1. INTRODUCTION

1.1 Biosensors

Analytical chemistry plays an important role in our everyday life because almost every sector of industry and public service relies on quality control. The majority of chemical analysis methods are time-consuming and heavily employ expensive reagents and equipment in order to achieve high selectivity and low detection limits. A serious breakthrough in this field was announced by professor Leland C. Clark in 1962 at a New York Academy of Sciences symposium who described the concept of construction of electrochemical devices for the rapid, cheap, selective, and sensitive glucose analysis produced by the immobilisation of the enzyme glucose oxidase in the vicinity of an electrochemical oxygen sensor [Clark and Lyons, 1962]. Then, having significantly improved the design of this sensor, Updike and Hicks [1967] reported the first functional biosensor for glucose.

According to the latest definition by IUPAC [Thevenot et al., 1999] an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element. Biosensors which do not need any additional reagents in a sample solution for the detection of an analyte, because they have all the components necessary for the reaction sequence, are called reagentless biosensors.

The first commercially available glucose biosensors, based on the amperometric detection of hydrogen peroxide generated by glucose oxidase, were delivered on the market by the Yellow Springs Instruments Company (Ohio, USA) in 1975. Since then many serious players in the field of medical diagnostics, such as Bayer, Boehringer Mannheim, Eli Lilly, Lifescan, DKK Corporation etc., invested in the development and the mass scale production of biosensors, which are utilised in health care [Alcock and Turner, 1994], environmental monitoring [Dennison and Turner, 1995], food and drink [Kress-Rogers, 1996], the process industries [White and Turner, 1997], defense and security.

Electrochemical biosensors employ redox enzymes as recognition elements. The use of oxidases and dehydrogenases has been extensively demonstrated in the literature. The biggest class of dehydrogenases includes dehydrogenases dependent on coenzymes I and II.

1.2 Coenzymes I and II

The full structure of codehydrogenase I was discovered by von Euler with coworkers [von Euler et al., 1936, von Euler et al., 1937] by hydrolysis that gave D-ribose-phosphoric acid, adenine and nicotinamide. The establishment of the constituents of codehydrogenase II was completed by Kornberg and Pricer [Kornberg and Pricer, 1950] through enzymatic degradation yielding adenosine-2′,5′-diphosphate and nicotinamide nucleotide. Therefore the Enzyme Commission of the International Union of Biochemistry and Biological Chemistry Nomenclature Commission of the International Union of Pure and Applied Chemistry recommended the name nicotinamide adenine dinucletide (NAD^+) for codehydrogenase I and nicotinamide adenine dinucleotide phosphate ($NADP^+$) for codehydrogenase II [Dixon, 1960]. The structure of NAD^+ and $NADP^+$ is depicted in Figure 1.

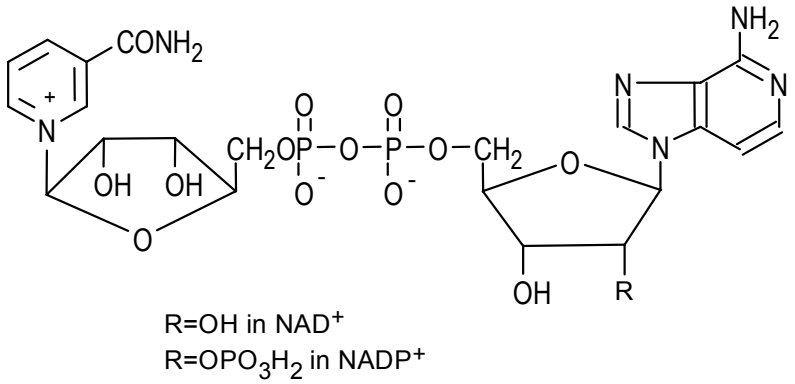


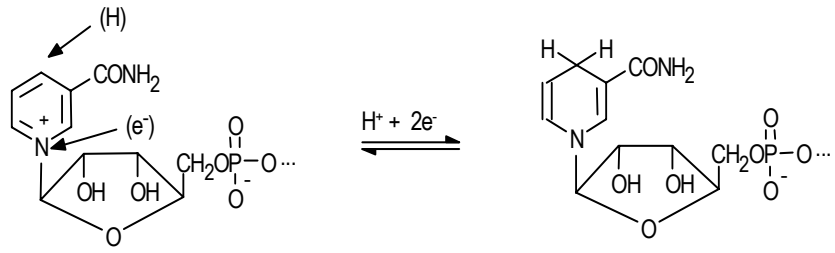
Figure 1. Structure of NAD^+ and $NADP^+$.

Many apoenzymes (dehydrogenases) combine with these coenzymes, performing the dehydrogenation of a great number of substrates. The structure of every apoenzyme controls the specificity of the reaction between $NAD(P)^+$ and corresponding substrate. More than 300 $NAD(P)^+$ dependent dehydrogenases have been discovered [Nomenclature Committee of the International Union of Biochemistry, 1984] and every year this list is extended.

All animal and plant cells, metabolising carbohydrates, contain NAD^+. For example the concentration of NAD^+ in fresh yeast is 0.5 g per kg. The heart muscle of man contains about 0.4 g of NAD^+ per kg. $NADP^+$ occurs in all living cells in association with NAD^+ and the ratio of these coenzymes depends on the type of tissue. Yeast cells contain very small amount of $NADP^+$, but there are animal tissues containing up to 80 μg of $NADP^+$ per g [Adler et al., 1939].

The study of the redox mechanism explaining the inter-convertibility of $NAD(P)^+$ and NAD(P)H was carried out by Karrer and coworkers [Karrer and Warburg, 1936; Karrer et al., 1936; Karrer and Benz, 1936; Karrer et al., 1937]. Their goal was to test the working hypothesis

that the redox behaviour of the two cofactors is caused by the presence of nicotinamide moiety. For that purpose a number of nicotinamide derivatives were synthesised as model compounds and their UV adsorption spectra were compared with these of coenzymes in oxidised and dihydro form. It was found that only the dihydropyridine derivatives with an sp^3 hybridised nitrogen atom in the ring and dihydro coenzymes have UV spectra with two absorption peaks at 260 and 340 nm, meanwhile the oxidised derivatives carrying an sp^2 hybridised nirogen atom in the aromatic pyridine ring and oxidised cofactors had only one peak at 260 nm. The group of Karrer arrived to the conclusion that the reduction of the coenzymes occurs in ortho position. Later, when deuterium was used as a tracer it was established that the reduction of $NAD(P)^+$ takes place in the para rather



than in the ortho position [Pullman et al., 1954] and can be represented according to Figure 2.

Figure 2. Redox transformation of $NAD(P)^+$.

The reduction of $NAD(P)^+$ to NAD(P)H requires two reducing agents per molecule: one electron (e^-) and one hydrogen atom ($H=H^+ + e^-$), which together can be regarded as a hydride ion (H^-). The redox properties of NAD^+ and $NADP^+$ are identical but in nature they act as coenzymes for enzymes participating in different metabolic pathways: $NADP^+$ is normally used by apoenzymes playing anabolic function, whereas enzymes reducing NAD^+ participate in catabolic transformations and the produced NADH serves as an energy source.

1.3 Reactions with participation of $NAD(P)^+$ and dehydrogenases

$NAD(P)^+$ dependent dehydrogenases need for operation a coenzyme, which can be regarded as enzymatic substrate, and at least one more substrate, hence the theory of two-substrate reactions can be applied here. Two substrate reactions can be divided into two categories.

(1). *Reactions with the participation of a ternary complex, containing enzyme and both substrates*

These reactions can be represented as follows

$$E + A + B \rightleftarrows EAB \rightleftarrows EPQ \rightleftarrows E + P + Q$$

This mechanism, can be further subdivided:

(1a). The reactions in which the ternary complex is formed in ordered manner. For example the substrate B can bind to the enzyme only if the substrate A had bound before:

$$E + A \rightleftharpoons EA$$
$$EA + B \rightleftharpoons EAB$$

For example, the oxidation of L-lactate by NAD^+ dependent lactate dehydrogenase. It was found that when NAD^+ binds to the enzyme there is no detectable binding of lactate, hence it was concluded that NAD^+ binding precedes that of lactate [Holbrook et al., 1975]. Binding of NAD^+ to the enzyme precedes also in the oxidation of L-malate by malate dehydrogenase, this fact was discovered by isotope labelling of NAD^+ with ^{14}C [Silverstein and Sulebele, 1969].

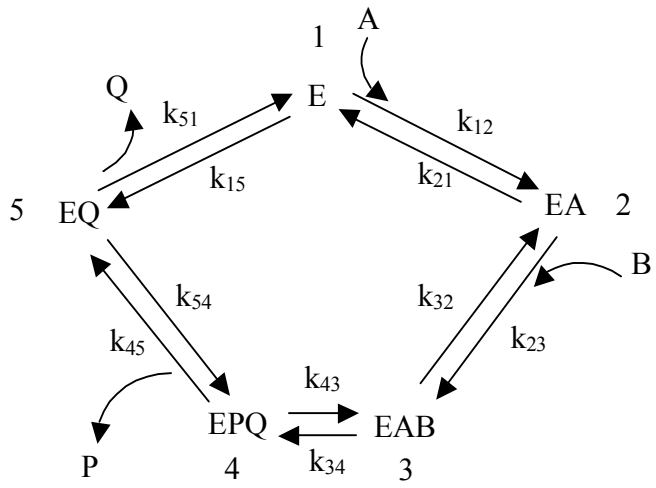
(1b). The reactions in which either substrate can bind first i.e. the ternary complex is formed in a random manner

$$E + A \rightleftharpoons EA \qquad E + B \rightleftharpoons EB$$

or

$$EA + B \rightleftharpoons EAB \qquad EB + A \rightleftharpoons EAB$$

The reactions catalysed by NAD^+ dependent alcohol dehydrogenase from yeast proceed via random ternary complex mechanism [Fromm, 1979].



For the ordered ternary complex mechanism the following equation the overall reaction rate ***v*** can be derived:

$$v = \frac{V_1([A][B] - [P][Q]/K_{eq})}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + K_{ia}K_b[P]/K_{ip1} + K_{ia}K_b[Q]/K_{iq} + K_b[A][P]/K_{ip1} + K_a[B][Q]/K_{iq} + K_{ia}K_b[P][Q]/K_pK_{iq} + [A][B][P]/K_{ip2} + K_a[B][P][Q]/(K_{ip3}K_{iq})}$$

Where: the Michaelis constants for the substrates A and B are $K_a=k_{34}k_{45}k_{51}/(k_{12}(k_{34}k_{45}+k_{34}k_{51}+k_{45}k_{51}+k_{43}k_{51})$, $K_b=k_{51}(k_{32}k_{45}+k_{34}k_{45}+k_{32}k_{43})/(k_{23}(k_{34}k_{45}+k_{34}k_{51}+k_{45}k_{51}+k_{43}k_{51})$, for the products P and Q $K_p=k_{21}(k_{32}k_{45}+k_{32}k_{43}+k_{34}k_{45})/(k_{54}(k_{21}k_{32}+k_{21}k_{34}+k_{21}k_{43}+k_{32}k_{43}))$, $K_q=k_{21}k_{32}k_{43}/(k_{15}(k_{21}k_{32}+k_{21}k_{34}+k_{21}k_{43}+k_{32}k_{43}))$, the maximum velocity in the forward direction $V_1=k_{33}k_{45}k_{51}[E_t]/(k_{34}k_{45}+k_{34}k_{51}+k_{45}k_{51}+k_{43}k_{51})$, $[E_t]$ is total enzyme concentration, the equilibrium constant $K_{eq}=k_{12}k_{23}k_{34}k_{45}k_{51}/(k_{15}k_{21}k_{32}k_{43}k_{54})$, the inhibition constants for A, Q and P are $K_{ia}=k_{21}/k_{12}$, $K_{iq}=k_{51}/k_{15}$, $K_{ip1}=k_{51}(k_{32}k_{45}+k_{34}k_{45}+k_{32}k_{43})/(k_{32}k_{43}k_{54})$, $K_{ip2}=(k_{34}k_{45}+k_{34}k_{51}+k_{51}k_{45}+k_{51}k_{43})/(k_{34}k_{54}+k_{43}k_{54})$, $K_{ip3}=k_{34}k_{45}/(k_{54}k_{34}+k_{54}k_{43})$

The initial rate of reaction (in the absence of P and Q) is:

$$v = \frac{V_1[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]}$$

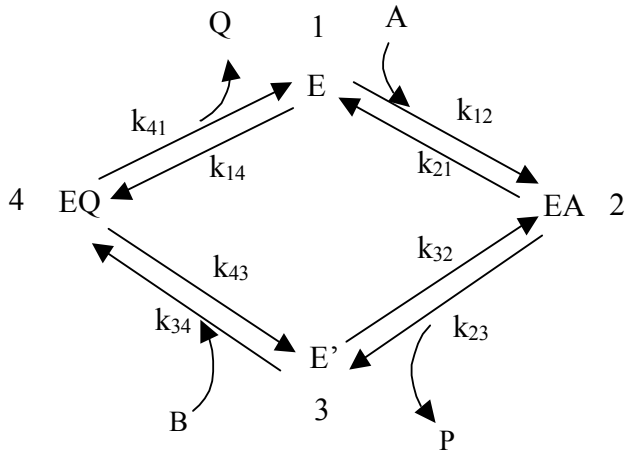
(2). Reactions which do not involve a ternary complex

These reactions are governed by enzyme substitution or ping-pong mechanisms. The second substrate interacts with the enzyme, which has been modified by the first substrate:

$$E + A \rightleftharpoons EA \rightleftharpoons E` + P$$

$$E` + B \rightleftharpoons EQ \rightleftharpoons E + Q$$

The rate equation for this reaction can be derived on the basis of the following mechanism:



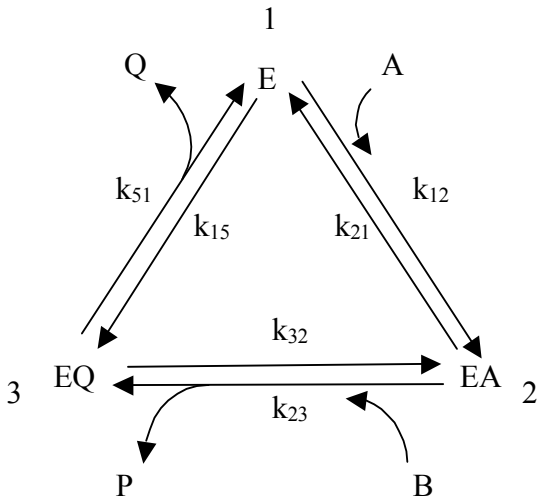
$$v = \frac{V_1([A][B] - [P][Q]/K_{eq})}{K_b[A]+K_a[B]+[A][B]+K_{ia}K_b[P]/K_{ip}+K_aK_{ib}[Q]/K_{iq}+K_b[A][P]/K_{ip}+K_a[B][Q]/K_{iq}+K_aK_{ib}(P)(Q)/K_pK_{iq}}$$

Where: the Michaelis constants for the substrates A and B are $K_a=(k_{21}k_{34}k_{41}+k_{23}k_{34}k_{41}+k_{12}k_{23}k_{43})/(k_{12}k_{23}k_{34}+k_{12}k_{23}k_{41})$, $K_b=k_{41}/(k_{34}+k_{41})$, for the product P $K_p=(k_{21}k_{43}+k_{23}k_{43})/(k_{21}k_{32}+k_{32}k_{43})$, the maximal velocity in the forward direction $V_1=k_{34}k_{41}[E_t]/(k_{34}+k_{41})$, $[E_t]$ is total enzyme concentration, the equilibrium constant $K_{eq}=k_{12}k_{23}k_{34}k_{41}/(k_{14}k_{21}k_{32}k_{43})$, the inhibition constants for A, B, Q and P are $K_{ia}=k_{21}/k_{12}$, $K_{ib}=k_{43}/k_{34}$, $K_{iq}=(k_{21}k_{34}k_{41}+k_{23}k_{34}k_{41}+k_{12}k_{23}k_{43})/(k_{14}k_{21}k_{34}+k_{14}k_{23}k_{34})$, $K_{ip}=k_{41}k_{23}/(k_{32}k_{41}+k_{32}k_{43})$.

In this case the initial rate of reaction becomes:

$$v = \frac{V_1[A][B]}{K_b[A]+K_a[B]+[A][B]}$$

Another reaction in which no central complex is formed at all proceeds through the mechanism proposed by Theorell and Chance for the oxidation of ethanol by NAD^+ dependent alcohol dehydrogenase from horse liver [Theorell and Chance, 1951; Dalzier and Dickinson, 1966]:



The reaction rate of a reaction governed by this mechanism follows:

$$v = \frac{(k_{12}k_{23}k_{31}[A][B] - k_{21}k_{32}k_{13}[P][Q])[E_t]}{k_{21}k_{31} + k_{12}k_{31}[A] + k_{23}k_{31}[B] + k_{12}k_{23}[A][B] + k_{21}k_{32}[P] + k_{21}k_{13}[Q] + k_{32}k_{13}[P][Q] + k_{12}k_{32}[A][P] + k_{23}k_{13}[B][Q]}$$

Or in the absence of P, Q

$$v = \frac{k_{12}k_{23}k_{31}[A][B][E_t]}{}$$

$k_{21}k_{31} + k_{12}k_{31}[A] + k_{23}k_{31}[B] + k_{12}k_{23}[A][B]$

1.4 Some applications of enzymes

In nature enzymes allow chemical reactions to occur within the homeostasis constrains of living systems operating as organic catalysts and enzymes are substrate specific. These properties make enzymes very useful in many areas of human activity.

The specificity of enzymes is widely exploited in analytical chemistry for quantitative and qualitative analyses. The capacity of enzymes to catalyse chemical reactions under mild conditions can be exploited in chemical reactors and fuel cells where enzymes or whole living cells are utilised as catalysts. The highest specific activity, in terms of weight of enzyme and support is desirable in enzyme reactors. The employed support also should perform the function of separation, because if this takes place simultaneously with reaction unfavourable equilibria can be displaced. Possible supports are molecular sieves, glass, silica; membranes of Celite, Bentonite, alumina, graphite and titanium oxide. Changes in physical and chemical properties of the immobilised enzymes have been seen. The stability of enzymes can either increase or decrease on insolubilisation, depending on whether the carrier and the procedure for the immobilisation denatures or stabilizes the proteins.

The specific activity of an enzyme usually decreases upon insolubilisation because of denaturation of the enzymic proteins. The apparent Michaelis constants of the immobilised enzymes are affected by the diffusion limitation of the substrate and normally are higher than those of free enzymes in a solution, but if a substrate has opposite charge to the carrier matrix then the apparent Michaelis constants of immobilised enzymes decrease. In some cases diffusional limitation may be an advantage, because the immobilised enzymes may be protected by inhibitor molecules.

1.5 Chemical regeneration of $NAD(P)^+$

In order to use the selective advantages of $NAD(P)^+$ dependent enzymes in bioreactors it was essential to solve the practical problem of coenzyme recycling. For example, it is known that the carbonyl group is one of the most significant functionalities in synthesis. The control of chirality of the reaction product in the course of reduction or oxidation of ketones and aldehydes is obviously very important. To achieve this goal the methods of classical organic synthesis use bulky groups in reagents with the purpose to hinder approach from one of the two enantiotropic faces of the carbonyl group. The use of enzymes could simplify the synthetic routes thanks to the presence of the proteic three-dimensional structure which controls the direction of attack but

dehydrogenases require quite expensive cofactors. Coenzyme recycling can be accomplished by use of added chemical oxidising or reducing agents. In case of need of NAD(P)H for the enzymatic reduction it can be regenerated by sodium dithionate. If $NAD(P)^+$ is required for the enzymatic oxidation then N-(2,3,4,6-tetraacetyl-D-glycopyranosidyl)-3-carbamoylpyridinium bromide, N-(2,3,4,6-tetraacetyl-β-D-glucopyranosidyl)-3-acetylpyridinium bromide, N-(2,6-dichlorobenzyl)-3-nitropyridinium bromide, N-(4-nitrobenzyl)-3-nitropyridinium bromide, N-benzoylmethyl-3-nitropyridinium bromide, and N-methyl-3,5-dinitropyridinium flourosulfonate can be applied to recycling [Jones and Beck, 1976; Jones and Taylor, 1976].

Recycling of NADH also can be performed by coupling the enzymatic reaction producing NAD(P) (where the substrate S1 is oxidised by $NAD(P)^+$ through the dehydrogenase *DH1* to give the product P1 and the reduced coenzyme NAD(P)H) with another enzymatic reaction which consumes NAD(P)H (where the substrate S2 is reduced by NAD(P)H through the dehydrogenase *DH2* to give the product P2 and the oxidised coenzyme $NAD(P)^+$ according to the scheme:

$$S1 + NAD(P)^+ \overset{DH1}{\rightleftharpoons} P1 + NAD(P)H$$

$$S2 + NAD(P)H \overset{DH2}{\rightleftharpoons} P2 + NAD(P)^+$$

For example galactose dehydrogenase, alanine dehydrogenase, and dextran bound NAD^+ were immobilised in a bioreactor, which produced alanine and galactonate from pyruvate and galactose [Davies and Mosbuch, 1974]. Another reactor containing rabbit muscle lactate dehydrogenase, horse liver alcohol dehydrogenase and poly(ethylene glycol)-bound NAD^+ was used for the production of lactate and acetic aldehyde from pyruvate and ethanol [Katayama et al., 1983].

1. 6 Electrochemical regeneration of NAD(P)H

1.6.1 Direct oxidation of NAD(P)H on bare electrode surfaces

An alternative to chemical and enzymatic regeneration of NAD(P)H in organic synthesis and enzymatic analysis is the electrochemical oxidation. The formal redox potential of the NAD^+/NADH couple is –0.56 V vs. SCE (at pH 7.0) but the first intents of direct NADH oxidation on solid carbon electrodes [Moiroux and Elving, 1979a; 1979b; 1980] demonstrated that very high overpotential must be applied (+0.5 - 0.7 V vs. SCE). The speculated mechanism [Blankespoor and Miller, 1984; Hapiot et al., 1990; Fukusumi et al., 1987] of the oxidation reaction on bare electrodes is:

$$NADH - e^- \rightleftharpoons NADH^{\cdot +}$$

$$NADH^{\cdot +} + B \rightleftharpoons NAD^{\cdot} + BH^+$$

$$NAD^{\cdot} \rightleftharpoons NAD + e^-$$

It is very possible that a disproportionation reaction also takes place:

$$NAD^{\cdot} + NADH^{\cdot +} \rightleftharpoons NAD^+ + NADH$$

where ***B*** is a base and $NADH^{\bullet +}$ and $NAD^{\bullet}$ are the intermediate radicals.

It is thought that the direct oxidation of NADH yields enzymatically active NAD^+ [Bonnefoy et al., 1988; Fassouane et al., 1990]. The solid carbon electrodes showed short lifetime due to fouling caused by adsorption of $(NAD)_2$ dimers (formed by the following reaction) on the electrode surface when the NADH concentration is more than 0.5 mM [Jaegfeldt et al., 1981].

$$2\ NAD^{\cdot} \longrightarrow (NAD)_2$$

Another probable reason for this deactivation is the formation of stable adducts between surface species and reaction radical intermediates [Jaegfeldt et al., 1983]. Probably the reason for the yields of NADH oxidation less then 100% and generally, the main limiting factor in the systems based on NADH recycling, is the low stability of this reduced cofactor due to its hydrolysis in aqueous solutions at low values of pH. It was shown that the half life time of NADH in solution at pH 6.0 is 9 hours [Hedemno et al., 1996] meanwhile at pH 7.5 half life-time is 17 hours [Wong and Whitesides, 1981; Bonnefoy et al., 1988].

The necessity to apply high overpotential is a real drawback for applications of carbon and platinum electrodes in biosensors because of the presence of interferents, having low redox potentials, in real samples. The use of silver as the electrode material helps to decrease the potential of direct NADH oxidation to +0.23 V vs. SCE [Li et al., 1996]. It is also reported that screen printed thick-film gold electrodes can be utilised for NADH detection at relatively low overpotential of +0.145 V vs. SCE with minimised fouling [Silber et al., 1995], but it has not reported if the reaction product, generated by silver or thick-film gold electrodes, retains enzymatic activity.

1.6.2 Oxidation of NAD(P)H via enzymes

One of the options for lowering the applied potential for NAD(P)H oxidation is to employ enzymatic recycling of reduced cofactors by diaphorases or NADH oxidases according to the following sequence of reactions:

$$NADH + Diaphorase_{ox} \rightleftharpoons NAD^+ + Diaphorase_{red}$$
$$Diaphorase_{red} + Mediator_{ox} \rightleftharpoons Diaphorase_{ox} + Mediator_{red}$$

and, finally, the reoxidation of a mediator on the electrode surface:

$$Mediator_{red} \longrightarrow Mediator_{ox}$$

The formation of enzymatically active $NAD(P)^+$ is secured by the fact that enzymatic NADH oxidation is the natural way for recycling of coenzymes.

The use of diaphorase in biosensors for NADH oxidation has been demonstrated for the first time in sensors incorporating glutamate and lactate dehydrogenase together with dextran bound NAD^+ in the 70's [Davies and Mosbach, 1974]. Next, an alcohol sensor based on alcohol dehydrogenase and diaphorase was reported [Smith and Olson, 1975].

NADH oxidases also proved to be suitable for construction of biosensors. NADH oxidase was immobilised in poly(vinyl alcohol) [Leca and Marty, 1997] or poly(4-styrene sulfonate) [Mizutani et al., 2000] to yield the system in which hydrogen peroxide, resulting from NADH oxidation, was detected. Thermostable NADH oxidase was immobilised on a immobilon AV membrane [Compagnone et al., 1995] by poly(vinylalcohol) bearing photocrosslinkable terminal groups on a carbon paste electrode and used as NADH sensor [Noguer et al., 1999] through the detection of hydrogen peroxide.

Not only NADH diaphorases and oxidases were used in biosensors. The electroplymerisation of flavin reductase-amphiphilic pyrrole ammonium mixture provides NADH biosensors based on the oxidation at 0.1 V vs. SCE of enzymatically generated dihydroriboflavin [Cosnier et al., 1997]. In another system NADH was oxidised by salicylate hydroxylase in an oxygen and salicylate dependent reaction, and the consumption of oxygen was monitored with a Clark electrode [Chen et al., 2000].

The use of diaphorases and oxidases is very promising if they become more widely available, stable and cheaper.

1.6.3 Oxidation of NAD(P)H by soluble mediators

Another possible solution to the problem of high overpotential is to introduce into the system homogeneous or heterogeneous catalysts for the oxidation of NADH. They increase the rate of processes by introducing new pathways (mechanisms) with lower activation energies mediating the transfer of electrons from NAD(P)H to the electrode surface and are referred as mediators which operate according to Figure 3. The function of a mediator is to serve as a catalyst for

NAD(P)H oxidation. NAD(P)H is oxidised by the mediator, and the mediator is oxidised in its turn on the electrode surface at low applied potential. It is desirable to use selective mediators for NADH oxidation in biosensors to avoid the oxidation of interfering substances. Another important requirement for them is their long lifetime.

The first reported water soluble mediators suitable for NADH oxidation in bulk solution were small organic molecules carrying aromatic moieties such as quinones, phenylene diimines, indophenols, phenazines and phenoxazines [Gorton, 1986].

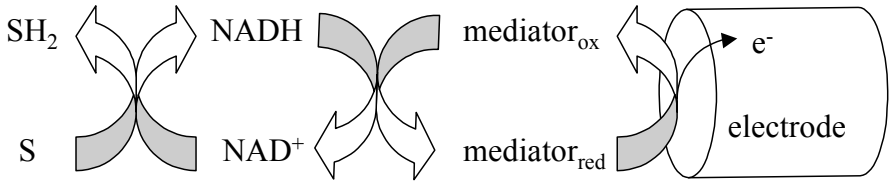


Figure 3. Schematic diagram of a system for electrochemical NADH recycling based on a mediator.

It is suggested that the NADH oxidation reaction proceeds via the formation of a mediator-NAD(P)H complex [Gorton, 1986; Katakis and Dominguez, 1997]. The mechanism of this reaction is represented in the following scheme:

$$NADH + M_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [NADHM] \xrightarrow{k_2} NAD^+ + M_{red}$$

Which is followed by the reoxidation of mediator at the electrode surface:

$$M_{red} \xrightarrow{k_s} M_{ox}$$

Oxygen molecules can compete with an electrode for the mediator reoxidation [Katakis and Dominguez, 1997] leading to a decrease in oxidation current in case of biosensors and fuel cells:

$$M_{red} + O_2 \longrightarrow M_{ox} + H_2O_2$$

At high pH another undesirable reaction occurs which adds to electrode fouling:

$$[NADHM] + B \rightleftharpoons BH + NAD\cdot + M\cdot$$

where ***B*** is a base. In case of ortho and para quinones (***Q***) and aromatic diamines the oxidation reaction goes through a net hydride transfer [Kitani et al., 1981; Jaegfeldt et al., 1983; Fukuzumi et al., 1984a; 1984b; 1989]:

$$NADH + Q \rightleftharpoons [NADHQ] \rightleftharpoons NAD^+ + QH^-$$

$$QH^- + H^+ \rightleftharpoons QH_2$$

and the reoxidation of the mediator

$$QH_2 - 2H^+ - 2e^- \rightleftharpoons Q$$

The high rate of the interaction between quinoid structures and NADH is caused by the formation of stabilised transition state complex [NADHQ]. Unfortunately this complex can decompose through a side reaction leading to poisoning of the catalyst.

$$[NADHQ] \longrightarrow NAD{:}QH$$

1.6.4 Oxidation of NAD(P)H on chemically modified electrode surfaces

The main disadvantage of homogenous mediators is the necessity to introduce them into a sample solution, therefore a good alternative is to find a way to modify the electrode surface with the objective to lower the applied potential for NAD(P)H oxidation.

One method is the modification of the electrode surface by pretreatment in order to achieve its activation. In a number of works it was shown that the drastic oxidation of a carbon electrode leads to the modification of its surface with ortho-quinone groups. The resulting electrodes showed NADH oxidation capacity at decreased applied potentials [Blaedel and Jenkins, 1975; Laval et al., 1984; Ravichandran and Baldwin, 1984]. Electrochemical pretreatment was combined with a novel polishing method for carbon fibre electrodes [Hayes and Khur, 1999] which generated a decreased overpotential and a high faradaic current of NADH oxidation. In another method the adenine moiety in NAD^+ was oxidised at carbon paste electrodes to modify carbon particles with redox active products capable of NADH oxidation at +0.05 V vs. $Ag/AgCl/KCl_{sat}$ [Alvarez et al., 2000], the surface of carbon fibre electrode also can be activated by electrochemical oxidation of NADH followed by exposure to ascorbic acid [Nowal and Kuhr, 1995] or peroxide [Nowall and Kuhr, 1997]. Another approach is to use new conducting materials for electrode preparation such as boron-doped chemical vapour deposited diamond film [Sarada et al., 1999; Fujishima et al., 1999; Rao et al., 1999]. Nothing was reported on enzymatic activity of NAD^+ produced at the surface of diamond electrodes.

The second method is the adsorption of water soluble or insoluble organic molecules, mediating NADH oxidation, onto the electrode surface. Electrochemical catalysis of NADH by mediators adsorbed on the electrode surface has been reported (Table I). The majority of mediators presented in the table suffer from short operational life time. Ortho-quinone is not stable in the process of NADH oxidation because it leads to the formation of a stable complex which poisons

and blocks the surface of the electrodes, which lose the capacity to oxidise NADH after several oxidative cycles [Jaegfeldt et al., 1983], and in many cases nothing was reported on the enzymatic activity of the oxidation product.

Electrodes covered with phenazine methosulphate are not stable in a stirred buffer solution and lost 60% of this mediator over 2 h due to desorption, the stability study in the presence of NADH by cyclic voltammetry has demonstrated consecutive decrease of the anodic current [Torstensson and Gorton, 1981] without showing enzymatic activity of the oxidation product. Electrodes modified with phenoxazinium salt, Meldola Blue in the oxidised form showed maximal half life-time of 12 h at neutral pH in the absence of NADH [Gorton et al., 1984], no data were presented on the NADH oxidising operational stability and enzymatic activity of resulting NAD^+. 1,2-benzophenoxazine-7-one modified electrodes showed higher stability than those modified with Meldola Blue [Gorton et al., 1985]. Toluidine blue, methylene blue, and brilliant cresyl blue are not stable at pH higher than 7, for example toluidine blue adsorbed on glassy carbon demonstrated half life-time of 10 h at pH 8 [Chi and Dong, 1995]. The majority of the above mentioned mediators are not stable at high pH due to the side reactions of oxidation intermediates with hydroxyls, an example of side polymerisation reaction for ortho-quinone is represented in Figure 4 [Katakis and Dominguez, 1997]. Phenoxazine dyes as well proved to be unstable in alkaline solutions [Huck, 1994].

The complexation of ortho-quinoid structures with transition metals leads to mediators having higher chemical stability in alkaline solutions. Graphite electrodes modified with complexes [Re(1,10-phenanthroline-5,6-dione)$(CO)_3Cl$], [Fe(1,10-phenanthroline-5,6-dione)$_3$ $(PF_6)_2$], [Ru(1,10-phenanthroline-5,6-dione)(vinylbipyridine)$_2$]$(PF_6)_2$ [Wu et al., 1996] and [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]$(PF_6)_2$ [Popescu et al., 1999], the latter yielding enzymatically active NAD^+ [Hedemno et al., 1996], showed electrocatalytic oxidation of NADH at potentials from 0.0 to +0.15 V vs. $Ag/AgCl/KCl_{sat.}$

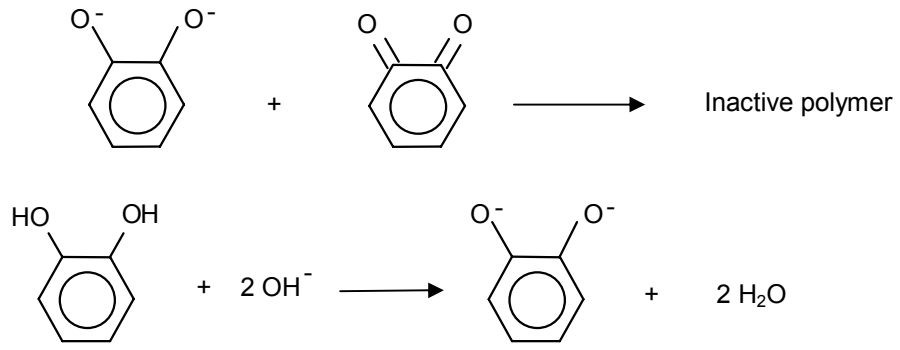


Figure 4. Reactions resulting in deactivation of orhto-quinone mediators.

One of probable explanations for the higher stability of complexed quinoid ligands is the greater delocalisation of the possible radical electrons on a positively charged central atom [Katakis and Domínguez, 1997]. Another example of stabilisation by complexation are complexes of terpyridine bearing catechol functionality with Co, Cr, Fe, Ni, Ru, Os for NADH oxidation [Storrier et al., 1999].

Table I. Immobilised NADH oxidising small molecules reported in the literature

Mediator	Working potential	$k_{[NADH]=0}/M^{-1} s^{-1}$ *	Reference
ortho-quinone	+0.1 V vs. SCE	$1x10^4$	Jaegfeldt et al., 1981, 1983
phenazine methosulphate	-0.05 V vs. SCE		Torstensson and Gorton, 1981
phenazine ethosulphate	-0.1 V vs. SCE		Torstensson and Gorton, 1981
meldola blue (phenoxazine)	-0.175 V vs. SCE	$2.7x10^4$	Gorton et al., 1984, Nagy et al., 1995
3-β–naphtoyl-nile blue	-0.22 V vs. SCE		Huck et al., 1982
1,2-benzophenoxazine-7-one	-0.21 V vs. SCE	$1.2x10^3$	Gorton et al., 1985
methylene blue	+0.3 V vs. SCE,	340	Chi and Dong, 1995
toluidine blue	+0.3 V vs. SCE,	320	Chi and Dong, 1995
brilliant cresyl blue	+0.4 V vs. SCE	180	Chi and Dong, 1995
methylene green	+0.1 V vs. SCE,		Han, et al., 1995, Chen et al., 1995
catechol violet	+0.1 V vs. SCE		Zhu et al., 1995
cobalt hexacyanoferrate	+0.18 V vs. SCE,		Cai et al., 1995
nickel hexacyanoferrate	+0.2 V vs. SCE,	$1.2x10^3$	Cai et al., 1995b
ferrocene derivatives	+0.6 V vs. Ag/AgCl	$4.68x10^3$	Tian and Dong, 1995
2,6-dichlorophen-olindo-phenol salts	+0.06 V vs. Ag/AgCl		Florou et al., 1998
nitro-fluorenone derivatives	-0.05 V vs. Ag/AgCl		Mano and Kuhn, 1999
riboflavin combined with zirconium phosphate	+ 0.25-0 V vs. SCE		Malinauskas et al., 1999
pyrocatechol sulfonephtalein	+0.3 V vs. SCE		Cai et al., 2000
phenothiazine derivatives			Dicu et al., 2000
[Re(1,10-phenanthro-line-5,6-dione)$_3$](PF_6), [Fe(1,10-phenanthro-line-5,6-dione)$_3$](PF_6), [Ru(1,10-phenanthro-line-5,6-dione)$_3$](PF_6),	+0.15 vs. Ag/AgCl	$2.7x10^3$ $6.8x10^3$ $4.3x10^3$	Goss and Abruña, 1985; Wu et al., 1996
[Os(4,4'-dimethyl-2,2'-bi-pyride)(1,10-phenanthroline-5,6-dione)](PF_6)$_2$	+0.15 vs. Ag/AgCl	$0.9x10^3$	Popescu et al., 1999

* $k_{[NADH]=0}$ is the value of the overall reaction constant k for the oxidation of NADH by the mediator extrapolated to zero NADH concentration. $NADH + M_{ox} \xrightarrow{k} NAD^+ + M_{red}$

The rate of reaction with NADH depends greatly on the mediator structure and Meldola Blue proved to be the fastest mediator. The dependence of reaction rate on the structure is represented in Figure 5 [Gorton, 1986]. Taking into concideration the mechanistic scheme for

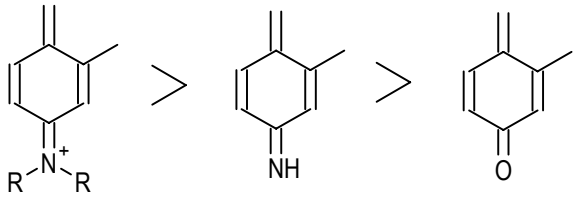


Figure 5. Observed dependence of reaction rate with NADH on structure of a mediator.

NADH oxidation (Figure 6) proposed by Bartlett with coworkers [Bartlett et al., 1997], it can be concluded that the mediators bearing positive amino groups demonstrated higher reaction rates because they have higher formal standard potential (which is the thermodynamic driving force for electron exchange) on the other hand nitrogen atom accepts a hydride more readily than oxygen.

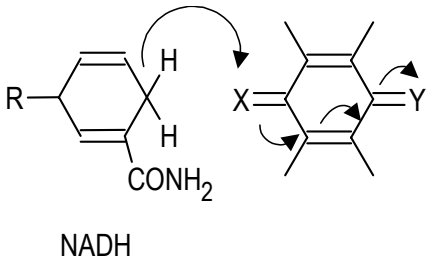


Figure 6. Proposed mechanistic scheme for NADH oxidation by reaction with a redox mediator. X is a group capable of accepting a hydride, Y is an electron-deficient group.

The most important disadvantage of electrodes modified with small molecules by adsorption is the leaching of the mediator from the electrode surface to the bulk solution. Hence different methods of mediator immobilisation via formation of chemical bonds with molecules of the electrode material have been developed. Ortho-quinone was chemically immobilised on the surface of solid carbon electrodes through covalent bonds [Tse and Kuwana, 1978; Ueda et al., 1982], toluidine blue was covalently attached to monolayers of cystamine or 3-mercaptopropionic acid on gold electrodes oxidising NADH at -0.1 V vs. $Ag/AgCl/KCl_{sat}$ [Schlereth et al., 1994], phenoxazine and phenothiazine derivatives were attached to monolayers of cystamine and cysteine [Schlereth et al., 1995], the imine derived from 3,4-dihydroxybenzaldehyde and 4-aminopyridine was adsorbed onto polycrystalline platinum electrodes through the pyridine nitrogen [Lorenzo et al., 1995], dopamine was covalently bound to self-assembled cysteamine monolayers on gold electrodes oxidising NADH at +0.15 V vs. SCE [Sun et al., 1997], thionine was attached to self-assembled cysteamine monolayer on gold to oxidise NADH at about 0 V vs. SCE [Chen et al.,

1997], graphite particles were exfoliated and subsequently covalently functionalised with toluidine blue [Ramesh and Sampath, 2000].

Another way to decrease leaching of mediators from the electrode surface is to boost the adsorption by using electroactive (redox) polymers for coating which contain the equivalent of many monomolecular layers. The electrodes modified with the redox polymer have much more electroactive sites per area, and in volume terms the concentration of electroactive sites in the polymer is higher therefore the electrocatalytic response to NADH of redox polymers can be much higher than this for monolayers of redox molecules due to the "volume effect" [Murray, 1984]. Two main methods for immobilisation of redox polymers on electrodes, which are adsorption of pre-synthesised polymer and electropolymerisation *in situ* of monomeric mediators, have been shown in literature. Examples of NADH oxidising polymers are reviewed in table II.

Study of charge propagation in quinoid polymers over electrode surfaces has shown that only the inner sublayer of the polymer is readily oxidised, whereas the outer sublayers are oxidised only very slowly. This phenomenon was explained in terms of slow charge propagation through polymeric films of quasi-reversible two-electron redox couples, so only the first monolayer of redox polymers is effective in NADH oxidation. Poly(dopaquinonone) proved to be very unstable on the electrode surface and about 70% of hydroquinone-quinone couples were inactivated after 22 consecutive cyclic voltammogramms at neutral pH because of the low stability of the oxidised form [Fukui et al., 1982]. Electropolymerised films of 3,4-dihydroxybenzaldehyde (3,4-DHB) showed higher stability, with 30% of redox couples lost after 30 min of continuous scanning. Electrodes modified with polymeric form of Meldola Blue showed high stability measured by repeated redox cycling and poor NADH sensitivity due to low permeability of NADH molecules through the polymeric film [Persson, 1995]. Electrodes bearing films of poly(ortho-phenylenediamine) were not stable immediately after preparation [Lobo et al., 1996]. Electropolymerisation of azure I gave electrodes which were losing their activity during 500 repetitive scans but after 6 h stabilisation was reached [Cai and Xue, 1996].

In general most polymeric mediators showed lower rate constants of reaction with NADH than the corresponding monomers because of "capping" by the polymer backbone of the active redox sites, oriented towards the electrode surface [Persson et al., 1995]. The majority of monomeric and polymeric mediators has pH dependent redox potential and rate of interaction with NADH. Both of them increase when pH decreases because protons participate in the redox conversion of $NAD(P)^+$ and many mediators and facilitate hydride transfer to accepting groups of the mediators.

Polymeric mediators proved to adhere more than small molecules on the electrode surface, but inherit from their corresponding monomers low chemical and hence operational stability at elevated values of pH, which is not convenient because the majority of the known $NAD(P)^+$

dependent dehydrogenases have optimal activity at pH higher than 7.0.

The reversible electrochemical oxidation of NADH to its enzymatically active form is the key to the application of mediators in reagentless biosensors. For example, the enzymatic activity of the product of NADH oxidation by [Os(4,4'-dimethyl-2,2'-bipyridine)(1,10-phenanthroline-5,6-dione)] has been demonstrated [Hedemno et al., 1996] though in the majority of the published works on NADH oxidising mediators the enzymatic activity of the resulting oxidation products has not been studied. Such regeneration can be questioned in many cases, for example, it is very doubtful that electrodes modified with $[Os(bpy)_2(PVI)_{10}Cl]Cl$ convert NADH to active NAD^+ [Ju and Leech, 1997].

Table II. Immobilised NADH oxidising polymers reported in literature

Polymer	Method of immobilisation	Working potential	$k_{[NADH]=0}$/ M^{-1} s^{-1}	Reference
poly(dopamine)	covalent coupling with poly(methacryloyl chloride) followed by adsorption on glassy carbon	+0.2 V vs. SCE		Degrand and Miller, 1980; Fukui at al., 1982
poly(toluidine blue)	inclusion in carbon paste	+0.2 V vs. SCE		Dominguez et al., 1993[a]
poly(3,4- dihydro-xybenzaldehyde)	electropolymerisation on glassy carbon	+0.2 V vs. SCE	$4.3x10^3$	Pariente et al., 1994; 1997
poly(meldola blue)	covalent coupling with poly(methylsiloxane) followed by adsorption on graphite	NADH at 0 V vs. SCE	$2.9x10^3$	Persson et al., 1995
poly(orthophe-nylenediamine	electropolymerisation on carbon paste	+0.15 V vs. Ag/AgCl		Lobo et al., 1996
poly(orthoamino-phenol)	electropolymerisation on carbon paste	+0.15 V vs. Ag/AgCl		Lobo et al., 1996
poly(azure I)	electoropolymerisation on glassy carbon electrodes	+0.1 V vs. SCE	$2x10^3$	Cai and Xue, 1997
poly(toluidine blue)	covalent coupling with poly(styrene) or poly(etheleneimine) followed by adsorption on graphite	0-+0.05 V vs. SCE		Huan et al., 1996
poly(methylene green)	electropolymerisation on glassy carbon	+0.1 V vs. SCE		Zhou et al., 1996
poly(methylene blue)	Electropolymerisation on gold	+0.2 V vs. Ag/AgCl		Silber et al., 1996
$[Os(bpy)_2(PVI)_{10}$ $Cl]Cl$	complexation of $[Os(bpy)_2Cl_2]$ with poly(vinylimidazol) followed by adsorption on carbon fiber	+0.5 V vs. Ag/AgCl		Ju and Leech, 1997
poly(nile blue A)	electropolymerisation on glassy carbon	+0.02 V vs. SCE	800	Cai and Xue, 1997; Malinauskas et

				al., 2000
poly(aniline)	electropolymerisation on glassy carbon	+0.1 V vs. SCE		Bartlett et al., 1997

Table II. (continued)

Polymer	Method of immobilisation	Working potential	$k_{[NADH]=0}$/ M^{-1} s^{-1}	Reference
poly(aniline)	electropolymerisation on poly(acrylic acid) film	+0.05 V vs. SCE		Bartlett and Simon, 2000
poly(1,2-, 1,3-, 1,4-diaminobenzene)	Electropolymerisation in the presence of PQQ on Pt, Au and graphite	+0.2 V vs. SCE		Curulli et al., 1997
poly(1,2-, 1,3-, 1,4-diaminobenzene)	Electropolymerisation in the presence of PQQ on Pt, Au and graphite	+0.2 V vs. SCE		Curulli et al., 1997
poly(4-aminobiphenyl)	electropolymerisation in the presence of PQQ on Pt, Au and graphite	+0.2 V vs. SCE		Curulli et al., 1997
poly(3-methyl-thiophene)	electropolymerisation on carbon fibre	+0.45 V vs. Ag/AgCl		Jaraba et al., 1998
poly(naphtol green B)	electropolymerisation on glassy carbon	+0.1 V vs. SCE		Cai and Xue, 1998
poly(toluidine blue O)	electropolymerisation on glassy carbon	+0.15 V vs. SCE		Cai and Xue, 1998b
poly(caffeic acid)	electroplymerisation on glassy carbon	+0.2 V vs. SCE		Zare and Golabi, 2000
poly(1,4-bis(3,4di-hydroxyphenyl)-2,3-dimethylbutane)	electropolymerisation on glassy carbon	+0.3 V vs. Ag/AgCl	$3.4x10^3$	Ciszewski and Milczarek, 2000

1.7 Dehydrogenase electrodes

The reaction of electrochemical oxidation of NAD(P)H can be coupled with the reaction of enzymatic oxidation by dehydrogenases to yield a great number of possible configurations applicable to the construction of biosensors, bioreactors and fuel cells. The following kinetic model for the conversion of substrate S to product P by a dehydrogenase modified electrode has been developed by Albery with co-workers [1987]:

$$E + NAD^+ \overset{K_0}{\rightleftharpoons} E\bullet NAD^+$$

$$S + E\bullet NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S\bullet E\bullet NAD^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P\bullet E\bullet NADH$$

$$P\bullet E\bullet NADH \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + NADH + P + H^+$$

The final step is the oxidation of NADH at the electrode:

$$NADH \xrightarrow{k`} NAD^+ + H^+ + 2e^-$$

For each step in this scheme

$K_n = k_n/k_{-n}$

The constant K_{TD} serves to describe the overall equilibrium between S + NAD^+ and P + NADH + H^+

$K_{TD} = K_0K_1K_2K_3$

The transport of S and P in the enzyme layer having the thickness L, is described by the mass transfer rate coefficients k'_S and k'_P.

Assuming that there is no product in the external bulk solution and the kinetics of binding of the enzyme to NAD^+ are rapid the following expression for the flux, j can be obtained:

$$\frac{e_\Sigma}{j} = \frac{1}{Lk_{cat}}\left(1 - \frac{j}{k'_S s_\infty}\right) + \frac{K_M}{Lk_{cat}s_\infty} + \frac{je_\Sigma}{k'_P s_\infty k' K_{TD}[NAD^+]}$$

$$+ \frac{j^2}{Lk'_P s_\infty k' K_{TD}[NAD^+]}\left(\frac{1}{k_{-1}`} + \frac{K_2}{k_{-1}`} + \frac{1}{k_{-2}`}\right) + \frac{e_\Sigma}{k'_S s_\infty}$$

where s_∞ is the substrate concentration in the external medium, e_Σ is the total enzyme concentration.

$e_\Sigma = [E\bullet NAD^+] + [S\bullet E\bullet NAD^+] + [P\bullet E\bullet NADH]$

$1/k_{cat} = 1/k_2` + 1/(K_2k_3`) + 1/k_3`$

$K_M/k_{cat} = 1/k_1` + 1/(K_1k_2`) + 1/(K_1K_2K_3)$

Many electrodes operating in the presence of enzymes, cofactors and mediators in a sample solution have been demonstrated in the literature, but the number of dehydrogenase electrodes which can detect an analyte without addition of any reagent to a sample solution, so called reagentless electrodes, is limited. Some reagentless electrodes based on dehydrogenases are represented in Table III. Most of these electrodes were produced by immobilisation of enzymes, NAD^+ and mediator for the coenzyme reoxidation on the surface of an electrode. Five strategies for the coenzyme immobilisation have been shown: (a) entrapment in hydrogels formed *in situ* by polymeric macromolecules, (b) adsorption onto pre-prepared polymeric membranes, (c)

entrapment in electropolymerised films, (d) entrapment in carbon paste, and (e) immobilisation in self-assembled monolayers.

a. Entrapment in hydrogels formed in situ by macromolecules

An attempt to entrap NAD^+ in gels formed by gelatine and poly(lysine) yielded very unstable biosensors for glycerol [Laurinavicius et al., 1996].

b. Immobilisation of NAD^+ under preprepared membranes

NAD^+ was crosslinked on a cellulosic dialysis membrane by glutarldehyde to yield unstable lactate biosensors [Blaedel and Jenkins, 1976]. Polymeric form of NAD^+ (NAD-dextran) was entrapped in the hydrogel formed by poly(vinyl alcohol) bearing styrylpyridinium groups and finally covered by a cellophane membrane to yield ethanol biosensors. The authors claim that they observed a good operational stability though they do not report the detailed results of their operational stability study [Leca and Marty, 1997]. Malate biosensors have been prepared by adsorption of NAD^+ onto nitrocellulose and cellulose acetate membranes which rapidly lost activity with time because of coenzyme leaching [Maines et al., 2000].

c. Entrapment of NAD^+ in electropolymerised films

An L-lactate biosensor based on NAD^+ entrapment in poly(pyrrole) electropolymerised on a platinum electrode [Ikariyama et al., 1990] showed poor stability and low sensitivity.

d. Entrapment of NAD^+ in carbon paste

L-lactate biosensors were fabricated on the bases of carbon paste, but nothing was reported on their operational stability [Yoon and Kim, 1996], glucose biosensors showed very high stability and lost 6-8% in their response during 3-8 h [Hedemno et al., 1996], alcohol biosensors protected with dialysis membrane [Lobo et al., 1996b] were stable during one day. Glutamate biosensors covered with poly(*o*-phenylenediamine) lost ½ of the initial response after 2 days of operation [Alvarez et al., 1997].

e. Immobilisation in self-assembled monolayers

This strategy has been pioneered by the group of Itamar Willner which reported L-lactate biosensors produced by attachment of NAD^+ to a self-assembled PQQ monolayer [Katz et al., 1998].

Biosensors in which NAD^+ was entrapped in hydrogels formed *in situ* have good sensitivities because of very easy transport of analyte to the enzyme, but suffer from low stability in the reagentless mode of operation due to easy desorption of the coenzyme and the enzyme. When NAD^+ was entrapped in electropolymerised films and pre-prepared membranes the sensitivity was bad due to the limited transport of analyte. The most stable biosensors were produced on the basis of carbon paste in which a "reserve pool" of the coenzyme was created, at the same time their sensitivity was high hence the local coenzyme concentration was high as well. Unfortunately it is not possible to opt for carbon paste configurations to fabricate miniaturised

sensors.

Table III. Configurations of reagentless electrodes based on $NAD(P)^+$ dependent dehydrogenases

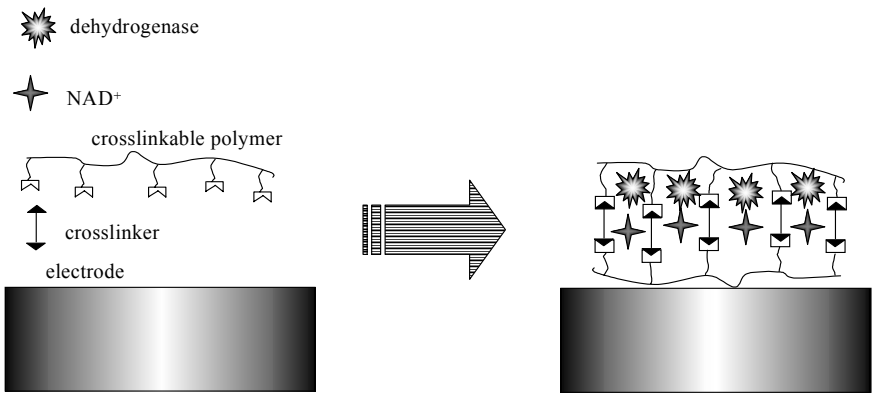
Analyte	Cofactor immobilisation	Dehydrogenase	Method of NADH oxidation / E_{app}	Time of operation without regeration	Reference
L-lactate	NAD^+ crosslinked on a cellulosic dialysis membrane by glutraldehyde	lactate dehydrogenase crosslinked on a cellulosic dialysis membrane by glutraldehyde	direct oxidation on glassy carbon +0.45 vs. Ag/AgCl	Response decreased over several hours by 20%	Blaedel and Jenkins, 1976
L-lactate	NAD^+ covalently attached to agarose	lactate dehydrogenase immobilised behind a dialysis membrane	direct oxidation on glassy carbon +0.45 vs. Ag/AgCl	Response almost unchanged over several hours	Blaedel and Jenkins, 1976
ethanol	NAD^+ immbolised by Schiff base	alcohol dehydrogenase in carbon paste	direct oxidation on carbon paste +0.4 V vs. SCE	unstable	Yao et al., 1979
L-lactate	NAD^+ immbolised by Schiff base	lactate dehydrogenase in carbon paste	direct oxidation on carbon paste +0.4 V vs. SCE	unstable	Yao et al., 1979
malate	NAD^+ immobilised in a dialysis membrane	malate dehydrogenase under dialysis membrane	direct oxidation on platinum electrode +0.875 V vs. SCE		Blaedel and Engstrom, 1980
L-lactate	NAD^+ in electropolyme-rised poly-(pyrrole)	lactate dehydrogenase in electropolymerised poly(pyrrole)	Methylene blue on platinum electrode +0.3 V vs. Ag/AgCl	unstable	Ikariama et al., 1990
glucose	NAD^+ in Eastman AQ™	glucose dehydrogenase in carbon paste	Cresyl blue 0.0 V vs. Ag/AgCl		Gorton et al., 1991
ethanol	NAD^+ in carbon paste	alcohol dehydrogenase in carbon paste	direct oxidation on carbon paste +0.58 V Ag/AgCl		Wang and Liu, 1993
ethanol	NAD^+ with poly(ethylene-imine) in carbon paste	alcohol dehydrogenase in carbon paste	poly(toluidine blue) +0.1 V vs. Ag/AgCl		Domínguez et al., 1993b
glucose	NAD^+ entrapped in ionic polymers on graphite	glucose dehydrogenase	toluidine blue on graphite 0 V vs. Ag/AgCl		Boguslavsky et al., 1995
glycerol	NAD^+ in gela-tine or polyly-sine crosslinked with glutaral-dehyde	glycerol dehydrogenase and lipase in gelatine or polylysine crosslinked with glutaraldehyde	meldola blue, nile blue, toluidine blue adsorbed on carbon 0 V vs. Ag/AgCl		Laurinavi-cius et al., 1996

Table III. (continued)

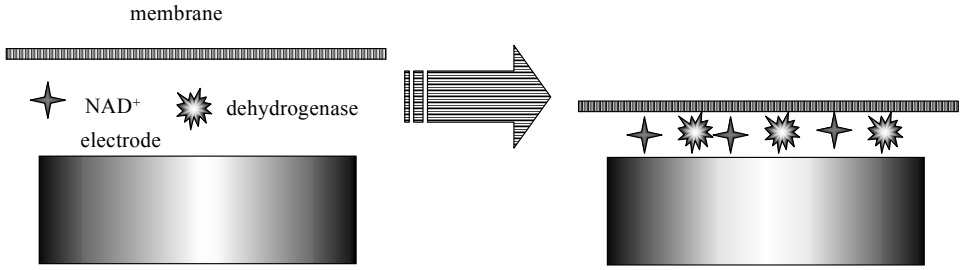
Analyte	Cofactor immobilisation	Dehydrogenase	Method of NADH oxidation / E_{app}	Time of operation without regeration	Reference
L-lactate	NAD^+ in carbon ink of screen printed electrodes	L-lactate dehydrogenase over carbon ink	meldola blue in carbon ink 0 V vs. Ag/AgCl		Sprules et al., 1996
glucose	NAD^+ in carbon paste	glucose dehydrogenase in carbon paste	[Os(4,4'dimethyl-2,2'-bipyridine)$_2$ (1,10-phenanthro-line -5,6-dione)] $(PF_6)_2$ in carbon paste +0.15 V vs. Ag/AgCl	loss of 6-8 % of response in 3-8 h	Hedemno et al., 1996
ethanol	NAD^+ in carbon paste	alcohol dehydrogenase in carbon paste	toluidine blue in carbon paste +0.05 V vs. Ag/AgCl	response is constant for 1 day, half life is 2 days.	Lobo et al., 1996b
L-lactate	NAD^+ in carbon ink of screen printed thick film	L-lactate dehydro-genase in carbon ink of screen printed thick film	thick film carbon electrode +0.35 V vs. Ag/AgCl		Yoon and Kim, 1996
L-lactate	NAD^+ in carbon paste	L-lactate dehydrogenase and glutamic pyruvic transaminase in carbon paste	poly(ortho-phenylenedia-mine) electro-polymerised on carbon paste +0.15 V vs. Ag/AgCl	response is constant for one day, 10 measurements	Lobo et al., 1997a
ethanol	NAD^+ in carbon paste	alcohol dehydrogenase	poly(orthophe-nylenediamine) electopolyme-rised on carbon paste +0.15 V vs. Ag/AgCl	useful time is 3 days provided periodic recalibration is performed	Lobo et al., 1997b
L-lactate	NAD^+ covalently attached to PQQ molayer	L-lactate dehydrogenase	PQQ monolayer on gold +0.1 V vs. SCE		Bardea et al., 1997; Katz et al., 1998
ethanol	NAD^+ in carbon paste	alcohol dehydrogenase	poly(orthophe-nylenediamine) electopolyme-rised on carbon paste +0.15 V vs. Ag/AgCl	useful time is 3 days provided periodic recalibration is performed	Lobo et al., 1997b
L-gluta-mate	$NADP^+$ in carbon wax	thermophilic L-glutamate dehydrogenase in carbon wax	poly(toluidine blue O) on carbon wax	response was constant during 4 days in the FIA system	Pasco et al., 1999

Table III. (continued)

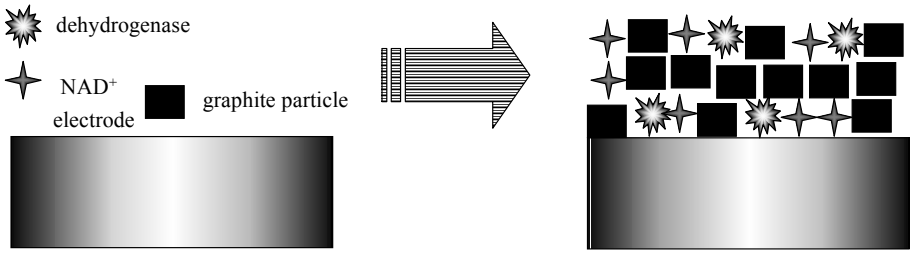
Analyte	Cofactor immobilisation	Dehydrogenase	Method of NADH oxidation / E_{app}	Time of operation without regeration	Reference
L-lactate	NAD^+ in carbon paste	L-lactate dehydrogenase in carbon paste	toluidine blue O in carbon paste 0.0 V vs. Ag/AgCl	this electrode in a flow cell retains 98% after 45 successive injections of L-lactate	Ramirez et al., 1999
ethanol	NAD^+ in carbon paste	alcohol dehydrogenase in carbon paste	[Fe(1,10-phenan-throline -5,6-dione)$_3$]$(PF_6)_2$ or [Re(1,10-phenan-throline -5,6-dione)$(CO)_3$Cl] in carbon paste 0 V vs. SCE	response was constant during 8 days of operational stability study	Tobalina et al., 1999
ethylene-bis(dithi-ocarba-mate) fungicides	NAD^+ in screen printed carbon ink	aldehyde dehydrogenase in screen printed carbon ink	NADH oxidase in poly(vinylalcohol) bearing styryl-pyridinium group		Noguer et al., 1999
ethanol	NAD^+ in carbon ink	alcohol dehydrogenase in carbon ink	bare thick film carbon electrode +0.47 V vs. Ag/AgCl	disposable sensors	Park et al., 1999
glycerol	NAD^+ in carbon paste under poly(ortho-phe-nylenediamine)	glycerol dehydrogenase in carbon paste under poly-(*o*-phenylene diamine)	Products of NAD^+ oxidation in carbon paste +0.15 V vs. Ag/AgCl	the signal is steady during 2 days	Alvarez et al., 2000
D-sorbitol	NAD^+ in carbon paste under poly(ortho-phe-nylenediamine)	D-sorbitol dehydrogenase in carbon paste under poly-(*o*-phe-nylenediamine)	products of NAD^+ oxidation in carbon paste 0.0 V vs. SCE	slope of calibration graph decreased by 15% and 40% during after 48 and 72h correspon-dingly	Saidman et al., 2000
malate	NAD^+ entrapped under ester cellulose membrane	malate dehydrogenase in ester cellulose membrane in conjunction with a PVC outer membrane	diaphorase mediated by hexacyanoferrate (III) or 2,4-dichloropheno-lindophenol +0.05 V vs. Ag/AgCl	this sensor retained almost 80% of its initial activity after 3 h	Maines et al., 2000



a. Entrapment in hydrogels formed *in situ* by macromolecules



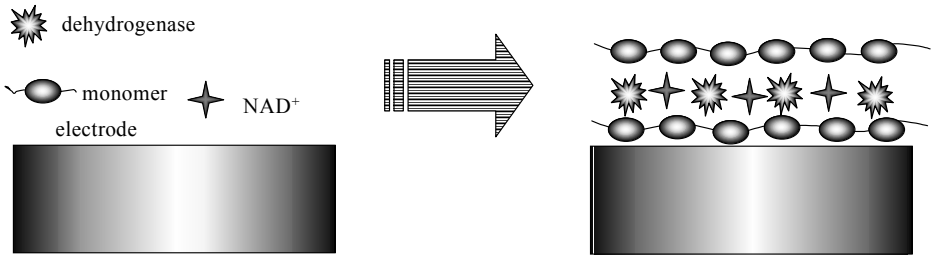
b. Immobilisation of NAD^+ under pre-prepared membranes



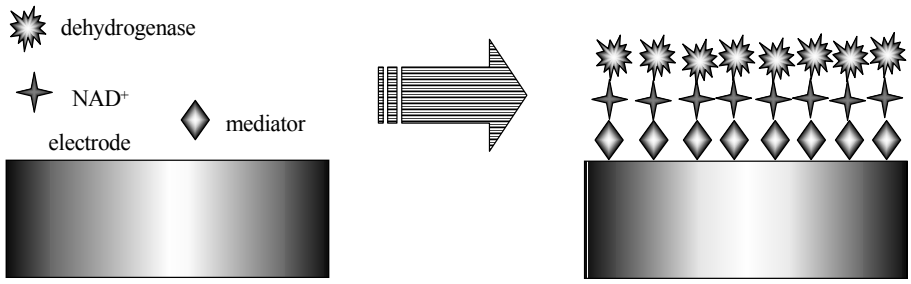
c. Entrapment in electropolymerised films

Figure 7. Different strategies for the fabrication of reagentless biosensors based on $NAD(P)^+$ dependent

dehydrogenases



d. Entrapment in carbon paste



e. Immobilisation in self-assembled monolayers

Figure 7. (continued)

1.8 Thermostable enzymes

The majority of enzymes utilised in organic synthesis and bioanalysis are obtained from organisms which live and reproduce optimally at temperatures below 45°C, so called mesophilic species or mesophiles. On the other hand a wide variety of natural habitats exist, including boiling or superheated springs and submarine volcanoes where the temperature of water is equal or higher than 100°C, inhabited by micro organisms termed as thermophilic species or thermophilies. The three main groups of thermophilic species were proposed by Farrell and Campbell [1969]:

(1) strict or obligate thermophiles, which have optimal growth at 65°C to 70°C but do not grow below 40°C

(2) facultative thermophiles having a maximal growth temperature between 50°C and 65°C

which are able to reproduce at room temperature

(3) thermotolerant bacteria having growth maxima at 45°C to 50°C, and grow at room temperature too

The first reports on enzymes extracted from thermophiles appeared as early as in 1949 when Militzer et al. demonstrated the thermostability at 65°C for 2 h of malate dehydrogenase from an obligate thermophile. It was found that the enzymes from the thermophile *Bacillus stearothermophilus* were significantly more resistant to heat inactivation than the homologous enzymes from the mesophile *Bacillus cereus* [Amelunxen and Lins, 1968]. The experiments comparing the 6-phosphogluconate dehydrogenase from the thermophile *Clostridium thermosacchrolyticum* and the mesophile *Clostridium pasteurianum* have shown greater thermostabilty of the enzyme extracted from the thermophile [Howell et al., 1969]. Different hypotheses explaining the elevated stability of enzymes from thermophiles have been proposed and tested.

The hypothesis of transferable protective factors suggested that those factors are present in thermophilic cells and impart thermostability to thermophilic enzymes and was tested by Koffler and Gale (1957). In these experiments they measured the extent of coagulation after heat treatment of free extracts from thermophiles, mesophiles and mixtures of both to find out that stabilising factors are non-transferable.

The hypothesis of rapid resynthesis of thermophilic macromolecules was based on a suggestion that thermophiles can very rapidly synthesise proteins in order to counteract thermal deactivation which was supported by the fact that the rates of protein and nucleic acid synthesis and turnover are higher in *B. stearothermophilus* than in *E. coli.* [Bubela and Holdsworth, 1966a, b].

Another group of hypotheses claims that the thermostability of thermophilic enzymes can be explained in terms of greater intrinsic stability of macromolecules in thermophiles. Physicochemical studies have demonstrated great homology between thermophilic and mesophilic proteins. Although there are some structural differences between very stable thermophilic and mesophilic enzymes, there is no pattern of systematic structural differences [Daniel and Cowan, 2000] but thermophilic proteins undergo only small conformational changes without denaturation when heated, in contrast to the mesophilic ones denaturing at about 60°C. The conformational changes are responsible for a break in the Arrhenius plot of many thermophilic enzymes [Ljungdahl and Sherod, 1976]. The secondary structure of proteins in aqueous environment is formed by hydrogen bonds, salt linkages, and hydrophobic interactions. Increase in temperature leads to a decrease in salt linkages and hydrogen bonding and an increase in hydrophobic interactions because of the increase of dielectric constant of water with temperature. It was estimated that hydrophobic interactions for large side-chains increase rapidly up to 75°C and then

slowly decrease [Brandts, 1967], therefore hydrophobic interactions could play a great role in the stabilisation of thermophilic enzymes. Nevertheless the calculations of relative hydrophobicity of thermophilic and mesophilic enzymes gave no correlation between hydrophobicity and thermostability [Singleton and Amelunxen, 1973; Bull and Breese, 1973]. The attempts to explain the thermostability in terms of hydrogen bonding also failed when no difference in hydrogen bonding was found in the secondary structure of thermophilic and mesophilic enzymes [Suzuki and Imahori, 1974; Cass and Stellwagen, 1975]. Other effort to understand the stability of thermophilic enzymes concentrated on finding a correlation between thermostability and secondary structure of globular proteins, particularly the helical and β-structure but spectropolarimetric measurements found no difference between the mesophilic and thermophilic proteins [Hibino et al., 1974; Hasegawa et al., 1976].

The hypothesis, connecting a decrease in cysteine content i.e. decrease of ancillary sulphydryl groups, subjected to oxidation, with enzymatic thermostability of glyceraldehyde-3-phosphate dehydrogenase from *Thermus aquaticus* seems to be more promising [Hocking and Harris, 1976]. Some thermophilic enzymes also have fewer sulphydryl groups, for example, phosphoglycerate kinase [Suzuki and Imahori, 1974] and adenosine triphosphatase [Yoshida et al., 1975], nevertheless the content of cysteine in thermophilic phosphofructokinase is much higher than in the mesophilic analogue [Cass and Stellwagen, 1975].

Another hypothesis relates thermostability with the increased number of acidic carboxyl moieties in thermophilic proteins [Singleton, 1976]. It is suggested that carboxyl groups can be protonated and form two hydrogen bonds with other groups, form an ion-dipole interaction with the phenolic hydroxyl of tyrosine, and a salt-linkage, therefore increasing the stability of the tertiary structure. Some other proposed factors contributing to the thermal stability of proteins are: increased number of ion pairs [Hennig et al., 1995], aromatic interactions in aromatic pairs and clusters [Burley and Petsko, 1985], stabilisation of the dipoles of the α–helices [Nicholson et al., 1988], high internal packing, and reducing the area of water accessible hydrophobic surface [Wigley et al., 1987]. Finally it can be concluded that there is not a unique way to obtain protein stability at elevated temperatures, every thermostable enzyme is individually adapted to environmental conditions by the combination of the above mentioned factors.

1.9 Application of Langmuir-Blodgett techniques films for electrode modification

The techniques allowing to form ordered monolayers and multilayers of molecules find wide applications in electronics and bioelectronics. One of the methods for building such layers is based on transferring them from the liquid-gas interface onto a solid substrate.

Monolayers of many insoluble compounds on liquid-gas interface can be created by

spreading their solutions in a volatile solvent over another liquid. The first systematic study of monolayers of insoluble compounds at the water-air interface was carried out by Langmuir [1917]. In his work he described a trough for creation and study of such films (Figure 8).

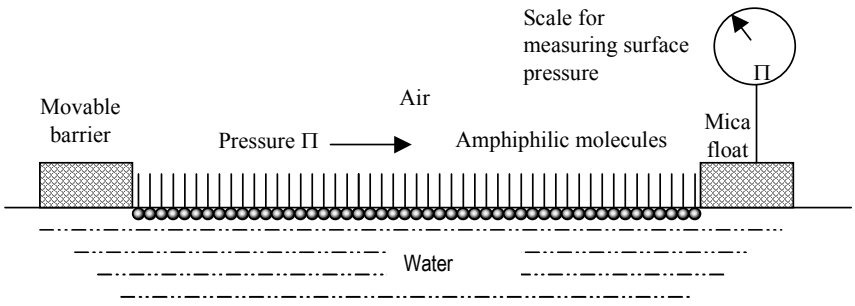


Figure 8. Diagrammatic representation of Langmuir-Blodgett trough in which the movable barrier compresses the insoluble monolayer. The scale, connected to the mica float by wire suspension, measures the surface pressure.

Typical representatives of amphiphilic compounds capable to form the above mentioned monolayers are aliphatic carboxylic acids which have two distinct moieties in the molecule: a hydrophilic headgroup (-COOH) and a hydrophobic long alkyl chain (C_nH_{2n+1}, where $n \geq 5$). If the distance between amphiphilic molecules on the surface of water is large then their interactions are small hence they can be regarded as forming a two-dimensional gas, having almost no effect on the surface tension. When a barrier system reduces the area of available surface for the monolayer molecules start to exert a repulsive effect on each other leading to the necessity to introduce the two-dimensional analogue of a pressure termed as the surface pressure Π, which is equal to the difference between the surface tension in the absence of a monolayer and that with the monolayer present.

The maximum value of the surface pressure for a monolayer of any amphiphilic compound at the water-air interface at room temperature is less than 73 mN/m. On compressing the surface areas beyond the limit of monolayer compressibility, the phenomenon of collapse takes place, which is explained in terms of breaking the monolayers with the formation of disordered multilayers.

A method for transferring multilayers of long chain carboxylic acids on solid substrate was invented by Blodgett [1934] who added to the trough of Langmuir a device, which lowers a solid substrate beneath a monolayer at the water-air interface and then withdraws it, therefore the resulting layers, deposited on the substrate, are referred to as the Langmuir-Blodgett (LB) films. An example of deposition of amphiphilic molecules on a hydrophilic substrate is shown in Figure 9.

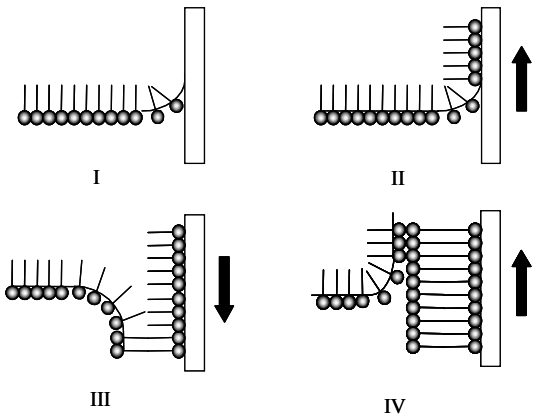


Figure 9. Deposition of Langmuir-Blodgett films on a solid substrate: (I) first dipping, (II) first withdrawal, (III) second dipping, (IV) second withdrawal.

During the first dipping (I) water wets the hydrophilic substrate's surface with no deposition at this stage because the meniscus is turned up. Only during the first withdrawal the first monolayer is being deposited in which hydrophilic tails are orientated toward the hydrophilic substrate. The second dipping (III) leads to the deposition of the second layer because the resulting surface now is hydrophobic and the meniscus is turned down.

The Langmuir-Blodgett (LB) technique is widely used to immobilise redox amphiphilic compounds on solid electrodes yielding photo-current generating devices, chemical sensors and biosensors. For example, photo-cells were produced by immobilisation of LB layers of pyrrolidinofullerene derivatives [Sheng et al., 2000], or ampiphilic stilbazolium dye dimer [Li et al., 2001] on a semiconducting transparent indium-tin-oxide.

Amphiphilic molecules also can be employed for the immobilisation of enzymes. The pioneering work demonstrating that enzymes retain their activity when entrapped into reversed micelles of surfactants were published in the beginning of the 80's [Martinek et al., 1981; Khmelnitskii et al., 1982]. Next a novel LB method was developed for producing and transferring a uniform protein thin film – a layer of a protein sandwiched between layers of a lipid [Vakula et al., 1995; Troitsky et al., 1996a,b; Berzina et al., 1996]. A glucose sensor was made on the basis of adsorbed layers of glucose oxidase on LB monolayers of N, N-dimethyl-acetylacetylmethacryloylethyl on platinum [Zaitsev et al., 1995]. Choline oxidase was inserted in a hydrophillic or hydrophobic environment of behenic acid LB films and the biosensing layer was directly coated on an especially designed amperometric transducer to yield a choline biosensor [Girardegrot et al., 1998]. An electroactive film was self assembled upon which a solution of

proteoliposomes was fused, the oxidation of reduced cytochrome c by the membrane bound cytochrome c oxidase being transduced to a gold electrode via a mediator bound to the polymer cushion where the lipid membrane rested [Lindholm-Sethson, 1998]. A glucose biosensor was fabricated by LB deposition of a phospholipid analogous vinyl polymer together with glucose oxidase [Yasuzawa et al., 2000]. LB films containing butyrylcholinesterase were fabricated to produce an enzymatic field effect transistor for the detection of organophosphorus pesticides in water [Wan et al., 2000].

The advantages of LB films deposition technique in the fabrication of biosensors are :

(a) creation of multilayered structure which allows to deposit all the components of a biosensor by one standard procedure leading to easy automation of the industrial production
(b) easy inclusion of protein layers in the desired position of the layered structure
(c) deposition of enzymes without any contact with air which allows to avoid protein denaturation
(d) minimisation of the amount of tensoactive materials consumed for the production of biosensors
(e) high stability in aqueous solutions of the deposited tansoactive bilayers

At the same time this technique suffers from:

(a) long time of deposition
(b) limitations of the transport of matter between hydrophobic chains of the deposited lipid bilayers, which in some cases can deactivate mediators requiring protons or hydroxyls for their reactions

1.10 Challenges

Nowadays a growing interest is demonstrated towards the development of *in-vivo* sensors for biomedical applications. These sensors should satisfy at least three requirements: to be oxygen independent; to be stable at temperatures of 36-40^{o}C; and to be miniaturisable. It can be seen that thermophilic NAD(P)$^{+}$ dependent dehydrogenases are the enzymes of choice for the construction of miniaturised biosensors provided that an efficient, simple and reagentless system for harnessing NAD(P)$^{+}$ dependent dehydrogenases is developed. Neither the carbon paste configurations nor the configurations based on the adsorption of NAD^{+} onto pre-prepared membranes are applicable because of size limitations. Only the strategies of entrapment of NAD^{+} in electropolymerised films, self-assembled monolayers and the entrapment in hydrogels formed *in situ* are applicable for the fabrication of biosensors operating *in-vivo*.

The main problems that should be solved in this field are (i) low response currents, (ii) low

operational and shelf stability of the resulting electrodes caused by three factors: (a) loss of $NAD(P)^+$/NAD(P)H redox couple, (b) loss of mediators for NAD(P)H oxidation, (c) loss of enzyme activity. How can these challenges be addressed?

(i) Low response currents can be increased by achieving an excess of NAD^+ using oxidising mediators with high rate of NADH oxidation and/or using an NADH oxidising redox polymer. The majority of reported NADH oxidising polymers are not stable at high pH and their capacity to produce enzymatically active NAD^+ has never been confirmed, consequently we opted for the synthesis and detailed characterisation of a novel NADH oxidising polymer (Os-phendione-PVP) by the complexation of [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] with poly(4-vinyl pyridine).

(ii) The reported reagentless dehydrogenase biosensors [Laurinavicius et al., 1996] produced by the entrapment of $NAD(P)^+$ in gelatine and poly(lysine) matrix showed low operational and shelf stability life because of the following three factors:

(a) Leaching of NAD(P)/NAD(P)H redox couple from the electrode surface which was very rapid in the previously reported reagentless sensors hence in the course of this thesis we developed a novel hydrogel matrix: poly(vinylpyridine) modified with amino groups which can be crosslinked with $NAD(P)^+$ by polyethylene glycol diglycidyl ether. Another type of gel was prepared by us on the basis of NAD^+ immobilised on alginic acid [Nakamura, et al., 1996]. It was also opted for creating highly organised layered architectures on the electrode surface. Many biosensors based on the organised layered architecture where an enzyme was sandwiched between lipid bilayers, have been reported in the literature but to our knowledge a reagentless dehydrogenase biosensor of this configuration has never been reported, probably due the absence of good amphiphilic mediators for the effective NADH oxidation at low working potential. Therefore a new NADH oxidising amphiphilic mediator having 1,10-phenathroline-5,6-dione ligands has been synthesised and used for the fabrication of a reagentless glutamate biosensor of the ordered architecture. The analogue of this surfactant carrying bipyridine ligands instead of 1,10-phenathroline-5,6-dione was synthesised too and its tensoactive properties were compared with those of the NADH oxidising surfactant.

(b) Low operational stability due to the loss of the NADH oxidising mediator can be amended: by covalent bonding of the mediator to the electrode surface or the macromolecules of the matrix; utilisation of redox polymers, and by employment of mediator with high chemical stability. The new NADH oxidising mediators, polymer Os-phendione-PVP and amphiphilic complex Os-phendione-surfactant were used in this work for the production of reagentless biosensors based on the three above mentioned

configurations.

(c) The poor operational and shelf stability due to the loss of the enzyme and its deactivation could be ameliorated by the formation of covalent bonds between the enzymes and the electrode surface and the macromolecules of a matrix, utilisation of stabilising additives and/or by utilisation of more stable thermophilic enzymes. Still, no detailed comparison of the operational and shelf stability of thermophilic and mesophilic enzyme based biosensors was published in the literature, so in the course of the present work such a study was performed using thermophilic and mesophilic glutamate dehydrogenases immobilised together with different additives, and glucose-6-phosphate dehydrogenases. For the first time biosensors based on thermophilic enzymes were applied to the detection of glutamate and glucose-6-phosphate at the temperatures higher than 60°C.

2. EXPERIMENTAL METHODS

2.1 The synthesis of new mediators for the oxidation of NADH

The synthesis of the redox amphiphilic and polymeric mediators was performed by the introduction of polymeric and amphiphilic ligands into osmium complexes [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] or [Os(2,2`-bipyridine)$_2$Cl$_2$]. These reactions were performed at high temperatures (above 180^{o}C) using reflux in ethylene glycol in a round bottom glass flask connected to a condenser. The structures of the resulting surfactants were established on the basis of ^{1}H NMR study in $CDCl_3$ using tetramethylsilane as a standard. NMR data confirmed the presence of long alkane chains: 1.29 p. p. m. (m) 1.26 (m) , 0.88 (t).

2.2 Electrochemical measurements in bulk solution

In the course in this work a three-electrode conventional thermostabilised cell (3 ml) equipped with an Ag/AgCl/KCl$_{sat}$ reference electrode, a platinum auxiliary electrode, and a graphite electrode modified according to one of the above mentioned methods as working electrode. The buffer, (0.1 M sodium phosphate containing 0.15 M sodium chloride) served as supporting electrolyte. The electrochemical measurements were performed in a three-electrode thermostabilised electrochemical cell (Figure 10) connected to the potentiostat Autolab PGSTAT 10 (Eco chemie, Holland) controlled by a computer.

Surface coverage of electroactive species was determined by linear potential sweep cyclic voltammetry in which the under peak areas served to calculate the charge. Taking into account the number of exchanged electrons per redox molecule and the Faraday´s constant the surface concentration was calculated. The peak current I_P is a function of scan rate υ, charge diffusion coefficient D_0, number of exchanged electrons n, surface concentration of redox active species C_0^*, surface coverage Γ, electrode surface area A, temperature T, Faraday´s constant F and gas constant R according to the equations:

$I_P = 0.4463(nF)^{3/2}(RT)^{-1/2}AC_0^*\upsilon^{1/2}D_0^{1/2}$ (for a thick film of redox species)

$I_P = n^2F^2\Gamma A\upsilon(4RT)^{-1}$ (for a monolayer of redox species)

Therefore in order to minimise the influence of charge diffusion limitations through the film of electroactive species on the surface low scan rates not exceeding 4 mV/s should be used for surface coverage measurements. Another factor affecting the credibility of surface coverage measurements is the reliability of determination of the electrode surface area.

In the course in the present work potential step chronocoulometry based on the Anson equation was employed to find the electrode surface area from the charge(Q) *vs.* time(t)

dependence in the presence of redox species with known concentration C:

$Q=2nFAD^{1/2}C\pi^{-1/2}t^{1/2}$

It is important to note that in any potential step experiment, there is a delay in attaining the step potential due to the finite rise time of the potentiostat. This non-ideal behaviour affects the validity of the data points measured during this delay time, and hence these data are discarded when calculating the slope and intercept. On another, hand the Anson equation is valid only within very short time periods hence in this study the pulse width not larger than 300 ms was used.

Linear potential sweep cyclic votammetry was employed also for the determination of heterogeneous rate constant k_S and the transfer coefficient α between the electrode surface and adsorbed redox mediators accordind to the Laviron equations:

$E_{pc}-E^{0'}=(2.303RT/\alpha nF)\log[\alpha nF\upsilon/(RTk_S)$

$E_{pa}-E^{0'}=(2.303RT/(1-\alpha)nF)\log[(1-\alpha)nF\upsilon/(RTk_S)$

Where E_{pc} and E_{pa} are cathodic and anodic peak potentials, $E^{0'}$ is the formal redox potential, υ is the scan rate, F is Faraday's constant, n is the number of electron. These equations are applicable only if the difference $(E_{pa} - E_{pc})/n$ is larger than 200 mV. In the case of quasi-reversible redox couple this condition can be satisfied by running cyclic voltammograms of an immobilised redox monolayer at scan rates higher than 10 V s^{-1}.

Chronoamperometric measurements with a rotating disk electrode were employed in the determination of rate constants of the reaction between NADH and mediators adsorbed on graphite using a solution having the volume of at least 10 mL. In order to eliminate the influence of oxygen on the response the modified rotating electrodes all solutions of NADH used in the measurements were deaerated with argon. The estimation of rate constants for the reaction between NADH and a mediator was based on the hypothesis that in thin polymeric films (which were obtained by the deposition of only one monolayer of a mediator) diffusional transport of both charge and NADH can be neglected and the substrate-mediator complex is formed in this interaction according to the following scheme:

$$\text{Os-phendione-PVP} + \text{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{Os-phendione-PVPNADH}] \xrightarrow{k_2} \text{Os-catechol-PVP} + \text{NAD}^+$$

The model proposed by Gorton to calculate the dependence of the catalytic current (I_{cat}) on experimental parameters in terms of a Michaelis-Menten like kinetic model were utilised in this work.

$1/I_{cat}=1/(nFA\Gamma kc_{NADH}) + [1/(0.62nFAD^{2/3} c_{NADH} v^{-1/6})](1/\omega^{1/2})$

$1/I_{cat}=1/(nFA\Gamma k_2) + [K_M/(nFA\Gamma k_2) + 1/(0.62nFAD^{2/3}v^{-1/6}\omega^{1/2})](1/c_{NADH})$

$K_M=(k_{-1}+k_2)/k_1$

where D (cm^2 s^{-1}) is the diffusion coefficient of NADH, v (cm^2 s^{-1}) the hydrodynamic viscosity, ω (rad s^{-1}) the speed of rotation of an electrode, A (cm^2) the electrode surface area, Γ(mol cm^{-2}) the surface coverage, c_{NADH} (M) the concentration of NADH, and k (M^{-1} s^{-1}) the rate coefficient of the formal overall chemical reaction:

$$\text{Os-phendione-PVP} + \text{NADH} \xrightarrow{k} \text{Os-catechol-PVP+NAD}^+$$

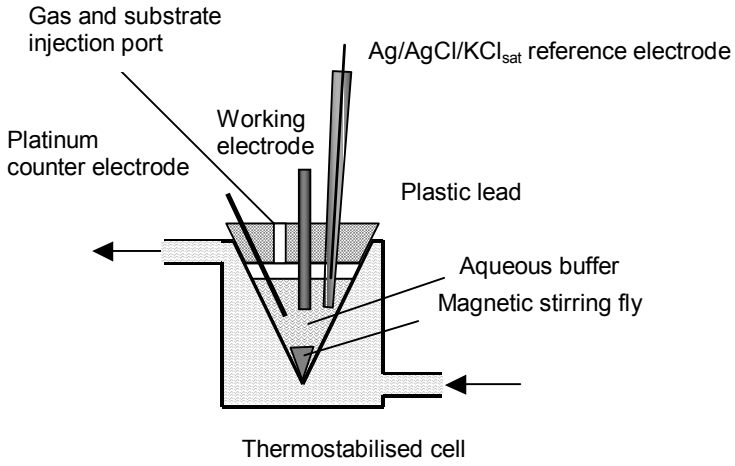


Figure 10. The electrochemical cell employed in electrochemical experiments

2.3 Shelf life study of glutamate biosensors

Disposable glutamate electrodes were prepared by deposition of the aqueous solution of mesophilic or thermophilic GLDH, NAD^+, water soluble mediator [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$ (1,10-phenanthroline-5,6-dione)]Cl_2 and an additive on screen printed electrodes depicted in Figure 11. A disposable glutamate biosensor was connected to the potentiostat Autolab PGSTAT 10 by the following way: the carbon electrode was connected to the working lead and Ag/AgCl reference electrode to the reference and auxiliary leads, next the biosensor was fixed during 5 min on a glass heat exchanger thermostabilised at 40^{o}C. In order to determine the response of the sensor 0.5 μL of a sample solution was deposited and after 20 s a potential of +0.2 V vs. Ag/AgCl electrode was applied to the working electrode.

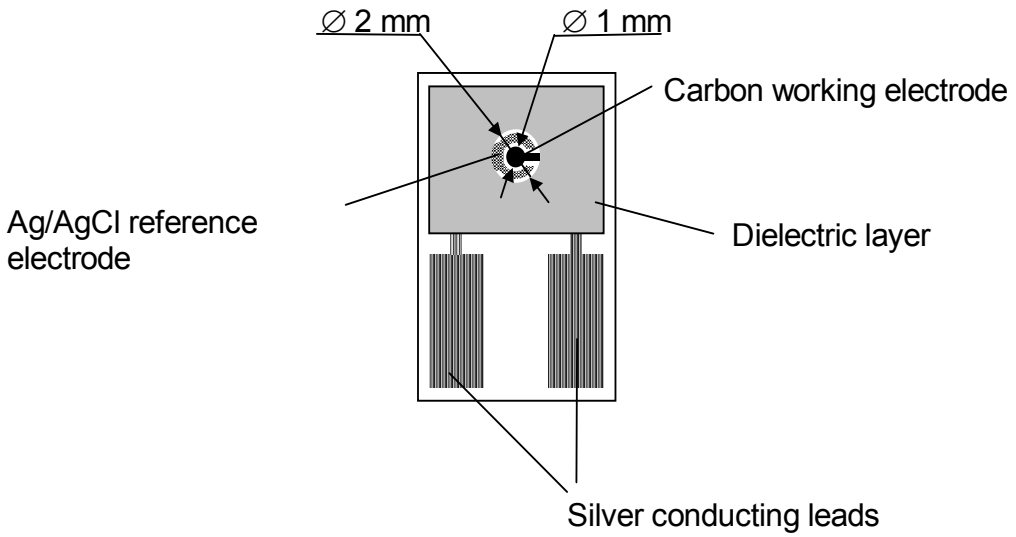


Figure 11. Screen printed electrode used for the preparation of disposable glutamate biosensors.

The value of current after 30 s (starting from the moment of applying the potential) was recorded for every electrode. From three to five sensors were used for measurements at every point. All sensors were assayed by depositing three different samples: 0.1 M Tris buffer, pH 7.4 (containing 0.15 M NaCl), or 0.6 M glutamate solution in this buffer, or 0.6 M glutamate solution with 0.18 M NAD^+ in the buffer.

2.4 Glutamate dehydrogenase (GLDH) assay method

Both mesophilic and thermophilic GLDH were tested spectrophotometrically by following the increase in absorbance at 340 nm at 30°C or 40°C. The following reagents were prepared: (A) 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride; (B) 0.1 M β-NAD^+ sodium salt solution in reagent A; (C) 0.3 M L-glutamic acid monosodium salt solution in reagent A; (D) mesophilic or thermophilic GLDH solution in reagent A. Next the following reagents : 884 μL of A, 33 μL of C, 33 μL of D were pipetted into a suitable 1 mL cuvette, mixed and equilibrated to 30 or 40°C. Then 50 μL of B were injected, mixed by inversion and the increase in A_{340} was recorded for 2 min. The ΔA_{340} (Au/min) was obtained using the maximal linear rate for both the test and blank (without enzyme) mixtures. The activity was calculated by the equation:

$$\text{Units/ml}_{\text{enzyme stock solution}} = \frac{\Delta A_{340}\text{ /min Test} - \Delta A_{340}\text{ /min Blank}}{\varepsilon_{NADH}\,(\text{ml}_{\text{enzyme stock solution}}/\text{ml}_{RM})}$$

Where ε_{NADH}=6.22 mM^{-1} cm^{-1} extinction coefficient of β-NADH, $ml_{enzyme\ stock\ solution}/ml_{RM}$ is amount of ml of stock enzyme solution per 1 ml of reaction mixture in a cuvette. One unit of GLDH oxidises 1 μmol of L-glutamate per minute at pH 7.4 at 30 or 40°C. Protein concentration was determined spectrophotometrically at A_{280} using an absorbance coefficient of 0.973 cm^2 mg^{-1}.

2.5 Glucose-6-phosphate dehydrogenase (G6PDH) assay method

Both mesophilic and thermophilic G6PDH were tested spectrophotometrically by following the increase in absorbance at 340 nm. For the G6PDG assay the following reagents were prepared: (A) 0.12 mM sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride; (B) 0.025 M β-NAD^+ sodium salt solution in reagent A; (C) 0.025 M glucose-6-phosphate solution in reagent A; (D) mesophilic or thermophilic G6PDH solution in reagent A. Next the following reagents: 650 μL of A, 50 μL of D, were pipetted into 1 mL cuvettes, mixed and equilibrated to 25°C (in case of mesophilic G6PDH) or 70°C (in case of thermophilic G6PDH) in the presence of a rotating micro-stirrer. Next 50 μL of C and 50 μL of B were injected and the increase in A_{340} was recorded for 2 min. The ΔA_{340} (Au/min) was obtained using the maximal linear rate for both the test and blank (without enzyme) mixtures. The activity was calculated by the equation mentioned in the description of the GLDH activity assay.

2.6 Instrumentation used for the study of amphiphilic compounds

A KSV 5000 film deposition system (KSV Instruments Ltd., Finland) was used for the preparation of the Langmuir-Blodgett films and the equipment was supervised with a KSV multitasking software package LB 5000 version 3.7. The area of the Teflon trough was 85 426 mm^2 and the surface pressure was monitored with a 20 mm wide Wilhelmy platinum plate suspended from a KSV microbalance. The Langmuir trough was extensively cleaned on a daily basis, which comprised soaking trough and barrier in 96 % ethanol, wiping them with Techni-Cloth texwipes, and rinsing for at least 20 min in ultrapure water. All electrochemical experiments on the transferred films were conducted at room temperature *in situ* in the Langmuir trough with a computer controlled (BAS, Bioanalytical Systems, West Lafayette, IN) CV-50W voltammographic analyser. A conventional three-electrode set up was employed involving a platinum wire counter electrode, a $Ag/AgCl/KCl_{sat}$ double junction reference electrode, and a planar gold working

electrode. The thin gold film electrodes (thickness ≈ 80 nm and electrode area 0.50 cm^2 connected with a 1 mm wide strip to a contact pad) were deposited by electron gun evaporation in ultra high vacuum onto glass slides using (3-mercapto propyl)-trimethoxy silane as a molecular glue between glass and glass and the gold working electrode.

3. GENERAL OVERVIEW OF THE PRESENTED PAPERS

The work performed in the course of elaboration of the present thesis was aimed at the development of new techniques for the fabrication of electrodes modified with dehydrogenases dependent on $NAD(P)^+$. The results of this investigation are presented in the following manuscripts:

1. **Electrocatalytic Oxidation of NADH at Graphite Electrodes Modified with Osmium Phenanthrolinedione.**
   I. C. Popescu, E. Domínguez, A. Narváez, V. Pavlov, I. Katakis
   J. Electroanal. Chem. 464 (1999) 208-214.

2. **A Novel [Os(1,10-phenanthroline-5,6-dione)$_2$(PVP)$_4$Cl]Cl Redox Polymer for Electrocatalytic Oxidation of NADH and its Application to Construction of β-glucose Biosensors.**
   V. Pavlov, O. Rincon, S. Sole, A. Narváez, E. Dominguez, I. Katakis
   Submitted for publication in *Anal. Chem.*

3. **New Reagentless Glutamate Biosensors Based on Mesophilic and Thermophilic Glutamate Dehydrogenases.**
   V. Pavlov, S. Kengen, I. Katakis
   Submitted for publication in *Anal. Chem*.

4. **The Use of Lipid Bilayers for the Construction of Reagentless Biosensors Based on NAD^+ Glutamate Dependent Dehydrogenase.**
   V. Pavlov, B. Lindholm-Sethson, G. Lindblom, I. Katakis
   Submitted for publication in *Langmuir*.

5. **Amperometric Sensing at High Temperature with a "Wired" Thermostable Glucose-6-Phosphate Dehydrogenase from *Aquifex aeolicus.***
   R. Iyer, V. Pavlov, I. Katakis, L. G. Bachas
   Anal. Chem. 75 (2003) 3898-3901.

In brief these articles can be summarized as follows:

Article 1. Electrocatalytic Oxidation of NADH at Graphite Electrodes Modified with Osmium Phenanthrolinedione.

OBJECTIVES: The investigation described in this article was carried out with the purpose to study the electrochemical behaviour of [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]$(PF_6)_2$ (Os-phendione) and assess its capacity to serve as a mediator for NADH oxidation.
METHODS: This compound was synthesised through complexation of [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$]Cl_2 with 1,10-phenanthroline-5,6-dione (phendione) by refluxing in ethylene glycol and converted into water insoluble form by ion exchage of the anions, next it was adsorbed on the surface of graphite electrodes. Cyclic voltammetry at in aqueous solutions at different scan rates and pH values was utilised to assess the number of protons and electrons participating in the redox process of Os-phendione. By evaluating the change in peak potentials with the potential scan rate the rate constant of heterogeneous electron transfer was calculated. Chronoamperometry using graphite rotating disk electrodes poised at +0.2 V vs. Ag/AgCl/KCl_{sat} was used for measurement of the second order rate constant of electrocatalytic oxidation of NADH.
MAIN RESULTS AND CONCLUSIONS: The redox potential of the complexed phendione was pH dependent because of the participation of protons according to the reaction:

$$\text{Os-phendione} + 2e^- + 2H^+ \rightleftharpoons \text{Os-catechol}$$

So at pH 6.0 the formal standard potential ($E^{0'}$) was +0.08 V vs. Ag/AgCl/KCl_{sat}, the voltammetric wave being quasi-reversible. The rate constant of heterogeneous electron transfer between the graphite electrode and adsorbed mediator was estimated to be 20.1 s^{-1}, the value was two times higher than this reported for Meldola Blue (10 s^{-1}). Voltammetric study of electrodes modified with Os-phendione in the presence of NADH in a solution at pH 6.0 demonstrated a dramatic enhancement of the anodic current indicating the electrocatalytic oxidation starting from +0.06 V vs. Ag/AgCl/KCl_{sat}. This potential was much lower than that for the NADH oxidation at bare graphite electrodes. The estimation of rate constants for the reaction between NADH and Os-phendione was based on the hypothesis that in a monolayer of the mediator adsorbed on a graphite surface the diffusional transport of both charge and NADH can be neglected and substrate-mediator complex is formed in this interaction according to the following scheme:

$$\text{Os-phendione} + \text{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Os-phendione}\bullet\text{NADH}] \xrightarrow{k_2} \text{Os-catechol} + \text{NAD}^+$$

The second order rate constants of electrocatalytic oxidation of NADH ($k_{[NADH]=0}$) were measured at different pH, and proved to be 3.1 x10^3, 1.9x10^3 , and 0.9 x10^3 M^{-1} s^{-1}, at pH 5.5, 6.1, and 7.0

respectively.

$$\text{Os-phendione-PVP} + \text{NADH} \xrightarrow{k} \text{Os-catechol-PVP} + \text{NAD}^+$$

The value of $k_{[NADH]=0}$ was lower than the corresponding value for Meldola blue at pH 7.0. It was observed that the linear correlation between $E^{0'}$ and log $k_{[NADH]=0}$ found for phenoxazine derivatives, extends to Os-phendione too. This mediator reversibly oxidises NADH through the formation of a charge transfer complex.

Article 2. A Novel [Os(1,10-phenanthroline-5,6-dione)$_2$(PVP)$_4$Cl]Cl Redox Polymer for Electrocatalytic Oxidation of NADH and its Application to Construction of β-glucose Biosensors.

OBJECTIVES: This investigation was performed in order to synthesise new NADH oxidising polymeric mediator [Os(1,10-phenanthroline-5,6-dione)$_2$(PVP)$_4$Cl]Cl where every fourth pyridine is complexed to osmium, (Os-phendione-PVP), to study its electrochemical behaviour, its capacity towards the electrocatalytical oxidation of NADH, and the possibility of its use in reagentless biosensors based on glucose dehydrogenase (GDH).

METHODS: The polymer (Os-phendione-PVP) was produced via derivatisation of poly(vinyl pyridine), having molecular weight of 50000, with [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] by reflux in ethylene glycol. The electrochemical study of this redox polymer adsorbed on graphite electrodes was performed by cyclic voltammetry at different values of scan rate and pH to assess the number of protons and electrons participating in the redox reaction, the influence of pH on its formal standard potential, and the rate constant of heterogeneous electron transfer. Amperometric experiments with graphite rotating disk electrodes poised at +0.2 V vs. Ag/AgCl/KCl$_{sat}$ were performed to find the rate constant for the interaction with NADH $k_{[NADH]=0}$. An electrochemical conversion experiment was carried out by the bulk oxidation of NADH at graphite electrodes modified with Os-phendione-PVP poised at +0.15 V vs. Ag/AgCl/KCl$_{sat}$. Spectrophotometry at 340 nm was used for measuring the depletion of the NADH concentration. The resulting NAD^+ solution was used as a cosubstrate of GDH and the recovery of the original NADH concentration was monitorised spectrophotometrically at 340 nm. Next reagentless glucose biosensors were built by the immobilisation of different amounts of glucose dehydrogenase and NAD^+ in the new hydrogel matrix formed by crosslinking of polyvinyl pyridine bearing amino groups with poly(ethylene glycol) diglycidyl ether.

MAIN RESULTS AND CONCLUSIONS: The wave of phendione in Os-phendione-PVP is quasi reversible and pH dependent, the formal redox potential being equal to +0.04 V vs. Ag/AgCl/KCl$_{sat}$ at pH 6.0. Cyclic voltammetry demonstrated also the voltammetric waves of $Os^{II/III}$

redox couple at +0.407 V vs. $Ag/AgCl/KCl_{sat}$, as expected for osmium atom coordinated with five pyridine ligands and one Cl^-. The comparison of peak areas of osmium and phendione indicated that two phendione moieties exchanged four electrons and four protons in the redox process at pH values lower than 6.5 according to the reaction:

$$Os(Q)_2PVP + 4e^- + 4H^+ \rightleftharpoons Os(QH_2)_2PVP$$

confirming the suggested structure of the new polymer. The cyclic voltammetry performed at high scan rates at pH 5, 6 and 7 demonstrated linear anodic and cathodic peak current (I_p) versus scan rate (ν) plots at scan rates up to 0.5 V s^{-1} which indicates that there are no limitations of charge propagation within the thin layer of Os-phendione-PVP adsorbed on the electrode surface. The heterogeneous electron transfer rate constant k_S (18.7 s^{-1}at pH 6.0), was very close to this measured for monomeric Os-phendione. Cyclic voltammetry performed with Os-phendione-PVP modified graphite electrodes in the presence of NADH in the bulk solution at pH 6.0 showed great increase in the oxidation current to show electrocatalytic NADH oxidation starting from the potential +0.07 V vs. $Ag/AgCl/KCl_{sat}$. This potential is much lower than that for NADH oxidation at bare electrodes (+0.33 V vs. $Ag/AgCl/KCl_{sat}$) under the same conditions.

The rate constant for the interaction with NADH $k_{[NADH]=0}$ was $(1.9\pm0.2)x10^3$ $M^{-1}s^{-1}$ at pH 6.0, which is practically equal to that Os-phendione because the standard potentials of both mediators are close. According to the data of the electrochemical conversion experiment, electrodes modified with Os-phendione-PVP generated 100% enzymatically active NAD^+ hence the new NADH oxidising polymer is suitable for the fabrication of reagentless biosensors based on NAD^+ dependent dehydrogenases.

Reagentless glucose biosensors were built using graphite electrodes modified with Os-phendione-PVP and a new hydrogel for the immobilisation of enzyme and cofactor. The response of glucose biosensors was dependent on the amount of immobilised enzyme and the amount of NAD^+. The maximum response current shown in reagentless mode of operation was 35.6 $\mu A\ cm^{-2}$ and in the presence of saturating NAD^+ concentration 92 $\mu A/cm^2$. The effect of pH on the response of reagentless and non reagentless glucose biosensors demonstrated the increase in the response current with pH until the value of 9.0. This data corroborates well with the effect of pH on the activity of free GDH. The kinetic analysis based on the equation relating the response current with the construction parameters of biosensors has demonstrated that the response current of all reagentless biosensors is limited by the amount of immobilised NAD^+. Thus new NADH oxidising polymeric mediator has been developed which generates enzymatically active coenzyme. This mediator is suitable for the construction of reagentless biosensors based on NAD^+ dependent dehydrogenases which demonstrate high current densities.

Article 3. New Reagentless Glutamate Biosensors Based on Mesophilic and Thermophilic Glutamate Dehydrogenases.

OBJECTIVES: This investigation was carried out to develop two new methods for the construction of reagentless biosensors based on NAD^+ dependent dehydrogenases and to compare the performance of resulting glutamate sensors prepared using mesophilic and thermophilic glutamate dehydrogenases (GLDH).

METHODS: Graphite electrodes were modified with a monolayer of new NADH oxidising polymer Os-phendione-PVP and utilised for the fabrication of reagentless glutamate biosensors by two methods. The first one consisted the immobilisation of mesophilic bovine GLDH or thermophilic GLDH from *Pyrococcus furiosus* in the hydrogel matrix formed by crosslinking of polyvinyl pyridine bearing amino groups ("binder" polymer) with poly(ethylene glycol) diglycidyl ether. The second method based on physical adsorption of polymeric form of NAD^+, alginate carrying NAD^+ moieties (NAD^+-alginate), together with thermophilic or mesophilic GLDH. The response curves, the effect of temperature and pH on the response current, and operational stability were studied. The second part of investigation was the accelerated shelf stability (at 40^{o}C) study of disposable glutamate biosensors prepared using thermophilic and mesophilic enzymes mixed together with different polymeric and monomeric additives, NAD^+, and mediator [Os(4,4′-dimethylbipyridine)$_2$(1,10-phenanthroline-5,6-dione)]Cl_2. The chronoamperometric response of disposable biosensors to analyte was determined by spreading 0.5 μl of sample solution on working carbon and Ag/AgCl/KCl_{sat} reference electrodes.

MAIN RESULTS AND CONCLUSIONS:

Cyclic voltammetry of the reagentless glutamate biosensors fabricated by the two above mentioned methods demonstrated clear electrocatalytic waves achieving almost a plateau at potentials more negative than 0.2 V *vs.* Ag/AgCl/KCl_{sat} in the presence of glutamate in sample solutions.

The effect of pH on the maximal response current of the glutamate biosensors was studied. The biosensors based on NAD-alginate prepared from mesophilic and thermophilic GLDH have maximum response at pH 9.0. This result is in good agreement with the pH optimum of 9.0 for glutamate oxidation by free bovine GLDH and GLDH from *Pyrococcus furiosus*. The biosensors based on immobilisation by crosslinking with "binder" polymer constructed using mesophilic and thermophilic GLDH achieved maximal response currents at pH 9.5 probably because of increased enzymatic stability caused by crosslinking. The study of the effect of temperature on the response to glutamate was also performed. The thermophilic enzyme based biosensors, independently of the immobilisation procedure, have shown increase in response to L-glutamate up to the maximum temperature of 88°C achieved in the thermostated electrochemical cell, this temperature value corroborates with optimal temperature of 95^{o}C for free thermophilic GLDH. Mesophilic

biosensors based on NAD-alginate showed the lowest optimal temperature of 52°C, "binder" polymer biosensors had the highest optimal temperature 56°C. This data is in good agreement with the published thermostability study of bovine GLDH according to which this enzyme starts to lose activity from the temperature of 52°C. The use of thermophilic GLDH instead of the mesophilic enzyme allowed to increase the operational stability of "binder" polymer based biosensors at 65°C, which showed the half life time of 16 min (in the presence of NAD^+ in a sample). In the reagentless mode of operation at 30°C the mesophilic enzyme based biosensors prepared using "binder" polymer demonstrated the half-life ($\tau_{1/2}$) of 12 h which is higher by 9 times than $\tau_{1/2}$ of the biosensors based on simple adsorption of NAD^+-alginate (1.5 h) due to better immobilisation of the biosensor components by forming covalent bonds.

The accelerated study of shelf life of mesophilic and thermophilic glutamate biosensors demonstrated that thermophilic dehydrogenase based sensors demonstrated the expansion of shelf half life by about 11 times in comparison with that of the mesophilic biosensors. The effect of different additives on the shelf life was studied too, to reveal that the best results were achieved by addition of the copolymer of vinyl-pyrrolidone and dimethylamino ethyl methacrylate termed as Gafquat® HS100 which enhanced the stability of mesophilic sensors by 4.6 times, and of thermophilic sensors by 3.3 times. This polyelectrolyte, promoting electrostatic interactions, forms a protein-polyelectrolyte adduct apparently stabilizing the enzyme.

With the purpose to take into consideration the loss of response to glutamate due to the decomposition of NAD^+ in biosensors during the shelf life study the controlled samples of glutamate containing fresh NAD^+ were utilised in the investigation but the response to pure glutamate proved to be higher by 1.5 times. The same phenomenon was encountered if NADH was used instead of glutamate i.e. the response to pure NADH samples was higher by 1.5 times than that to NADH samples containing NAD^+. This was related with the hypothesis of the formation of a parasite complex between NAD^+ and the mediator. According to the kinetic analysis the effect of NAD^+ on the response to NADH can be observed at high NAD^+ concentration and low rate of oxidation of the reduced mediator at the electrode surface.

Finally it can be concluded that two new methods for the fabrication of reagentless biosensors based on NAD^+ dependent dehydrogenases have been developed which can be utilised in miniaturised sensors. The advantages of using thermophilic glutamate dehydrogenase instead of mesophilic enzyme is the improvement of operational stability at extreme temperatures and the extension of shelf life. The operational stability of the resulting sensors at temperatures more than 40°C is limited by leaching of NAD^+.

Article 4. The Use of Lipid Bilayers for the Construction of Reagentless Biosensors Based on NAD^+ Dependent Glutamate Dehydrogenase.

OBJECTIVES: The study described in this work was performed to synthesise a new tensoactive mediator for the electrochemical oxidation of NADH, asses its tensoactive properties, include it in lamellar phase, and use it for the construction of reagentless biosensors based on NAD^+ dependent bovine glutamate dehydrogenase (GLDH).

METHODS: A novel NADH oxidising surfactant [Os(1,10-phenanthroline-5,6-dione)$_2$4,4'-(n-$C_{18}H_{37}NHCO)_2$bpy)]$(PF_6)_2$ (Os-phendione-surfactant) was produced by complexation of [Os(1,10-phenanthroline-5,6-dione)$_2$]Cl with the hydrophobic ligand 4,4'-(n-$C_{18}H_{37}NHCO)_2$bpy under reflux in ethylene glycol. Its structure was confirmed by ^{1}H NMR investigation. Langmuir-Blodgett (LB) monolayers of Os-phendione-surfactant were studied in a Langmuir-Blodgett trough. The redox surfactant together with NAD^+ and GLDH was included into a lamellar phase based on the lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine and ^{31}P NMR investigation proved the presence of lipid multilayers. Reagentless biosensors were constructed by adsorption of the lamellar phase on graphite electrodes. The effect of analyte concentration, temperature, and pH was studied.

MAIN RESULTS AND CONCLUSIONS:

Surfactant for the NADH oxidation can be produced by the complexation of a hydrophobic ligand with redox active head group bearing 1,10-phenanthroline-5,6-dione leading to Os-phendione-surfactant.

This surfactant forms Langmuir-Blodgett monolayers in the air-water interface. The surface pressure vs. area per molecule isotherm showed a take off surface pressure at 240-250 Å^2 per molecule, and the monolayer collapsed at 78-80 mN m^{-1}. The results obtained from this isotherm point out to the mean molecular area of 190 Å^2. This high value can be explained by the presence of bulky phenanthroline ligands. Some reorganisation of the monolayer takes place starting from surface pressure value of 40 mN m^{-1}. LB layers of this compound can be deposited on gold planar electrodes but the redox reactions at the phendione moiety are passivated due to the limitation on the transport of protons through the layers of hydrophobic tails. The mixture of this mediator with 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine forms a lamellar phase, (confirmed by the presence of a broad peak from –7 to –15 ppm in ^{31}P NMR spectra) in which phendione retains electrochemical activity. NAD^+ and GLDH can be included in this lamellar phase to give the suspension which is adsorbed on graphite electrodes to yield reagentless glutamate biosensors which showed clear electrocatalytic cyclic voltammograms in the presence of glutamate. Their response time was 7-8 min, and was limited by the diffusion of analyte through the lamellar phase. The operational half-life was 30 min, because of leaching of GLDH or NAD^+.

Therefore Os-phendione-surfactant can be utilised to create a lamellar phase in the presence of a "helper" lipid at room temperature. The resulting lamellar phase can include NAD^+ and dehydrogenase offering a new method for the fabrication of reagentless biosensors. Their response is limited the rate of the NADH oxidation at the electrode surface and by the transport of the analyte and the reaction products through the layers of hydrophobic aliphatic tail groups. In order to be useful for the construction of biosensors this new technique needs to improve the entrapment of NAD^+ and enzyme and the stability of the lamellar phase.

Article 5. Amperometric Sensing at High Temperature with a "Wired" Thermostable Glucose-6-Phosphate Dehydrogenase from *Aquifex aeolicus.*

OBJECTIVES: This investigation was performed with the purpose to develop the system operating at temperatures up to 83°C for enzymatic sensing based on NAD^+ dependent dehydrogenase and to study the advantages of biosensors based on thermophilic glucose-6-phosphate dehydrogenase over the mesophilic enzyme.

METHODS: Graphite electrodes were modified with NADH oxidising polymer [Os(1,10-phenanthroline-5,6-dione)$_2$(PVP)$_4$Cl]Cl. Then thermophilic glucose-6-phosphate dehydrogenase (tG6PDH) from *Aquifex aeolicus* and the mesophilic analogue from *Leuconostoc mesenteroides* (mG6PDH) together with NAD^+ were immobilised on the modified electrodes through crosslinking with poly(ethylene glycol) diglycidyl ether in a hydrogel matrix formed by poly(vinylpyridine) bearing amino groups. The response of the resulting biosensors to glucose-6-phosphate in the presence of NAD^+ at temperatures ranging from 25 to 83 °C was measured by chronoamperometry at applied potential +0.15 V vs. Ag/AgCl/KCl$_{sat}$.

MAIN RESULTS AND CONCLUSIONS: The resulting glucose-6-phosphate biosensors based on the thermophilic enzyme showed Michaelis-Menten type response with an apparent K_M of 2.9 mM for glucose-6-phosphate (the free enzyme shows K_M of 0.18 mM in homogenous solution at 70 °C). The increase in the apparent K_M of the immobilised enzyme can be explained by transport limitations. The current densities at 0.05 M concentration of glucose-6-phosphate reached a plateau of 390 μA cm^{-2} at 70 °C. Biosensors based on mG6PDH had an apparent K_M of 6.1 mM (free mG6PDH showed K_M of 0.069 mM in a homogenous solution at 25 °C) with current densities reaching a plateau of 120 μA cm^{-2} at 0.05 M glucose-6-phosphate concentrations at 25 °C. Electrodes based on tG6PDH showed clear electrocatalytic activity up to 83 °C, proving that the enzyme exhibits high thermostability. In contrast, when mG6PDH was used, maximum activity at the temperature of 50 °C was observed above which there was a steady drop in current densities due to enzyme denaturation. The operational half life of tG6PDH immobilised system was 2 h at

60°C whereas the biosensors based on mG6PDH did not show any response to glucose-6-phosphate at this temperature due to total enzyme denaturation.

In summary, a robust system demonstrating the possibility of recycling of NAD^+ at extreme temperatures for using in conjunction with a recombinant thermophilic G6PDH from *Aquifex aeolicus* was developed. The scheme of mediation reported here can be used for many thermostable dehydrogenases that use the co-factors β-nicotinamide adenine dinucleotide (NAD^+) and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) thereby opening a wide spectrum of applications at high temperatures in biosensing and bioreactors.

4. GENERAL OVERVIEW OF THE PAPERS NOT INCLUDED IN THE THESIS

Summary of the articles about the use the NADH oxidising complexes of osmium and 1,10-phenanthroline-5,6-dione)$_2$Cl$_2$ in electrochemical biosensors: (Parellada et al., 1998), (Hedemno et al., 1996), (Fernández et al., 1998).

OBJECTIVES: These investigations concentrated on the development of phosphate biosensors and glucose biosensors in which electrocatalytic oxidation of NADH was performed using complexes of osmium containing 1,10-phenanthroline-5,6-dione.

METHODS: The phosphate sensors were prepared by the immobilisation under membrane of glassy carbon electrodes of the composition containing [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$], NAD^+, glucose-6-phosphate dehydrogenase, glycogen, phosphorylase A, phosphoglucomutase, Oxidation current was monitored at +0.2 V *vs*. SCE.

The glucose biosensors were fabricated using electrodes based on carbon paste containing [Os(4,4´-dimethyl-2,2`-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)](PF$_6$)$_2$, glucose dehydrogenase and NAD^+. The sensors were operated at the applied potential of +0.15 V *vs.* Ag/AgCl/KCl$_{sat.}$

MAIN RESULTS AND CONCLUSIONS: The resulting phosphate electrodes were based on the trienzymatic configuration consisting of phosphorylase A (enzyme that produces glucose 1-phosphate from glycogen), phosphoglucomutase (glucose 1-phosphate gives glucose 6-phosphate) and glucose 6-phosphate dehydrogenase (that oxidises glucose 6-phosphate in the presence of NAD^+ producing NADH). This cofactor is reoxidised electrocatalyticlly by the mediator [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$ at the applied potential of +0.2 V *vs.* SCE. The optimised phosphate sensors demonstrated maximum current densities of 1.97 μA cm^{-2} and the detection limit of 6 μM. The phosphate biosensors showed a catalytic current that is independent of the pH between pH 6.5 and 7.5. and the optimal temperature of operation of 41^{o}C. When an Arrehenius plot is made of the temperature data, an activation energy of 84.5 kJ mol^{-1} can be calculated. This high value shows that a kinetic step, probably the phosphoglucomutase reaction, limits the overall response of the system.

The use of [Os(4,4´-dimethyl-2,2`-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)](PF$_6$)$_2$ mediator helped to achieve a more than 0.5 V lowering of the overpotential for NADH oxidation at carbon paste electrodes allowing to use the potential of +0.15 V *vs.* Ag/AgCl/KCl$_{sat}$. Electrochemical conversion experiments yielded a 100% conversion of NADH into enzymatically active NAD^+. The reagentless glucose biosensors were based on the electrocatalytic oxidation through the above mentioned mediator of NADH generated by glucose dehydrogenase. The optimised reagentless glucose sensors demonstrated maximum current density of 469.8 μA cm^{-2}

which was limited by enzyme kinetics. The operational stability study of these biosensors showed a loss of 6-8% in their response in 3-8 h. The stability was so good due to the creation of a 'pool' of NAD^+ and mediator in the carbon paste.

Summary of the article about electrochemical and spectroscopic characterization of a Langmuir-Blodgett film based on an $Os(bpy)_3$ surfactant mediator: (Maestre et al., article in preparation).

OBJECTIVES: The investigation presented in the article was aimed at characterisation of the redox surfactant $[Os(bpy)_2$4,4´-$[n\text{-}(C_{18}H_{35})_{17}NHCO]_2bpy](PF_6)_2$ (Os-bpy-surfactant) by studying its monolayers at the water-air interface, and Langmuir-Blodgett (LB) multilayers on solid electrodes.
METHODS: Os-bpy-surfactant was produced by complexation of $[Os(bpy)_2]Cl_2$ with hydrophobic ligand 4,4'-$(n\text{-}C_{18}H_{37}NHCO)_2bpy$ under reflux in ethylene glycol. Its structure was confirmed by ^1H NMR investigation. A monolayer Os-bpy-surfactant in the air-water interface was obtained by spreading its solution in chloroform on water surface. For electrochemical studies by cyclic voltammetry in the LB trough layers of this mediator were transferred on planar gold electrodes pre-modified with self-assembled monolayer of 3-mercapto 1-propane sulfonic acid.

For spectroscopic characterisation layers of Os-phendione-surfactant were deposited on glass slides modified with a monolayer of 3-mercaptopropyltrimethoxysilane. The thickness and organization of the Os-surfactant film on the substrate were estimated by the electron density projection (EDP) method using the molecular electron density surface.
MAIN RESULTS AND CONCLUSIONS:
Os-bpy-surfactant forms Langmuir-Blodgett layer at the water-air interface. The surface pressure vs. area per molecule isotherm demonstrated a take off surface pressure at 160-170 $Å^2$ per molecule, and the collapse of the monolayer at the surface pressure 65 mN m^{-1}, the largest estimate of the mean molecular area being 150 $Å^2$. The treatment of data according to MacDonald and Simon has indicated the organisation of the monolayer in a perfectly fluid liquid expanded phase with all head groups solvated in the aqueous phase. Cyclic voltammograms showed that the formal standard potential of the mediator was affected by the value of surface pressure used for the deposition, so when the layers were deposited at 30 mN m^{-1} E_o' was +0.827 V *vs.* $Ag/AgCl/KCl_{sat}$ meanwhile at surface pressures higher than 40 mN m^{-1} the potential was more positive +0.845 V *vs*. $Ag/AgCl/KCl_{sat}$. The ratio of electroactive mediator in multilayers decreases with surface pressure and hence with the surface coverage, due to increase in hydrophobicity within the layers.

Spectroscopic measurements based on Beer-Lambert law allowed to find the surface concentration of Os-phendione-surfactant in LB layers deposited on glass slides. The average orientation and thickness of the first layer were estimated by the EDP method. Assuming a uniform

film and that the chosen Fe-derivative resembles the Os-surfactant optimised geometry, the fitted projection of the EDP onto the surface indicates a dense monolayer, where the individual molecules are slightly oriented away from the surface normal. If the amide functionalities are used as a reference for the orientation, the average tilt of the molecules should then be around 20° from the surface normal at 30 mN m^{-1} and the thickness can be estimated to be about 32 Å.

It can be concluded that the Os-surfactant can form stable electroactive films on gold and glass surfaces modified with LB technique. The described redox system might provide a new transduction design permitting a rigorous surface control for incorporation in bioelectronic configurations.

5. CONCLUSIONS

1. The NADH oxidising polymer [Os(1,10-phenanthroline-5,6-dione)$_2$(PVP)$_4$Cl]Cl (Os-phendione-PVP) can be synthesised by the complexation of [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] with poly(vinyl pyridine) in ethylene glycol. The physical adsorption of this polymer onto graphite electrodes from its solution in ethylene glycol leads to the formation of a thin film without limitations on charge propagation.

2. The quasi-reversible redox process of this mediator involves 4 electrons and 4 protons within the pH range of 3-6.5. The mediator loses its chemical stability at pH values higher than 6.5. Three different lineal parts in the plot of $E^{0'}$ *vs*. pH with different slopes having two breaking-points are caused by the fact that pK of pyridine moieties is pH 4.5 and pK of Os-catechol-PVP is 6.4.

3. The heterogeneous electron transfer rate constant (k_S) of Os-phendione-PVP is higher or of the same order of magnitude as that of other reported NADH oxidising mediators (k_S= 18±2 s^{-1}).

4. Os-phendione-PVP is an efficient electrocatalyst for the oxidation of NADH. The modification of graphite electrodes with Os-phendione-PVP leads to the decrease in overpotential for the electrochemical oxidation of NADH from +0.33 V *vs*. Ag/AgCl/KCl$_{sat}$ for bare electrodes to +0.11 V *vs.* Ag/AgCl/KCl$_{sat}$. Measurement of the kinetic rate constant for the interaction of the oxidised redox polymer with NADH by rotating disk electrodes gave the value of $k_{1,[NADH]=0}$ equal to (1.9±0.2)x10^3 $M^{-1}s^{-1}$ at pH 6. This value practically coincides with that for Os-phendione suggesting that the number of phendione ligands of the osmium complex changes proportionally the response current to NADH but does not effect the electrochemical kinetic constants.

5. The amphiphilic mediator for NADH oxidation can be synthesised by the interaction of [Os(1,10-phenanthroline-5,6-dione)$_2$Cl$_2$] with 4,4′-[$CH_3(CH_2)_{17}NHCO]_2$bpy.

6. This amphiphilic NADH oxidising mediator forms stable Langmuir-Blodgett monolayers at the water-air interface. Multilayers of this compound deposited on the solid electrode surface by Langmiur-Blodgett technique lose electrochemical activity of 1,10-phenanthroline-5,6-dione due to the transport limitations of protons through bilayers formed by hydrophobic aliphatic chains.

7. The analogue of the above mentioned compound, which does not bear phenanthroline ligand, forms stable monolayers at the water-air interface and can be deposited on a solid electrode with partial loss of electrochemistry of the osmium atom, possibly, due to high hydrophobicity of the resulting multilayers.

8. Three new configurations, based on these mediators, were developed for the fabrication of reagentless dehydrogenase biosensors for glucose and L-glutamate. The first two fabrication methods rely on the adsorption of Os-phendione-PVP on the surface of graphite electrodes, followed by either adsorption of dehydrogenase and alginic acid modified with NAD^+ moieties (NAD-alginate) or by the entrapment of dehydrogenase and NAD^+ in a hydrogel formed by poly(vinyl pyridine) bearing amino groups. The third fabrication method relies on the entrapment of enzyme and coenzyme in lamellar phase created by a mixture of the NADH oxidising amphiphilic mediator and a lipid in aqueous solutions.

9. Reagentless glucose biosensors were fabricated by the immobilisation of biosensor's components in the above mentioned hydrogel deposited on graphite electrodes modified with a thin film of Os-phendione-PVP. The response to glucose of these sensors bearing different amount of glucose dehydrogenase in reagentless mode of operation was governed by the kinetics of the cycling of the $E{\cdot}NAD^+$.

10. Reagentless glutamate biosensors were built by the three above mentioned methods using thermophilic and mesophilic glutamate dehydrogenase. Mesophilic reagentless glutamate biosensors, fabricated with the use of NAD-alginate showed J_{max} 14.26 μA cm^{-2}, apparent K_M 14.3 mM, and $\tau_{1/2}$ 1.5 h, glutamate biosensors produced on the basis of the hydrogel demonstrated J_{max} 7.8 μA cm^{-2}, K_M 10.1 mM, and $\tau_{1/2}$ 12 h, glutamate sensors based on vesicle showed showed J_{max} 3.5 μA cm^{-2}, K_M 47.0 mM, and $\tau_{1/2}$ 0.5 h. Thermophilic glutamate biosensors based on the adsorption together with NAD-alginate demonstrated J_{max} 13.0 μA cm^{-2}, apparent K_M 39 mM and the sensors constructing using the hydrogel had shown J_{max} 19.1 μA cm^{-2}, apparent K_M 55 mM. In general the biosensors having higher maximal response current demonstrated the higher apparent Michaelis constants. The response of those biosensors was determined by the rate of electrochemical oxidation of NADH by the mediator and diffusional limitations.

11. The response of lamellar phase based biosensors was determined by the rate of electrochemical oxidation NADH and diffusional limitations.

12. The use of the hydrogel helps to increase the operational stability of produced reagentless sensors by improving enzyme and NAD^+ immobilisation through formation of covalent bonds between the enzyme proteins, NAD^+ and amino group born by modified poly(vinyl pyridine).

13. The effect of pH on the response of the present glutamate and glucose biosensors is defined by the kinetics of enzymatic reaction but not by the kinetics of NADH oxidation as one can see from the plots of response currents *vs.* pH.

14. The operational stability of biosensors at elevated temperatures can be improved by the use of thermophilic enzymes instead of mesophilic ones. It was proved by comparing performance of glutamate and glucose-6-phosphate prepared using mesophilic and thermophilic dehydrogenases in the presence of NAD^+ in the external solution. The use of thermophilic enzymes also allows to improve drastically the shelf stability of biosensors as it was shown by comparing shelf half-life time of glutamate biosensors constructed with the use of mesophilic and thermophilic glutamate dehydrogenases. This can be explained in terms of higher intrinsic stability demonstrated by the thermophilic enzymes. Some additives such as a copolymer of vinyl-pyrrolidone and dimethylamino ethyl methacrylate termed as Gafquat® HS100 can extend the shelf life of biosensors by forming a protein-polyelectrolyte complex which mimicks aqueous environment of enzymes in dry state. Other tested additives such as glycerol , trehalose and poly(ethylene imine) proved to be useless for the improvement of shelf life because they fail to form the protective complex with the enzyme

15. The presence of NAD^+ in a sample solution can decrease the response of disposable glutamate biosensors to glutamate and NADH because of the formation of the parasite com-plex between the mediator and NAD^+. This effect is evident if the concentration of the reduced cofactor is high and/or the rate of reoxidation of the mediator is low.

6. FUTURE WORK

This work, although demonstrated that the reagentless biosensors based on NAD^+ dependent dehydrogenases can be constructed with the use of new NADH oxidizing mediators and methods for the NAD^+ immobilization, leaves open questions that could be answered with the following types of studies:

1. The possibility to apply Os-phendione-PVP and the "binder" polymer to the construction of the reagentless glutamate microsensors should be investigated. The resulting microsensors can be employed to detect glutamate anions in the vicinity of axons of living neurons.

2. The rotating disk electrode (RDE) should be used to evaluate the response of the reagent glutamate biosensors to glutamate in order to assess experimentally the influence of the controlled mass transport on the shapes of the Eadie-Hofstee plots and the resulting values of the Michaelis constants. This study could further confirm the mathematical model describing the effect of different factors on the response of the biosensors to their analytes.

3. The detailed study of the electrochemical behavior of Os-phendione-surfactant adsorbed on graphite electrodes should be carried out with cyclic voltammetry at different scan rates and pH values to determine the rate constant for the heterogeneous electron transfer of the phendione redox couple in this new complex. Next the rotating disk electrode should be employed to evaluate the second order rate constant for NADH oxidation.

4. The above-mentioned investigation should be also performed with Os-phendione-surfactant in the lamellar phase immobilized on the electrode surface.

5. The inhibiting effect of NAD^+ on the NADH oxidation by [Os(4,4'-dimethyl-2,2'-bipyridine)$_2$(1,10-phenanthroline-5,6-dione)]$(PF_6)_2$, Os-phendione-PVP, and Os-phendione-surfactant can be studied in details by varying concentrations of NAD^+ and NADH in the electrolyte to determine the constants of inhibition. Next, the effect of the pH values on those inhibition constants can be investigated.

6. An interesting additional study can be carried out by using Os-phendione-surfactant as the redox catalyst for the reduction of $AuCl_4^-$ anions by NADH leading to the formation of colloidal gold in the enzymatic reaction of dehydrogenases with their substrates and NAD^+. Theoretically, this amphiphilic mediator is capable of forming stabilizing micelles

around the resulting gold nanoparticles.

7. BIBLIOGRAPHY

E. Adler, H. von Euler, G. Gunther, M. Plass, *Biochem. J.,* **1939**, *33*, 1028.

W. J. Albery, P. N. Bartlett, A. E. G. Cass, K. W. Sim, *J. Electroanal. Chem.*, **1987**, *218*, 127.

S. J. Alcock , A. P. E. Turner, *IEEE Engin. Med. Biol.,* **1994**, 319.

S. L. Alvarez, M. J. Lobo, A. J. Miranda, P. Tuñon, *Biosens. Bioelectron.*, **1997**, *12*, 739.

M. I. Alvarez, S. A. Saidman, M. J. Lobo, A. J. Miranda, P. Tunon, *Anal. Chem.,* **2000**, *72*, 520.

R. E. Amelunxen, M. Lins, *Arch. Biochem. Biophys.*, **1968**, *125*, 765.

A. Bardea, E. Katz, A. F. Buckmann, I. Willner, *J. Am. Chem. Soc.*, **1997**, *119*, 9114.

P. N. Bartlett, P. R. Birkin, E. N. K. Wallace, *J. Chem. Soc., Faraday Trans.*, **1997**, *93*, 1951.

P. N. Bartlett, E. Simon, *Phys. Chem. Chem. Phys.*, **2000**, *2*, 2599.

T. S. Berzina, V. I. Troitsky, A. Petrigliano, D. Alliata, A. Yu. Tronin, C. Nicolini, *Thin Solid Films,* **1996**, *284/285*, 757.

R. L. Blankespoor, L. L. Miller, *J. Electroanal. Chem.,* **1984**, *171*, 231.

W. J. Blaedel and, R. C. Engstrom, *Anal. Chem.*, **1980**, *52*, 1691.

W. J. Blaedel and R. A. Jenkins, *Anal. Chem.,***1975**, *47,* 1337.

W. J. Blaedel and R. A. Jenkins, *Anal. Chem.,***1976**, *48,* 1240.

K. B. Blodgett, *J. Am. Chem. Soc.*, **1934**, *56*, 495.

L. I. Boguslavsky, L. Geng, I. P. Kovalev, S. K. Sahni, Z. Xu, T. A. Skotheim, *Biosens. Bioelectron.*, **1995**, *10*, 693.

J. Bonnefoy, J. Moiroux, J. -M. Laval. C. Bourdillon, *J. Chem. Soc., Faraday Trans.,* **1988**, *84*, 941.

J. F. Brandts, *In Thermobiology,* Ed. A. H. Rose, Academic Press, New York and London, **1967**, pp. 25.

B. Bubela, E. S. Holdsworth, *Biochim. Biophys. Acta*, **1966a**, *123*, 364.

B. Bubela, E. S. Holdsworth, *Biochim. Biophys. Acta*, **1966b**, *123*, 376.

H. B. Bull, K. Breese, *Arch. Biochem. Biophys.*, **1973**, *158*, 681.

S. K. Burley, G. A. Petsko, *Science*, **1985**, *229*, 23.

C. X. Cai, H. X. Ju, H. Y. Chen, *Anal. Chim. Acta,***1995a**, *310,* 145.

C. X. Cai, H. X. Ju, H. Y. Chen, *J. Electroanal. Chem.,***1995b**, *397,* 185.

C. X. Cai, K. H. Xue, *J. Electroanal. Chem.*, **1997a**, *427*, 147.

C. X. Cai, K. H. Xue, *Anal. Chim. Acta*, **1997b**, *343*, 69.

C. X. Cai, L. H. Yin, K. H. Xue, *J. Mol. Catalysis, A. Chem.,* **2000**, *152*, 179.

C. X. Cai, K. H. Xue, *Microchem. J.*, **1998a**, *58*, 197.

C. X. Cai, K. H. Xue, *Talanta*, **1998b**, *47*, 1107.

K. H. Cass, E. Stellwagen, *Arch. Biochem. Biophys.*,**1975**, *171*, 682.

H. Y. Chen, A. M. Yu, J. L. Han, Y. Z. Mi, *Anal. Lett.,* **1995**, *28*, 1579.

H. Y. Chen, D. M. Zhou, J. J. Xu, H. Q. Fang, *J. Electroanal. Chem.*, **1997**, *422*, 21.

Q. Chi, S. Dong, *Electroanalysis,* **1995**, *7*, 147.

A. Ciszewski, G. Milczarek, *Anal. Chem.*, **2000**, *72*, 3203.

L. C. Clark, C. Lyons, *Ann. N. Y. Acad. Sci.*, **1962**, *102*, 29.

D. Compagnone, C. J. McNeil, D. Athey, C. Dillio, G. G. Guilbault, *Enz. Micr. Techn.*, **1995**, *17*, 472.

S. Cosnier, M. Fontecave, C. Innocent, V. Niviere, *Electroanalysis*, **1997**, *9*, 685.

A. Curulli, I. Carelli, O. Trischitta, G. Palleschi, *Biosens. Bioelectron.*, **1997**, *12*, 1083.

K. Dalzier, F. M. Dickinson, *Biochem. J.,* **1966**, *100*, 34.

R. M. Daniel, D. A. Cowan, *Cell. Mol. Life Sci.*, **2000**, *57*, 250.

P. Davies, K. Mosbach, *Biochim. Biophys. Acta.*, **1974**, *370*, 329.

C. Degrand, L.L. Miller, *J. Am. Chem. Soc.*, **1980**, *102*, 5728.

M.J. Dennison, A. P. F. Turner, *Biotechnol. Adv.,* **1995,** *13*, 1.

J. G. Dewan and D. E. Green, *ibid.,* **1937**, *31*, 1074.

D. Dicu, L. Muresan, I. C. Popescu, C. Cristea, I. A. Silberg, P. Brount, *Electrochim. Acta,* **2000**, *45*, 3951.

M. Dixon, *Nature*, **1960**, *188*, 464.

E. Domínguez, H. L. Lan, Y. Okamoto, P. D. Hale, T. A. Skotheim, L. Gorton, *Biosens. Bioelectron.*, **1993a**, *8*, 167.

E. Domínguez, H. L. Lan, Y. Okamoto, P. D. Hale, T. A. Skotheim, L. Gorton, B. Hahn-Hägerdal, *Biosens. Bioelectron.*, **1993b**, *8*, 229.

H. von Euler, P. Karrer, B. Becker, *Helv. Chim. Acta,* **1936**, *19*, 1060.

H. von Euler, F. Schlenk, R. Vestin, *Naturwiss.*, **1937**, *25*, 318.

H. von Euler, E. Bauler, *Ber.*, **1938**, *71*, 411.

J. Farrell, L. L. Campbell, *Adv. Microbial Physiol.*, **1969**, *3*, 83.

A. Fassouane, J. -M. Laval, J. Moiroux, C. Bourdillon, *Biotechnol. Bioeng.,* **1990**, *35,* 935.

J. J. Fernández, J. R. López, X. Correig, I. Katakis, *Sens. Actators*, **1998**, *47*, 13.

A. B. Florou, M. I. Prodromidis, M. I. Karayannis, S. M. Tzoueara-Karayanni, *Electroanalysis,* **1998**, *10,* 1261.

H. J. Fromm, *Methods Enzymol.,* **1979**, *63*, 42.

A. Fujishima, T. N. Rao, E. Popa, B. V. Sarada, I.Yagi, D. A. Tryk, *J. Electroanal. Chem.,* **1999**, *473*, 179.

M. Fukui, A. Kitani, C. Degrand, L. L. Miller, *J. Am. Chem.Soc.*, **1982**, *104*, 28.

S. Fukuzumi, Y. Kando, T. Tanaka, *J. Chem. Soc. Perkin Trans. II*, **1984a**, *4*, 673.

S. Fukuzumi, N. Nishizawa, T. Tanaka, *J. Org. Chem.*, **1984b**, *49*, 3571.

S. Fukuzumi, S. Koumitsu, K. Hironaka, T. Tanaka, *J. Am. Chem. Soc.,* **1987**, *109,* 305.

S. Fukuzumi, S. M. Ishikawa, T. Tanaka, *J. Chem. Soc. Perkin. Trans. II*, **1989**, *11*, 1811.

A. P. Girardegrot, R. M. Morelis, P. R. Coulet, *Bioelectrochem. Bioenerg.*, **1998**, *46*, 39.

L. Gorton, *J. Chem. Soc., Faraday Trans.,* **1986**, *82*, 1245.

L. Gorton, G. Bremle, E. Csöregi, G. Jönsson-Pettersson, *Anal. Chim. Acta*, **1991**, *249*, 43.

L. Gorton, A. Torstensson, H. Jaegfeldt, G. Johansson, *J. Electroanal. Chem.,***1984**, *161*, 103.

L. Gorton, G. Johansson, A. Torstensson, *J. Electroanal. Chem.,***1985**, *196*, 81.

C. A. Goss, H. D. Abruña, *Inorg. Chem.*, **1985**, *24*, 4263.

D. E. Green, J. G. Dewan, L. F. Leloir, *Biochem. J.*, **1937**, *31*, 934.

J. L. Han, A. M. Yu, H. Y. Chen, *Acta Chim. Sinica,* **1995**, *53,* 362.

P. Hapiot, J. Moiroux, J. -M. Savient, *J. Am. Chem. Soc.,* **1990**, *112*, 1337.

D. C. Harrison, *ibid.,* **1931**, *25*, 1016.

J. P. Hart, A. K. Abass, D. C. Cowell, A. Chappel, *Electroanalysis*, **1999**, *11*, 406.

A. Hasegawa, N. Miwa, T. Oshima, K. Imahori, *J. Biochem.*, **1976**, *79*, 34.

M. A. Hayes, W. G. Kuhr, *Anal. Chem,,* **1999**, *71*, 1720.

M. Hedemno, A. Narváez, E. Dominguez, I. Katakis, *Analyst*, **1996**, *121*, 1891.

M. Hennig, B. Darimont, R. Sterner, K. Kirshner, J. N. Jansonius, *Structure*, **1995**, *3*, 1295.

Y. Hibino, Y. Nosoh, T. Samejima, *J. Bioche.*, **1974**, *75*, 553.

J. D. Hocking, J. I. Harris, *FEBS Lett.*, **1973**, *34*, 280.

J. J. Holbrook, A. Liljas, S. J. Steindel, M. G. Rossmann, *Enzymes*, **1975**, *11,* 281.

N. Howell, J. M. Akagi, R. H. Himes, *Can. J. Microbiol.*, **1969**, *15*, 461.

Zh. Huan, B. Persson, L. Gorton, S. Sahni, T. Skotheim, P. Bartlett, *Electroanalysis*, **1996**, *8*, 575.

H. Huck, *Fresenius J. Anal. Chem.,* **1982**, *313*, 548.

H. Huck, *Fresenius J. Anal. Chem.*, **1994**, *350*, 599.

Y. Ikariyama, T. Ishizuka, H. Sinohara, M. Aizawa, *Denki Kagaku,* **1990**, *58*, 1097.

H. Jaegfeldt, *Bioelectrochem. Bioenerg.,***1981**, *8*, 935.

H. Jaegfeldt, A. B. C. Torstensson, L. Gorton, G. Johansson, *Anal. Chem.*, **1981**, *53*, 1979.

H. Jaegfeldt, T. Kuwana, G. Johansson, *J. Am. Chem. Soc.,* **1983**, *105*, 1805.

P. Jaraba, L. Agui, P. Yañez-Sedeño, J. M. Pingarron, *Electrochim. Acta*, **1998**, *43*, 3555.

J. B. Jones, J. F. Beck, *Applications of Biochemical Systems in Organic Chemistry, Techniques of Organic Chemistry Series* (eds. J. B. Jones, C. J. Sih, D. Perlman), Wiley Interscience, New York, **1976**, p. 107.

J. B. Jones. K. E. Taylor, *J. Am. Chem. Soc.,* **1976**, *98*, 5689.

H. Ju, D. Leech, *Anal. Chim. Acta*, **1997**, *345*, 51.

P. Karrer, O. Warburg, *Biochem. Z.,* **1936**, *225*, 297.

P. Karrer, G. Schwarzenbach, F. Benz, U. V. Solmssen, *Helv. Chim. Acta,* **1936**, *19,* 811.

P. Karrer, F. Benz, *ibid.,* **1936**, *19*, 1028.

P. Karrer, G. Schwarzenbach, G. E. Utzinger, *ibid.,* **1937**, *20*, 72.

Y. Kashiwagi, Q. Pan, F. Kurashima, C. Kikuchi, J. Anzai, T. Osa, *Chem. Lett.*, **1998**, 143.

I. Katakis, E. Dominguez, *Mikrochim. Acta*, **1997**, *126*, 11.

N. Katayama, I. Urabe, H. Okada, *Eur. J. Biochem.*, **1983**, *132*, 403.

E. Katz, V. Heleg, A. Bardea, I. Willner, H. K. Rau, W. Haehnel, *Biosens. Bioelectron.*, **1998**, *13*, 741.

A. Kitani, Y. H. So, L. L. Miller, *J. Am. Chem. Soc.*, **1981**, *103*, 7636.

I. L. Khmelnitskii, A. V. Levashov, N. L. Kliachko, V. I. Cherniak, K. Martinek, *Biokhimiia*, **1982**, *47*, 86.

H. Koffler, G. O. Gale, *Arch. Biochem. Biophys.*, **1957**, *67*, 249.

A. Kornberg, W. E. Pricer, *J. Biol. Chem.*, **1950,** *186*, 557.

E. Kress-Rogers,. "Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment", CRC Press, Boca Raton, USA, 1996.

I. Langmuir, *J. Am. Chem. Soc.*, **1917**, *39*, 1848.

V. Laurinavicius, B. Kurtinaitiene, V. Gureviciene, L. Boguslavsky, L. Geng, T. Scotheim, *Anal. Chim. Acta.*, **1996**, *330*, 159.

J. M. Laval, C. Bourdillon, J. Moiroux, *J. Am. Chem. Soc.,* **1984**, *106*, 4701.

B. Leca, J. L. Marty, *Biosens. Bioelectron.*, **1997**, *12*, 1083.

G. X. Li, J. J. Zhu, H. Q. Fang, H. Y. Chen, D. X. Zhu, *J. Electroanal. Soc.,* **1996**, *143*, L141.

F. Y. Li, L. P. Jin, C. H. Huang, J. Zheng, J. Q. Gou, X. S. Zhao, T. T. Liu, *Chem. Mater.*, **2001**, *13*, 192.

B. Lindholm-Sethson, J.C. Gonzalez, G. Puu, *Langmuir*, **1998**, *14*, 6705.

L. G. Ljungdahl, D. Sherod, *In Extreme Environments: Mechanisms of Microbial Adaptation,*Ed. M. R. Heinrich, Academic Press, New York and London**,** p. 147.

M. J. Lobo, A. J. Miranda, J. M. López-Fonseca, P. Tuñon, *Anal. Chim. Acta.*, **1996a**, *325*, 33.

M. J. Lobo, A. J. Miranda, P. Tunon, *Electroanalysis*, **1996b**, *8*, 932.

M. J. Lobo, A. J. Miranda, P. Tunon, *Anal. Chim. Acta*, **1997a**, *346*, 165.

M. J. Lobo, A. J. Miranda, P. Tuñon, *Biosens. Bioelectron.*, **1997b**, *12*, 511.

E. Lorenzo, L. Sanchez, F. Pariente, J. Tirado, H. D. Abruña, *Anal. Chim. Acta*, **1995**, *309*, 79.

A. Maines, M. I. Prodromidis, S. M. Tzouwara, M. I. Karayannis, D. Ashworth, P. Vadgama, *Anal. Chim. Acta*, **2000**, *408*, 217.

A. Malinauskas, T. Ruzgas, L. Gorton, *Bioelectrochem. Bioenerg.*, **1999**, *49*, 21.

A. Malinauskas, G. Niaura, S. Bloxham, T. Ruzgas, L. Gorton, *J. Coll. Interface Science,* **2000**, *230*, 122.

N. Mano, A. Kuhn, *J. Electroanal. Chem.,* **1999**, *477*, 79.

K. Martinek, A. V. Levashov, N. L. Klyachko, V. I. Pantin, I. V. Berezin, *Biochim. Biophys. Acta*, **1981**, *657*, 277.

W. Militzer, T. B. Sonderegger, L. C. Tuttle, C. E. Georgi, *Arch. Biochem.*, **1949**, *24*, 75.

F. Mizutani, Y. Sato, Y. Hirata, T. Sawaguchi, S. Yabuki, *Sens. Actuat. B Chem.,* **2000**, *65*, 46.

J. Moiroux; P. J. Elving, *J. Electroanal. Chem.,* **1979a**, *102***,** 93.

J. Moiroux; P. J. Elving, *Anal. Chem.,* **1979b**, *51*, 346.

J. Moiroux; P. J. Elving, *J. Am. Chem. Soc.,* **1980***, 102,* 6533.

R. Murray, *Electroanal. Chem.,* **1992**, *13*, 297.

G. Nagy, I. Kapui, L. Gorton, *Anal.Chim. Acta.,* **1995**, *305*, 65.

Yo. Nakamura, S. Suye, J. Kira, H. Tera, I. Tabata, M. Senda, *Biochim. Biophys. Acta*, **1996**, *1289*, 221

H. Nicholson, W. J. Becktel, B. W. Matthews, *Nature*, **1988**, *336*, 651.

Nomenclature Committe of the International Union of Biochemistry, *Enzyme Nomenclature,* Academic Press, New York, 1984.

T. Noguer, A. Gradinaru, A. Ciucu, J. L. Marty, *Anal. Lett.*, **1999**, *32*, 1723.

W. B. Nowal, W. G. Kuhr, *Electroanalysis*, **1997**, *9*, 102.

W. B. Nowal, W. G. Kuhr, *Anal. Chem.,* **1995**, *67*, 3583.

Y. Okamato, T. Kaku, R. Shundo, *Pure & Appl. Chem.,* **1996**, *68*, 1417.

J. Parellada, A. Narváez, M. A. López, E. Domínguez, J. J. Fernández, V. Pavlov, I. Katakis, *Anal. Chim. Acta*, **1998,** *362*, 47.

F. Pariente, E. Lorenzo, H. D. Abruña, *Anal. Chem.*, **1994**, *66*, 4337.

F. Pariente, F. Tobalina, G. Moreno, L. Hernández, E. Lorenzo, H. D. Abruña, *Anal. Chem.*, **1997**, *69*, 4065.

J. K. Park, H. J. Yee, K. S. Lee, W. Y. Lee, M. C. Shin, T. H. Kim, S. R. Kim, *Anal.Chim. Acta.*, **1999**, *390*, 83.

N. Pasco, C. Jeffries, Q. Davies, A. J. Downard, A. D. Roddick-Lanzilotta, *Biosens. Bioelectron.*, **1999**, *14*, 171.

B. Persson, H. S. Lee, L. Gorton, T. Skotheim, P. Bartlett, *Electroanalysis*, **1995**, *7*, 935.

I. C. Popescu, E. Dominguez, A. Narvaez, V. Pavlov, I. Katakis, *J. Electroanal. Chem.,* **1999**, *464*, 208.

M. E. Pullman, A. San Pietro, S. P. Golowick, *J. Biol. Chem.,* **1954**, *206*, 129.

P. Ramesh, S. Sampath, *Anal. Chem.,* **2000**, *72*, 3369.

C. Ramirez, M. Boujtita, N. El-Murr, *Anal. Chim. Acta*., **1999**, *401*, 155.

K. Ravichandran, R. P. Baldwin, *Anal. Chem*., **1984**, *56*, 1744.

S. B. Saidman, M. J. Lobo, A. J. Miranda, P. Tunon, *Anal. Chim. Acta*, **2000**, *424*, 45.

D. D. Schlereth, E. Katz, Schmidt, *Electroanalysis,* **1994**, *6*, 725.

D. D. Schlereth, E. Katz, Schmidt, *Electroanalysis,* **1995**, *7*, 46.

A. Silber, C. Brauchle, N. Hampp, *J. Electroanal. Chem.,* **1995**, *390*, 83.

A. Silber, N. Hamp, W. Schuhmann, *Biosens. Bioelectron.*, **1996**, *11*, 215.

E. Silverstein, G. Sulebele, *Biochemistry*, **1969**, *8*, 2543.

R. Singleton, In *Extreme Environments: Mechanisms of Microbial Adaptation,* Ed. M. R. Heinrich, Academic Press, New York and London, **1976**, p. 189.

Z. Sheng, L. B. Gan, C. H. Huang, *Chem. Phys. Lett.*, **2000**, *331*, 143.

M. Hennig, B. Darimont, R. Sterner, K. Kirshner, J. N. Jansonius, *Structure*, **1995**, *3*, 1295.

R. Singleton, R. E. Amelunxen, *Bacteriol. Rev.*, **1973**, *37*, 320.

M. D. Smith, C. L. Olson, *Anal. Chem.*, **1975**, *47*, 1074.

S. D. Sprules, J. P. Hart, R. Pittson, S.A. Wring, *Electroanalysis*,**1996**, *8*, 539.

G. D. Storrier, K. Takada, H. D. Abruña, *Inorg. Chem.,***1999**, *38*, 559.

J. J. Sun, J. J. Xu, H. Q. Fang, H. Y. Chen, *Biolectrochem. Bioenerg.,* **1997**, *44*, 45.

K. Suzuki, K. Imahori, *J. Biochem.*, **1974**, *76*, 771.

H. Theorell, B. Chance, *Acta Chem. Scand.,* **1951**, *5*, 1127.

M. Tian, S. J. Dong, *Electroanalysis,* **1995**, *7*, 1995.

D.R. Thevenot, K. Toth, R.A. Durst, and G.S. Wilason, *Pure Appl. Chem.*, **1999**, *71*, 2333.

F. Tobalina, F. Pariente, L. Hernandez, H. D. Abruña, E. Lorenzo, *Anal. Chim. Acta*, **1999**, *395*, 17.

A. Torstensson, L. Gorton, *J. Electroanal. Chem.*, **1981**, *130*, 199.

V. I. Troitsky, T. S. Berzina, A. Petrigliano, C. Nicolini, *Thin Solid Films*, **1996a**, *284/285*, 122.

V. I. Troitsky, M. Sartore, T. S. Berzina, D. Nardeli, C. Nicolini, *Rev. Sci. Instr.*, **1996**, *67*, 4216.

D. C-S. Tse, T. Kuwana, *Anal. Chem.,* **1978**, *50*, 1315.

B. Ueda, D. C-S. Tse, T. Kuwana, *Anal. Chem.,* **1982**, *54,* 850.

S.J. Updike, J.P. Hicks, *Nature*, **1967**, *214,* 986.

S. Vakula, V. Erokhin, C. Nicolini, P. Facci, F. Antolini, patent WO 9509058.

K. Wan, J. M. Chovelon, N. Jaffrezic-Renault, *Talanta*, **2000**, *52*, 663.

J. Wang, J. Liu, *Anal. Chim. Acta*, **1993**, *284*, 385.

O. Warburg and W. Christian, *Helv. Chim. Acta*, **1936**, *19*, E79.

D. B. Wigley, A. R. Clarke, C. R. Dunn, D. Barstow, T. Atkinson, W. Chia, H. Muirhead, J. Holbrook, *Biochim. Biophys. Acta*, **1987**, *916*, 145.

S.F. White, A. P. F. Turner In: "Encyclopedia of Bioprocess Technology: Fermentation,

Biocatalysis and Bioseparation"(Eds. M C Flickinger and S W Drew). Wiley, New York, USA, 1997.

C. H. Wong, G. M. Whitesides, *J. Am. Chem. Soc.*, **1981**, *103*, 4890.

Q. Wu, M. Maskus, F. Pariente, F. Tobalina, V. M. Fernandez, E. Lorenzo, H. D. Abruña, *Anal. Chem.,***1996**, *68*, 3688.

T. Yao, S. Musha, *Anal. Chim. Acta*, **1979**, *110*, 203.

M. Yasuzawa, M. Hashimoto, S. Fujii, A. Kunugi, T. Nakaya, *Sens. Actuat. B Chem.*, **2000**, *65*, 241.

H. C. Yoon, H. S. Kim, *Anal. Chim. Acta*, **1996**, *336*, 57.

M. Yoshida, N. Sone, H. Hirata, Y. Kagawa, *J. Biol. Chem.*, **1975**, *250*, 7910.

S. Y. Zaitsev, *Sens. Actuat. B Chem.*, **1995**, *24*, 177.

H. R. Zare, S. M. Golabi, *J. Solid State Electrochem.*, **2000**, *4*, 87.

Z. Zheng, L.B. Gan, C. H. Huang, *Chem. Phys. Lett.*, **2000**, *331*, 143.

D. Zhou, H. Q. Fang, H. Chen, H. Ju, Y. Wang, *Anal. Chim. Acta*, **1996**, *329*, 41.

R. G. Zhu, J. L. Han, H. Y. Chen, *Chem. J. Chin. Univ.*, **1995**, *16*, 35.