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Electroenzymatic Synthesis (Regeneration of NADH Coenzyme): Use of Nafion Ion Exchange Films for Immobilization of Enzyme and Redox Mediator

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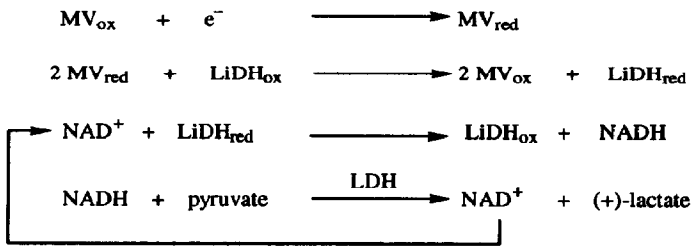
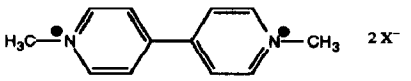
Abstract: A carbon electrode coated with lipoamide dehydrogenase and methyl viologen (1) under a Nafion® film provides the basis for a convenient system for electrochemical regeneration of NADH.

The high cost of the coenzymes NAD^+ and NADH required in a number of biological redox processes makes it necessary to recycle these substances in any synthetic scheme intended to carry out enzymatic syntheses on a practical scale. In recent years a number of procedures have been reported for electrochemical regeneration of enzyme cofactors.[1] A representative process of this type, in which electrochemical regeneration of NADH is used to drive the enzymatic reduction of pyruvate to lactate, is shown in Scheme I.[2] In principle, one need only reduce an NAD^+-accepting enzyme such as lipoamide dehydrogenase (LiDH) at a cathode; the reduced enzyme would then reduce NAD^+ to NADH. Unfortunately, direct electron transfer between electrode and enzyme is slow with most enzymes, so one must reduce the enzyme indirectly. A redox mediator is introduced into the system; the electrode reduces it, and the mediator then reduces the enzyme, which in turn reduces NAD^+. Methyl viologen salts (MV^{2+}, 1) have frequently been used as redox mediators. Salts of 1 which undergo rapid reversible reduction at electrodes have been shown to reduce LiDH.[3] The latter enzyme reduces NAD^+ to NADH, the coenzyme for enantiospecific enzymatic reduction of pyruvate to (+)-lactate by L-lactate dehydrogenase (LDH). Reduction of pyruvate by NADH regenerates NAD^+ to continue the catalytic cycle. This reiterative process is driven by the continued reduction of 1 at the cathode. NAD^+ cannot be reduced by direct electrochemical reduction because of the formation of a mixture of inactive dimers in the absence of LiDH.[4]

Scheme I



LiDH = lipoamide dehydrogenase; LDH = L-lactate dehydrogenase



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Separation and isolation of the products from practical syntheses of this type carried out on large scale are difficult when all components are present in bulk solution. This suggests the use of a system in which one or more of the components is immobilized. A number of such approaches have been reported. For example, one or both of the enzymes used in the process may be immobilized in a semi-permeable gel immersed in the medium; the electrode, redox mediator, and enzyme(s) can be contained in a "membrane reactor" whose walls are sufficiently permeable to permit substrates and products to pass; or the enzyme and/or mediator may be chemically bound to or otherwise physically attached to the electrode surface (e.g., in the form of an insoluble but porous polymer). Thus, Matsue, *et al.*, immobilized LiDH and horse liver alcohol dehydrogenase together on a carbon electrode by cross-linking with glutaraldehyde, and used this electrode (with a viologen derivative in solution) to effect the reduction of cyclohexanone derivatives.[3a] Simon, *et al.*, have used both covalently immobilized mediators at carbon electrodes with viologen accepting pyridine nucleotide oxidoreductase (VAPOR) in solution[5] and co-immobilized VAPOR and viologen for direct electron transfer in regeneration of NADH.[1c] We felt it to be desirable to physically separate the two enzymes, which are likely to have considerably different stability, permitting the less stable enzyme to be replaced as necessary. Furthermore, we felt that the mediator and the enzyme ought to be co-immobilized on the electrode, removing the biotoxic viologen from solution and bringing the mediator **1** in close proximity to the enzyme LiDH.

We wish to report an experimentally simple, fast, and effective procedure for immobilizing both enzyme and mediator on an electrode surface, and the use of this electrode for the reduction of pyruvate to lactate. Lipoamide dehydrogenase and methyl viologen dichloride (**1**, X=Cl^-) are dip-coated onto a reticulated vitreous carbon (RVC®) electrode from aqueous solution. After air drying, the electrode is immersed in an aqueous alcohol solution containing the ion-exchange polymer Nafion® in its neutralized form. After drying again,[6] the enzyme-mediator modified electrode is connected as the cathode in an electrochemical cell containing pyruvate ion, NAD^+ (catalytic quantity), and LDH in phosphate buffer at pH 7.0. Controlled-potential electrolysis is then carried out at -0.9 V vs S. C. E., a potential corresponding to the first reduction potential of **1**, and the reaction progress is monitored by NMR spectroscopy. After an induction period of one to two days, the progress of the electroenzymatic process can be observed both by the appearance of lactate and the disappearance of pyruvate. For optimal rates, we found that addition of small amounts of pyruvate to the medium at regular intervals is preferable to less frequent addition of larger amounts, to minimize both self condensation of pyruvate[7] and inhibition of LDH by high concentrations of pyruvate.[8] It is necessary to add fresh lactate dehydrogenase every two days due to its relatively short half-life in solution.[9,10]

The lifetime of LiDH is considerably extended by immobilization on the electrode in this manner. We normally run these experiments for about two weeks, but in one run lactate production from pyruvate was still taking place, though at only 11% of the rate observed at the end of the induction period (*vide infra*), after *fifty-one days* when the experiment was interrupted. In contrast, we find that in solution LiDH has a half-life of only 4 days at room temperature in phosphate buffer at pH 7.0, and no measurable activity after fifty-one days. A control experiment using an electrode containing the redox mediator but not LiDH produced only a small amount of lactate after eighteen days of electrolysis, consistent with recent findings by Drueckhammer and Grimes.[1b] Immobilization in the Nafion film therefore extends the lifetime of the enzyme dramatically. Furthermore, 100% of the enzyme activity is retained in this immobilization procedure, i. e., the coating with Nafion has no detrimental effect on LiDH. The slow loss of activity by the enzyme electrode is not due to

loss of viologen to solution; we have not detected viologen in solution by spectrophotometry or NMR spectroscopy.

Under these conditions, the film as initially produced contains about 0.14 μmol cm^{-2} of **1** and 0.5-1.5 μmol cm^{-2} of active LiDH. The amount of viologen was determined by integration of the current under the linear sweep voltammetric wave with the electrode immersed in phosphate buffer and the amount of LiDH was determined by use of the Bradford assay for protein quantitation.[11] The electrode lifetime is considerably longer when the electrode is used to produce lactate electrochemically than when it is simply stored in phosphate buffer (as determined by spectrophotometric determination of LiDH activity).[12,13]

At this stage these experiments are carried out on small scale and reaction is complete in eight days. The current efficiency is close to 100% and the electrode efficiency, in terms of lactate produced (analysis by HPLC), is 700 nmol hr^{-1} cm^{-2}. (This is competitive with other modified electrodes intended for similar purposes.[5]) We are exploring a number of ways in which the electrolysis scale can be improved, e.g., improved convective stirring, loading of increased amounts of mediator and LiDH into the film, alterations in film thickness, etc.[14] Although this system produces lactate efficiently, NMR spectroscopic analysis shows that there is a one to two day induction period after the beginning of electrolysis before measurable lactate formation takes place. The rate determining step for the coupled process of electrochemical regeneration of NADH by LiDH in solution is reduction of LiDH by the mediator.[2] This process presumably begins taking place in the film electrode as soon as passage of current begins, but subsequent formation of lactate in solution may be delayed because of the time required for diffusion of NAD^+ into the film for reduction and subsequent diffusion of NADH out of the film into solution for reaction with LDH; it may take some time to build up steady-state concentrations of NAD^+ and NADH in the film at the surface of the electrode. However, inclusion of a small amount of NAD^+ in the film did not eliminate the induction period.

Electrocatalytic behavior is generally readily diagnosed by cyclic or linear sweep voltammetry; the faradaic current corresponding to reduction of the electrocatalyst increases substantially in the presence of the substrate with which it reacts.[15] A small glassy carbon electrode was modified with an LiDH-**1**-Nafion film in the manner described above. The shapes of the two voltammetric reduction waves of **1** when immobilized on the electrode are both characteristic of diffusion-controlled reduction,[15] indicating that **1** can diffuse freely within the film on the voltammetry time scale. However, and surprisingly, the height of the cyclic voltammetric wave corresponding to the first reduction step of **1** was found to be *diminished* upon addition of NAD^+ to the medium. This observation has been replicated many times. We are studying this phenomenon further. It may be related to the induction period observed in the preparative electrolyses.

Typical procedure for preparing and using the co-immobilized electrode. The working electrode is a *ca.* 1 cm^3 piece of 20 ppi RVC. This is dip-coated in a 10 mL solution containing 0.6 mM FAD, 2 mM methyl viologen, and 325 U of LiDH (Type V; from Torula Yeast, EC 1.8.1.4; activity 34 U/ mg protein) for one hour and then air dried for one to two hours. The polymer film is then added by dipping the electrode in a 2.5% solution of Nafion®, obtained by dilution of the commercial 5% solution with water and the Nafion® film is air dried. [The pH of the Nafion® solution is adjusted to 7.0 with concentrated ammonium hydroxide prior to coating.] The cell is constructed with a platinum wire counter electrode and a calomel reference electrode. The electrolyte is an 0.20 M phosphate buffer (pH=7.0), containing 60 mM imidazole, 2 mM NAD^+, 30 U LDH [from rabbit muscle (EC 1.1.1.27)], and 1.6 g (14.5 mmoles) sodium pyruvate in a total volume of 100 mL. The cell is degassed by nitrogen flow for 30 min and the electrolysis is run under

nitrogen. The cathode potential is maintained at -0.9V vs S.C.E. during the course of the reaction. The reaction is monitored by NMR spectroscopy and determined to be complete after 8 days.[16]

In conclusion, we report here the development of a long term stable system for electroenzymatic regeneration of NADH using an easily prepared electrode with MV (1) and LiDH co-immobilized under a Nafion® film.

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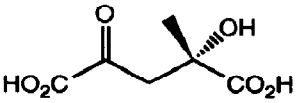
7. The self-condensation product **2** of pyruvic acid [Wolff, L. *Annalen*, **1899**, *305*, 154] was produced in most experiments in competition with lactate formation. To avoid adding pyruvate at intervals to the electrolysis solution, we tried starting with larger quantities of pyruvate, but under these conditions an appreciable amount of **2** is formed, especially at pH > 7.0 or with amine-based buffers such as Tris. The rate of formation of **2** is the rate of non-enzymatic enolization of pyruvate in solution. Any condition that increases the concentration of (non-enzymatically) enolized pyruvate produces a larger quantity of **2**.



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Condensation product **2** is useful as a marker for the decline in LiDH activity. For example, at the end of our 51-day electrolysis, addition of pyruvate led primarily to formation of **2**. Condensation product **2** is readily identified by the distinctive AB pattern at δ 3.20 and 3.33 (J = 17.9 Hz) in its ^{1}H NMR spectrum.

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10. The half-life of LDH is greater (five days) in the presence of 1% bovine serum albumin.

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16. Even though lactate production involves consumption of protons, the pH of the solution increases only slightly (to ca. 7.1) during electrolysis because of concomitant production of protons at the anode.

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