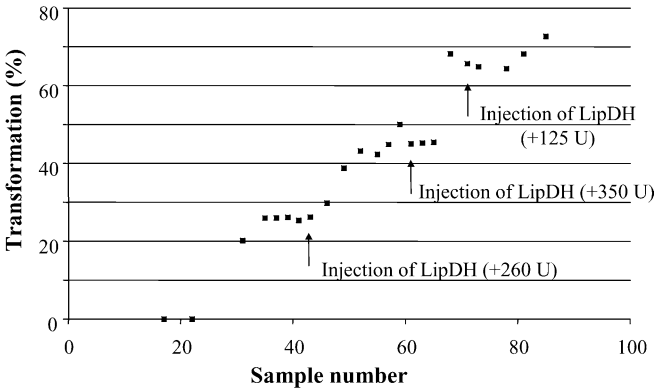

Fig. 6 Influence of LipDH on the transformation of cyclohexanone into cyclohexanol in the UF-MER in continuous mode. Regeneration of NADH with methyl viologen as mediator. Phosphate buffer 0.1 M pH 8.0, cyclohexanone 100 mM, MV 5 mM, NAD^+ 1 mM, HLADH 60 U, LipDH 590 U, flow 0.47 mL/h

The differences in the internal arrangement of the two reactors should be invoked (Figs. 2 and 3) to explain the difference observed here. In the D-MER, the solution circulates tangentially to the dialysis membrane. Under the effect of concentration gradient, the solute diffuses through the membrane towards the reaction layer, which contains the two enzymes. The electrode was placed at the bottom of this reaction layer. The order in which the reactions take place is firstly the enzymatic reaction and then the electrochemical reaction. In the UF-MER, the solution crosses the electrode before the reaction layer that contains the enzymes. In this reactor, the electrochemical reaction occurs first, before the enzymatic reaction. On the other hand, the reaction system is made up of three successive steps: the electrochemical step on the electrode surface (reaction 1a, 1b), and the two enzyme-catalysed reactions (reactions 2a and 3), which occur in the reaction layer. In the UF-MER,

Table 2 Results obtained with the different configurations of reactors

Regeneration system	Reactor volume or flow rate	Transformation ratio	nmol/h/U	nmol/h/cm^2	Space-time yield (g/L/h)
Pt/MV/LipDH Cyclohexanone concentration 0.1 M	D-MER recycling	0	0	0	0
	UF-MER continuous 0.47 mL/h	72%	564	1128	–
C/Rh complex Initial cyclohexanone concentration 0.1 M	Beaker 8 mL	75%	Max 965 Average 147	Max 1180 Average 179	Max 0.27 Average 0.04
	UF-MER continuous 0.27 mL/h	75%	337	675	–
	UF-MER recycling 100 mL	100%	1957	4762	0.14
		75%	2568	6250	0.19
Pt/H_2O_2 [43] oxidation Initial glucose concentration 0.3 M	D-MER recycling 100 mL	28%	28000	93330	5.49

this sequence was respected, i.e., the electrochemical reaction 1a, 1b occurred first, and the solution reached the reaction layer with a high concentration of the reduced species $MV^{\circ +}$. On the contrary, the sequence occurred in the opposite order in the D-MER because the electrode was at the bottom of the reaction layer. This difference in the sequence of the events may partly explain the difference in the performance of the two reactors.

The experiments were carried out with nitrogen bubbling in the storage tank, but traces of oxygen may still remain. Any trace of oxygen can drastically decrease the synthesis yield via two parasitic reactions. On one hand, oxygen is known to react very quickly with $MV^{\circ +}$:[42]:

$$2\ MV^{\circ +} + O_2 + 2H^+ \rightarrow 2MV^{2+} + H_2O_2 \qquad (4)$$

This reaction likely competes with reaction 2a, which regenerates NADH.

On the other hand, LipDH catalyses the reaction between NADH and oxygen:

$$2\ NADH + O_2 + 2\ H^+ \xrightarrow{LipDH} 2\ NAD^+ + 2\ H_2O \qquad (5)$$

This reaction competes with the synthesis reaction. Occurrence of reaction 5 was quickly verified experimentally. 20 U lipoamide dehydrogenase were added to a 3 mL aerated solution containing 0.24 mM NADH. Addition of LipDH provoked a NADH consumption rate of 0.21 mmol min^{-1}, measured spectrophotometrically at 340 nm.

In this respect also, the sequence of events achieved by the UF-MER was more efficient because any traces of oxygen were electrochemically reduced on the electrode before entering the reaction layer. The reaction layer was thus absolutely anaerobic. On the contrary, oxygen was reduced only at the bottom of the reaction layer in the D-MER. The internal arrangement of the UF-MER was more suitable to carry out this synthesis, because it avoided all the side-reactions due to the presence of oxygen traces.

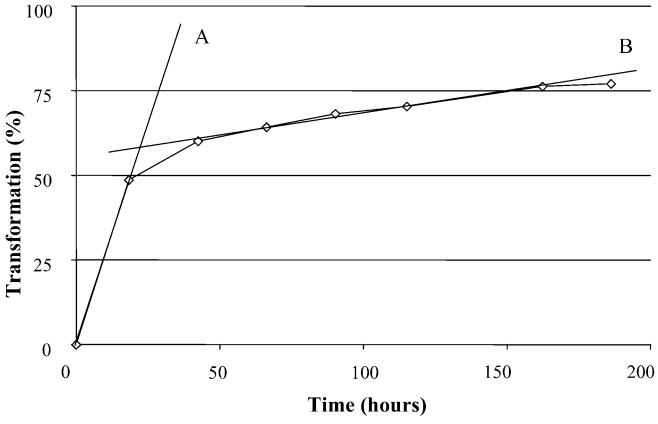

Fig. 7 Transformation of cyclohexanone into cyclohexanol in a beaker (volume 8 mL). Regeneration of NADH with rhodium complex as mediator. Tris-HCl buffer 0.1 M pH 7.5; cyclohexanone 100 mM; rhodium complex 1 mM; NAD^+ 1 mM; HLADH 22 U

Regeneration with rhodium complex

Before being tested in the UF-MER, the regeneration of NADH with the rhodium complex was carried out in a beaker. The working electrode was a 5 mm thick carbon felt rolled to have the form of a cylinder with a geometric surface area of 18 cm^2. In the experimental conditions reported in Table 1, electrolysis at –0.8 V/SCE led to 75% transformation after 186 h. The form of the curve transformation ratio as a function of time (Fig. 7) allowed two phases to be distinguished with two very different reaction rates. During the first hours, the transformation rate derived from the slope of the straight line A was 965 nmol/h/U. After a few hours, it decreased to reach an approximately stable value of 46 nmol/h/U (slope of straight line B), which is called the ''steady rate'' later on. The average production rate after 186 h was 147 nmol/h/U (Table 2). This first experiment showed the efficiency of the rhodium complex for NADH, but the volume processed was low (8 mL), and the enzyme should be separated and recuperated to be further reused in order to process higher

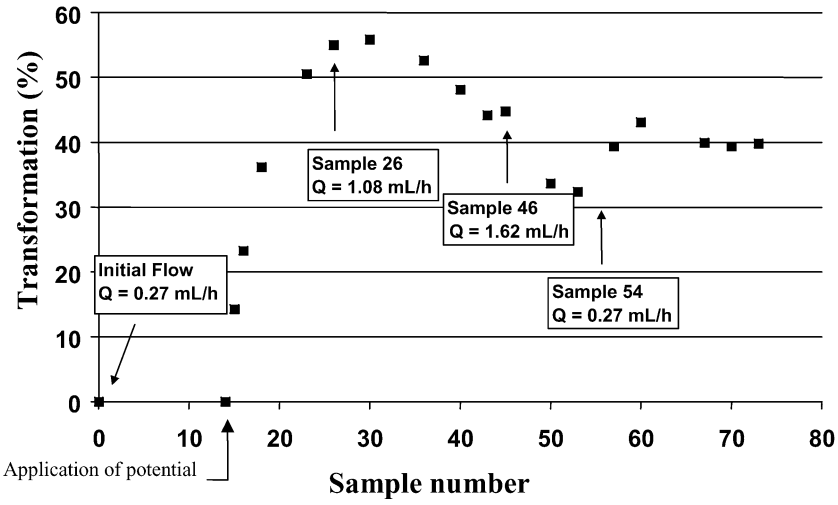

Fig. 8 Influence of flow on the transformation rate of cyclohexanone into cyclohexanol in the UF-MER in continuous mode. Regeneration of NADH with the rhodium complex as mediator. Tris-HCl buffer 0.1 M pH 7.5; cyclohexanone 100 mM; Rh complex 1 mM; NAD^+ 1 mM; HLADH 60 U

volumes. Moreover, a drastic decrease of the initial production rate was observed.

100 mL of solution (composition in Table 1) were treated in the UF-MER equipped with a carbon felt electrode and a 5 kDa cut-off UF-membrane. The reactor was processed in continuous mode, with an initial flow rate of 0.27 mL/h. The transformation started as soon as the potential of –0.8 V/SCE was imposed and the transformation ratio continuously increased (Fig. 8). When it reached approximately 55%, the flow rate was increased, and the transformation ratio decreased to 45% for 1.08 mL/h, and to 33% for 1.62 mL/h. When the flow rate was restored at the initial value 0.27mL/h the transformation ratio increased again up to 40%. The transformation ratio significantly decreased when the circulation rate increased. It can consequently be concluded that the transformation ratio was directly controlled by the residence time in the reaction layer. It should be noted that the initial and final flow rates were both equal to 0.27 mL/h but a lower transformation ratio was obtained at the end of the experiment than at the beginning. To verify whether this ratio decreased with time, a synthesis was carried out with a constant flow of 0.27 mL/h and with the same concentration of reagents (Table 11 and Fig. 9). The potential of –0.8 V/SCE was imposed after 24 h. The transformation ratio kept a constant value of 75% over 36 h. The decrease observed in the previous experiment was probably due to the different changes of flow rate imposed in the course of the experiment, and not to an intrinsic ageing of the reaction system. The highest values of the flow rate may disturb the morphology of the porous electrode or the adsorption of the enzyme on the electrode.

As the transformation ratio was at least partly controlled by the residence time, it may be hoped to reach 100% transformation with a lower flow. But the duration of the reaction would become far too long. With the view of increasing the transformation ratio, the UF-MER was used in the recycling configuration. The 5 kDa cut-off membrane was replaced by a membrane with 30 kDa cut-off, and the circulation was driven by a pump to increase the solution flow rate through the reactor. The initial conditions and the results are reported in Table 1 and Fig. 10, respectively. After 70 h of electrolysis, transformation of cyclohexanone into cyclohexanol was complete. The average production rate was 1957 nmol/h/U.

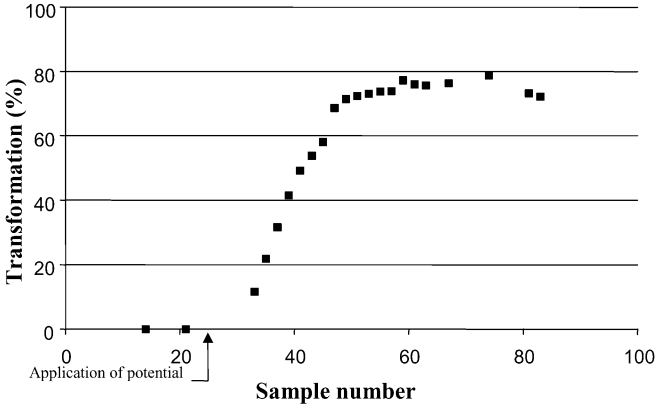

Fig. 9 Transformation of cyclohexanone into cyclohexanol in the UF-MER in continuous mode at constant flow (0.27 mL/h). Regeneration of NADH with the rhodium complex as mediator. Tris-HCl buffer 0.1 M pH 7.5; cyclohexanone 100 mM; Rh complex 1 mM; NAD^+ 1 mM; HLADH 60 U

Discussion

First of all it must be emphasized that the drastic failure observed here with the D-MER is linked to the nature of the reactions to be processed; it is absolutely not an intrinsic characteristic of the reactor. For instance, the D-MER has proveded remarkably efficient when applied to the production of gluconic acid by the glucose oxidase (GOD)-catalysed oxidation of glucose [43]:

$$C_6H_{12}O_6 + O_2 + H_2O \xrightarrow{GOD} C_6H_{12}O_7 + H_2O_2$$

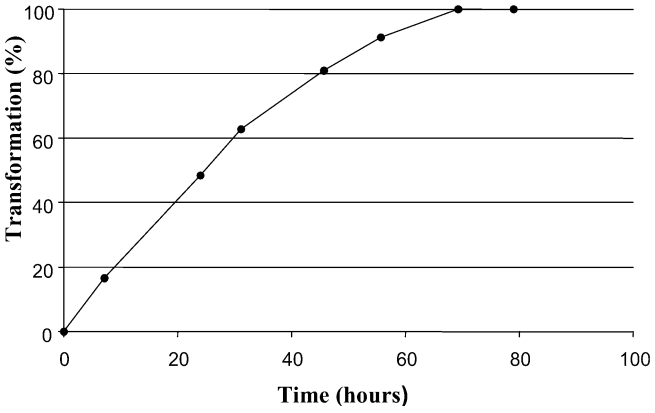

Fig. 10 Transformation of cyclohexanone into cyclohexanol in the UF-MER in recycling mode. Regeneration of NADH with the rhodium complex as mediator. Tris-HCl buffer 0.1 M pH 7.5; cyclohexanone 100 mM; Rh complex 1 mM; NAD^+ 1 mM; HLADH 73 U

In this case the role of the electrochemical step is to consume as fast as possible the hydrogen peroxide produced, which strongly inhibits the enzyme. This reaction has been processed in a D-MER with 100 mL of 0.3 M glucose and 100 U GOD behind the dialysis membrane. Very good results have been obtained as reported in Table 2. In this case the absence of solution flow around the enzyme is an essential key for success, because it allows GOD to remain very close to the electrode surface, or adsorbed on it, which is the only location where the concentration of hydrogen peroxide can be maintained at zero.

The good results obtained here with the UF-MER with a reaction that cannot be processed in the D-MER perfectly illustrate the great importance of reactor type when enzyme-catalysed reactions are processed. Two causes were identified: the order of successive reactions was not favoured in the D-MER, and the D-MER was not efficient in keeping the reaction environment absolutely anaerobic. The experiments conducted in the UF-MER with successive additions of LipDH clearly showed that this enzymatic reaction was the limiting step of the synthesis. Another disadvantage is the high sensitivity to oxygen of the reduced methyl viologen, particularly when LipDH is present in solution because of side-reaction 5. In all cases, the use of rhodium complex seems more suitable since it is not necessary to eliminate oxygen and no enzyme is required to catalyse the reaction between NAD^+ and the rhodium complex.

The results obtained with the different configurations of reactors are reported in Table 2 in three forms:

- The production rate with respect to the enzyme activity (nmol/h/U) evaluates the capability of the reactor to effectively exploit the enzyme catalysis
- The production rate with respect to the electrode surface area (nmol/h/cm^2) is currently used to compare the effectiveness of the electrochemical reactors
- The space-time yield for recycling and batch processes (g/L/day) gives the raw production yield of the reactor.

For the beaker, two different values were derived either from the initial maximal production rate (slope A in Fig. 7), or from the average production rate. In the continuous configuration the UF-MER is clearly not effective enough, because of the very low flow rates that were necessary to increase the residence time was drastically detrimental to the mass transfer through the electrode and the membrane. The recycling configuration revealed the great effectiveness of the UF-MER. Actually, the evolution observed in the beaker, from the ''initial rate'' to the ''steady rate'', was certainly due to the accumulation of the products inhibiting the enzymatic transformation. In the UF-MER the products were continuously removed from the reaction layer by the circulation, and no accumulation occurred. Moreover, the UF-MER, which can process higher volumes of solution than in a simple beaker with the same quantity of enzyme, is obviously economically more efficient (1957 nmol/h/U instead of 147 nmol/h/U). A more pertinent comparison of the results obtained with the beaker and with the UF-MER in recycling configuration should be done by calculating the production rates and the yield obtained with the UF-MER when 75% transformation were reached, as was the case in the beaker. These values, reported in Table 2, clearly demonstrated the efficiency of this reactor. Good results were obtained even with the methyl viologen based regeneration system, which is difficult to process.

As can be seen through the brief survey proposed in the introduction, the capability of the reactors to reach complete transformation is generally considered as an essential advantage. The complete transformation of 0.1 M in 70 h achieved here may be considered as a very good result with respect to the performances commonly reported in the literature. Comparison with the electrochemical studies cited in the introduction also shows that the production rates with respect to the surface electrode area obtained here are among the higher values encountered in the literature. The electrodes were consequently exceptionally efficient, as could be expected thanks to the combination of the solution flow through the electrode with the confinement of the enzyme in the strict vicinity of the electrode surface.

The space-time yields obtained here are markedly lower than the values reported in the introduction for classic membrane processes. This situation seems logical because the high space-time yields reported elsewhere have been obtained with homogeneous processes. Space-time yields are rarely calculated for heterogeneous processes as was the case here. For instance, the high space-time yield that has been obtained for glucose oxidation (last line in Table 2) corresponded to an homogeneous process (see the common values reported in the introduction) because, in this case, the electrochemical step did not drive the reaction, but its role was only to consume hydrogen peroxide to prevent the enzyme from inhibition.

The higher space-time yield obtained in the simple beaker (initial maximal value of 0.27 g/L/h or 6.5 g/L/day) than in the UF-MER, indicates the possibility to improve the UF-MER. Actually, the surface/volume ratio in the beaker was 2.25 cm^2/cm^3 instead of 0.3 cm^2/cm^3 for the UF-MER. Increasing the surface/volume ratio in the UF-MER would be a simple way to improve more its performance.

Conclusion

The reactors proposed here greatly improved the results commonly obtained with classic electrochemical reactors. The combination of the solution flow through the porous electrode with the confinement of the enzymes against the electrode surface leads to remarkably good performances. Because no preliminary modification or immobilisation of the enzymes are required, these reactors can be used with all kinds of enzymes, and more importantly with all kinds of catalysts that can be retained by a membrane. Raw enzyme preparations can, for instance, be used as soon as they are obtained without any losses due to chemical immobilisation. This innovative technology may open new and promising opportunities for electrochemical processes in the field of enzyme-catalysed synthesis.

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