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CO-ELECTROPOLYMERIZATION OF A VIOLOGEN OLIGOMER AND LIPOAMIDE DEHYDROGENASE ON AN ELECTRODE SURFACE. APPLICATION TO COFACTOR REGENERATION

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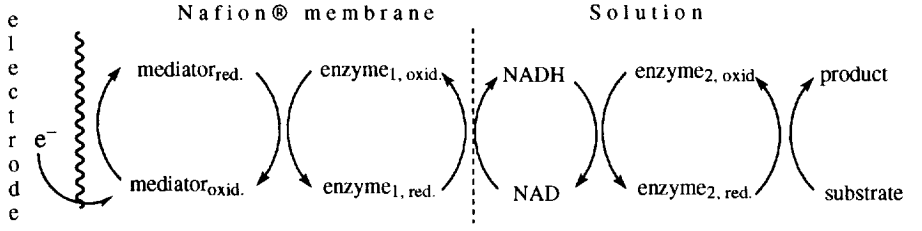
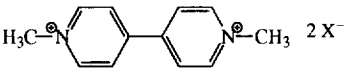
Abstract: We report here a novel enzyme electrode obtained by electrochemically copolymerization of a redox enzyme with an oligomeric organic redox mediator on the surface of a glassy carbon electrode. This electrode serves as an efficient catalyst for the regeneration of the cofactor in enzymatic syntheses requiring NADH. The reaction shows a relatively short induction period and the rate of product formation is higher than for similar electrodes upon which the enzyme and redox mediator are physically trapped under a Nafion membrane.

Enzyme electrodes have received increasing attention during the last decade due to their use in the field of biosensors and for the synthesis of chiral compounds. We have previously reported that an electrode coated with a mixture of lipoamide dehydrogenase (LiDH) and the redox mediator methyl viologen (MV_{ox}, **1**) under a Nafion coating is active for conversion of NAD^+ to NADH.[1] Use of this electrode considerably improves and simplifies the synthesis of α-hydroxy and α-amino acids via electrogenerated NADH (Scheme I). Leakage of **1**, a toxic substance, from the electrode is undesired because it would contaminate the final product. To solve this problem, we have been interested in chemically binding the viologen species to LiDH. We previously reported that a viologen-modified LiDH is effective for preparation of NADH from NAD^+ without leakage of **1** into solution.[1c] We now report an alternate approach that is experimentally more convenient and affords a highly active enzyme electrode without the need for the Nafion film. This new electrode does not exhibit the induction period for lactate formation which we observed with electrodes constructed with Nafion films.[1]



Scheme I



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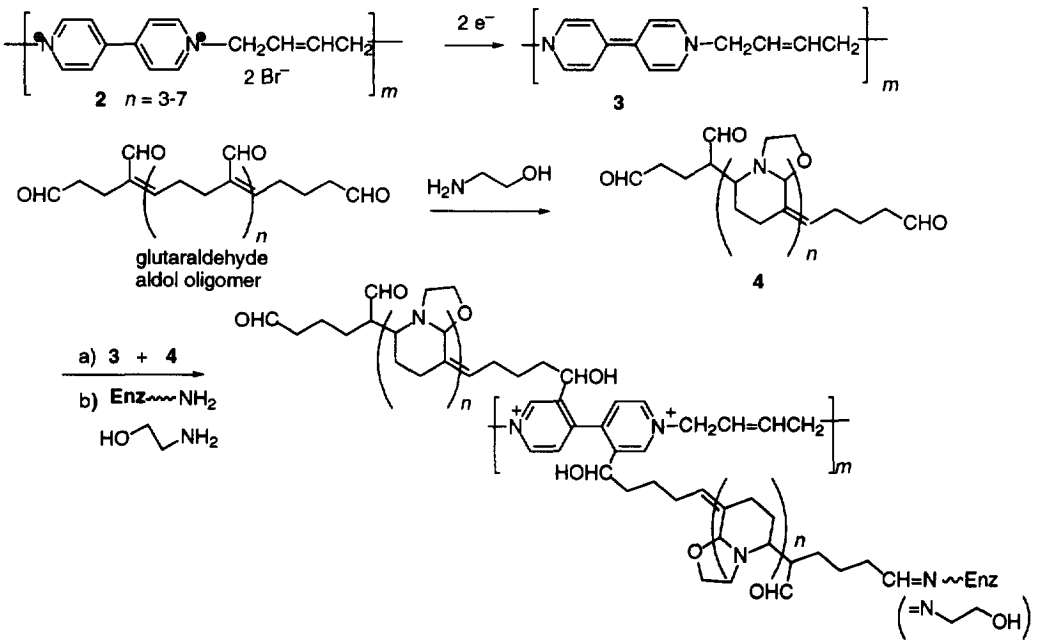
Polymer-modified electrodes are usually fabricated by dip- or spin-coating of redox polymers followed by drying.[2] Electropolymerization has been used to prepare electrodes with enzyme coatings.[3] Since many polymers can be synthesized electrochemically a variety of structural types can be incorporated into electrodes in this way. Immobilization of biological species via electrochemical polymerization appears to be a promising approach for preparation of biochemically active electrodes. This technique makes uniform and reproducible

spatial distribution of the enzyme in the electropolymerized film possible. Since the polymerization proceeds only at the surface of the electrode, it has been used for the modification of area-defined electrodes, such as microdevices used for sensing purposes.[2,3] Polymer-modified electrode surfaces contain the equivalent of many molecular layers.[4] This is advantageous when the film contains redox centers; higher currents are obtained than with electrodes with monolayers immobilized by covalent binding. The immobilized enzyme can be either physically entrapped within the polymer matrix and/or chemically linked to the polymer backbone.

We were intrigued by a report describing a redox polymer electrode prepared by electrochemical reduction of an oligomeric polyviologen oligomer (**2**) and its subsequent cross-linking (*via* the nucleophilic enamine moieties of the reduced oligomer) with a glutaraldehyde (GA) aldol oligomer and an amino alcohol.[5] We decided to extend this work by incorporating a redox enzyme into the electrolysis mixture such that the enzyme, with its nucleophilic amino groups, would also become part of the electrogenerated polymer. By integrating the biological element (the enzyme) into the copolymer with the organic redox mediator (the viologen), we hoped to eliminate leakage of the mediator from the electrode and perhaps also to improve the efficiency of electron transfer between mediator and enzyme. The electropolymerization was carried out as previously reported,[5] except that LiDH was included in the medium. A complex series of transformations[5] (see below) results in formation of a polymeric film containing all of the redox species of interest.



Experimental

Electrochemical co-crosslinking. The oligomeric polyviologen **2** was prepared from 4,4'-dipyridyl and 1,4-dibromo-2-butene.[6] The electropolymerization was carried out by immersing a glassy carbon (RVC) electrode (1 cm^3) in 100 mL of a solution of **2** (0.25% w/v) and GA (0.75% v/v) in deaerated tris(hydroxymethyl)aminoethane (Tris) buffer (50 mM, pH=7.4). After a 30 minute incubation period

controlled potential electrolysis was carried out at -0.8 V (vs. SCE) until the value of the current had decreased to about 10% of its original value.[5] Lipoamide dehydrogenase (233 U) was then added to the solution and the electrolysis was continued until the current had decayed to a constant low base-line value. The enzyme was not added at the beginning of electrolysis to avoid excessive cross-linking and possible loss of activity. The modified electrode obtained was washed, air-dried, and subsequently used in the synthesis of lactate. An incubation period in which the mixture was left at room temperature for 30 minutes before applying a potential was found to be desirable. The cross-linking process is improved by the use of aged GA in order to have a certain degree of oligomerization of GA before reaction with the amino component begins.

Cyclic voltammetry. The polyviologen **2** was characterized by cyclic voltammetry at a 3 mm diameter glassy carbon cathode. The substance shows the same reduction waves as **1** (- 0.68 V and -0.88 V). If the polymerization is performed at -0.8 V the resulting film shows excellent time stability: in fact, the voltammetric behavior was constant over a period of 62 hours of repeated cycling of the potential between 0 V and -0.8 V.

Enzyme assays and analysis of lactate were carried out as previously described in ref. 1.

Electrosynthesis of lactate. 100 mL of pH 7.0 phosphate buffer (0.2 M) containing LDH (20 U), pyruvate (0.145 M), and NAD^+ (2 mM) were placed in an undivided electrolysis cell equipped with platinum wire anode, SCE reference electrode, and working electrode (prepared as described above). After the cell was flushed with nitrogen, controlled potential electrolysis was carried out at –0.8 V vs SCE (a potential corresponding to the first reduction step of **1**. Aliquots (1 ml) were removed periodically, lyophilized, and dissolved in D_2O. Analyses for lactate were carried out by ^1H NMR and HPLC as previously described.[1] An additional 20 U of LDH was added to the electrolysis every two days.

Results and Discussion

The electrode obtained by electrochemical copolymerization of **2**, GA, and LiDH exhibits excellent activity for conversion of NAD^+ to NADH. (It was not *a priori* evident that this would be so. For example, redox communication between nitrate reductase and a viologen derivative continues after they are incorporated into a polymer matrix, but similar immobilization of glutathione reductase does not afford an active assembly.[7]) Leakage of the mediator into solution is negligible. This is a very important consideration due to the toxicity of viologen derivatives. The yield of lactate is not higher than with our previous electrodes employing Nafion films;[1] however, the induction period of up to two days for lactate formation which we observed in our earlier work[1] is largely absent. The improvement in this respect is spectacular: the cell began producing lactate with minutes after electrolysis was begun. This is probably due to both the elimination of the time necessary for the dry Nafion film to be equilibrated with water and to the more facile diffusion of reacting substances through the uncharged GA-based polymeric film. The current yield and turnover number (TN) are better for this electrode and only one-third the time is necessary to generate a given amount of lactate than with our previous electrodes in which LiDH and **1** were either (a) co-immobilized, but not chemically bound to each other[1a,b] or (b) chemically linked ("wired")[1c] (Table 1). On the debit side, LiDH is shorter lived when covalently linked with the oligomeric viologen **2** through GA in this manner. The currents produced by this electrode are far higher during the first 15 days' operation than the 0.5 - 1 mA current produced by our original electrode under the same conditions.[1a,b] However, the electrolysis must be interrupted at the end of 15 days due to increasing by-product formation, which is seen when the electrode loses its LiDH activity.[1a] As with our original system,[1b] no evidence for electrocatalysis was observed when a smaller electrode containing **2** and LiDH (co-immobilized in

this fashion) was analyzed by cyclic voltammetry in a solution containing NAD^+ and pyruvate. The electrocatalytic activity of the electrode, as with our Nafion film electrodes, had to be established by actually performing the preparative electrolysis.

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Table 1. Comparison between cells with different working electrodes

Electrode/cell (product)	Yield (%)[a]	Rate of Product formation[b]	Total turnover number, TTN[c]	Turnover number, TN[d]	Literature Reference
LiDH physically immobilized (lactate)	49	716	42	60	1a,b
LiDH physically immobilized (glutamate)	30	194	18	15	present work
"Wired" enzyme (lactate)	53	1419	39	57	1c
"Wired" enzyme (malate)	50	107	25	10	1c
LiDH coelectro-polymerized with viologen oligomer (lactate)	35	7842	38	219	present work

[a]Mol product produced/mol substrate consumed. [b] nmol of product produced/cm^2/h. [c] Mol product produced/ mol cofactor [d] Mol product produced/mol cofactor/sec.

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