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Powering artificial enzymatic cascades with electrical energy

Ammar Al-Shameri[a], Marie-Christine Petrich[a], Kai junge Puring[b,c], Ulf-Peter Apfel[b,c], Bettina M. Nestl[d] and Lars Lauterbach*[a]

Abstract: We developed a scalable platform that employs electrolysis for an *in vitro* synthetic enzymatic cascade in a continuous flow reactor. Both H_2 and O_2 were produced by electrolysis and transferred *via* a gas permeable membrane into the flow system. The membrane enabled the separation of the electrolyte from the biocatalysts in the flow system, where H_2 and O_2 served as electron mediators for the biocatalysts. We demonstrated the production of methylated *N*-heterocycles from diamines with up to 99% conversion yield as well as excellent regioselective labeling with stable isotopes. Our platform can be applied for a broad panel of oxidoreductases to exploit electrical energy for the synthesis of fine chemicals.

The use of electrical energy to perform biological and chemical reactions gained extensive interest in recent years. Electro-driven reactions offer the advantage of being clean, easy to tune, and sustainable when coupled with renewable energy sources. Biological electron transfer reactions are performed predominantly by cofactor-dependent oxidoreductases. These represent a highly interesting and versatile class of biocatalysts for specific reduction, oxidation, and oxyfunctionalization reactions in organic synthesis.[1] Electrical energy was applied in various biotechnological approaches to drive whole cells or immobilized enzymes on electrodes towards recycling of their cofactor and production of biofuels and fine chemicals.[2] In this context, electrochemical water splitting delivers H_2 as an electron mediator required for redox reactions with H_2 splitting enzymes. However, performing electro-driven enzymatic cascades with isolated enzymes (*in vitro*) are still hampered by the high potentials required for water splitting, the low pH and the high temperature generated in the process. These crucial aspects will eventually lead to denaturation of metal-dependent enzymes, unspecific side reactions and formation of undesired reactive oxygen species (ROS).[2c]

Thus, we herein set out to design a novel platform to perform an electro-driven *in vitro* enzymatic cascade. The one-pot enzymatic cascade exploited the redox power of H_2, which is generated by water splitting in order to produce *N*-heterocycles in a flow system. Utilizing a gas selective permeable membrane,[3] we could decouple the electrochemical H_2 generation from enzyme transformations in one setup, thereby establishing an unhindered transfer of gases from the liquid to liquid phase. This ensured a safe H_2 handling by avoiding the formation of explosive gas mixtures and enabled higher stability of the biological system. Furthermore, we established an integrated platform to monitor online the relevant parameters within the system.

The bioelectrochemical system (upper part Figure 1) was subsequently validated with a synthetic enzymatic cascade consisting of an immobilized oxidase, reductase and hydrogenase to produce *N*-heterocycles from diamines in a continuous process. *N*-heterocycles belong to a highly important class of compounds, which are found in various natural products, biologically active structures, and medicinally relevant compounds.[4] In previous work, we have successfully shown that a NADPH-dependent imine reductase (IRED) can be combined with an oxygen-dependent diamine oxidase variant (PuO^{E203G}) and a $NADP^+$-reducing hydrogenase in a one-pot process for the selective synthesis of *N*-heterocycles.[5]

For this study, we decided to couple a NADH-dependent IRED variant[6] from *Myxococcus stipitatus* with the O_2-tolerant, NAD^+-reducing hydrogenase (SH) from *Ralstonia eutropha*[7] using molecular H_2 as reductant. We also exploited the versatility of this electro-driven approach for (1) the synthesis of methylated *N*-heterocycles and (2) the isotopic labeling of *N*-heterocycles, which gave insights into the reaction mechanism of IREDs. Electrolysis was performed using a pentlandite/Ni catalyst for the hydrogen-evolving reaction (HER) and the oxygen-evolving reaction (OER), respectively. Pentlandite is a Ni and Fe-rich metal sulfide. It is a cheap and a sustainable alternative to platinum for HER in operations performed at conditions poisoning to other catalysts.[8] The pentlandite/Ni system was benchmarked against the more classical Pt-system. Notably, both systems were comparable in terms of efficiency with a variable dependency of H_2 and O_2 evolution on the pH and voltage (Figure 1S-3S). In our preliminary experiments, we observed that pentlandite could produce extensive amounts of H_2 at a low voltage of 0.6 V leading to displacement of the trace amounts of O_2 generated. Thus, the required amounts of O_2 could only be generated at high voltages with Ni as OER. Moreover, the low solubility of the gases in the electrolyte negatively affected the transfer efficiency of the gases *via* the membrane. Thus, increasing gas concentrations in the electrolyte by applying high voltages was crucial to enhance the transfer efficiency.[9] Therefore, we decided to perform the electrolysis at 2 V and pH 2.0 for Pt/Pt as well as 3.5 V and pH 1.3 for pentlandite/Ni.

The conversion of the model substrate 1,5-diamino-2-methylpentane (**1**) into 3-methylpiperidine (**2**) was subsequently investigated with *in-situ* produced H_2 and O_2 in the enzymatic cascade. A mixture of purified enzymes was thus injected to the circulating system to start the transformation of the test substrate (**1**). First results showed the successful formation of (**2**) with a production formation of 53% within 16 hours (Figure 4S) independent of the electrodes used. The general applicability of the flow reactor was then tested for the preparation of piperidine (**6**) and its derivatives using immobilized enzymes in the cascade reaction. While SH and catalase were immobilized on Amberlite FP54™ [10], enzyme carrier EziG™ from EnginZyme [11] was used

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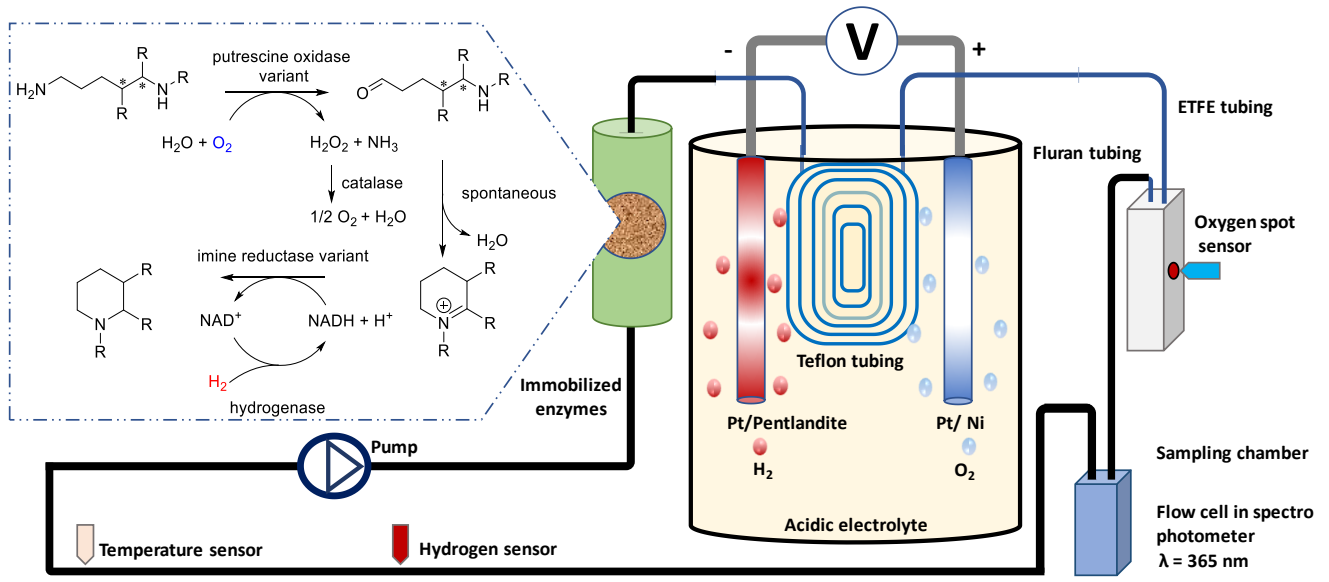


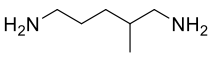
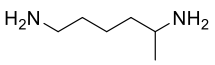
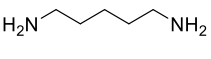
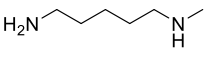
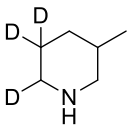
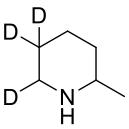
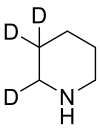
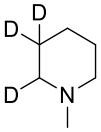
Substrates	 **1**[(a)]	 **3**	 **5**[(a,b)]	 **7**[(b)]
Products	 **2**	 **4**	 **6**	 **8**
Yield [%]	99	17	31	20
Deuteration (%)*	3 D (66), 2 D (86), 1 D (99)	3 D (63), 2 D (88)	3 D (78), 2 D (95)	3 D (83), 2 D (99)

Figure 1: Platform of the electro-driven *in vitro* enzymatic cascade for the synthesis of *N*-heterocycles from diamines in a flow reactor. Both H_2 and O_2 are delivered by electrolysis using Pt/pentlandite as HER and Pt/Ni as OER in an acidic electrolyte. A gas permeable tube transfers H_2 and O_2 from the electrolysis chamber (250 ml) into the flow system (16 ml). Sensors for H_2, O_2 and temperature plus a spectrophotometer (NADH) are integrated into the flow reactor to monitor online the evolution and consumption of H_2 and O_2 and NADH, respectively. The enzymes of the cascade (left side) are immobilized and packed into a column within the flow system. Electro-driven cascades were performed in deuterated Tris-HCl buffer (50 mM, pD 8.0) with 5 mM diamine substrate and 2 mM NAD^+ cofactor in the flow reactor at 22°C for 16 h. O_2 and H_2 were generated by performing electrolysis for 5 hours using Pt/Pt electrodes at 2 V and pH 2.0. Product formation in (%) was determined via GC-FID. The regioselective labeling was verified by ^{1}H-NMR (Figure 10-18S). The labeling yield was detected by comparing the signals in LC-MS spectra (Figure 6-9S). (a) Electrolysis with pentlandite /Ni electrodes at 3.5 V in pH 1.3. (b) substrate concentration 8 mM. * *x* D indicates incorporation of *x* deuterium atoms into products. R= methyl.

for immobilization of PuO^{E203G} and $IRED^{NADH}$. Specific activities per carrier ranged between 0.64 and 1 U/mg (Table 1S). The immobilized enzymes were packed in a column that was integrated into the flow reactor. The evolution and consumption of O_2, H_2 and NADH, respectively, were monitored during the cascade using *N*-methylcadaverine (**7**) as the model compound (Figure 2). The online monitoring enabled the recording and tracking of each step of the catalysis by checking if O_2 and H_2 concentrations were sufficient to start/proceed the reaction. It also gave a comprehensive picture whether each enzyme was functioning correctly and ensured no parameter was limiting within the cascade. It is worth mentioning that such a monitoring system (Figure 1) was until now only used *in vivo* during fermentations.

Notably, we prepared various methylated *N*-heterocycles with product formations of up to 99%. The results in the conversion of **1** and **3** were comparable to those from previous work using native IRED and $NADP^+$-reducing SH.[5] Interestingly, in this approach 1,5-diaminopentane (**5**) was poorly converted. The poor conversion might be explained by the application of the $IRED^{NADH}$ variant. The $K_{M,NADH}$ value of this variant is 10 times higher than the K_M value of its wildtype for NADPH. The k_{cat} of $IRED^{NADH}$ is 41% lower than wildtype IRED for reducing the model substrate 2-methylpyrroline.[6] We have also observed a product inhibition on $IRED^{NADH}$ for **2** and **6** (Figure 22S). Herein, a scale-up of the reaction was easily made possible by increasing the reaction volume (up to 300ml). We produced 69 mg of **8** and 55 mg of **2** in 300 mL and 150 mL setup, respectively, using the 3-fold and 2-fold excess amounts of immobilized enzymes (Table 2S). Furthermore, we could produce 3 mg of *N*-methylpyrrolidine from linear *N*-methylputrescine (11 % yield) by using the same set of immobilized enzymes in a 150 mL scale.

The new setup was then used to test the reusability of the immobilized enzymes. The same set of immobilized enzymes could produce **6** from **5** with 26% remaining product forming of the maximum product formation after six cycles (Figure 5S). The total turnover numbers were very high for all biocatalyst (Table 1) and (Table 3S), demonstrating the operational stability of our system.

Finally, we studied the labeling of piperidines with stable isotopes such as deuterium in the electro-driven cascade. The preparation of organic compounds labeled with hydrogen

Table 1. Total turnover numbers (TTN $n_{product}/n_{enzyme}$) of each biocatalyst based on the sum of product formed after the six cycles using the same set of immobilized enzymes

Biocatalyst	SH	$IRED^{NADH}$	PuO^{E203G}	Catalase
TTN	$1.1 X10^6$	$1.6 X10^4$	$1.2 X10^4$	$1.6 X10^8$

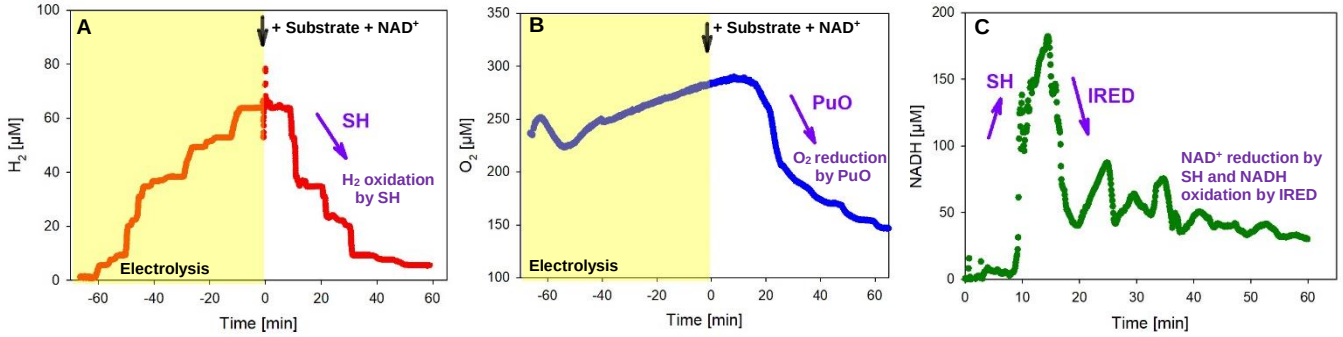
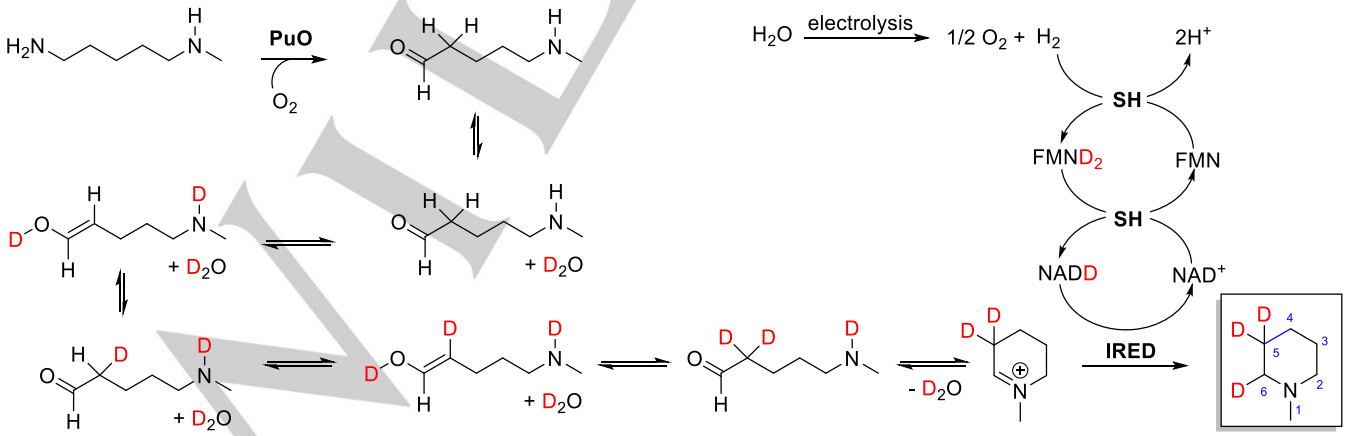


Figure 2: Online monitoring of H_2, O_2 and NADH during electro-driven biotransformation. The concentrations of H_2 (A), O_2 (B and NADH (C) were followed during the first hour to transform **7** to **8** as an exemplary biotransformation. The electrolysis was performed for 1 hour (yellow box) until both H_2 and O_2 reached a plateau and stopped. Then substrate and NAD^+ were added (black arrows). Purple arrows indicate the activity of each enzyme: the activity of putrescine oxidase (PuO^{E203G}) is indicated by the consumption of O_2 after adding the substrate (B), the activity of imine reductase ($IRED^{NADH}$) is shown in the consumption of NADH (C) and the activity of soluble hydrogenase (SH) is shown in the consumption of H_2 (A) and the evolution of NADH (C). The formation of H_2O_2 was monitored over time, no H_2O_2 formation was detected. The experiment was performed as described in Figure 1.

isotopes is of essential in the chemical, biological and environmental sciences. Hence, deuterated fine chemicals are valued molecules for spectroscopy analysis, pharmaceuticals and analytical tracing.[12] Herein, we exploited the unique characteristic of the SH to synthesize deuterated NAD(D). SH splits H_2 at the hydrogenase module separately from the NAD^+ reduction at the diaphorase module. H_2-driven labeling can be achieved using D_2O by providing two electrons from H_2 splitting and D^+ from D_2O-generating NAD(D).[13] NAD(D) can then be utilized as a cofactor for various NADH-dependent reductases like IRED to produce deuterated compounds (Scheme 1).

The labeling was performed with D_2O in the biotransformation and H_2O in the electrolysis units. We produced various labeled piperidine derivatives from diamines with up to 99% of deuterium labeling. Only a small fraction of the products was partially labeled (Figure 1). This inhomogeneity can be explained by the presence of water traces in D_2O (purity 98%), protons released form H_2 splitting (~1%), residual water on gases, remaining H_2O from immobilized enzymes and natural proton abundance of NAD^+. We expected to observe a labeling with a single D at the C6 carbon atom of the imine. Interestingly, we observed further labeling with two D at C5 (MS and NMR Figure 6-18S). We hypothesize that the deuteration at C5 might have been caused either by a possible keto-enol tautomerization, followed by the oxidation of the substrate by $PuOE^{203G}$, or an imine-enamine tautomerization with subsequent spontaneous cyclization of the aminoaldehyde intermediate. To examine this hypothesis, we tested the isotopic labeling using imines (2-methylpyrroline and 3,4-dihydroisoquinoline) and $IRED^{NADH}$. Here, the labeling is facilitated only by the activity of SH. Mass and NMR spectra revealed a 85% labelling corresponding to the C6 carbon atom of the imine (Figure 19-21S) without any labeling on further atoms. In addition, we used formate dehydrogenase (FDH) for cofactor regeneration in control experiments. No labeling was detected, when FDH was used. This indicates that the deuteration at C5 was merely caused by the keto-enol tautomerization (Scheme 1).

We determined that only ~4 % of electrical energy emerged in the product (see chapter 2.11 in the SI). While this seems very low, this finding can be rationalized by a low solubility of the gases in water, reduction of O_2 at the HER, heat production by overpotentials, inefficient transfer throughout the gas membrane and product inhibition of $IRED^{NADH}$. Our system can be further optimized by using suitable gas exchange membranes, sophisticated electrodes, and further engineered biocatalysts with improved features and broader substrate spectra.



Scheme 1: Proposed mechanism of isotopic labeling *via* several rounds of keto-enol tautomerization (C5) and SH activity (C6) with the model compound (**7**). The iminium ion is formed during cyclization. SH enzyme catalyzed the combined H_2 oxidation and NAD^+ reduction activity. Electrons are transferred *via* the prosthetic Fe-S clusters and flavins to the NAD binding site. The FMN at the NAD^+-binding site is converted by two electrons and two deuterium ions from water to $FMND_2$. A deuteride transfer from $FMND_2$ to NAD^+ generates NAD(D).[14] PuO is putrescine oxidase variant E203G, IRED is NADH-dependent imine reductase and SH is NAD^+-reducing hydrogenase.

In conclusion, we have designed a scalable platform to power enzymatic cascades with electricity. We furthermore demonstrated the potential of the novel design by producing various piperidine derivates from diamines. We extended the applicability of the system towards performing regioselective isotopic labeling and providing useful insights into imine reduction by IREDs. This platform represents an important advance in the field for biocatalytic synthesis, and it can be expanded for powering various cofactor-dependent oxidoreductases.

Conflict of interest:

There are no conflicts to declare.

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Keywords: Isotopic labelling • Electrochemical biocatalysis • Imine reductase • Hydrogenase, *N*-heterocycles • Immobilisation

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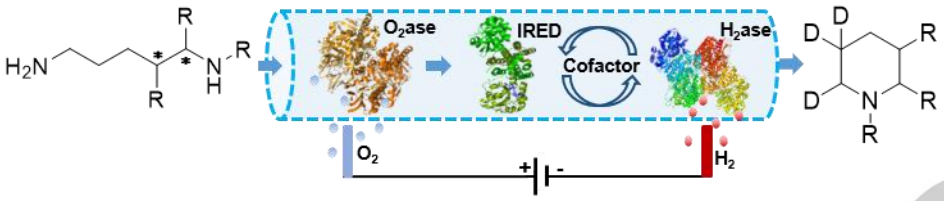

We developed a novel flow bioelectrochemical system to power enzymatic cascades *in vitro*. H_2 and O_2 were generated by electrolysis and served as electron mediators for oxidoreductases. We synthesized methylated *N*-heterocycles from diamines with up to 99% conversion yield and 99% regio-selective labelling. This platform can be used for various oxidoreductases to exploit the electrical energy for production of fine chemicals.