J|A|C|S
COMMUNICATIONS

Published on Web 07/12/2005

Laminar Flow-Based Electrochemical Microreactor for Efficient Regeneration of Nicotinamide Cofactors for Biocatalysis

Seong Kee Yoon,[†] Eric R. Choban,[‡] Cheikhou Kane,[§] Theodore Tzedakis,[§] and Paul J. A. Kenis*[,†,‡]

Departments of Mechanical & Industrial and Chemical & Biomolecular Engineering, University of Illinois at Urbana Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, and Chemical Engineering Laboratory, Université Paul Sabatier, Toulouse, France

Received April 21, 2005; E-mail: kenis@uiuc.edu

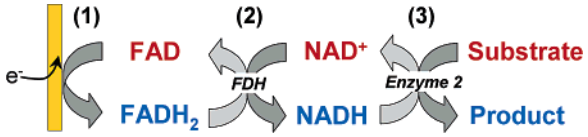
This communication reports a microfluidic electrochemical method for efficient regeneration of cofactors such as NADH for biocatalysis. The use of more environmentally friendly and stereoselective enzymatic transformations for the industrial synthesis of fine chemicals such as pharmaceutical intermediates, food additives, and cosmetics continues to gain interest.[1] For example, oxidoreductases are enzymes that catalyze the asymmetric reduction of carbonyl groups to alcohols and amines or promote the oxygenation of C−H bonds in the presence of nicotinamide cofactors such as NAD(P)H. Stoichiometric use of these cofactors, however, is cost prohibitive, leaving in situ regeneration of the cofactor as one of the few remaining alternatives. Yet, the development of efficient methods of cofactor regeneration is one of the long-standing challenges that prevent the more widespread use of biocatalysis.[2] Here, we utilize multistream laminar flow in a microreactor to perform efficient regeneration of the cofactor NADH, and we demonstrate its use in the conversion of the achiral substrate pyruvate into the chiral product L-lactate within the same microreactor through a coupled enzyme reaction. To date, only a few examples of such coupled enzyme reactions in microfluidic systems have been reported,[3,4] and these examples do not involve chemistries that are hard to achieve at the macroscale, unlike the NADH cofactor regeneration challenge addressed in this work.

Various in situ chemical, photochemical, enzymatic, biological, and electrochemical regeneration methods have been developed with mixed success.[5] Comtat et al. have shown that NADH can be regenerated electrochemically from NAD^+ using a mediator (e.g., flavin adenine dinucleotide, FAD[6]) that shuttles electrons from a cathode to an enzyme (e.g., formate dehydrogenase, FDH) that regenerates NADH from NAD^+ following Michaelis−Menten kinetics (reactions 1 and 2 of Scheme 1).[7,8] FAD is a mediator that is known to remain stable for numerous oxidation and reaction cycles.[6] $FADH_2$ rather than formate serves as the substrate for FDH.[8] Enough $FADH_2$ is generated at the electrode to shift the normally unfavorable equilibrium (reaction 2) to the desired direction of NADH formation. Unfortunately, the reverse reaction, the oxidation of the desired NADH species by the FAD mediator (eq 1), is spontaneous at pH 7 ($\Delta G'^{\circ} = -20$ kJ/mol).[9]

$$NADH + FAD + H^+ \leftrightarrow NAD^+ + FADH_2 \qquad (1)$$

Due to this reverse reaction, the concentration of electrochemically generated $FADH_2$ is never sufficiently high in the *bulk* solution in classical batch reactors to shift the equilibrium in the direction of NADH regeneration. At a smaller scale, Bergel et al. were able to regenerate NADH at an initial rate of 12 μM/min in a nonflowing thin layer electrochemical cell, which unfortunately is not suitable for continuous regeneration.[8]

Scheme 1. Indirect Electrochemical Regeneration of NADH Using FAD/$FADH_2$ as the Mediator and Subsequent Biocatalytic Conversion



The microreactor reported here exploits the occurrence of *laminar flow* in microscale channels: *Multistream laminar flow* enables focusing of a reagent-containing stream close to the electrode by adjustment of the flow rate ratio Q_2/Q_1 of the reagent and the buffer stream flowing in parallel without turbulent mixing (Figure 1). $FADH_2$ can be produced in sufficiently high concentrations at the electrode to drive the subsequent reaction 2 (Scheme 1) toward NADH regeneration. Bergel et al. showed that the [FAD·NADH]/[$FADH_2$·NAD^+] ratio has to be lower than 3×10^{-4} at pH 7.0 to shift reaction equilibrium 2 (Scheme 1) toward NADH formation.[8] The lack of a bulk phase in this microreactor prevents the undesired reverse eq 1 from occurring, while its continuous flow characteristics allow for continuous operation.

In a typical configuration, two liquid streams are guided into a Y-shaped 3-cm-long and 250-μm-wide square microchannel at a flow rate ratio Q_2/Q_1 of 12 (Figure 1b), thereby focusing the stream containing all necessary reactants closely to the cathode (Figure 1a). This reactant stream contains 1−10 mM of FAD and NAD^+ in a phosphate buffer. The enzyme FDH is present at a concentration of 2 g/L (= 1.86 U/mL). Earlier work has shown that the rate of NADH formation was highest when an initial NAD^+ concentration of 5−10 mM and an initial FAD concentration of 10 mM is used.[8] The microreactor consists of a polymer sheet carrying a Y-shaped channel geometry obtained via replica molding in poly(dimethyl-

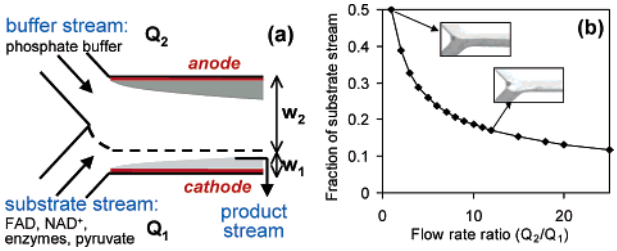


Figure 1. (a) Schematic of the electrochemical microreactor for cofactor regeneration and subsequent biocatalytic substrate conversion. In gray: reaction depletion zones (not to scale). (b) Adjustment of the width or focusing of the substrate stream by change of the ratio of volumetric flow rates, Q_2/Q_1. Insets: optical micrographs of two differently dyed aqueous streams in laminar flow at $Q_2/Q_1 = 1$ and 12.

[†] Mechanical & Industrial Engineering, University of Illinois at Urbana Champaign.
[‡] Chemical & Biomolecular Engineering, University of Illinois at Urbana Champaign.
[§] Université Paul Sabatier.

10.1021/ja052613n CCC: $30.25

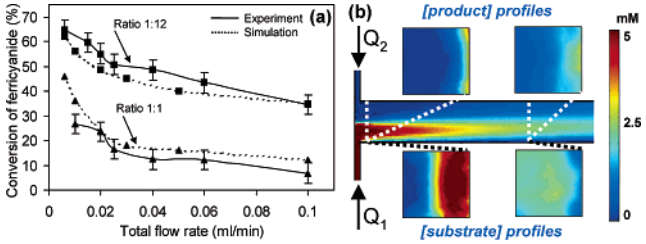


Figure 2. (a) Conversion of ferricyanide to ferrocyanide as a function of the flow rate at flow rate ratios Q_2/Q_1 of 1 and 12. (b) FEMLAB visualization of the substrate and product concentrations at the flow rate ratio Q_2/Q_1 of 1 (total flow rate of 0.01 mL/min).

siloxane) (PDMS)[10] or by micromachining in polycarbonate sheets, sandwiched between gasket layers (PDMS), and polycarbonate capping layers to produce a sealed and robust system. The Au electrodes that line opposing sides on the inside of the main microfluidic channel are deposited via sputtering. (Fabrication details: see Supporting Information.)

Before pursuing actual cofactor regeneration, we characterized the microreactor using the ferri/ferrocyanide redox couple. The microreactor was run in a potentiostatic mode at −0.1 V versus a Pt electrode placed in the cathodic stream near the inlet of the Y-junction. As expected, the conversion of ferricyanide increases as a function of lower flow rate (Figure 2a). Moreover, the 30% higher conversion observed at a Q_2/Q_1 of 12 shows the beneficial effect of focusing of the reactant stream on the electrode. A large fraction of the ferricyanide leaves the microreactor without reacting at $Q_2/Q_1 = 1$, whereas at $Q_2/Q_1 = 12$ as much as 67% reacts. We also performed 3D finite element simulations (FEMLAB[11]) of the relevant physical (i.e., convection and diffusion, Navier−Stokes equations) and chemical processes (i.e., electrochemical reaction at the cathode, Butler−Volmer equation) taking place in the microreactor (see Supporting Information for details). The resulting concentration profiles (e.g., Figure 2b for $Q_2/Q_1 = 1$) are in agreement with the experimental data obtained for this electrochemical microreactor (Figure 2a).

Next, we proceeded with the cofactor regeneration experiments (reactions 1 and 2 of Scheme 1) using the just identified operation conditions of a total flow rate of $<\sim 0.01$ cm^3/min and a flow rate ratio Q_2/Q_1 of ∼12 to enhance the conversion efficiency. Using cyclic voltammetry, we first showed that indeed high concentrations of $FADH_2$ are generated at the electrode, and an operation point of −0.55 V versus Pt was determined. To determine the NADH regeneration efficiency, we analyzed the product stream using UV−vis spectroscopy.[8] The FAD concentration was found using the absorbance at $\lambda = 450$ nm where only FAD absorbs. From this, the absorbance of FAD at $\lambda = 340$ nm was calculated using a correlation obtained from calibration curves (see Supporting Information). This A_{340} for FAD was subtracted from the measured absorbance at 340 nm, where both FAD and NADH absorb. From the remaining absorbance at 340 nm, the [NADH] can be calculated using a second correlation. We determined a maximum conversion efficiency of 31% NADH, which is significantly better than any cofactor conversion efficiency reported previously. For example, the previously reported rate of NADH regeneration of 12 μM/min in a stagnant thin film cell corresponds to less than 5% conversion efficiency.[8]

After establishing the promise of multistream laminar flow-based electrochemical microreactors as excellent tools for efficient cofactor regeneration, we also attempted the in situ conversion of an achiral substrate (pyruvate at 2−4 mM) into a chiral product (L-lactate), using lactate dehydrogenase (LDH at 5 U/mL) as enzyme 2 in reaction 3 (Scheme 1). The enzyme and substrate concentration were chosen so they matched the rate of NADH regeneration. HPLC analysis of the product stream showed a stoichiometric yield of 41% in L-lactate using the same microreactor under the same conditions (see Supporting Information). The maximum yield one theoretically could obtain in this microreactor is about 50%, since some of the substrate molecules will diffuse away from the cathode, thereby never having a chance to react in this single microreactor unit.

The "turnover number" (TN, moles of product formed per mole of cofactor per unit of time) for this biocatalytic experiment is 21 mmol L-lactate/mol NAD^+/s (= 75.6 h^{-1}), which is higher than the TN of 2.7 h^{-1} for a 14-day experiment reported in the literature.[12] For an actual biocatalytic process, one would envision connecting multiple microreactors in parallel while recirculating all reagents to increase the TN. The TN also can be significantly increased within the individual microreactors by engineering of the depletion concentration boundary layer formed at the electrode through periodic reactant replenishment or removal of the depleted boundary layer.[13] Research along these lines as well is ongoing. Also, we will confirm that cofactor regeneration in this microreactor still follows Michaelis−Menten behavior.

In this work, a novel microfluidic electrochemical method that enables efficient regeneration of the cofactor NADH has been developed. The ability in multistream laminar flow to focus a stream of reactants close to the electrode enables a reversal of a normally unfavorable reaction equilibrium essential for enzyme/cofactor regeneration. Moreover, we were able to perform a subsequent actual biocatalytic process: the conversion of the achiral substrate pyruvate into the chiral product L-lactate. While further research and development is needed to utilize this cofactor regeneration methodology in actual biocatalytic processes, the challenge has now shifted from the long-standing problem of cofactor regeneration to a more tangible engineering challenge of how to integrate a large number of these microreactors in a recirculating system for the biocatalytic synthesis of chiral fine chemicals in larger quantities (i.e., kilograms).

Acknowledgment. This work was supported by the University of Illinois and by CNRS, France.

Supporting Information Available: Detailed descriptions of microreactor fabrication, calibration curves, and details of FEMLAB simulations including the visualization for $Q_2/Q_1 = 12$. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 232−240. (b) Bommarius, A. S.; Riebel, B. R. *Biocatalysis*; Wiley-VCH: Weinheim, Germany, 2004.
(2) Chenault, H. K.; Whitesides, G. M. *Appl. Biochem. Biotechnol.* **1988**, *6*, 221−270.
(3) Seong, G. H.; Crooks, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 13360−13361.
(4) Holden, M. A.; Jung, S.-Y.; Cremer, P. S. *Anal. Chem.* **2004**, *76*, 1838−1843.
(5) Zhao, H.; van der Donk, W. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 421−426.
(6) Durliat, H.; Barrau, M. B.; Comtat, M. *Bioelectrochem. Bioenerg.* **1988**, *19*, 413−423.
(7) Bergel, A.; Comtat, M. *Bioelectrochem. Bioenerg.* **1992**, *27*, 495−500.
(8) Bergel, A.; Comtat, M. *J. Electroanal. Chem.* **1991**, *302*, 219−231.
(9) Edsall, J. T.; Gutfreund, H. *Biothermodynamics*; Wiley & Sons: New York, 1983.
(10) Duffy, D. C.; McDonald, J. C.; Schueller, O. J. A.; Whitesides, G. M. *Anal. Chem.* **1998**, *70*, 4974−4984.
(11) *FEMLAB Modeling Guide*; Comsol Inc.: Burlington, MA, 2004.
(12) DiCosimo, R.; Wong, C. H.; Daniels, L.; Whitesides, G. M. *J. Org. Chem.* **1981**, *46*, 4622−4625.
(13) Yoon, S. K.; Fichtl, G.; Kenis, P. J. A. University of Illinois at Urbana Champaign, IL, 2004. Unpublished results.

JA052613N