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# Hybrid flow bioreactor with all catalysts immobilized for enzymatic electrosynthesis

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## Abstract

The electrochemical regeneration of the NADH cofactor was realized in a hybrid flow reactor coupling fuel cell technology and redox flow device, paying attention to the robust immobilization of all catalysts. The rhodium catalyst  $\text{Rh}(\text{Cp}^*)(\text{bpy})\text{Cl}^+$  was covalently immobilized on a MWCNT layer and the association with the gas diffusion electrode was carefully optimized. High stability and activity of the electrochemical system were assessed by cyclic voltammetry and amperometry in the flow reactor. Afterwards, the optimal cofactor regeneration was applied to NADH-dependent biosynthesis using immobilized lactate dehydrogenase for the conversion of pyruvate to lactate in the flow cell in the presence of cofactor concentration as low as 10  $\mu\text{M}$ . 79 % faradaic efficiency was achieved and remarkable total turnover number (TTN) were reached: 2500 and 18000 for the NADH cofactor and the Rh complex, respectively.

**Keywords:** immobilization, electrochemical regeneration, redox flow, gas diffusion electrode,  $\beta$ -nicotinamide adenine dinucleotide, bioconversion

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## 1. Introduction

The catalytic hydrogenation using heterogeneous or homogeneous transition metal catalysts is well set up in industrial fine chemicals synthesis<sup>[1]</sup> where 10 % to 20 % of chemical steps involves catalytic hydrogenations.<sup>[2]</sup> Noble metals are highly exploited as catalysts in this area<sup>[3,4]</sup>. In these processes, high pressures of hydrogen are generally required for the efficient catalytic hydrogenation (>80 bars)<sup>[4]</sup>. Alternatively, the synthesis of fine chemicals, *e.g.* the reduction of the “C double bond X” in the ketones, alkenes, and imines, requires the use of biological catalysts, *e.g.*, oxidoreductases (known for their high productivity, enantio- and chemo-selectivity).<sup>[5]</sup> This particularity is valuable in the chemical and pharmaceutical sectors where 90 % of the chiral molecules were sold as racemic mixture before 1993<sup>[6]</sup> and 75 % of new drugs in 2002 were single enantiomers.<sup>[7]</sup>

However, oxidoreductases applied in bioconversions cannot use molecular hydrogen directly. Hydrogen can be transferred through enzymatic cofactors such as NADH which plays an important role in biological systems and can be applied as electron carrier in enzymatic systems catalyzing stereo- and regio- selective reactions having a great biotechnological potential.<sup>[5,8]</sup> The use of enzymatic cofactors in biosynthesis applications (bioconversion) requires their regeneration due to their high costs.<sup>[9]</sup> The NADH cofactor can be indirectly regenerated *in situ* by electrochemistry (using suitable redox mediators), which offers an easy separation of products and a high control of the process.<sup>[9,10]</sup> Other NAD(P)H regeneration techniques can be applied such as the chemical, photochemical, enzymatic, and biological ones.<sup>[11–13]</sup> The application of the electrochemical regeneration of the NAD(P)H cofactor requires the use of an electrochemical mediator in order to lower the cathodic overpotentials associated to the reduction of  $\text{NAD(P)}^+$ , thus preventing the formation of the inactive dimer form  $(\text{NAD(P)})_2$  occurring at more cathodic potential values.<sup>[14]</sup> One efficient mediator for the electrochemical regeneration of 1,4-NAD(P)H, the active form of NAD(P)H, is chloro(2,2'-bipyridyl)

(pentamethylcyclopentadienyl)-rhodium (III) chloride complex  $[Cp * Rh(bpy)Cl]^+$ .<sup>[15–17]</sup> Catalysts applied in the electrochemical or the electroenzymatic regeneration of the NAD(P)H cofactor, either molecular or enzymatic, *e.g.*, the aforementioned rhodium complex or Ferredoxine NADP(+) reductase (FNR), can be immobilized on electrode surfaces,<sup>[18][19]</sup> which is important for the easy recovery of the products formed (*i.e.*, no need for separation) and the possible re-use of the electrocatalyst.

There is expanding interest in implementing hydrogenation in continuous flow reactors, where reactants are continuously fed to the reactor and emerge as a continuous stream of products.<sup>[20,21]</sup> Advantages of continuous flow reactors include increased yields of bioconversion due to a high mass transport between reactants and increased selectivity through refined cell design and technology.<sup>[22,23]</sup> The electrochemical regeneration of the NADH cofactor has been previously reported for biocatalytic hydrogenations and dehydrogenations.<sup>[9][24]</sup> However, the translation into flow electrochemical reactor has suffered from different constraints such as the reactor design<sup>[25]</sup> and low conversion efficiencies related to slow kinetics of regeneration of NADH at conventional electrodes<sup>[26]</sup>.

Previously, we have described a new modular route to combine efficiently the oxidation of hydrogen to the regeneration of NADH using a mediator in solution.<sup>[27]</sup> The idea was to couple the two redox reactions in a hybrid reactor that combines a gas diffusion electrode and a redox flow device with the help of an ion-exchange membrane for protons conduction from the anodic to the cathodic compartment. More precisely, the reactor allows the electrochemical regeneration of the NADH cofactor mediated by a rhodium complex in solution at the cathode with continuous flow reaction, and hydrogen oxidation at the gas diffusion anode. Several parameters were optimized such as hydrogen and solution flow rates (20 mL.min<sup>-1</sup>), pH (7.2), and the molar ratio between NAD<sup>+</sup> and the rhodium complex.<sup>[27]</sup> The efficiency of the approach was demonstrated first in H<sub>2</sub>-driven NADH production, then to supply an NADH-dependent

lactate dehydrogenase immobilized by adsorption on multi-walled carbon nanotubes for reduction of pyruvate to lactate. The lactate dehydrogenase is unable to use electrons directly and, therefore, only operated if supplied with reducing equivalents in the form of NADH regenerated by the rhodium complex mediator added in solution. The next challenge is thus the immobilization of all catalysts, compatible with the use of a sustainable flow bioreactor using low amount of enzymatic cofactor.

In the context of immobilized complex for NADH electrochemical regeneration, two essential aspects have been investigated in the present work. First, the method for immobilization of the rhodium complex  $[Cp * Rh(bpy)Cl]^+$  have been optimized using carbon-based supports to obtain Rh-grafted matrices exhibiting high stability and activity in the redox flow reactor. Carbon materials has been selected because of their low cost, large potential window, relatively inert surface, and active electrochemical properties for redox reactions,<sup>[28]</sup>. Then, the immobilization of the lactate dehydrogenase has been achieved in order to promote rapid transfer of  $NAD^+/NADH$  from the molecular catalyst to the enzymatic catalyst.  $NAD^+$  amounts as low as 10  $\mu M$  were shown sufficient for the bioconversion of 25 mM pyruvate to lactate in the optimal cell. The original design of the cathode chamber led to a total turnover of NADH larger than the best values ever reported in the literature for electrochemical regeneration.<sup>[29]</sup>

## 2. Results and Discussion

### 2.1. Immobilization of $[Cp * Rh(bpy)Cl]^+$

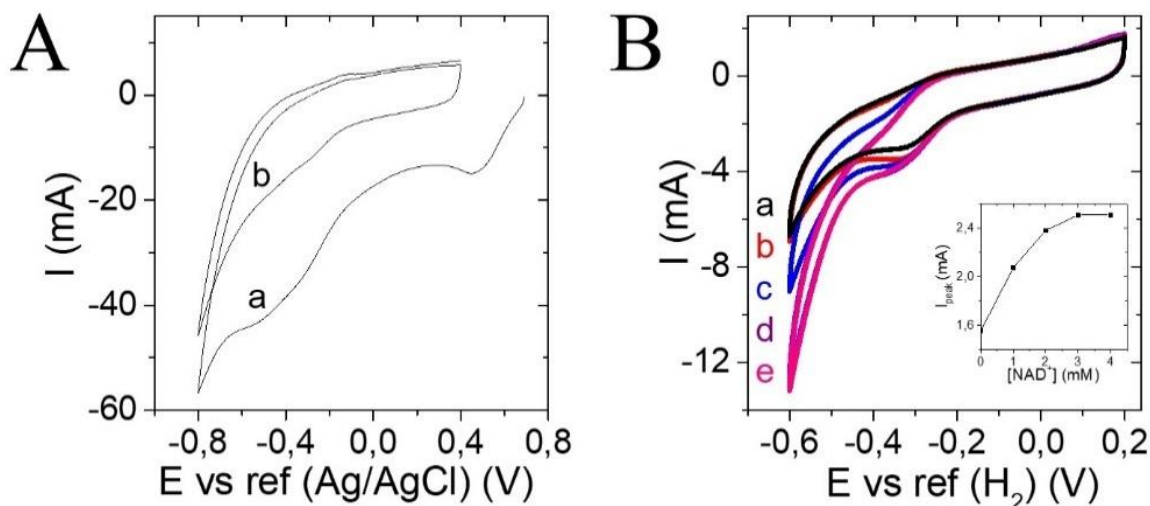
The immobilization of the rhodium complex on a carbon support involves a two-step process as described in the literature.<sup>[17]</sup> First, 4-amino-2,2-bipyridine reacts quantitatively in solution to produce bipyridyl diazonium cations. Then, the functionalization of the carbon support with 2,2-bipyridine (bpy) moieties is carried out by cyclic voltammetry, involving the electroreduction of the diazonium cations with concomitant release of dinitrogen and generation of aryl radicals prone to be grafted on the carbon support. The rhodium complex is then formed after a complexation reaction of  $(RhCpCl_2)_2$  with the bipyridyl-functionalized electrode, giving rise to rhodium complexes covalently bonded to the carbon support. (**Figure S1**)

#### Selection of the carbon electrode material

Multi-walled carbon nanotubes (MWCNTs) can allow significant improvement of the efficiency of the electroenzymatic synthesis system by tuning some electrochemical properties, *e.g.*, by enhancing the electroactive surface area.<sup>[30]</sup> On the basis of this opportunity, MWCNTs had been identified as suitable supporting materials for Rh complex immobilization.<sup>[29,31]</sup> Based on the perspective of combination between fuel cell technology and redox flow technique, a carbon paper (CP) coated with MWCNTs (noted CP-MWCT) was chosen for the construction of the catalytic layer after comparing it with bucky paper and graphite felt electrodes.

Diazonium electrografting on a 16 cm<sup>2</sup> CP-MWCNT electrode was performed by cyclic voltammetry from 0.4 V to -0.8 V vs Ag/AgCl, as shown in **Figure 1A**. During the first scan, an irreversible wave was observed at -0.35 V vs Ag/AgCl (curve a) corresponding to the grafting of 2,2-bipyridine and the release of N<sub>2</sub>. A cathodic reduction peak of lower intensity was observed in the second scan (curve b) due to the partial blockage of the CP-MWCNT electrode surface, indicating successful electrografting. The CP-MWCNT electrografted with

2,2-bipyridine moieties was then immersed in a solution of  $(\text{RhCpCl}_2)_2$  for the formation of rhodium complexes covalently immobilized onto the electrode surface.



**Figure 1.** (A) (a) first and (b) second cyclic voltammograms for the reduction of diazonium cations generated ‘in situ’ from 1 mM 4-amino-2,2’-bipyridine and 2 mM NaNO<sub>2</sub> in 0.5 M HCl, as recorded on CP-MWCNT electrode at 20 mV.s<sup>-1</sup>. (B) Cyclic voltammograms recorded at a potential scan rate of 5 mV.s<sup>-1</sup> using a CP-MWCNT-bpy-Rh electrode in 50 mM PBS buffer at pH 7.2 under nitrogen and in the presence of increasing concentrations of NAD<sup>+</sup> (a) 0 mM (b) 1 mM (c) 2 mM (d) 3 mM and (e) 4 mM. The geometric surface area of the electrode was 16 cm<sup>2</sup> and hydrogen and solution flow rates were at 20 mL.min<sup>-1</sup>.

The electrocatalytic behavior of the so-formed CP-MWCNT-bpy-Rh electrode was evaluated through cyclic voltammetry (**Figure 1B**), in a configuration where the working electrode has been arranged in the redox flow reactor, at a scan rate of 5 mV.s<sup>-1</sup> in 50 mM phosphate buffer solution (PBS) at pH 7.2, with hydrogen and solutions flow rates of 20 mL.min<sup>-1</sup>. As shown in scheme S1 in Supporting Information, a graphite felt (16 cm<sup>2</sup>) was placed on the top of the CP-MWCNT-bpy-Rh electrode in order to ensure a uniform distribution of the solution in the cathodic compartment and to maintain good electrical conduction with the graphite bipolar plate. A gas diffusion electrode, allowing hydrogen oxidation was used in the anodic

compartment A Nafion membrane (N-212) was placed between the two electrodes for separation of the two compartments and to permit a uniform proton transport rate from the anode to the cathode. In the absence of  $\text{NAD}^+$  in the medium, a cathodic current was observed at -0.31 V vs ref. ( $\text{H}_2$ ) (see curve **a** in **Figure 1B**), which was less negative than the potential reported in previous works for the same Rh complex immobilized on a bucky paper (BP) electrode (-0.65 V vs ref. ( $\text{Ag}/\text{AgCl}$ )  $\approx$  -0.45 V vs ref. ( $\text{H}_2$ ))<sup>[32]</sup> or in solution (-0.4 V vs ref. ( $\text{H}_2$ ))<sup>[27]</sup>. This potential shift can be explained by the influence of the electrode material on the electron density of the rhodium complex center<sup>[33]</sup> or due to the minimized distance between the anodic and the cathodic electrodes separated by the Nafion membrane. The addition of increased amounts of  $\text{NAD}^+$ , from 1 mM to 4 mM (see curves **b**, **c**, **d** and **e**, in **Figure 1B**), led to the increase in the cathodic current showing the mediated electrocatalytic activity of the Rh complex towards  $\text{NAD}^+$  reduction. A maximum cathodic current of 2.6 mA was observed at the concentration of 3 mM  $\text{NAD}^+$ . Alternatively, for comparison with a previous work where  $[\text{Cp} * \text{Rh}(\text{bpy})\text{Cl}]^+$  was reacting on graphite felt,<sup>[27]</sup> the direct immobilization of the Rh complex on a graphite felt electrode (GF-bpy-Rh) was evaluated. The cathodic current peak observed at -0.35 V vs ref ( $\text{H}_2$ ) decreased with increasing  $\text{NAD}^+$  concentrations, expressing a lower electroactive surface of the GF than with MWCNT<sup>[34]</sup> and indicating the poor stability of the support-Rh complex system under high flow rate conditions (**Figure S2A&B**), confirming the advantage of CP-MWCNT-bpy-Rh electrode for the present study.

In addition, the catalytic activity of CP-MWCNT-bpy-Rh towards NADH regeneration was also confirmed by amperometry at constant potential (-0.3 V vs ref ( $\text{H}_2$ )) and the corresponding current variation for the conversion of 1 mM  $\text{NAD}^+$  in solution ( $V=20$  mL) is shown in **Figure S3A** (curve **a**). Complete conversion of  $\text{NAD}^+$  into NADH was observed within 50 min electrolysis (**Figure S3B**, curve **a**) with an averaged faradaic efficiency of 71 % (**Figure S3C**, experiment **a**) using the CP-MWCNT-bpy-Rh cathode. The amount of rhodium complex on the



CP-MWCNT electrode,  $4.125 \text{ nmol.cm}^{-2}$ , was estimated from cyclic voltammetry by integrating the cathodic peak. From that amount, a turnover frequency of  $1.7 \text{ s}^{-1}$  was calculated during the first 5 min of NADH electrochemical regeneration. This value of turnover frequency is very close to the highest value reported in the literature ( $3.6 \text{ s}^{-1}$ ) for an immobilized Rh complex on MWCNTs.<sup>[31]</sup> Furthermore, total conversion of  $\text{NAD}^+$  into NADH was obtained with a ratio of  $\text{NAD}^+$  over rhodium complex concentrations in the order of 300, which is much larger than the maximal value achievable with dissolved Rh complex (ratio between  $\text{NAD}^+$  and Rh complex not exceeding 40).<sup>[27]</sup> Such improvement in terms of performance came in addition to the advantage of CP-MWCNT-bpy-Rh leading to effective NADH regeneration while avoiding the use of dissolved rhodium complex.

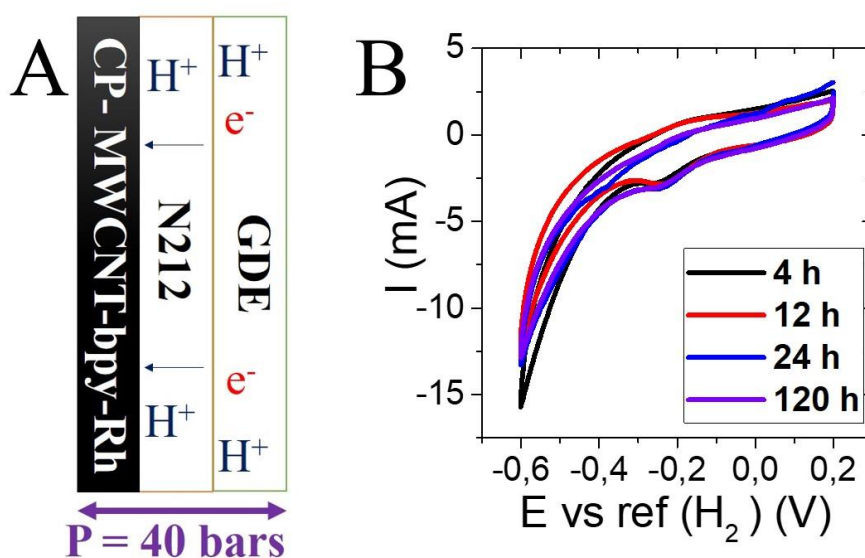
To complete this study, bucky paper functionalized by the rhodium complex was also evaluated for the conversion of  $1 \text{ mM NAD}^+$  into NADH in the flow bioreactor. Its catalytic activity for  $\text{NAD}^+$  reduction was confirmed by cyclic voltammetry (**Figure S2D**), and the conversion reached 92 % after 50 min (**Figure S3B**, curve **b**) with a faradaic efficiency at only 56 % (**Figure S3C**, experiment **b**). In addition, with the BP-bpy-Rh system, residual amounts of the dissolved  $(\text{RhCpCl}_2)_2$  could not be removed by dissolution in the solvent (DCM) which renders the technique poorly attractive.

Among the three functional electrodes that were tested, a carbon paper modified with a layer of MWCNT and functionalized with  $[\text{Cp} * \text{Rh}(\text{bpy})\text{Cl}]^+$  (CP-MWCNT-bpy-Rh) was thus selected here as the best one for regeneration of the NADH cofactor.

### Stability and sustained activity

In order to ensure the robustness of the CP-MWCNT-bpy-Rh electrode, a cell assembly was prepared by pressing the GDE, the Nafion membrane (N212), and the CP-MWCNT-bpy-Rh together at 40 bars at room temperature, as illustrated in **Figure 2A**. The technique was expected

to improve mechanical and electrical contacts in the whole flow cell (anode, membrane and cathodic chamber). CP-MWCNT-bpy-Rh system was characterized by cyclic voltammetry (**Figure 2B**) for different times under flow conditions after the electrode preparation: 4 h, 12 h, 24 h, and 120 h. The electrode was left inside the reactor and was tested each time with a new solution of phosphate buffer (50 mM; pH = 7.2). The very similar cathodic currents observed revealed that the electrode has maintained its activity for more than five days, and more precisely that the rhodium complex remained immobilized under flow conditions ensuring a long-term operational stability of the electrode. After confirming the electrode activity towards NADH regeneration by mediated  $\text{NAD}^+$  electroreduction, its application in electroenzymatic biosynthesis was further explored.

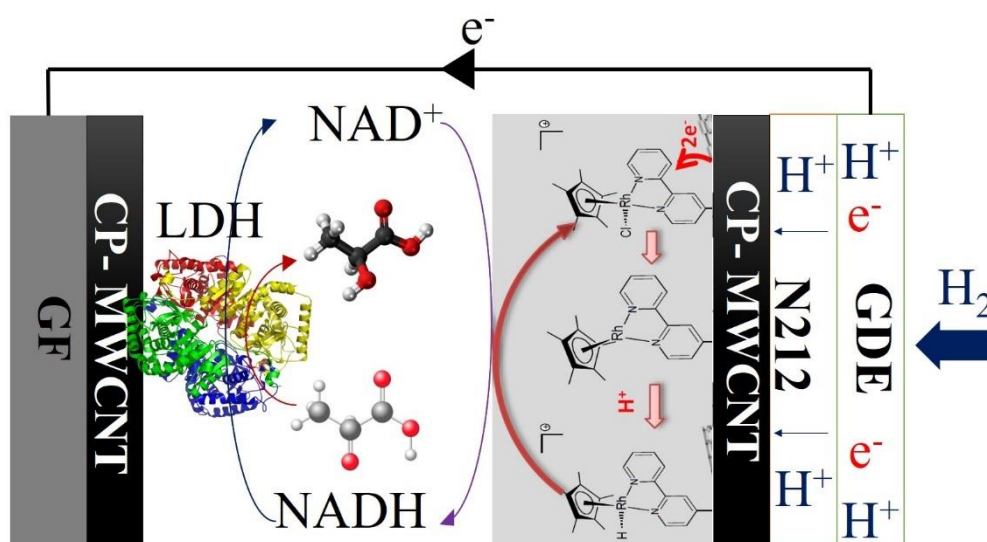


**Figure 2.** (A) Schematic representation of GDE-N212-(Cp-MWCNT-bpy-Rh) system pressed together at 40 bars. (B) Cyclic voltammograms recorded at a potential scan rate of  $5 \text{ mV.s}^{-1}$  using a  $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]^+$  functionalized CP-MWCNT (using the pressed system in **Figure 2A**) electrode in 50 mM PBS buffer at pH 7.2 under nitrogen after 4 h, 12h, 24 h, and 120 h of electrode preparation. The geometric surface area of the electrode was  $16 \text{ cm}^2$  and hydrogen and solution flow rates were at  $20 \text{ mL.min}^{-1}$ .

## 2.2. Application of regenerated NADH in an enzymatic reaction

## Parameters optimization for the efficient bioconversion of pyruvate

Coupling an enzymatic reaction to NADH regeneration should allow demonstrating that the active form of NADH (1,4-NADH)<sup>[35]</sup> was really produced by the redox flow reactor, and would be compatible with the production of fine chemicals with electrochemically regenerated NADH as a reactant involved in the biocatalytic reduction of the organic substrate. NAD-dependent lactate dehydrogenase LDH was used here as a convenient example (robust enzyme, commercially available, with reliable analytical methods for lactate and pyruvate).



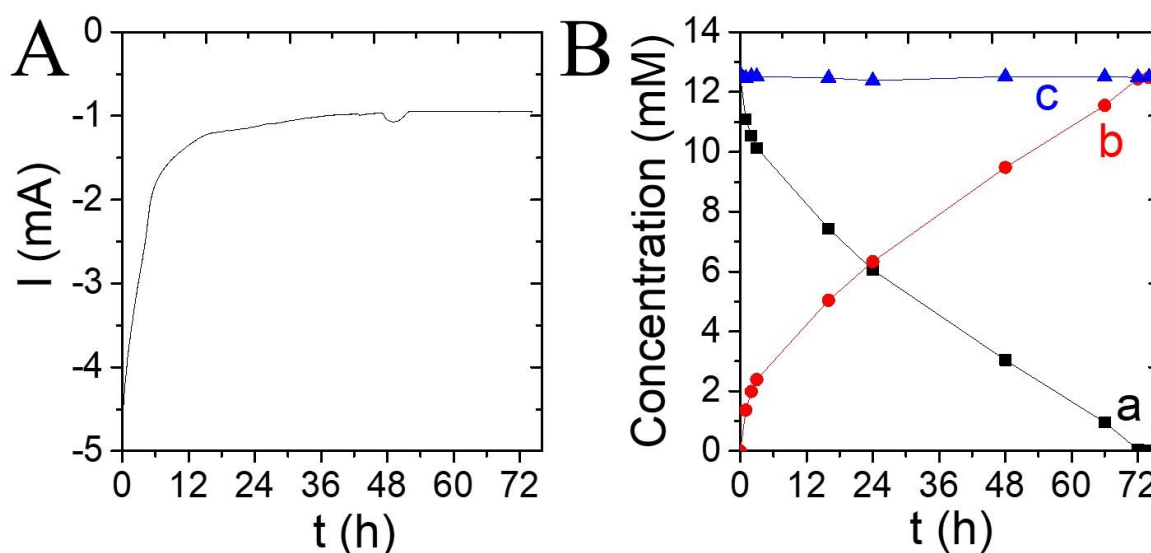
**Scheme 1.** Schematic representation of the electroenzymatic reactor including CP-MWCNT-bpy-Rh electrode and CP-MWCNT-LDH enzymatic system reactions with the LDH-MWCNT-CP system.

**Scheme 1** illustrates the NADH regeneration on a CP-MWCNT-bpy-Rh electrode coupled to hydrogen oxidation and pyruvate bioconversion. LDHs were immobilized on a second CP-MWCNT layer placed inside the electrochemical cell and stacked to the CP-MWCNT-bpy-Rh as shown in scheme 1. This design has the great advantage to connect electrically the current collector and the active electrode pressed on the Nafion membrane. The  $\text{NAD}^+$  and pyruvate-containing solution was circulated in the cathodic compartment, where NADH was regenerated by the Rh complex, then consumed continuously in the biosynthesis of lactate.

While the cofactor in its oxidized or reduced form is used as an active mediator leading to the bioconversion, we worked with low amounts of the cofactor (10  $\mu\text{M}$ ) which is economically desired and proves the stability of the cofactor with time. In addition, low amounts of cofactor are targeted to reach high TTNs.<sup>[36]</sup>

### Effect of the arrangement of supported Rh complex and LDH in the cell

The bioconversion of 12.5 mM pyruvate (in 100 mL solution containing 10  $\mu\text{M}$   $\text{NAD}^+$ ) applying the process described in **Scheme 1** was carried out potentiostatically, at -0.3 V vs ref. ( $\text{H}_2$ ) for 72 hours, with the two electrodes CP-MWCNT-bpy-Rh and CP-MWCNT-LDH onto which 250 units of LDH were immobilized ( $\approx 6.6$  nmol). The current near -4 mA in the first instants, decreased gradually to reach -1 mA after 72 hours (**Figure 3A**) whereas 100 % of pyruvate introduced was converted to lactate within this period (**Figure 3B**). The sum of the two species concentrations was nearly constant at 12.5 mM (**Figure 3B**) indicating the high selectivity of the enzymatic reaction.



**Figure 3.** (A) Variation of sampled currents with time during the electroenzymatic synthesis of lactate from 12.5 mM of pyruvate in 100 mL solution containing 10  $\mu\text{M}$   $\text{NAD}^+$  (and 50 mM PBS, pH 7.2) in the flow reactor provided with one CP-MWCNT-bpy-Rh electrode and one CP-MWCNT-LDH (250 units) layer. (B) Variation of concentrations with time of (a) Pyruvate, (b) Lactate, and (c) the sum pyruvate and lactate, as determined by HPLC.

The faradaic efficiency was equal to 73 %, a value comparable to that of NADH regeneration without enzymatic reaction (**Figure S2C**). TTNs of Rh complex,  $\text{NAD}^+$ , and LDH were respectively larger than  $1.8 \times 10^4$ , 1250, and  $1.8 \times 10^5$ . TTN for the Rh complex is – to our state of knowledge – the highest value with rhodium complex applied in electroenzymatic synthesis.<sup>[32,37]</sup> The turnover frequency was  $0.28 \text{ s}^{-1}$  after 3 hours of the bioconversion. This value is higher than the value reported in our previous work,<sup>[17]</sup> thanks to the flowing system allowing of the performance cofactor regeneration coupled to the bioconversion of pyruvate.

On the other hand, setting the CP-MWCNT-LDH layer in another reactor downstream of the “electrochemical cell” provided with the CP-MWCNT-bpy-Rh layer for NADH regeneration (see **Scheme S2** in Part 5 of supporting information) led to a lower bioconversion of 12.9 % with only 49% faradaic efficiency (**Figure S4**), far below the performances obtained with the two modified CP layers (CP-MWCNT-Rh and CP-MWCNT-LDH) stacked in the same cell.

This reported experiment leads to the conclusion that the bioconversion of pyruvate is facilitated by close interaction between the Rh complex and LDH, under flow conditions for efficient mass transport rates, leading to increased overall conversion kinetics.

As a control experiment and following reported works in the literature where both catalysts for the regeneration of the cofactor and the production of fine chemicals were placed on the same electrode,<sup>[38]</sup> LDH enzymes were adsorbed directly on the Rh-immobilized CP-MWCNT layer forming the cathode. The efficiency of the single layer system, where both the Rh complex and LDH were immobilized on the same CP-MWCNT, was tested by studying the conversion of 20 mM pyruvate (50 mL at pH=7.2) with 500 units LDH adsorbed, in the presence of 0.5 mM  $\text{NAD}^+$ , at -0.3 V vs ref ( $\text{H}_2$ ). The recorded currents decreased rapidly (in absolute value) from -1.5 mA to -0.25 mA after 5 hours (**Figure S5A**). Chemical analysis of the solution showed that the bioconversion had not occurred (**Figure S5B**), whereas  $\text{NAD}^+$  was entirely reduced to

NADH as revealed by UV-visible measurement at 340 nm. This result suggests that LDH was totally deactivated while being placed in a direct contact with rhodium complex, in agreement with former works reporting deactivation of the rhodium complex or lactate dehydrogenase.<sup>[16]</sup> In conclusion, the best strategy for optimal activity of these two catalysts, the molecular one and the biological one, is to associate them closely while keeping a separation, as it is illustrated here with the two catalytic layers.

### **Towards higher production rate of lactate**

Production rate of the designed system (scheme 1) is a quite important criterion to address. For this purpose, four experiments have been designed and carried out, in particular for optimization of the amount of Rh complex in relation to LDH immobilized on the separated CP-MWCNT material. Conditions and results of these tests are reported in Table 1.

**Table 1.** Ratio optimization between Rh complex and LDH for the optimal bioconversion of pyruvate (V = 100 mL). (\*) Current efficiency is for lactate production.

Experiment	[Pyruvate] at t=0 (mM)	LDH (nmol)	Rh (nmol)	$n_{Rh}/n_{LDH}$ ratio	[Lactate] after 72 h (mM)	(*) Current efficiency (%)
1	20	13.8	66	5	7.2	76.7
2	12.5	6.6	66	10	12.5	73
3	25	13.8	138	10	25	78
4	25	2.76	138	50	16.5	79

First, in order to evaluate the effect of LDH amount on the productivity, Experiment (1) and Experiment (2) were performed with the same amount of immobilized Rh complex and different amounts of LDH (Table 1). The current obtained with the system in Experiment (2), where the ratio between the Rh complex and LDH is equal to 10, led to a total (100 %) bioconversion (**Figure 3B**) approximately 1.5 times higher than the one obtained in Experiment

(1) where the ratio between the Rh complex and LDH is equal to 5 for which the bioconversion was restricted to 59 % (see **Figure S6** in Part 7 of supporting information). Faradaic efficiencies for both systems were in the same order of magnitude. This result leads to the consequence that an equilibrium between the regeneration of NADH by the Rh complex and its oxidation by LDH is important in order to reach a total bioconversion within an optimal time.

Later, for the sake of higher lactate production within an optimal time and to obtain a system for the electrochemical regeneration of NADH comparable with the electroenzymatic one involving enzymatic mediators, e.g. FNR,<sup>[37,38,39]</sup> additional tests (not reported in Table 1) were carried out by inserting two CP-MWCNT-bpy-Rh layers in the electrochemical bioreactor instead of one. As a matter of fact, the immobilization of the Rh complex on the same CP-MWCNT with a larger amount of Rh complex allowed by a larger concentration of 2,2-bipyridine moieties led only to the blockage of the electrode.

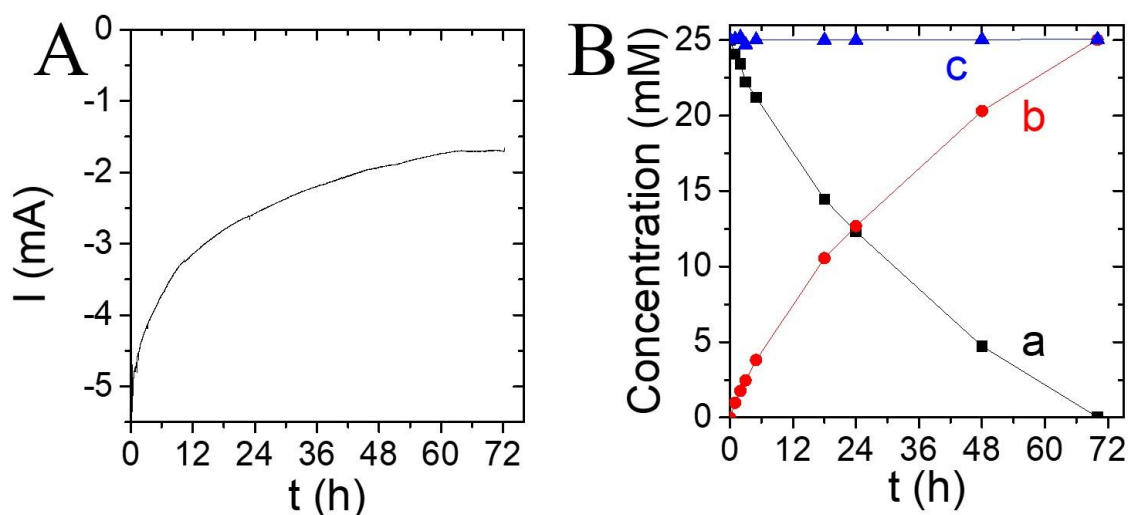
Further, the electrocatalytic activity of the two coupled CP-MWCNT-bpy-Rh electrodes was evaluated by cyclic voltammetry (**Figure S7A**). Without NAD<sup>+</sup> in the flow cell, the cathodic peak was located at -0.3 V vs ref. (H<sub>2</sub>) and a cathodic current near 3.5 mA was measured, as compared to 1.6 mA with a single CP-MWCNT-bpy-Rh layer (**Figure S7B**), in agreement with the higher amount of rhodium complex immobilized in the former case. The calculated surface coverage with the rhodium complex estimated by charge balance and Faraday's law from voltammograms was found equal to 138 nmol. The surface concentration near 4.3 nmol.cm<sup>-2</sup> was very close to that exhibited by a single CP-MWCNT-bpy-Rh layer. Then, when adding increasing amounts of NAD<sup>+</sup> in the flow cell, significant increases in the cathodic currents were observed, much larger than for the single CP-MWCNT-bpy-Rh layer (**Figure S7B**). In the NAD<sup>+</sup> concentration range investigated, the cathodic current measured at -0.3 V increased gradually with the increase of NAD<sup>+</sup> until levelling off with NAD<sup>+</sup> concentration higher than 3

mM. The improved activity of the bilayer electrochemical system appears promising for more efficient regeneration of NADH coupled to pyruvate bioconversion.

Two experiments of lactate biosynthesis were performed in the presence of the two layer CP-MWCNT-bpy-Rh electrode associated to one CP-MWCNT-LDH on which distinct amounts of LDH were immobilized (Table 1, Experiment 3 and Experiment 4).

Let's first consider the case where 500 units (approximately 13.8 nmol) of enzyme were adsorbed on a CP-MWCNT, corresponding to Rh-over-LDH molar ratio near 10, for which the bioconversion of 100 mL solution of 25 mM pyruvate was carried out in the presence of 10  $\mu$ M NAD<sup>+</sup>, under usual flow and potential conditions (Experiment 3 in Table 1). The current monitored at -0.3 V vs ref. (H<sub>2</sub>) decreased regularly from -5.5 mA to reach a fairly stable current value at -1.8 mA after 72 hours (**Figure 4A**). This current was larger than that measured with a single Rh electrode (**Figure 3A**) in correlation with the two-time larger amount of immobilized rhodium complex. Pyruvate was continuously converted along the run, until its disappearance after 72 hours. In the meanwhile, lactate was formed quantitatively (**Figure 4B**). These results show the positive impact of increasing the amount of immobilized Rh complex on the overall performance of the regeneration of the cofactor coupled to the enzymatic reaction. Furthermore, these results showed that the regeneration system of NADH coupled to pyruvate bioconversion can be scaled up and can lead to a high bioconversion yield following a ratio of 10 between the rhodium complex and lactate dehydrogenase both immobilized on separated CP-MWCNT. TTNs obtained with the bilayer system for the Rh complex, NAD<sup>+</sup>, LDH were respectively calculated at  $1.81 \times 10^4$ ,  $2.5 \times 10^3$  and  $1.8 \times 10^5$ . The TTN for the rhodium complex is in the same order of magnitude as the one reported in the literature for the FNR-mediated regeneration of NADPH.<sup>[19]</sup> In addition, the TTN obtained for NAD<sup>+</sup> is – to our state of knowledge – the highest value among those reported in the literature.<sup>[19,38]</sup>





**Figure 4.** Electroenzymatic synthesis of lactate from 25 mM pyruvate in 100 mL solution containing  $10\ \mu\text{M}$   $\text{NAD}^+$  (and 50 mM PBS, pH 7.2) in the hybrid flow cell provided with two CP-MWCNT-bpy-Rh electrodes and one enzymatic CP-MWCNT-LDH (500 units) layer (Experiment 3). (A) Variations of the current in the run. (B) Time variations of the concentrations of (a) pyruvate, (b) lactate, and (c) the sum pyruvate and lactate, as determined by HPLC.

On the other hand, the bioconversion of the 25 mM lactate in the presence of two CP-MWCNT-bpy-Rh layers, corresponding to 13.8 nmol of immobilized Rh complex, and one CP-MWCNT-LDH layer with only 100 LDH units (2.76 nmol) showed a bioconversion current (Experiment 4 in Table 1) starting near -5 mA and decreasing to -1 mA after 72 h (**Figure S8A**). This bioconversion current is lower in absolute value than the one obtained in Experiment 3 with 500 units of LDH. As a consequence, 16.5 mM of lactate were synthesized after 72 h (**Figure S8B**) with a faradaic efficiency of 79 %. Moreover, extending the electrosynthesis up to 120 h resulted in the bioconversion of 21.5 mM of pyruvate (**Figure S8B**).

To conclude, an optimal ratio between the Rh complex and LDH of 10 allows a total bioconversion of pyruvate within an optimal time (72 hours).

### 3. Conclusion

Thanks to the robust immobilization of all catalysts and fine optimizations, the presented continuous flow electrochemical reactor shows remarkable performances for catalytic hydrogenation of pure chemicals through NADH regeneration. The work deals with different challenges such as the choice of the cathode material, the immobilization of both the redox catalyst and the enzymes, their association or separation, the molar ratio between each of them, and the use of low amounts of the enzymatic cofactor (10  $\mu$ M).

Different aspects have to be taken into consideration when coupling efficient electrochemical regeneration of the NADH cofactor to enzymatic bioconversion of chemicals:

- 1) Immobilizing the Rh complex on carbon paper with MWCNT, and pressing at 40 bars this catalytic layer with a GDE and Nafion membrane led to an active system for the efficient electrochemical regeneration of the NADH cofactor system with a stability of more than 5 days under continuous flow.
- 2) Reducing the distance between the Rh complex and LDH by immobilizing each of them on separated, stacked layers led to an active loop for the continuous regeneration of the cofactor and pyruvate bioconversion.
- 3) The ratio between the immobilized Rh complex and enzymes units has to be at least equal to 10 in order to reach the highest faradaic efficiency (79 %), productivity (1.08  $\mu$ mol.cm<sup>-2</sup>.h<sup>-1</sup>) and TTN for cofactor (2500), rhodium catalyst (18000) and enzyme (180000).
- 4) Finally, hydrogen and solution flow rates can be fixed at 20 mL.min<sup>-1</sup> in the bench cell as described previously<sup>[27]</sup> and the applied electrolysis potential at -0.3 V ref. (H<sub>2</sub>). In addition, the flow cell was built advantageously using a graphite felt electrode allowing uniform distribution of the solution in the cathodic compartment and ensuring electrical contact between the functional layers and the current collector of the cell.

Lactate produced from the bioconversion of pyruvate can have a wide range of applications in cosmetics, food, and pharmaceutical industries.<sup>[41]</sup> Although we have focused on the hydrogenation of a ketone catalyzed by an alcohol dehydrogenase, the approach presented here should be applicable to many other NADH-dependent bioconversions.

## **4. Materials and methods**

### **4.1. Chemical and reagents**

All chemicals and LDH from porcine heart (250 units.mg<sup>-1</sup> protein) used were from Sigma-Aldrich. Multi-walled carbon nanotubes (MWCNT, NC7000™ series) were from Nanocyl (Belgium) and carbon papers (SpectraCarb 2050L-0550 Carbon Paper) were acquired from Fuel Cell Store (USA). MWCNT were dispersed in a solution of ethanol (96 %). High purity water (18 MΩ.cm) was used to prepare all solutions.

### **4.2. Immobilization of $[Cp * Rh(bpy)Cl]^+$ on a carbon support electrode**

#### **4.2.1. Graphite felt electrode pretreatment**

A piece of graphite felt (GFD4.6EA, SGL, Germany) being 4 cm x 4 cm x 0.4 cm in dimensions was used as cathode in the first part of the study. In order to reduce its hydrophobicity, the graphite felt electrode was washed and ultrasonicated with different proportions of ethanol and water at a temperature of 25 °C following the adopted protocol in our previous work.<sup>[27]</sup>

#### **4.2.2. Carbon paper with MWCNT (CP-MWCNT) preparation**

A piece of SpectraCarb carbon paper (CP) (4 cm x 4 cm x 0.05 cm) covered with MWCNT was prepared following a reported work in the literature <sup>[42]</sup> where 10 mg of MWCNT were ultrasonicated in a solution of ethanol (50 mL) for 5 hours. This suspension was then vacuum filtered on the CP and the resulting material was dried overnight. The CP coated with MWCNT

was then denoted CP-MWCNT and was used as cathode for the rhodium complex covalent immobilization and as a support material for the immobilization of L-lactate dehydrogenase.

#### **4.2.3. Carbon support electrode functionalization with $[Cp * Rh(bpy)Cl]^+$**

The carbon support was functionalized following a protocol from the literature.<sup>[17]</sup> 1 mM 4-amino-2,2-bipyridine (synthesized following a reported procedure<sup>[17]</sup>) was mixed with 2 mM sodium nitrite in 0.5 M HCl under gentle stirring for 5 min in order to generate 2,2-bipyridyl diazonium cations. A negative potential was then applied by running 2 cyclic voltammograms between 0.4 V and -0.8 V (vs Ag/AgCl (3M)) at a scan rate of 20 mV.s<sup>-1</sup>. The electrode was then rinsed with ultra-pure water and left to dry.

The functionalized electrode with 2,2-bipyridine moieties was then placed into a solution of DCM containing 0.15 mM of ((RhCp\*Cl<sub>2</sub>)<sub>2</sub>) for 4 h with a gentle stirring. The functionalized electrode with the rhodium complex was then rinsed with DCM for 5 min in order to remove the unreacted residues.

#### **4.3. Enzymatic carbon paper with MWCNT (CP-MWCNT-LDH) preparation**

CP-MWCNT was prepared following the same protocol in 4.2.2. LDH enzyme was adsorbed on the MWCNT surface by depositing 50 µL suspension of LDH and keeping for 2 hours at a temperature of 3°C.

#### **4.4. Electrochemical reactor**

Scheme S1 shows the bioelectrochemical bioreactor used in this study. The bioreactor has been presented in a previous work.<sup>[27]</sup> A gas diffusion electrode (4 cm \* 4 cm), used as anode, was placed on a grooved graphite bipolar plate allowing the introduction and the distribution of hydrogen in the anodic compartment at flow rate of 20 mL.min<sup>-1</sup>.<sup>[27]</sup>

At the same time, the reaction solution containing 50 mM phosphate buffer (pH = 7.2),  $\text{NAD}^+$ , and pyruvate was flowed continuously to the cathodic compartment using peristaltic pump at flow rate of  $20 \text{ mL}\cdot\text{min}^{-1}$ .<sup>[27]</sup> A CP-MWCNT on which is immobilized the rhodium complex was used as cathode. On the top of the CP-MWCNT-Rh was placed a graphite felt electrode known as a good electrical conductor and acting as a three-dimensional cathode, with homogeneous distribution of the solution. For pyruvate bioconversion tests, a CP-MWCNT-LDH was placed on the top of the CP-MWCNT-Rh electrode in addition to the graphite felt electrode.

Finally, a Nafion membrane (N-212) was placed between the two compartments to separate them and to transport protons from the anodic to the cathodic compartment.

Cyclic voltammograms and batch electrolysis were performed in a two-electrode configuration, the hydrogen anode acting also as reference electrode. Potential scan rate and electrolysis potential were fixed at  $5 \text{ mV}\cdot\text{s}^{-1}$  and  $-0.3 \text{ V}$  vs ref ( $\text{H}_2$ ).

Quantitative analysis of pyruvate bioconversion was performed by High Performance Liquid Chromatography (HPLC), using the Aminex HPX-87H HPLC column (300 mm x 7.8 mm, Phenomenex) thermostated at  $45^\circ\text{C}$ , and a refractive index detector. The aqueous phase was flowed at  $0.7 \text{ mL}\cdot\text{min}^{-1}$ . 200  $\mu\text{L}$  aliquots were taken at different intervals from the reaction mixture. These aliquots were further analyzed by HPLC to determine the pyruvate and lactate concentrations.

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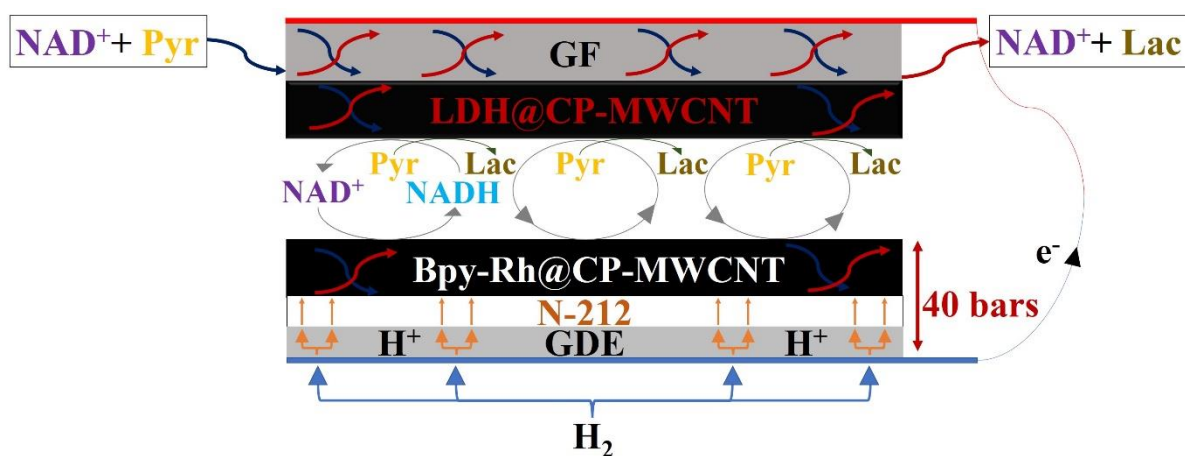
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## Graphical Abstract



High total turnover of NADH cofactor can be achieved with using all-immobilized catalysts in a hybrid flow bioreactor allowing efficient transfer of electrons and protons from  $\text{H}_2$  reacting in a gas diffusion electrode to the enzymatic reaction with only  $10\ \mu\text{M}$  enzymatic cofactor