STUDIES ON ENZYME ELECTRODES

A THESIS
SUBMITTED
FOR THE DEGREE OF

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IN LIFE SCIENCES

BY

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STATEMENT

I hereby declare that the matter embodied in this thesis entitled "Studies on Enzyme Electrodes" is the result of investigations carried out by me in the School of Life Sciences, University of Hyderabad, Hyderabad - 500 134, under the supervision of **Dr.** Chanchal **K. Mitra** and this work has not been submitted for any degree or diploma of any other University.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whereever the works described are based on the findings of the other investigators.

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CERTIFICATE

Certified that the work embodied in this thesis has been carried out by Mr. D. Narasaiah under my guidance for the full period prescribed under the Ph.D. ordinance of this University and the same has not been submitted elsewhere for any degree or diploma of any other University.

I recommend his thesis entitled **"Studies on Enzyme Electrodes"** for consideration for the award of the degree of Doctor of Philosophy of this University.

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ABBREVIATIONS

NHE	=	Normal hydrogen electrode
SCE	=	Saturated calomel electrode
mV	-	millivolts
CME	=	Chemically modified electrode
CV	=	Cyclic voltammetry
BSA	=	Bovine serum albumin
NAD	=	Nicotlnamlde adenine dinucleotide (oxidized form)
NADH	=	Nicotinamide adenine dinucleotide (reduced form)
FAD	=	Flavin adenine dinucleotide
FMN	=	Flavin mononucleotide
PQQ	=	Pyrrolo-quinoline quinone
ADH	=	Alcohol dehydrogenase
GOD	=	Glucose oxidase
LOD	=	Lactate oxidase
LDH	=	Lactate dehydrogenase
HRP	=	Horse-radish peroxidase
ARP	=	Arthromyces ramosus peroxidase
FIA	=	Flow-injection analysis
GA	=	Glutaraldehyde
PT	=	Polytryptophan
PTyr	=	Polytyrosine
FFT	=	Fast fourler transformation
A/D	=	Analog to digital
D/A	=	Digital to analog

CONTENTS

1. MODIFIED ELECTRODES

The rate of electron transfer across an electrode/solution interface is dependent on the physical and chemical properties of the electrode surface. Control of reactivity of the electrode/solution interface, If achieved, can influence the process of electrocatalysls. One variable which has been commonly used for controlling reaction at the surface is the applied potential. The other ways of controlling the electrochemical processes is the modification of the electrodes.

The electron transfer reactions of biological molecules are often very slow at ordinary electrodes. To overcome this problem and to facilitate the direct coupling of biological redox reactions to electrodes for biosensor or bioelectronic applications, various types of modified electrodes have been used. In various domains, and especially in the biomedical field, there is a constant demand for obtaining reliable information in real time. This explains the strong interests dedicated to sensors and more specifically to biosensors.

The advantages offered by biosensors, devices that transduce a selective biological response into an analytical signal, are many. It is to understand why this relatively new field of chemical sensing has received such interest, particularly when one considers that the majority of analytical measurements made today are concerned with the qualitative and quantitative analysis of species in multi-component matrices. Because of their inherent specificity, biosensors can be used in these complex milieu without the need for any complicated and time consuming separation steps. Not only does this offer advantages of both a financial and temporal nature but it also allows for real-time analysis of particular importance where fluctuations in determinant concentration over short-term periods are critical. The ability of biosensors to provide real-time analysis is also important

in the field of clinical biochemistry, especially for the purpose of providing information about the levels of key analytes in metabolically unstable patients. Such in *vivo* sensors could in theory provide sufficient real-time data to direct drug release via associated mechanical devices, thereby restoring normal metabolic status in the patient. Further, biosensors are seen as playing an important part in the routine *in vitro* analysis of marker biochemicals, an essential prerequisite for the diagnosis of possible maladies and the subsequent administration of patient care.

1.1. **Oxidation-reduction processes**

An oxidation is defined as loss of electrons to give a higher oxidation state (more positive) and **reduction** is defined as a gain of electrons to give a lower oxidation state (more negative).

A reduction-oxidation reaction (commonly called a redox reaction) is one that occurs between a reducing and an oxidizing agent.

$$Ox + Red \rightleftharpoons Red + Ox \qquad (1)$$

Ox is reduced to Red and Red is oxidized to Ox . Ox is **the** oxidizing agent and Red is the reducing agent. The reducing or oxidizing tendency of a substance will depend on its reduction potential. An oxidizing substance will tend to take on an electron or electrons and be reduced to a lower oxidation state.

$$M^{a+} + ne^- \longrightarrow M^{(a-n)+} \qquad (2)$$

Conversely, a reducing substance will tend to give up an electron or electrons and be oxidized to a higher oxidation state:

$$M^{a+} \longrightarrow M^{(a+n)+} + ne^- \qquad (3)$$

If the oxidized form of a metal ion is complexed, it is more stable and will be more difficult to reduce, so its tendency to take on an electrons will be decreased. If the reduced form is complexed, the opposite may be true.

1.2. Electrochemical cells

There are two kinds of electrochemical cells, galvanic (voltaic) and electrolytic. In the first case, a chemical reaction spontaneously occurs to produce electrical energy. The lead acid storage cells and the ordinary dry cells are examples. In the second case electrical energy is used to force a non-spontaneous chemical reaction to occur, that is, to go in the reverse direction as it would be in a galvanic cell. An example is the electrolysis of water. In both types of cells, the electrode at which oxidation occurs is the anode and that at which reduction occurs is the cathode.

A cell consists of two half-cells which are electrically connected to enable electrochemical measurements to be made. Each half cell has an electrode potential (but cannot produce current). For the measurement of potential difference, electrical continuity is required within the cell. To satisfy the conditions of Ohm's law the measured voltage or potential of a cell is determined as the product of the current and resistance of the circuit (E=IR). If there is a lack of electrical continuity the resistance would be infinite and the current would be zero. The resulting potential would be indeterminate. For a cell potential to be measured a small but finite current must flow around the circuit. In practice the current may be as low as 10^{-10} amps (1). In the following reaction:

$$Fe^{2+} \longrightarrow Fe^{3+} + e^{-} \qquad (4)$$

If a solution containing Fe * is mixed with one containing Ce^{4+} there is a certain tendency for the ions to transfer electrons. Assume the

Fe^{2+} and Ce^{4+} are in separate beakers connected by a **salt bridge,** as shown in Fig. 1.1. (A salt bridge allows charge transfer (ionic passage) through the solutions **but** prevent mixing of the solutions). No reaction can occur, since the solutions do not make contact. The **set** up **now** constitutes a galvanic cell. If an ammeter is connected in series, it will indicate **that a** current **is** flowing. The Fe* is being oxidized at the platinum wire (at the anode).

$$Fe^{+2} \longrightarrow Fe^{3+} + e^{-} \qquad (5)$$

The released electrons flow through the wire to the other beaker where the Ce^{+} is reduced (at the cathode)

$$Ce^{4+} + e^{-} \longrightarrow Ce^{3+} \qquad (6)$$

This occurs because of the tendency of these ions to transfer electrons. The net result is the reaction written in equation (4) which would occur if Fe and Ce were added together in a single beaker. The platinum wires can be considered as **electrodes.** Each will adopt an electrical **potential** that is determined by the tendency of the ions to give off or take on electrons and this is called the **electrode potential.** A voltmeter placed between the electrodes **will** indicate the difference in the potentials between the two electrodes. The larger the potential difference, the greater the tendency for the reaction between Fe^{2}* and Ce^{4}*. The driving force of **the** chemical reaction (the potential difference) can be used to perform work such as lighting a light bulb or running a motor, as in a battery. Equations (5) and (6) are **half-reactions.** No half-reaction can occur by itself. There must be an **electron donor** (a reducing agent) and an **electron** acceptor (an oxidizing agent). In this case Fe^{*2} is the reducing agent and Ce^{4}* is the oxidizing agent. Each half-reaction will generate a definite potential that would be adapted by an inert electrode dipped in the solution.

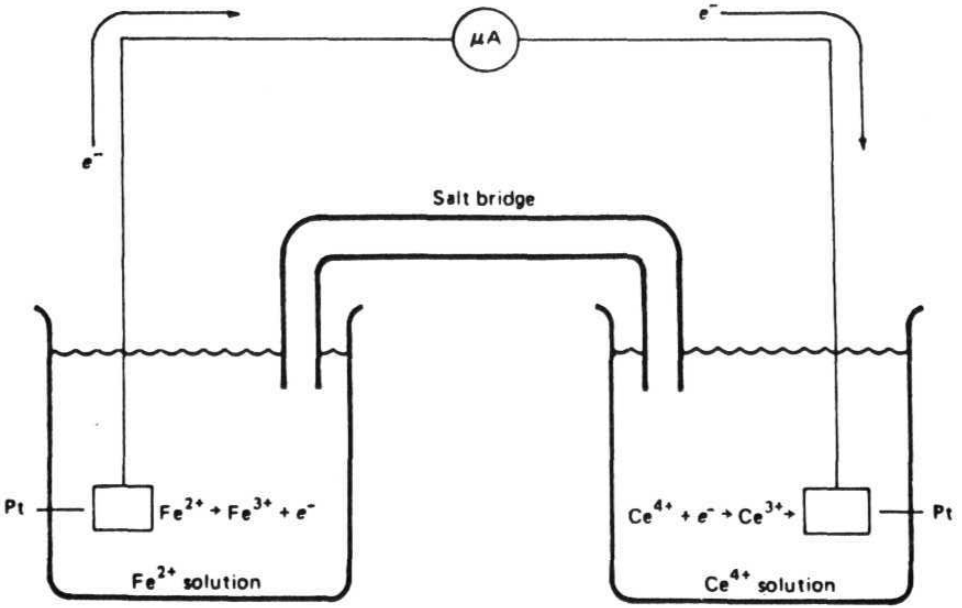


Fig. 1.1. Galvanic cell.

If the potentials of all half-reactions could be measured, then we could determine which oxidizing and reducing agents will react. Unfortunately there is no way to measure individual electrode potentials, but as described above the difference between two electrode potentials can be measured. The electrode potential of the following half-reaction has arbitrarily been assigned a value of 0.0V.

$$2H^+ + 2e^- \rightleftharpoons H_2$$

This is called the **normal hydrogen electrode (NHE) or the standard** hydrogen **electrode** (SHE). The potential differences between this half-reaction and other half-reaction have been measured and arranged in decreasing order (2).

1.3. The Nernst equation

The standard **redox potentials** designated by E are determined when the concentrations of both the oxidized and reduced form (and all other species) are at unit activity. The actual potential is dependant on the concentration of the species. This concentration dependence of potential is described by the Nernst equation.

$$Ox + ne^- \rightleftharpoons Red$$

$$E = E^0 - \frac{2.3026RT}{nF} \log \frac{[Red]}{[Ox]}$$

Where E is the reduction potential at the specific concentration, **n** is the number of electrons involved in half-reaction, R is the gas constant (8.3143V $Coul.deg^{-1}/mol^{-1}$) T is the absolute temperature and F **is** the Faraday constant (96,487 $coul.eq^{-1}$). At 25°C (298.16K), the value of 2.3026 RT/F is 0.05916. The concentration of pure substances such as precipitates and liquids (H 0) is taken as unity.

The potential of an electrode placed in a solution containing the ions of two half-reactions at equilibrium can be calculated using the Nernst equation for either half-reaction. This is because when the reaction comes to equilibrium, the potentials at the two half-reactions become identical; otherwise, two reactions would be still going on. An electrode dipped in the solution will adopt the equilibrium potential.

A basic system consists of a potentiostat for potential control of an electrode and a means of detecting the response (usually the current through the electrode). All redox reactions occur in the interface region between the bulk solution and the electrode surface. When a potential difference is applied between two electrodes in an electrolyte solution the potential gradient exists only in the narrow interface regions near the two electrodes with the bulk of the solution remaining electroneutral. Therefore molecules in the bulk solution cannot feel the presence of the electrodes or the potential gradients. Only molecules with in the potential gradient (typically less than 10 m from the electrode) are probed during an electrochemical experiment.

It is also important to understand how the potential gradient between an electrode and the bulk solution is established and controlled. Because the potential difference between the electrode and the bulk solution is not measurable, a second electrode must be employed and the potential difference between two electrodes in the solution can be determined. If the solution electrode potential difference at one of the electrodes is held constant by maintaining a rapid redox couple such as silver-silver chloride or mercury-mercurous chloride (calomel) then the potential difference between the solution and the second electrode can be directly related to the potential difference between the two electrodes. Although the potential difference between the electrode and solution is not known in an absolute sense, it is established in a relative sense.

In the two electrode system described above the electrode of maintained potential difference is known as the counter electrode while the electrode at which the potential difference is established relative to the counter electrode is termed the working electrode. It is at the working electrode where the electrolytic processes of interest to electroanalytical chemistry occur. The counter electrodes serves the second purpose of completing the electrical circuit and allowing charge to pass through the cell. The two roles of the counter electrode are not independent because a current through the electrode can cause a change in its potential difference with the solution. This problem is circumvented by splitting the role of the counter electrode between two electrodes to give an overall three electrode system. In the three electrode system a reference electrode is used to maintain a constant solution potential difference. An auxiliary electrode is used to complete the electrical circuit and a working electrode is used to apply a potential difference to drive electrolytic reactions. Fig. 1.2. In this arrangement there is negligible current through the reference electrode so its solution potential difference is constant regardless of the current through the electrochemical cell (working to auxiliary or counter electrode).

In a high dielectric constant medium (i.e. water) where a potential gradient is established between an electrode and the solution a charge excess will develop on the electrode surface which must be balanced by solution species. Solvent molecules and other species (i.e. electrolyte) orient themselves at the electrode surface to counter the electrode charge. This region is known as the double layer because of the arrangement of ions. This electrode-solution double-layer region behaves in a manner similar to a capacitor. when a potential difference is applied between the electrode and the solution, charge will accumulate near the electrode and the amount of charge will be a function of the potential difference applied "Double-layer charging currents" are thus produced whenever the potential difference is rapidly changed.

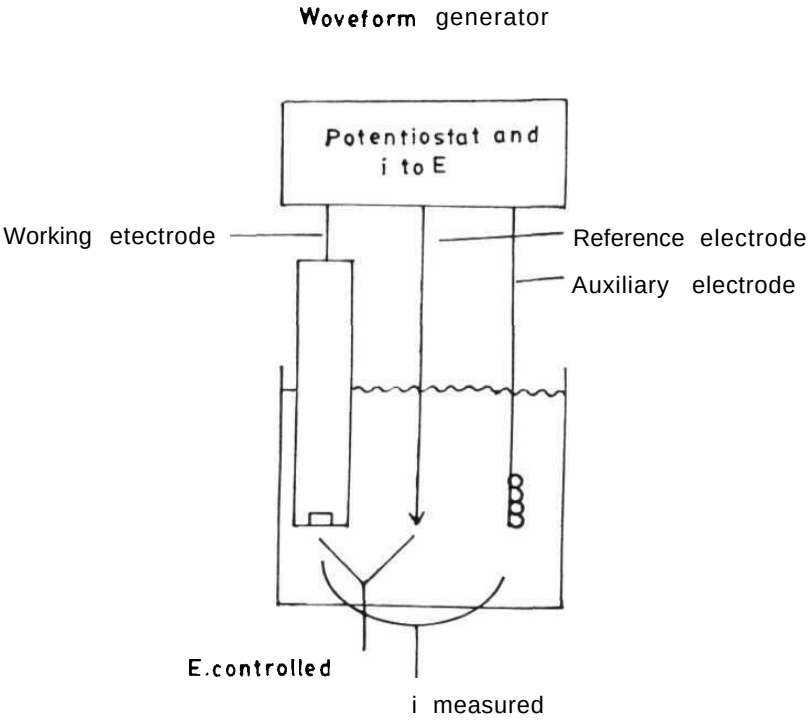


Fig. 1.2. Three electrode voltammetry system.

All Faradaic electrochemistry ultimately depends on Faraday's law:

$$Q = nFN$$

Where Q is the charge (coulombs) n is the number of electrons transferred (equivalents/mole), F is the Faraday constant (96,485 coulombs/equivalents) and N is the number of moles of reactant. Faraday's law is the most fundamental concept in electroanalytical chemistry because it directly relates the degree of chemical reaction to an electrical current (charge).

1.4. Electrode Materials

The reactive support is the electrode surface and thus must be a reasonably good conductor of electricity. Platinum, glassy carbon, different forms of graphite, tin oxide and a variety of pyridine-type polymers have been used widely in the preparation of chemically modified electrodes and thus are suitable supports for attachment of oxidation-reduction enzymes or co-factors. Metal electrodes, such as platinum, need to have an oxide film on the surface. Most metal oxide films will bind to an alkyl silane. By proper choice of the functional group on the end of the alkyl chain, a variety of different linkages between the alkyl silane and the enzymes or cofactors can be utilized.

Glassy carbon is a gas-impermeable form of solid carbon that has a very low porosity and high strength. It is highly desirable for use as an electrode because it can be polished to give electrochemically reproducible surfaces. Most carbon surfaces contain low levels of various carbon-oxygen functional groups. With glassy carbon the levels of such groups normally is very low so that some type of surface activation is required. Oxidation of the glassy carbon surface to form carboxylic acids is usually the favoured method of activation, although in reality a variety of functional groups

probably form. Functionalization of glassy carbon can be carried out by electrochemical (3), radio frequency oxygen plasma (4) or hot acid treatments (5) with the level of functionalization varying somewhat between research groups and the technique used.

Most graphite materials consists of several small crystals. They can differ from each other with respect to density, specific electrical resistance, hardness, strength, porosity, pore size, grain size, heat resistance and ash content. Possible impurities are species such as Ca, Cu, Fe, Mg and Si. Spectrographic graphite is a high purity grade. Pyrolytic graphite is obtained through pyrolysis of hydrocarbon (e.g., natural gas) at high temperatures (1000-2000 C) with deposition on a colder base from the gaseous phase. Graphite crystals consist of more or less well ordered planar 6-ring systems where the carbon is sp -hybridized. The electron orbitals which point out from the ring system will combine to give delocalized π-orbitals. The carbon layers are loosely bound together by van der Waals forces. Graphite electrodes are especially effective at chemisorbing reagents that have extended π-bond systems. The delocalized electrons of graphite can interact with delocalized electrons of other aromatic (unsaturated) structures forming new electron orbitals. Cofactors can be immobilized by adsorption on graphite electrodes (6).

Carbon paste electrodes made from a mixture of carbon powder and an inert oil are sometimes used for electroanalytical applications. The paste electrodes are a very good method for the preparation of organic conducting salt electrodes because they are easy to fabricate and give low background currents.

The choice of electrode material is also influences by the practical working range of applied potentials. For example, Hydrogen evolution, electrolyte electrode interactions and buffer-electrode interactions can produce enhanced background currents that limit the useful potential range for many types of electrodes. The practical range for

platinum at highly acid pH is about -200mV to 1100mV, while at pH 7 the range is shifted to about -600 mV to 600mV. For various forms of carbon, the useful range varies roughly from -1100 to 1100 mV.

In addition to the working electrode, a counter and reference electrodes are required to complete the three electrode system. The counter electrode is necessary to complete the circuit by passing an equal and opposite current to that flowing at the working electrode. To avoid problems in driving the current through the counter electrode it should ideally be large in area when compared with the working electrode. In addition, the counter electrode should not get corroded and contaminate the solution. For these reasons the most common choice for the counter electrode is a large area platinum gauze. An alternative is to use a carbon rod as the counter electrode.

The reference electrode can be any electrode which has a stable and reproducible redox potential. In practice the most commonly used electrodes are the saturated calomel electrode (SCE) and the Ag/AgCl electrode. Both are commercially available and widely used. The saturated calomel electrode is an excellent general purpose reference electrode. However it is not ideal if a very small size reference electrode is required. In these circumstances the Ag/AgCl electrode is a much better choice since it can be prepared from a piece of silver or silver plated platinum wire (7).

2. CHEMICALLY MODIFIED ELECTRODES

Adsorption on an electrode surface can often cause difficulties in the investigations of electrochemical reactions. It can interfere with electron transfer process and also can make the determination of potential uncertain. Irreversible adsorption for a particular redox couple can, however, be very useful with covalent coupling or polymer coating of electroactive molecules to an electrode surface. A new interesting area has now opened, leading to the development of

chemically modified electrodes (CMEs) to describe electrodes that have molecules intentionally immobilized on their surfaces. Murray et al (8) introduced the term modified electrodes. Lane and Hubbard (9) were among the first to use adsorption of olefinic compounds on platinum and to study in detail the electrode reactions. Miller et al (10) coupled optically active amino acids to graphite. The modified electrode was called "Chiral Electrode" and could be used to produce optically active alcohols by the electrochemical reduction of ketones. Anson and coworkers discovered that some organic redox reagents and complexes could be irreversibly bound to graphite electrodes by strong adsorption (11, 12) while maintaining excellent redox characteristics.

Some of the beneficial properties that may be obtained by electrode modification are listed below.

a) The possibility to study intramolecular and electrochemical electron transfers within the attached molecules;

b) No mass transfer of reactant or product to and from the electrode is required during the electrochemical oxidation or reduction, as in the case of a solution mediator;

c) The CMEs can be used in electrocatalysis, i.e., the attached molecule acts as fast electron transfer mediator for a substrate dissolved in the solution. If the substrate itself reacts slowly on the unmodified electrode, a favoured reaction path through the mediating groups will result in the desired improvement of electrode performance. A catalytic electrode combines specificity with sensitivity and convenience of electroanalytical techniques in a compact form to facilitate analysis.

d) Electrosynthesis of new compounds;

e) Selective molecular recognition (biosensor) (13)

f) Selective preconcentration (14);
(ion-exchange, complexation or covalent reactions);

g) Separation via permselectivity through membrane barriers (14);
(occlusion and exclusion based on charge or size);

h) Electroreleasing properties (15);

1) Chromic properties (16);

j) Photogalvanic properties (17).

2.1. Classification and preparation methods:

Modified electrodes can be classified into various categories as represented in Fig. 1.3. These can be divided into monolayer or multilayer structures depending on the thickness of material present on the electrode surface. A multilayer structure has the advantage that many more catalytic (or binding) sites are present and the stability may be higher but has the concomitant disadvantage that electrons must propagate through the multilayer film if the sites at the outside are to be of any practical use in analysis. Monolayer modified electrodes are subdivided into those prepared by the covalent attachment of species to the electrode surface and those in which the molecules are absorbed at the surface. Multilayer electrodes on the other hand are frequently based on polymeric films either produced at the electrode surface by in *situ* polymerization or polymerized ex situ and subsequently applied to the electrode (dip or drop coat) (18).

2.1.1. Covalent coupling:

For the covalent coupling of the species of interest a number of methods based on rather simple organic reactions are available. For carbon electrodes the introduction of oxygen functionalities at the surface is often the first step for possible coupling reactions. This can be performed in various ways e.g., thermal, 0 plasma or chemical oxidation. The molecule to be coupled must have a peripheral functionality for the attachment. In this case it is often an NH_2-group which then yields an amide linkage with an activated carboxylic group at the electrode. The covalent coupling can also be performed by using an intermediate bifunctlonal molecule such as alkyl-silanes or cyanuric chloride, which will act as a linkage between the electrode and the redox couple. In a good covalent method the attachment must be

1) stable in the potential region of interest
2) chemically stable in the solution
3) electrochemically inert.

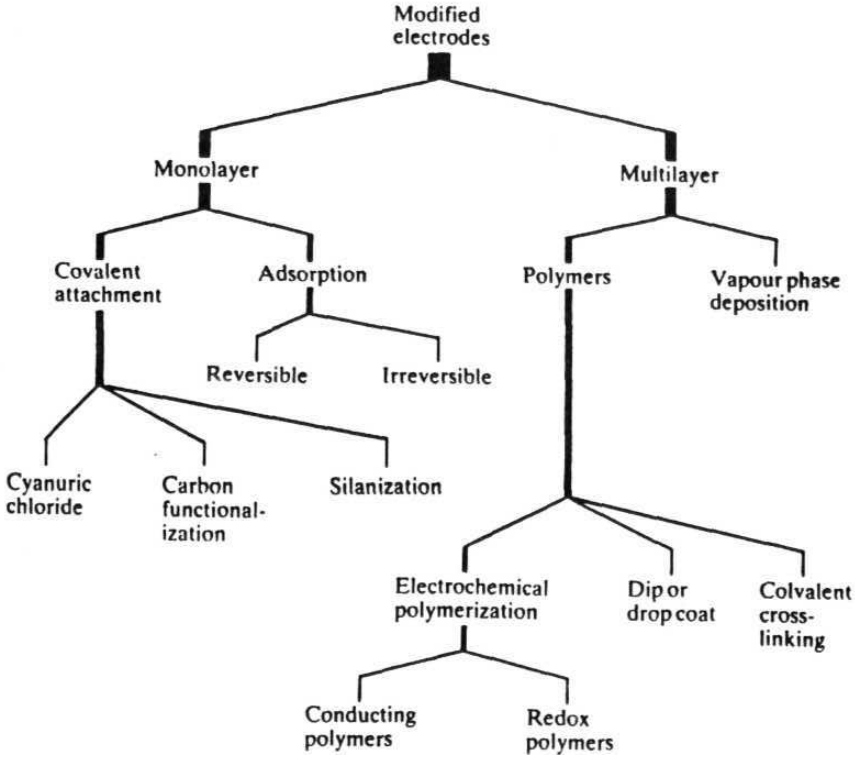


Fig. 1.3. The various types of modified electrodes.

The combinations of 0 plasma oxidation and cyanuric chloride invented by Kuwana fulfills the above mentioned requirements. This approach has the disadvantage of being rather time-consuming due to the excessive number of steps and long reaction times.

2.1.2. Polymer deposition:

Polymer deposition is obtained by applying preformed polymers on the electrode surface followed by evaporation of the solvent, by spin or dip coating, or by bonding via covalent linking agents. Polymerization of monomers resulting in precipitation of the polymer on the electrode surface can take place through plasma discharge or through chemical or electrochemical polymerization. The redox active modifier may be part of the monomer or be entrapped in the polymer matrix. The polymer itself may be conducting or non-conducting. Polymer consisting of the equivalent of several thousands of monomers are easily prepared while the other methods usually give coverage from one to a few monolayers.

Three broad categories can be distinguished among the polymeric films, electronically (π-conjugated) conducting, redox and ion-exchange polymers (19). Electronically conducting polymers, e.g., polypyrrole (20), may be partially oxidized or reduced electrochemically. The charge transport in either configuration occurs in a metal like fashion. A conducting state can also be made by reductive or oxidative doping with chemical agents. Redox polymers, e.g., polyvinylferrocene (21), conduct current by electron self-exchange reactions between neighboring redox sites. Ion-exchange polymers, e.g., protonated polyvinyl pyridine film exchanging counterions for the redox active compounds (22), conduct current both by self-exchange reactions and ion diffusion. However, in the case of multilayer polymers, the control of the overall rate of the charge transfer is complex and involves contributions by the flow of solvent and

reagents, the motion of redox centers, the rate of the current reactions, and the diffusion of counterions to maintain electroneutrality within the polymeric layer (23). Theoretical models of the electrochemistry of extended polymer films have reported given where the contributions of the propagation of charge within the film are treated as diffusive processes (24,25).

2.1.3. Adsorption:

The adsorption method of immobilization uses the ability of many organic compounds to "stick" to a surface. The attraction forces are caused by the high polarizability of the surface and the aromatic rings in an organic compound. It has been found that an increasing number of aromatic rings leads to an increase of the strength of adsorption (11,12). The lower solubility of these compounds in aqueous solutions also contributes to what will appear as a stronger adsorption. The method of preparation is quite simple and fast. The compound to be adsorbed is dissolved in an appropriate solvent after which the electrode is dipped into the solution. The amount adsorbed is governed by several factors (26). Carbon electrodes are especially effective when the modifier consists of a large aromatic system resulting in an extended π-bond interaction. The degree of adsorption will depend on the following factors:

a) concentration in solution of the species

b) time spent in the solution

c) degree of convection or stirring during adsorption

d) strength of adsorption

e) No adsorption is perfectly irreversible and the important factor contributing to the stability of the adsorbed layer depends on the solubility of the compound in the contacting solution.

3. METHODS OF IMMOBILIZATION

The strong adsorption of organic molecules to graphite has been used practically since the early days of chemistry. Graphite consists of more or less well ordered planar 6-ring systems where the carbon is sp^2- hybridized. The π-electron orbitals which point out perpendicularly from the ring system will combine to form delocalized π-orbitals. The basis for the ability of graphite to adsorb unsaturated ring systems could be explained by n-electron interactions. It has been suggested that polycyclic aromatic hydrocarbons adsorb on metal electrodes with the plane parallel to the surface. The type of bonding involved should be of the similar kind as in π-complexes (27).

The best way to get a flat reproducible surface is to polish gently on a machine with a wet fine grade emery paper. Other polishing methods, which could produce a more shining surface turned out to increase the background current without increasing the electrochemically available area. The CMEs also resulted in a less reversible electrochemical behaviour. Oxygen containing functionalities could be incorporated by holding the electrode at a positive potential (>1.1V vs SCE) in a buffer solution (28). There are some clear drawbacks to work with a material which is inhomogeneous and porous and shows irreproducibility between batches. The easiness with which electrodes could be prepared and modified has so far overweighed this.

3.1. Direct method

A CME could be prepared in a few minutes by Just dipping the graphite electrode in a solution containing the substrate to be adsorbed. The amount adsorbed could be changed either by changing the time for adsorption or the concentration of the substance in the solution. Adsorption of water insoluble redox systems could be easily take place from organic solvents or solvent mixtures to give irreversible voltammograms unless the film formation had been controlled.

3.2. Voltage facilitated method

Phenazonium ions could not functionalize at an untreated graphite surface to a large extent by direct adsorption. The immobilization occured only when the electrode was held at a positive potential (+1.1 +1.3V vs SCE) in a solution containing the phenazonium salt. The mechanism has not been explored. The electrode surface was saturated after 15 min (29). The maximum coverage reached a value close to 10^{-8} mole. cm ^{2}. Adsorption has been stated to be less controlled approach for electrode modification (30). The modification techniques outlined above turned out to be quick, relatively reproducible, and stable when compared to other methods. The maximum coverage is 2.10 mole.cm , which is one to two orders of magnitude lower than the highest reported for polymer coatings (31) and also much more than that reported for covalently attached groups (32). With a polymer coating the redox molecules will be spaced apart between the polymerizing agent while in an adsorption layer only the molecule of interest will be present. The electron transfer rate of an adsorbed species should be faster than in a polymer matrix where the redox sites are at greater distance from the electrode (33). It has been presumed that incorporating the redox compound in polymers improves the life time compared to covalent binding and adsorption even if the polymer framework is very stable. The electroactive component need not be more stable than when directly immobilized onto the electrode surface. Adsorption in many cases will be a well suited method to attach functionalities to an electrode surface.

4. INVESTIGATIONS OF CMEs

4.1. Cyclic Voltammetry (CV)

Voltammetry is the electrochemical technique in which the current at an electrode is measured as a function of the potential or voltage

applied to the electrode. The potential is varied in some systematic manner and the resulting current-potential plot is called a **voltammogram**. The most common application of **voltammetry** is for analytical purposes.

Voltammetry can be used to analyze any chemical species that is **electroactive,** i.e, that can be made to undergo oxidation or reduction. The potential of the electrode is the controlled parameter that causes the chemical species to be oxidized or reduced. The potential can be thought of as "electron pressure" which either forces a species in solution to gain an electron (reduction) or lose an electron (oxidation). As the potential of the electrode becomes more negative, it becomes more strongly reducing. Conversely as the potential becomes more positive, it becomes more strongly oxidizing. Therefore, the redox reaction taking place on the electrode can be controlled by controlling the electrode potential (34). A single CV experiment only hints at the event that constitute the electrochemical reaction at the electrodes. However, multiple CV experiments can be used for a variety of applications including:

1. the determination of Nernstian (reversible) or **non-Nernstian** (irreversible) behavior of a redox couple
2. the number of electrons transferred in an oxidation or reduction
3. formal potentials
4. rate constants
5. formation constants
6. reaction mechanisms
7. diffusion coefficients (35).

A typical cyclic voltammogram is shown in Fig. 1.4. for an electroactive species present in an unstirred solution (when the electrode does not adsorb the electrode species). With an oxidized species present in solution, the scan would be started **in** this example at about 0.02V and varied at a rate of 1-100 **mV/sec** in the direction

of more negative potentials (i.e., stronger reducing capability). As the potential becomes more negative it finally reaches a value about -0.20V (in Fig. 1.4) when the oxidized species begins to undergo reduction. This transfer of electrons produces an increase in current. The current peaks at i because the cofactor solution in the vicinity of the electrode surface gets consumed (i.e., reduced) and because the rate of influx of additional species cofactor from the bulk solution is diffusion controlled. At still more negative potentials, reduction current is limited by the rate of diffusion of oxidized species to the electrode surface. The direction of the scan is reversed and the species cofactor now undergoes oxidation, in Fig. 1.4. at about -0.35V. The formal potential E for the species oxidation-reduction reaction is defined as follows:

$$C_{ox} + ne = C_{rd}$$

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{[Cox]}{[Crd]} \qquad \text{eqn 2}$$

Where the terras in brackets refer to concentrations. T Is temperature, R the gas constant, F is the Faraday constant and E is the measured or actual potentials. E^{o} is the the measured potential (reference to a normal hydrogen electrode) with [Cox] = [Crd]. For cyclic voltammetry, with the electroactive material in solution as in Fig. 1.4. the oxidation and reduction peak potentials, Epa and Epc, are separately by 0.059/n volts because the process is operating under diffusion controlled conditions. Thus E° is located midway between Epc and E_{pa}. Here n is the number of electrons transferred. The peak current, i_{pa} or i_{pc}, varies according to equation 3 at 25°C for a reversible reaction

$$i_{pc} = (2.69 \times 10^{5}) n^{3/2} A D_{0}^{1/2} v^{1/2} [C_{0}^{*}] \qquad \text{eqn 3}$$

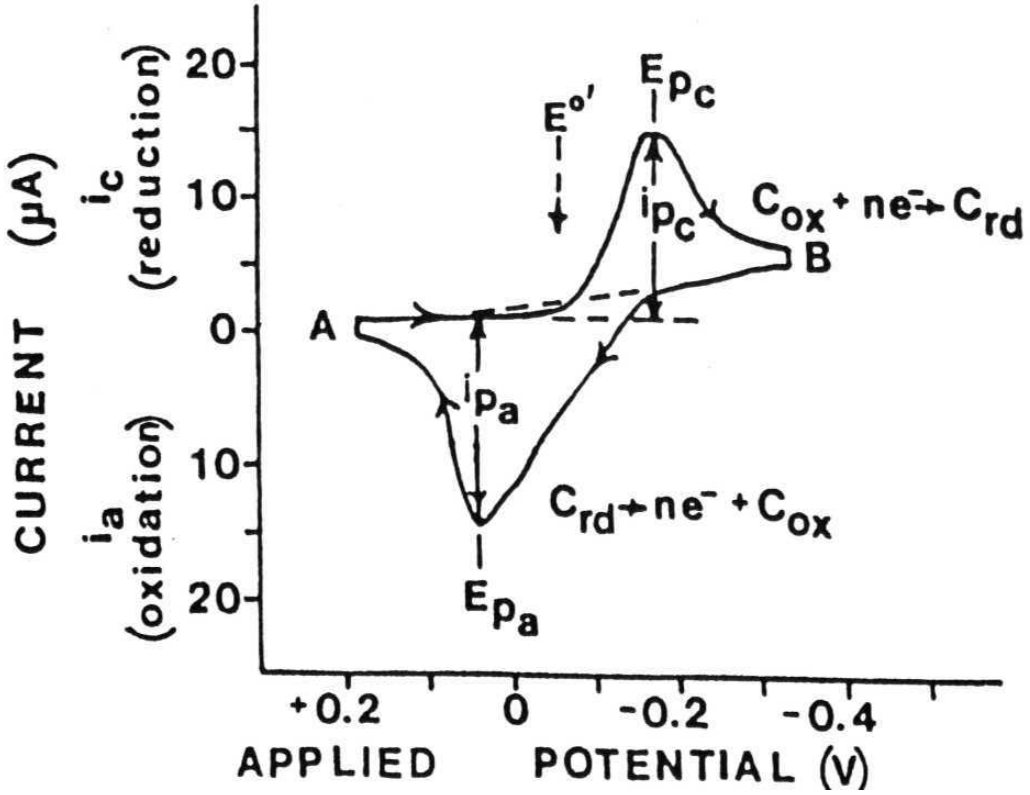


Fig. 1.4. Typical current-potential plot of a single scan cyclic voltammogram for an electroactive species in solution taken between +0.02 V and -0.35 V. Arrows show the scan direction. Measurements at A for an oxidized material or at B for reduced material. Results are shown for a constant scan rate.

Where A is electrode area (cm), D is diffusion coefficient of oxidized cofactor (cm^2/sec), v is scan rate (v/sec), and $[C_o]$ is the bulk phase concentration of oxidized cofactor. Two points are especially relevant. First, i_p is proportional to the square root of the scan rate and second, $(E_{pa} - E_{pc}) > 0.059/n$ if the process is not fully reversible.

When the electroactive species is attached to the electrode surface with no free material present in solution, the cyclic voltammograms have a different shape. Since all of the oxidation-reduction material is on the electrode surface, we no longer have diffusional effects. Thus, the oxidation and reduction peaks are symmetrical and centered about E° , as shown in Fig. 1.5. Another difference is that i_p is proportional to the scan rate for the attached case, as compared with the square root of the scan rate for the solution case. Therefore the log-log plot of i_p versus scan rate is a good way of showing whether an electroactive species is in solution or immobilized on the electrode surface. The method of immobilization can be covalent or ionic bonding or adsorption. The non-reversible conditions lead to increased separation between E_{pa} and E_{pc}, for both the solution and immobilized cases. The shifts in peak position with change in scan rate can be used to determine the kinetic rate constant (36).

5. ELECTROCATALYSIS AT CMEs

Electron transfer (ET) between a redox compound and an electrode, although thermodynamically feasible, may be obstructed by **a large** kinetic barrier (overpotential) or steric hindrances. Only in a few cases a direct ET between the bound cofactor of an oxidoreductase and an electrode has been reported (37-40). Promoted electrocatalysis between bound cofactors and an electrode may be obtained by the immobilization on the electrode surface of compounds that facilitate a direct ET by diminishing the steric hindrances. Mediated electrocatalysis takes place by the use of a low molecular weight

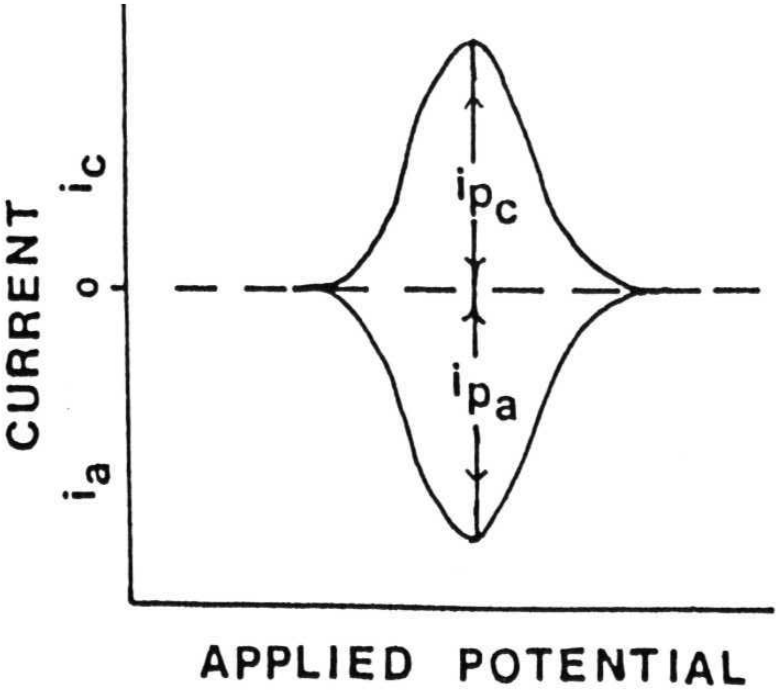


Fig. 1.5. Cyclic voltammogram for electroactive material attached to an electrode surface with none in solution and rapid, reversible electron transfer.

redox mediator acting as an electron shuttle between a compound in solution and the electrode. Mediated electrocatalysis may overcome steric hindrances and large overpotential. The potential at which the mediated ET occurs between the substrate and the electrode will mainly be dictated by the E° of the mediator.

The simplest case of mediated electrocatalysis at a surface modified electrode is when the current is limited only by the mass transport of a redox compound to the surface and/or the reaction rate between the compound and the mediator at the surface. The potential at which an anodic catalytic peak appears in CV, provided that the reaction rate is not too low, is then described by

$$E_p = E^{O'} + \frac{RT}{nF}\left[0.78 + \ln\left(\frac{D^{1/2}}{K_{obs}\tau}\right) + \ln\left(\frac{nFv}{RT}\right)\right]^{1/2}$$

Where D is the diffusion coefficient, (cm s), of the compound in solution, and K_{obs} is the second order overall rate constant, (M s) , of the reaction between the compound and the modifier at the surface. The difference in the peak potentials of the direct and the mediated electrochemical reactions is a measure of the decrease in the overpotential (41).

The electrochemical properties of the immobilized species turned out to be dependent on several parameters.

a) type and batch of graphite
b) electrode pretreatment
c) adsorption procedure
d) coverage
e) pH
f) buffer capacity
g) buffer components
h) ionic strength

6. ENZYME ELECTRODES AND BIOSENSORS

A biosensor may be defined as "a biochemically active material immobilized in intimate contact with a suitable transducing system to convert the biochemical signal into a quantifiable electrical signal".

The modern concept of a biosensor owes much to the ideas of Leland C. Clark and co-workers (1962). They proposed that enzymes could be immobilized at electrochemical detectors to form "enzyme electrodes" that would expand and/or modify the analyte range of the base sensor. The term "enzyme electrode" was introduced by Updike and Hicks who coated a layer of polyacrylamide gel containing glucose oxidase entrapped onto an oxygen electrode. Biosensors are extensively described in a number of books and reviews (13, 42-61). Fig. 1.6 shows a typical schematic of a biosensor (62).

Biosensors promise to provide a powerful and inexpensive alternative to conventional analytical strategies for assaying chemical species present in complex matrices. They do so by being able to discriminate the target analyte from a host of inert and potentially interfering species without the requirement for separating and, subsequently, identifying all the constituents of the sample.

The requirement for accurate and fast chemical intelligence is particularly conspicuous in human health care but is becoming increasingly important in several other areas like veterinary medicine, the agri-food, horticultural, pharmaceutical and petrochemical industries, environmental surveillance, defense and security. For example, it is now generally recognised, that inexpensive and reliable sensors for monitoring key metabolites, hormones, drugs, gases or ions in the ward, surgery, homework place, outpatients department and central laboratory are essential for the delivery of effective patient care. Biosensor technology is eminently

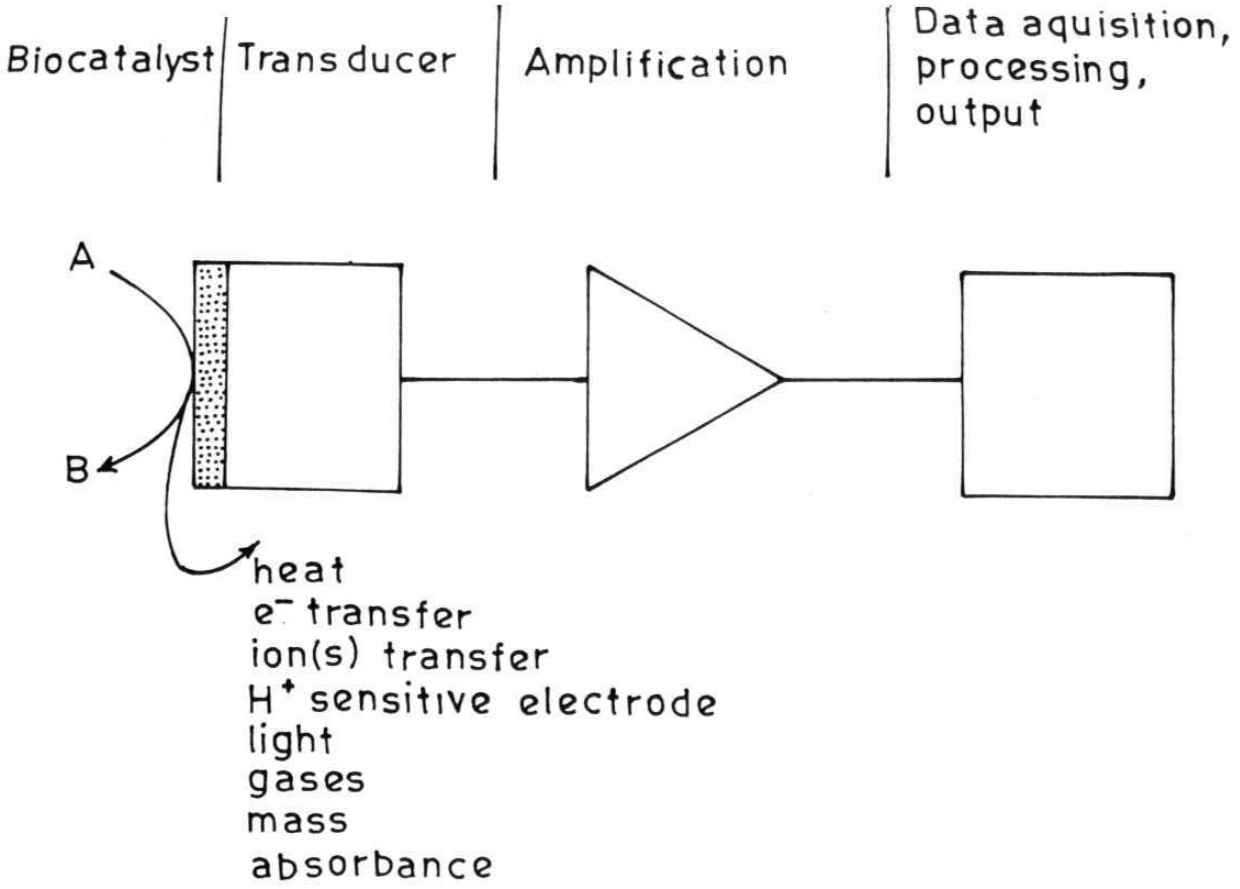


Fig. 1.6. Schematic of a generalized biosensor. The biocatalyst converts substrate A into product B with a concurrent change in a physico-chemical parameter which is converted into electrical signal by the transducer, amplified and suitably processed and outputted.

suitable for satisfying the needs of "alternate" site diagnosis and is particularly opposite in circumstances where there are advantages in obtaining immediate analytical results. For example, assessing cancer marker in tissue proximal to an excised tumor within an operating theater or in assessing the nature of the drug in patients suspected of an overdose. In circumstances such as these, the sensor output could be qualitative whereas for therapeutic drug monitoring where the "window" between the therapeutic and the toxic effects could be relatively narrow, the sensor must display high precision even at the expense of longer assay times. These differences in performance criteria for biosensors also extend to other parameter such as price. For example, biosensors designed for monitoring glucose in the home by diabetics will be extremely price-sensitive while similar devices for critical care units and industrial bioreactors could possibly be quite price-insensitive. Thus, the features required for particular sensors will depend on the individual application although in all cases the device should be sufficiently specific, sensitive and reliable to permit analysis of the target species (63).

7. EVALUATION OF CATALYTIC CMEs:

Three main properties are to be taken in to account when a catalytic CME is considered for performance:

a) The decrease of the overvoltage of the catalyzed reaction

b) The rate of its catalytic effect

c) The life-time and stability of the catalytic layer

advantages and Disadvantages of Biomolecules in CMEs:

7.1. **Advantages**

1) analysis is rapid

2) instrumentation is often simple, inexpensive and portable

3) optically clear solutions are not required (whole blood may be used)

4) analysis is simple

5) analysis is often non-destructive

7.2. Disadvantages:

1) primary calibration is not-possible or is difficult

2) junction potentials may vary between samples in indirect systems

3) analysis may be affected by ionic or chemical interference

4) response is usually non-linear (except for several amperometric methods)

5) response is sometimes slow, particularly at low concentrations, limiting the use of electrodes in automated analysis

6) some electrodes have a limited life or easily poisoned (2).

Many different biomolecules and transducer combinations have until now been developed to detect molecules ranging from hydrogen gas to large proteins. The majority of biosensors are based on the combination of immobilized enzymes and classical sensors such as photometers and amperometric or potentiometric electrodes. Table 1.1 shows classification of biosensors according to the type of transducer, and components that may be used to construct a biosensor(64).

8. OPERATIONAL CHARACTERISTICS OF ENZYME ELECTRODES OR BIOSENSORS:

An enzyme electrode operates via a five-step process (36):

1) the substrate must be transported to the surface of the electrode

2) the substrate must diffuse through the membrane to the active site

3) reaction occurs at the active site at a specific rate

4) the product formed in the enzymatic reaction is transported through the membrane to the surface of the electrode

5) product is measured at the electrode surface

Table 1.1. Classification of biosensors according to the type of transducer.

Biological elements	Type of transducer	Measuring principle or property measured
Organisms	Electrochemical	potentiometry
Cells		amperometry
Organelles		voltammetry
Tissues		impedimetric
Membranes		field effect (ChemFET, ISFET)
Enzymes	Electrical	surface conductivity
Enzyme components		electrolyte conductivity
Receptors	Optical	fluorescence
Antibodies		absorption
Antigens		reflection
Nucleic acids		luminescence
Organic molecules		light scattering
		evanescent wave
	Magnetic	paramagnetism
	Mass-sensitive	resonance frequency of a Piezoelectric crystal (PZ)
		Surface Accoustic Wave (SAW)
	Thermal	heat of reaction
		heat of adsorption

8.1. Important features of biosensors.

Several important features are given in Table 1.2 (65).

Generally speaking, when biological molecules interact specifically and reversibly there is a change in one or more physico-chemical parameters associated with the interaction and especially so if accompanied by catalysis. For example, there may be a change in proton concentration, release or uptake of gases (0_2, CO_2, NH_3), specific ions (NH_4^+, CN^-, I^-) heat, absorbance, mass, conductance and electron transfer which, if generated in close proximity to a suitable transducer, may be converted into electrical signals.

9. ELECTRON TRANSFER IN BIOLOGICAL SYSTEMS:

Redox enzymes are primarily responsible for biological electron transfer. Redox proteins are relatively small molecules and catalyze specific chemical reactions. They contain a redox active site, the role of which is simply to transfer electrons between biotransforming molecules. Example of such redox proteins are ferredoxin (an iron-sulfur protein), cytochrome C (a heme-protein), and plastocyanin (a Cu-protein)

Redox enzymes (oxidoreductases) also contain a redox active site but are usually of higher molecular weight than redox proteins. Their role is to catalyze the oxidation or reduction of a particular substance in cells by transferring H atoms, 0 atoms or electrons from one substrate to another. Oxidases transfer hydrogen, H, to 0_2, either two ($2H^+ + 2e^-$) or four ($4H^+ + 4e^-$) hydrogens forming H_2O or H_2O_2 respectively. Dehydrogenases transfer hydrogen to an acceptor other than molecular oxygen (NAD*, substrate). peroxidases transfer hydrogen to peroxides (H_2O_2 or organic peroxides).

Table 1.2. Important Characteristics of Biosensors (65).

Features	Requirement
Selectivity	Ideally specific, no matrix interference
Sensitivity	Linear concentration-response curves, ability to reliably detect less than 1% concentration change
Reversibility	Recovery of full analytical response within seconds of clean-up cycles
Detection limit '	Better than nanomolar for most biochemical analytes of interest
Response time	99% maximum signal development with in few seconds
Size	Miniaturized laminar flow systems generally provide improvement of response time and reversibility, possibility for implantation
Ruggedness	Insensitive to minor physical or electrical shock, no calibration drift
Biocompatibility	Desirable for *in vivo* use, and direct sampling" of biological fluids
Reliability	Calibrated system of minimal drift, with lifetime of months, should be easily used by untrained personnel
Cost	Low cost for disposable or continuous wide spread use, possible advantageous for lithographic technology
Signal recovery	Signal must be reliable, free from electrical or magnetic interference, easily transmitted

A great majority of the oxidases contain FAD. Some oxidases, with their respective prosthetic group(s) in brackets, are exemplified below:

Glucose oxidase	produces H_2O_2
Xanthine oxldase (FAD, Fe, Mo)	produces H_2O_2
Galactose oxldase (Cu)	produces H_2O_2
Lactate oxidase (FMN)	produces H_2O_2
Ascorbate oxldase (Cu)	produces H_2O_2

10. COFACTORS/COENZYMES

Oxidoreductases depend for their function on a non protein organic molecule or a metal ion, called coenzyme or cofactor. This cofactor can be either soluble or tightly bound within the enzyme and in the latter case it is called a prosthetic group. Coenzymes usually function as intermediate carriers of functional groups, atoms or electrons. In some cases the coenzyme is only temporarily and loosely bound to the enzyme and is therefore sometimes viewed as a co-substrate, the concentration of which can limit the rate of the reaction catalyzed by the enzyme. The following scheme, exemplified in Fig. 1.7 with an oxidation reaction of a substrate, describes how the electron flow can take place in an enzyme catalyzed reaction.

Dehydrogenases are unaffected by molecular oxygen and they can have either soluble cofactors such as $NAD^+/NADH$, $NADP^+/NADPH$ (Fig. 1.8(a)) or bound cofactors, FMN, FAD (Fig. 1.8(b)), PQQ, (pyrrolino-quinoline quinone) and other Heme containing cytochromes.

The cofactors in the oxidases are strongly bound (prosthetic group) within the enzyme structure, e.g. FMN, FAD or metal containing structures (e.g. Cu, Mo, Fe). The prosthetic group of the vast majority of the peroxidases is protoporphyrin IX containing an iron

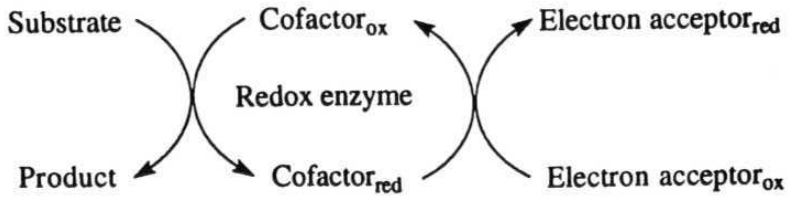


Fig. 1.7. The electron flow in an enzyme catalyzed reaction.

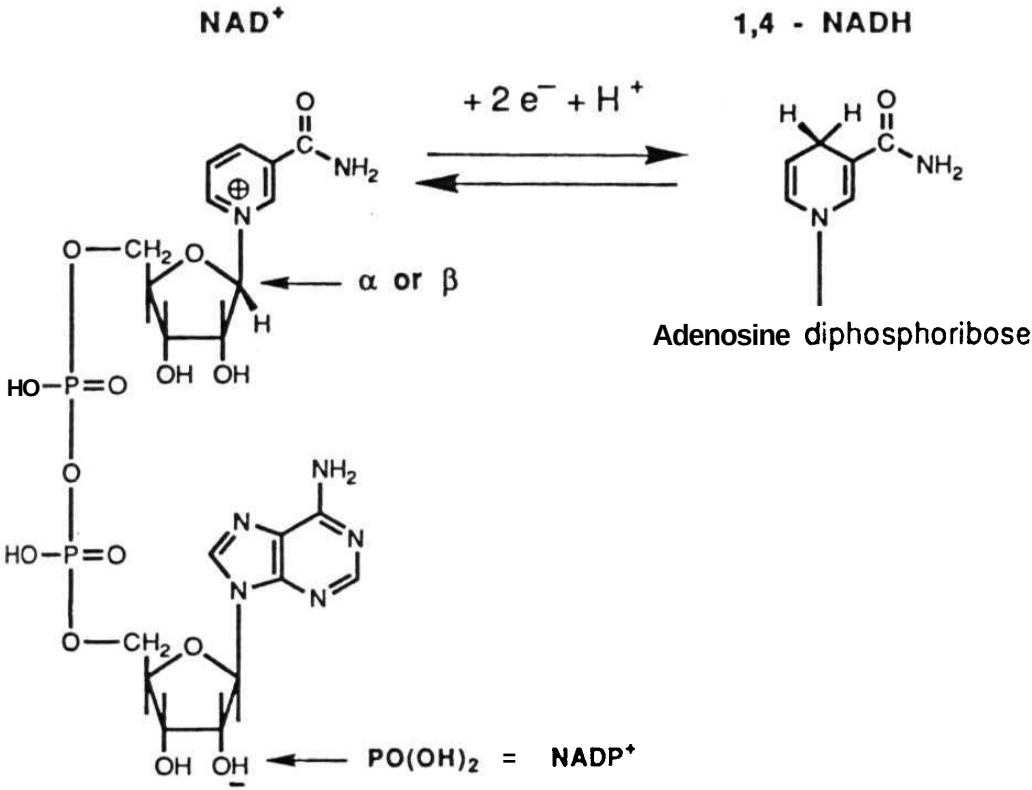


Fig. 1.8(a). Structural formulae (β-form shown) and overall redox reaction of nicotinamide adenine dinucleotide, NAD . and its reduced form, the enzymatically active 1,4-NADH.

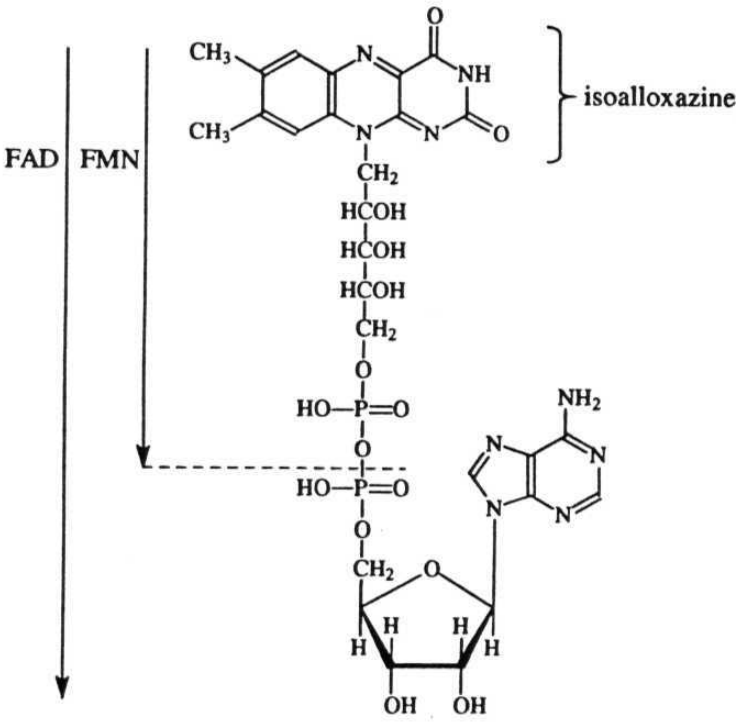


Fig. 1.8(b). Structures of flavin nucleotides.

atom. The porphyrin skeleton provides four ligands for the iron and is equivalent with the porphyrin moiety present in hemoglobin, cytochromes, vitamin B and chlorophyll, although the attached substituents may differ and in vitamin B and chlorophyll the central ions are Co and Mg respectively (Fig. 1.9).

11. THE BIOCATALYST:

A key feature of the biosensor is the interface between the biochemically sensitive coating and the transducer and an intimate contact is generally achieved by immobilizing the biocatalyst at the surface of the transducer. The substrate(s) to be determined diffuses through the membrane (M) into the enzyme layer where the enzymatic reaction occurs.

$$S + R \longrightarrow P + R'$$

Producing a product (P or R') or consuming a coreactant (R) which can be measured electrochemically. The coreactant could be oxygen or a cofactor such as NAD(H). A steady state electrochemical signal is reached after a short delay, when the rate of product formation equals the rate at which the product diffuses out the enzyme membrane. Alternatively, a kinetic measurement can be used in which the rate of change at the electrochemical signal is monitored as a function of time.

12. **TRANSDUCER:**

It is physicochemical device that transforms one form of signal into another. A photo cell that converts light energy into electrical energy is an example. The ideal transducer should display a reasonably fast response time, be amenable to facile fabrication and of miniaturization, be reliable and should compensate for adverse environmental effects such as temperature dependency and drift.

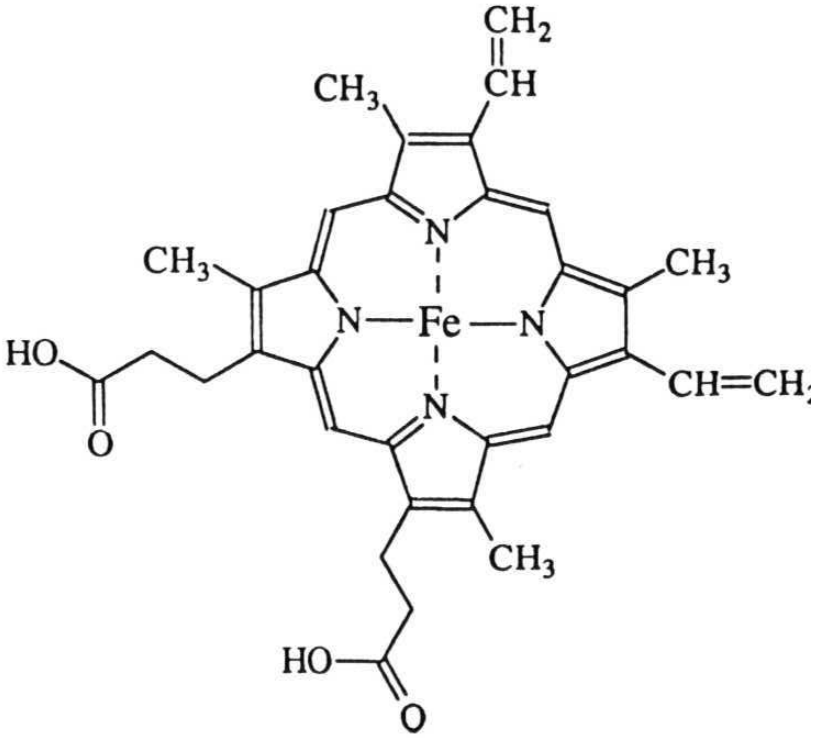


Fig. 1.9. Structure of protoporphyrin IX.

12.1. Electrochemical Transducers:

Electrochemical transducers are essentially of two types, Potentiometric and Amperometric electrodes.

12.1.1. Potentiometric Transducers

Potentiometric electrodes such as ion selective electrodes, produce a voltage which is dependent on the logarithm of the concentration of a selected ionic substance. The pH electrode, which measures the activity of hydrogen ions, is the classic example of an ion selective electrode (Table 1.3). Electrodes that are selective to a wide range of other ions have also been commercially available for a number of years. The limitations of such transducers are their relatively large size in some applications, their voltage drift and their fragility. Most of the devices contain an inner electrolytic solution separated from the test solution by a thin ion-selective membrane based on glass or plasticized polyvinyl chloride, and are therefore not robust enough to be used in many industrial applications. Attempts are being made to develop small solid state transducers whose readings will be sufficiently stable and reproducible so that they will not need frequent calibrations.

12.1.2. Amperometric Transducers

Amperometric transducers are based chiefly on chemically inert materials such as platinum, gold, or carbon (Table 1.4). If an electrode composed of one of these substances is kept at a selected electrical potential with respect to the test solution it will oxidize or reduce chemical substances in the solution. An electrical current is thus produced whose magnitude is related to the concentration of the substance in question. The voltage dependence of the electrochemical reaction gives a certain degree of selectivity.

Table 1.3. Potentlometrlc biosensors.

substrate	enzyme	product(s)	range	references
aspartam	L-aspartase	NH_4^+	0.1-0.6mmol L^{-1}	(66)
fats	lipase	fatty acids	5-50 10^{-6}mol L^{-1}	(67)
glucose	glucose oxidase	gluconic acid	0.2-2gm L^{-1}	(68)
urea	urease	NH_4^+, CO_2	0.01-10mmol L^{-1}	(69-71)
nitrite	nitrite reductase	NH_4^+	10^{-3}mol L^{-1}	(72,73)
penicillin	pencillinase	H^+	0.2-70mmol L^{-1}	(74)

Table 1.4. Amperometric biosensors.

substrate	enzyme	products	range	references
alcohol	dehydrogenase	NADH	10-150mmol L^{-1}	(75,76)
malate			5-100 10^{-6} mol l	
formiate				
choline	choline oxidase	H_2O_2	500mmol L"	(77)
glucose	glucose oxidase		0-7g L^{-1}	(78-84)
glutamine	glutaminase	"	0-25mmol L^{-1}	(85)
hypoxanthine	xanthine oxidase		4-180 x 10^{-6}mol L^{-1}	(86,87)
oligo-	glucoamylase	"	0.1-2.5mmol L^{-1}	(88)
saccharides	glucose oxidase			

However, other means of discriminating against interfering substances are also often essential for proper performance of an amperometric transducer, such as the introduction of semipermeable membranes and so called redox mediator substances. Considerable progress has been made in the area of modified electrodes, where mediators, promoters and electrochemically active materials are attached to the electrode surfaces.

12.2. BIOSENSORS based on other non-electrochemical transducers:

12.2.1. Heat Measurements

Enzymatic reactions are often accompanied by a considerable heat evolution, generally in the range of 25 to 100 kJ mole^{-1}. Thus, enzyme calorimetry is a highly versatile technique, which can be the basis for a workable biosensor. Almost any enzyme system can be used to fashion the biosensor, since most enzyme-substrate reactions will give a measurable heat change. Excellent reviews on the theory and applications of calorimetric sensors have been written by Danielsson (89).

12.2.2. Optical Methods

Optical methods are defined as those which are based on the measurement of the absorption or emission of electromagnetic radiation by either a reactant or product of the biological system. Spectrophotometric methods have been used to construct biosensors since 1966. In this first paper a column packed with immobilized enzyme was prepared, and the absorbance of the product was measured. The real advances in the field came from the work of Hornby, Hovarth, Sundaram and Camblaghl, who constructed nylon colls with immobilized enzymes, which could be coupled directly to flow injection or bubble (Technicon) analysers (90).

12.2.3. Piezoelectric (PZ) and Surface Accoustic Wave (SAW) Detectors:

The piezoelectric crystal is a piece of oscillating quartz, onto which is placed on an adsorbent that selectively interacts with the analyte compound. The basic frequency of oscillation of the crystal, generally 9 or 14 MHZ, is changed by the adsorption, due to a resulting mass increase on the crystal.

A frequency change qualitatively indicates the presence of the analyte, and the total frequency change, ΔF, is proportional to the amount of analyte, according to the basic equation developed by Sauerbrey:

$$\Delta F = -K\Delta mass$$

selectivity is developed by the coating, and sensitivity is characteristic of the crystal. The limit of detection in this technique is estimated to be about 10 g.

In a similar fashion a surface accoustic wave (SAW) detector can be used. The oscillation of the crystal in this case, is at a higher frequency, several hundred MHz instead of 9-14 MHz.

King first reported the use of a piezoelectric crystal as a detector (91). Excellent reviews have been written by Guilbault and others (92-94). Most previous analytical work with such detectors has centered on the use of inorganic and organic coatings for gaseous environmental pollutants, such as HCl, isocyanate, CO, NO , SO , etc.

12.2.4. The Chemically Sensitive Field Effect Transistor (CHEMFET):

This was born of the integration of two technologies- the solid state integrated circuit and ion-selective electrodes. The first CHEMFET was demonstrated by Bergveld who used (95) a silicon dioxide layer to

impart sensitivity to hydrogen ions upon an insulated gate field-effect transistor. Several reviews of this field have appeared (96-98). The primary advantages of the FET device is its small size. An enzyme sensitive field effect transistor (ENFET) can be fabricated by applying a thin layer of enzyme gel over the ion selective membrane. Thus a miniature biosensor can be easily obtained.

13. MICROBIAL BASED BIOSENSORS:

The majority of biosensors based on immobilized microorganisms function with amperometric oxygen sensors as transducers. Biosensors for the determination of phosphate, nitrate, nitrite, sulfite, methane and phenol have recently been described, but none has yet achieved a commercial breakthrough. It is more difficult to devise selective sensors that use microorganisms to respond to a variety of different substances. On the other hand, there is a number of rather 'fastidious' microorganisms which are highly specific. An example of exploitation of such species is a sensor for urea constructed by the Tokyo Institute of Technology group in which both urease and Nitrosomonas are immobilized on an oxygen electrode. The microorganism is highly specific in its response to ammonia produced by the enzyme in the presence of urea. This gives improved selectivity compared with that of the enzyme alone immobilized on an NH_3-selective electrode although response time is considerably longer.

Whole organism electrodes usually exhibit considerably longer response times than do enzyme electrodes commonly being in the range 1-20 min. whereas many enzyme sensors respond typically within less than 1 min. There are great differences in stability between different whole organism sensors although some, e.g., a methane biosensor can be made stable for many months (99).

14. BIOSENSORS BASED ON TISSUE SECTIONS OR CELL CULTURES:

Not only isolated enzymes but also entire groups of intact cells can be used as the basis for the biosensors (Table 1,5). Compared with the isolated enzymes, these often have the advantage of being active for longer periods, as the enzymes are kept in their natural environment. In this case additional immobilization step for the coenzymes or cofactors is not necessary.

The principle of these devices can be explained by talcing as an example a biosensor for H_2O_2. As bovine liver contains relatively high concentrations of the enzyme catalase, a section of fresh tissue only 0.1mm thick is immobilized with nylon mesh on the surface of a membrane-covered O_2 electrode. The hydrogen peroxide whose concentration is to be determined undergoes enzymatic decomposition to oxygen and water, and the oxygen thus produced is measured amperometrlcally. This sensor is less sensitive to fluctuations in temperature and pH, and has a longer lifetime than a biosensor containing an isolated enzyme (100).

15. BIOSENSORS BASED ON RECEPTORS:

A very promising approach is the development of biosensors in which individual receptors or receptor structures from living organisms are immobilized on transducers. The advantages of receptors over enzymes, for example, lie in very high sensitivities and short response times. However, the problems of isolating receptor molecules and of their instability when immobilized are still so great that this idea must be regarded at present as an interesting approach with potential for future developments.

A sensor for the determination of glucose which is based on a receptor protein has developed by Schultz and Meadows (40). This makes use of

Table 1.5. Biosensors based on tissues and related materials.

substrate	biocatalytic material	sensing element
Glutamine	Porcine kidney cells	NH -sensor
Adenosine	Mouse small intestine mucosal cells	NH -sensor
Adenosine 5 -mono phosphate	Rabbit muscle	
	Rabbit muscle acetone powder	
Guanine	Rabbit liver	"
Hydrogen peroxide	Bovine liver	0 -sensor
Glutamate	Yellow squash	CO -sensor
Pyruvate	Corn kernel	CO -sensor
Urea	Jack bean meal	NH -sensor
Phosphate/fluoride	Potato tuber/ glucose oxidase	0 -sensor
Dopamine	Banana pulp	0 -sensor
Tyrosine	Sugar beet	0 -sensor
Cysteine	Cucumber leaf	NH -sensor
Glutamine	Porcine kidney mitochondria	NH -sensor

the competition between glucose and a glucose analogue, a dextran labelled with FITC for binding sites on the glucose receptor protein concanavalin A labelled with Rhodamine (RH-Con A). FITC-dextran and RHh-Con A are sealed in a length of dialysis tube at the end of a bifurcated light guide with a diameter of 100μm. Light of a suitable wavelength excites FITC, which fluoresces. If the FITC is connected via the dextran to the Rh-Con A, the energy absorbed by the FITC is transmitted to the rhodamine, which then also fluoresces. If glucose diffuses through the dialysis membrane into the measuring cell, the dextran is partly displaced from its binding sites by the glucose and the measured FITC fluorescence increases, because less energy is transferred from FITC to rhodamine. From increase in fluorescence intensity the glucose concentration can be determined. Acetyl choline (Ach) receptor protein was isolated from Torpedo electric organ was incorporated into asolectin liposome and immobilized non covalently on the surface of a planar interdigitated capacitive sensor to produce an Ach receptor-based biosensor **(41).**

16. APPLICATIONS OF BIOSENSORS:

16.1. Clinical Chemistry and Diagnosis:

A rapid test for substances such as amylase, glucose, paracetamol, sallcylate, creatlne kinase, aspartate amino transferase, creatinine, and urea would be invaluable in emergencies. Biochemical measurements of substances such as lactate, glucose, urea and creatinine would be useful for continuous use in intensive care units.

16.1.1. AIDS:

H. Worf (Germany) has described a biosensor based on piezoelectric quartz crystals for direct detection of HIV specific antibodies. **For this** purpose, the oscillator crystal is coated with **peptide-antigens** derived from the core protein p24 and the **glycoprotein** gpl20 of HIV.

The protein p24 forms the nucleocapsid of HIV and is synthesized as a precursor molecules together with pl7 and pl5. Antibodies against p24 are of particular importance for diagnosis of the early phase of infection. The envelope protein gpl20 of the HIV virus is responsible for the specific binding of HIV to the CD4 receptor present on the surface of T-helper lymphocytes mediating the virus into the cell and elicitates neutralizing immune response.

16.1.2. Glucose sensor:

A majority of biosensor researchers both in academia and industry have concentrated their development efforts on one goal: the commercialization of a portable glucose sensor for use by diabetics. Many diabetics currently use blood glucose testing strips and meters and they are willing to switch to biosensors provided the units are fast, reliable and cheap. Considerable academic and commercial interest also exists in producing a continuous glucose sensor for *in vivo* monitoring and for automated blood gas and electrolyte analysers.

16.2. Agriculture / Veterinary:

The diagnosis of infectious diseases in crops and live stock will be the primary application of biosensors in agriculture. These devices will be used by farmers for rapid, single-use, on-site testing. Of secondary importance will be soil testing for microorganism, plant nutrients, and pesticides. For example, monitoring the soil nutrient content will assist farmers in the optimum application of chemical fertilizers.

16.3. Food:

People are increasingly concerned about the content of the food eaten. They want to be informed about the levels of additives, growth hormones and cholesterol in food. Contamination in the food

production chain, such as the presence of Salmonella in the poultry and egg industry, is spurring the need for faster and more sensitive testing instrumentation. Also, the consumer demand for fresh food and the industry's desire to reduce spoilage, will make food testing one of the more interesting biosensor application field.

16.3.1. Food Processing

Modified human blood glucose sensor can be used to monitor glucose levels in potato and snack food manufacture. The sugar content of fruit and vegetables is an important indicator of ripeness and an important indicator of browning and sweetening properties. Several groups have explored the possibility of measuring sugars and alcohol in fruit juices. The major problem remains interference from components such as ascorbic acid. Other substances under investigation includes devices for monitoring lactate, sulfur dioxide and aldehyde, the alternative for monitoring rancidity of oils and fats. Cranfield Institute of Technology have patented the concept of the organic phase enzyme electrode which can be used to detect peroxides in inorganic solvents and may also be useful in detecting rancidity in oils and fats.

16.3.2. Food Testing

The Leatherhead Food Research Association (LFRA), U.K., in collaboration with Cranfield Institute of Technology has developed a device for measuring the shelf-life of a chilled meat joints and carcasses. The unit senses the depletion of meat glucose by bacterial surface flora during chill storage. The point when this glucose reduction starts is approximately two days before the onset of actual spoilage in pork, lamb and beef. The LFRA/ Cranfield team designed a simple, four-point electrode array which monitors glucose concentration levels between the meat's surface and at a depth of 8mm. The disposable knife-head contains the sensor and plugs into a hand

held instrument. In the handle of the instrument is a "traffic-light" indicator. A green signal indicates that the meat is fresh, amber means that spoilage will commence within two days and a red light warns that the meat is no longer edible. Pegasus (Canada) offers fish freshness meter to determine the state of any seafood.

16.3.3. Toxin Testing

The LFRA is developing immunosensors for identifying salmonella, listeria, enterotoxins and moulds. The salmonella detector is the most advanced effort. The projects goal is to devise a system which will reduce the detection time from 7 days to just 8 hours. A surface plasmon resonance (SPR) device is employed. The greater sensitivity of SPR Technology will dramatically shorten the time required to culture the salmonella. The LFRA device will be able to detect the 1000 organisms/ml, three order's of magnitude better than existing equipment.

16.4. Environmental/Military:

In many ways biosensor requirements for environmental and military applications are quite similar. The detection principles for industrial gases are the same as for mustard gas and nerve agents. Monitoring hydrocarbon emissions from a chemical plant are not fundamentally different from monitoring diesel fumes from a tank.

16.4.1. Explosives

Workers at Cranfield Institute of Technology have investigated monoclonal antibodies for TNT used in conjunction with a piezoelectric quartz crystal biosensor. The sensitivity obtained was however, insufficient for security applications even with a significant preconcentration phase.

16.4.2. Microorganism

There is an interest in adapting medical technology such as the fluorescence capillary fill device and surface plasmon resonance based sensors for detecting bacteria in the environment.

16.4.3. Toxic Gases

Imperial College, London is working "Chemical Canaries", biosensors which detect and measure traces of toxic gases, such as hydrogen cyanide and hydrogen sulfide. The enzyme used is cytochrome oxidase. Each molecule of toxic gas inactivates one enzyme molecule. Since one enzyme molecule normally reacts with over one thousand substrate molecules every second, the system is very effective in amplifying the effects of toxic gases and is therefore very sensitive in detecting them. Cranfield Institute of Technology and Sutherland Polytechnic have published work on a carbon monoxide sensor using carbon monoxide oxidoreductase in a ferrocene-mediated format.

16.4.4. Water pollutants

Environmental concerns are fueling research into biosensors capable of detecting toxic chemicals, metals and pesticides in water. Several enzyme biosensors have been developed to detect phenol in sewage.

Nitrilotriactetic, which is used as a component of detergent formulations, is detected with whole cells in conjunction with an ammonia electrode. However, this biosensor is subjected to interference from other non-toxic compounds.

Biosensors have also been developed for monitoring acids, alcohols, nitrates and ammonia. Butyrylcholine esterase immobilized on a glass

electrode has proven effective in monitoring low concentration of organophosphorous pesticides in river water.

One of the most advanced amperometric biosensors for detecting herbicides in drinking water supplies has been developed at the Luton College of Higher Education (Luton, U.K.) in cooperation with the Water Research Council and Cranfield Institute of Technology. The device uses the photosynthetlc cyanobacteria to a number of herbicides, including atrazine and metoxuron. The major limitation at this device is its short operating life less than two weeks with constant use.

16.5. Process Control and Monitoring:

There are several potential applications of biosensors in the industrial fermentation processes for monitoring substrate levels, product formation and microbial biomass. Currently there are several off line methods but these are both cumbersome and time consuming since samples have to be withdrawn for analysis. The development of biosensors for fermentation is however hampered by the need of many food and pharmaceutical fermentations for steam sterilization to prevent microbial contamination.

16.5.1. Antibiotic production:

Penicillin sensors have been the subject of several papers. These have been based mainly on pH electrodes coupled with pencillinase. These devices are still at an experimental stage.

16.5.2. Chemical processes:

Only a few experimental biosensors have been developed to monitor chemical processes. Most applications involve aqueous solutions, but Cranfield Institute of Technology has designed and patented enzyme electrodes for operation in organic solvents expanding monitoring

possibilities to the petrochemical and pharmaceutical Industries. In one example, polyphenol oxidase was immobilized at an amperometric electrode for the detection of phenols directly in chloroform.

16.5.3. Fermentation:

Cranfleld Institute of Technology has developed a probe for measuring glucose directly inside the fermentation vessel. The biosensor had a useful life time of between 2 and 14 days and a response time of approximately 60 sec.

The yellow springs 2700 (USA) is an amperometric biosensor detector instrument is available in the market. It is a microprocessor controlled unit, that permits application of any potential to a biosensor and can be used for assay of glucose, lactate, lactose, sucrose, ethanol, starch and galactoase in fermentation broth and all culture extracts.

An autosampler for the sterile determination of glutamine, glucose and oxygen in animal cell culture has been developed by Jean Romette and is patented by the MIT (Cambridge, USA). In one configuration a membrane is immobilized with glutaminase and glutamine oxidase. L-glutamine is determined by the product produced by the two stage enzymatic reaction.

17. IMMOBILIZATION OF BIOLOGICAL COMPONENTS:

The most common biological components used in biosensors are enzymes, hence the discussion will focus mostly on their immobilization. However, many of the methods and techniques are generally applicable to most biocomponents.

For practical and fundamental reasons, the use of immobilizsed enzymes that are confined close to an electrode surface offers several

attractive features when compared with conventional methods. When the enzyme is immobilized, it can be readily separated from the reaction mixture and easily reused. Furthermore, by appropriate control of the mlcroenvironment at the immobilized enzyme, more desirable properties, such as increased stability, high sensitivity, greater response, high sampling rate (in flow injection analysis), great flexibility in the choice of electrode dimensions and shapes (allowing easy miniaturization or use of complex surfaces, such as reticulated vitreous carbon), and prevention of electrode fouling and interferences from other species in solution can be achieved.

There are several ways to immobilize a biocomponent onto the electrode surface (Fig 1.10). The most common techniques include (1) adsorption, (ii) covalent attachment, (iii) gel/polymer entrapment and (iv) cross linking.

Several factors must be considered before choosing an immobilization technique:

i) applicability for a variety of surfaces

ii) the biocomponent should exhibit high activity in its immobilized state

iii) pH range for desirable activity for the immobilized biocomponent

iv) the need for greater stability (half-life) after immobilization

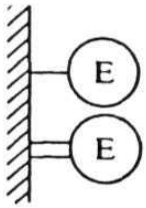
v) the ability to co-immobilize more than one biocomponent

vi) the necessity for no or little leakage of biocomponent

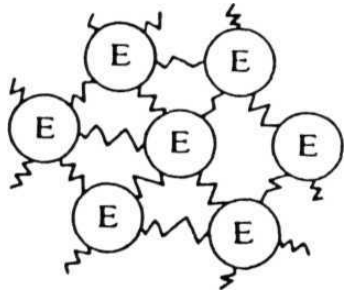
vii) sufficient surface loading for operation (103).

17.1. Adsorption:

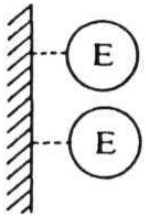
Physical adsorption of the biocomponent on the electrode surface is a popular technique. The advantages of physical adsorption are that it requires virtually no reagents, minimal activation and clean up steps are needed, and less disruption to the enzyme occurs than with other, generally harsh chemical methods. It suffers from greater susceptibility to changes in pH, temperature, ionic strength, and



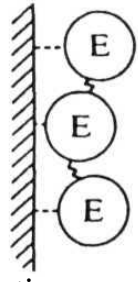
Covalent binding



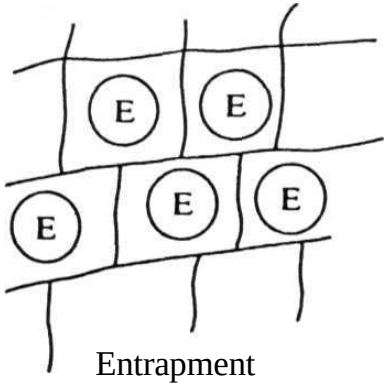
Cross-linking



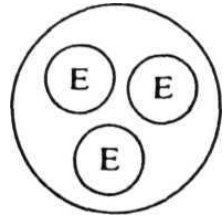
Adsorption



Adsorption-cross-linking



Entrapment



Micro-encapsulation

Fig. 1.10. Methods of enzyme immobilization.

substrate; it also requires considerable optimization. This method is not widely applicable because of the possibility of the biocomponent leaching out of the electrode surface, but this can be minimized by cross linking.

Physical adsorption of an enzyme onto an electrode is usually done by dipping the clean electrode in a buffered solution containing the enzyme of interest. It is common to do the adsorption at 4° C, so the enzyme does not thermally degrade.

Ikeda and coworkers (104) have adsorbed glucose oxidase on the surface of carbon paste electrodes by solvent evaporation followed with a collodion ethanol treatment. The collodion ethanol forms a thin nitro-cellulose film on the surface which helps to keep the enzymes on the surface. The electrode retained its original response after one week and could be operated in the pH range 5-8.

Gorton et. al. (105) have shown, with meladola blue, that the order of adsorption of mediator and enzyme can affect the electrode response, when the mediator is adsorbed first followed by the enzyme, a greater response to glucose can be achieved with less noise, than if the reverse is done.

Suad-Chagny and Gonon (106) used a carbon mlcroelectrode for the adsorption of lactate dehydrogenase, in an electrochemically deposited protein sheath of BSA. The sensor has a stable and reproducible NADH response for about 150 hrs, which is an improvement over previous sensors.

17.2. Covalent attachment:

Covalent attachment to an electrode surface is more difficult than adsorption, but it provides a more stable immobilized biocomponent, and is more widely applicable. Many factors must be taken into

account when covalently attaching a biocomponent to a surface. The attachment often involves three steps: activation of the support, enzyme couplings and removal of loosely bound enzyme.

Activation of the support is usually accomplished with chemicals such as sllanes **(107)** or cyanuric chlorides. The enzyme is then attached to the activated surface directly or is linked to a bifunctional agent which has been attached to the surface. Both of these methods have been shown to be effective procedures for covalently attaching enzymes to the surface of electrodes.

Pretreatment of platinum electrodes has an effect on the covalent attachment of enzymes (108). Thomas and co workers have shown that appropriate pretreatment before immobilization enhances electrode response and life time. The effect on electrode life is apparent between anodized and thermally oxidized platinum, and the effect on response between anodized platinum and anodized platinum black.

Narasimhan and Wingard (109) have developed a method for the covalent attachment of glucose oxidase which uses a (aminophenyl boronic acid) modified glassy carbon electrode as a support. This attachment has been reported to allow direct electron transfer between the enzyme and the electrode, although, due to high noise, no catalytic current has been observed for the sensor.

17.3. Gel/Polymer Entrapment:

By far the most popular technique is the use of gel/polymer entrapment. This technique uses mild conditions, and wide variety of gels/polymers are available. Other advantages are that most biocomponents can be easily incorporated into a polymer, the entrapment can be improved by using other immobilization techniques, such as covalent attachment and cross-linking and a high concentration of active biocomponent can be immobilized.

As with any technique, it suffers from several disadvantages and many experimental factors must be controlled. Deactivation of the biocomponent by radicals created during polymer formation is possible. The success is limited by the size and structure of polymer to relatively large species and there is an increase in response time due to a large diffusional barrier. One of the most important problems with this technique is the common use of physical, rather than chemical methods for confining the gel/polymer to the electrode surface.

Foulds and Lowe (110) developed a very interesting method of immobilizing glucose oxidase onto electrodes. Their method involves the co-immobilization of GOD and polypyrrole onto a platinum electrode, which takes place at a pH above the isoelectric point of polypyrrole, and the electropolymerized polypyrrole film builds up an anionic charge. Although the exact mechanism is unknown, this anionic charge may electrostatically entrap the enzyme in the film. Electropolymerized polyaniline films have also been effective. Aizawa and co workers (111) have shown that the electropolymerization of aniline, in the presence of glucose oxidase can be effective way to entrap GOD, and also provide protection from larger interferences.

17.4. Cross linking:

Physical adsorption and gel/polymer entrapment are often supplemented with cross linking to prevent leaching of the biocomponent. Cross linking alone can sometimes also be used for immobilization to an electrode surface.

The difficulty with this method is that conditions for membrane formation are not easily determined, and must be carefully controlled. One must control the pH, ionic strength, temperature, and reaction

time. Wingard (112) has shown that the thickness and glutaraldehyde content of cross linked membranes can have a significant effect on the response of the sensor. Also, bifunctional agents may not be selective, and could bond intra as well as intermolecularly.

18. ENHANCEMENT OF ELECTRODE RESPONSE:

Enhancement of the electrode response has taken a variety of forms to solve a diversity of problems. Electron mediators are used to shuttle electrons between the electrode and a species in solution, which is difficult to electrolyte directly. The use of new electrode materials, such as organic metals, have improved electrolysis.

18.1. Electron Mediators:

Electron mediators are used for various reasons. Amperometric biosensors, that used oxidoreductase enzymes, normally rely on oxygen as an electron acceptor to recycle the enzyme after conversion of the substrate to the product. The requirements on an ideal mediator are the following (Fig. 1.11):

1) Well defined electron stoichiometry (n value)

2) a formal potential E, near or below 0 mV vs SCE for an oxidation process and near or above 0 mv Vs SCE for a reduction process.

3) Fast heterogeneous (at the electrode surface) and homogeneous (with the enzyme) electron transfer.

4) Stable in both oxidized and reduced forms.

5) no auto oxidation (by 0) of the reduced form

6) regeneration independent of pH

7) non-toxic

8) poor solubility in the surrounding medium (immobilized mediators).

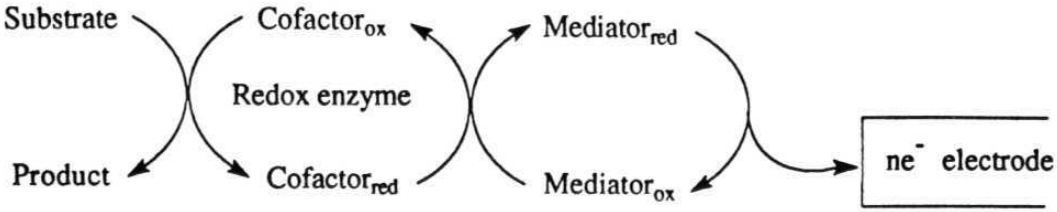


Fig. 1.11. The sequential electron transfer at a mediator/enzyme modified electrode.

There are several mediators which can be used but ferrocene and its derivatives have received most attention. Ferrocenes are popular because they are hydrophobic, exhibit good electrochemistry, and can be structurally altered to change their redox potential. Hydrophobicity is important because it prevents the mediator from leaching from the electrode surface when used in aqueous systems. Green and Hill (113) have noted that positively charged ferrocenes are much better mediators for glucose oxidase than negatively charged ferrocenes. This research shows how the structure can be altered to facilitate a specific approach. Also, ferrocenes can be made to act as electron donors or acceptors.

Ferrocene modified polypyrrole has been used to incorporate a mediator and enzyme attachment into one step. As noted previously, Foulds and Lowe (114) have been able to immobilize enzyme by entrapment in electropolymerized polypyrrole. They also found that this can be accomplished with ferrocene modified polypyrrole. The ferrocene modification allows the polymer to act as an electron mediator. A disadvantage is the markedly slower electron transfer that occurs at the modified polymer compared with soluble ferrocene, which could be due to the limited mobility of the immobilized ferrocene.

Mizutani and Asai (115) have used a combination of polymer films and polymerization methods to create a glucose-sensing biosensor. The work illustrates that combinations of polymers may be effective. They have shown that a mixture of electrically conducting polypyrrole and insulating polyvlnyl alcohol, with enzyme and ferrocene present, creates a stable sensor which shows an increased response for glucose. The sensor has increased stability compared with using polypyrrole alone and increased response over the use of polyvinyl alcohol alone.

The use of meladola blue as a mediator has been shown by Gorton and co-workers (105). This mediator adsorbs spontaneously to graphite or carbon, and oxidizes reduced NADH at about 0.0 V (Vs SCE). The

mediator does not depend on diffusion for charge transfer, and the molecules of meladola blue must be in contact with each other, so mediation can be achieved by intermolecular electron transfer. Unfortunately, the mediator coverage decreases by 20-30% over the course of a day, but the decrease in response is small for high mediator coverages.

19. ORGANIC METAL ELECTRODES:

Organic metals can be used as mediators, for a variety of enzymes, or as the electrode material itself. Kulys (116) has shown that these types of electrodes can transfer electrons in two ways: for cytochrome b , direct electron transfer takes place but for oxidases mediation occurs. Mediation is accomplished by the dissociated ions of the organic metal. The organic metal electrodes which were constructed could be operated at 0.05V (vs Ag/AgCl), which greatly reduce the possibility of interferences. The efficiency of these electrodes is determined by the catalytic activity of the enzyme, and the electron exchange rate between the enzyme and the organic metal.

Albery and Bartlett (117) have shown that organic metal electrodes made from N-methyl phenazinium tetracyanoquinodimethamide (NMP^+, $TCNQ^-$) are also useful for the oxidation of NADH. This is a good electrode material with rapid electrode kinetics. It shows no deterioration of response for days and has a stable potential range from -0.1 to 0.3 V (vs SCE) over which the oxidative current of NADH is virtually independent of potential. Its surface can be renewed, in a controlled fashion by sweeping the potential outside of the stable range, which dissociates the organic metal, causing it to dissolve in solution, leaving a new layer of organic metal.

20. REMOVAL OF INTERFERENCES:

Ascorbic acid, uric acid, tyrosine, cysteine, bilirubin, glutathione, catecholamines, and acetaminophen (paracetamol) are examples of easily oxidized compounds often present in biological samples and may therefore interfere with the electrochemical signal. Proteins, e.g., albumin, and other macromolecules can adsorb on the electrode surface forming a diffusion barrier for the analyte and possible reagents. An interfering compounds can interact with the biochemical reaction, i.e., inhibiting or activating enzyme catalysis (e.g. metal ions), acting as a false substrate or as an alternative electron mediator. Electrodes can be protected by several methods including chemical removal of interferences and the use of polymer films as barriers. Polymer films have emerged as the predominant method because they provide protection against both fouling and interferences. The major advantage is that most of these polymer films are attached physically, which is not suitable for all chemical approach to biosensor construction.

Fouling, which is passivation of the electrode surface by the adsorption of non-electroactive species, is a major problem in the analysis of biological samples. High relative molecular mass species, such as proteins, are a major source of fouling in these cases. Electrode fouling results in a decreasing biosensor response over time.

The most common way of minimizing interfering reactions in amperometric sensing is to design an electrode with high activity within the optimal potential range (-200 to OmV Vs SCE), where most compounds are electrochemically inactive. Electrochemical interference can be measured and subtracted from the signal by the use of parallel equally prepared electrodes, one of them with denatured enzyme or no enzyme at all (118). However, differential measurements cannot eliminate biochemical, chemical or electrode fouling

interferences. Yao et al immobilized GOD together with benzoquinone as electron mediator in carbon paste electrodes (119). The electrode was first covered with a nitrocellulose film and then with a gold net, the latter with an applied potential of +0.5 V Vs SCE. Ascorbic acid and Uric acid were oxidized and thereby removed at the gold net, whereas glucose could pass and reach the enzyme. Ascorbic acid was effectively repelled from the electrode surface of an GOD and ferrocene modified carbon past electrode through the incorporation of negatively charged stearic acid into the paste (120). Another method for the removal of ascorbic acid is to co-immobilize the analytical enzymes and ascorbic acid oxidase, the latter producing an electrochemically inactive derivative of ascorbic acid and H 0. The influence of electrochemical interferences on the background current can be eliminated through measuring the initial rate (current increase) instead of the steady state current, as suggested by Guilbault et al (121). Palleschi et al (122) investigated the influence of possible interfering compounds on the oxidation of H 0 at different pH. Unfortunately, the oxidation of the investigated compounds displayed the same dependence on pH in the solution on as the oxidation of H 0 and could therefore not be eliminated through a pH shift. However, the concept could be advantageous for the reagentless HRP modified electrodes. HRP is highly active between pH 4 and 5 where as the rate of the electrochemical oxidation of e.g., ascorbic acid is very slow at low pH. Nevertheless, the optimal conditions for an electrode bearing HRP co-immobilized with an oxidase are often obtained at a somewhat higher pH due to the pH dependence of the oxidase.

20.1. Membranes:

An extended usefulness of chemically modified electrodes including biosensors, as selective, analytical devices has put demands on the application at some sort of membrane covering the electrode surface bearing the catalytic sites. The membrane can have one or several of the following functions

i) entrapment of enzyme and/or electron mediator

ii) anti-fouling of the electrode surface (resulted adsorption of macromolecules)

iii) size exclusion (perm-selectivity)

iv) ion exchange (charge specific separation)

v) partition across the membrane/solution interface (elimination or enhancement of the response)

Depending on the nature of the membrane, its application onto the electrode surface can be made through adsorption from a polymer solution, electropolymerization or fixation as a premade membrane. The mass transport of analytes and interferents through the membrane layer is determined by their diffusion coefficients in the film and by their partition coefficients between the film and the solution. The enzyme layer and membrane film thickness are important parameters affecting the electrode response. Electropolymerization at the membrane makes it easier to control film thickness, homogeneity and reproducibility. The resulting structure and permeability of the polymeric membrane are very much dependent on the anodization conditions. O-phenylenediamine electropolymerized on top of GOD covalently bound to platinized carbon was shown to drastically eliminate electrochemical interferences such as ascorbic acid, uric acid, L-cystein, and also prevented fouling by proteins (123). Moreover, the film increased the thermal stability of GOD.

Nafion covered lactate oxidase modified electrodes almost totally excluded the signal for ascorbic acid (124). Other negatively charged membranes, used in combination with oxidases, are e.g., polyurethane (125) polymethacrylic acid ester membrane (126) and Eastman AQ (polyster sulfonic acid) films (127).

21. MULTISTEP ENZYME SYSTEMS:

Multiple enzyme systems can be used to enhance the response of biosensors. When the product at a second enzyme can be used as the

substrate for the first, enzyme cycling can occur. This enzyme cycling allows a small amount of analyte to create a larger amount of electrochemically active product than would be possible if a direct conversion was made. This creates a greater analytical signal and allows a lower detection limit. Co-immobilization of GOD and GDH, with an excess of NADH, for the detection of glucose can be given as an example (128). GOD oxidizes glucose to δ-glucanolactone, which in the presence of high concentration of NADH is rereduced by GDH to glucose. The enzyme substrate can also be electrochemically regenerated. The two enzymes may be intimately mixed or immobilized on different layers in an ordered fashion.

22. ENZYMES:

22.1. Horseradish Peroxidase CHRP) (E.C.1.11.1.7)

This enzyme produced by horse radish is an oxidoreductase with tightly bound protohemin IX as prosthetic group, an iron-porphyrin derivative. The iron content makes the enzyme sensitive to strong iron-complexing agents like cyanide ions. It exhibits high specificity for the catalysis of the reduction at hydrogen peroxide to water. MW of this enzyme is 40,000. Seven isozymes have been described by Shannor et al (1966). All contain protohemin IX as prosthetic group. Neutral and amino sugars account for a approximately 18% of the enzyme. Weineryb (1966) indicates that the active site involves apoprotein as well as the heme group. HRP is reversibly inhibited by cyanide and sulfide at a concentration of 10^{-} M. The enzyme is quite stable (129).

22.2. Alcohol dehydrogenase (ADH) (1.1.1.1):

Alcohol dehydrogenase catalyzed the reaction

$$RCH_2OH + NAD^{*} \rightleftharpoons RCHO + NADH + H^{+}$$

The common reaction in yeast cells is reduction of acetaldehyde to ethanol. *In vitro,* however, the enzyme is generally assayed and or utilized in a more alkaline pH region a condition which favors a shift equilibrium towards the oxidation of ethanol.

This enzyme has four subunits each with MW of 35,000. This is a metalloenzyme containing four tightly bound zinc atoms per molecules. There are two distinct active site sulfhydryl groups in each subunit which can be distinguished on the basis of differential reactivity with iodoacetate and butyl isocyanate. A histidine residue is considered to have an essential role. Optimum pH for the oxidation of ethanol is 8.6-9.0 (the enzyme becomes increasingly unstable at higher pH). Yeast ADH which has a more narrow specificity than that of liver enzyme, is somewhat more active on the straight chain primary alcohols, and acts to a very limited extent on certain secondary and branched chain alcohols.

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2. SOME ELECTROCHEMICAL TECHNIQUES

1. POTENTIOSTAT:

An instrument widely used in electrochemical research which controls the potential applied to the working electrode, and allows one to measure the current. Fig. 2.1. shows a circuit for a simple potentiostat which is perfectly adequate for general purposes. In addition to the potentiostat one requires a voltage source to provide the input potential to the potentiostat. We have used EG&G, PAR 174A model polarographic analyzer in our work. This unit contains a fixed voltage source as well as a triangular wave generator suitable for cyclic voltammetry measurement and is very well suited to the type of measurements described in this thesis.

In addition to the potentiostat, some form of chart recorder is required to record the data. Ideally this should be an X-Y-t recorder so that both current-potential (Cyclic voltammograms) (X-Y) and current-time responses at constant potential (Y-t) can be recorded. The Rikadenki digilogue model RY 201T recorder is used in our experiments (1).

2. IMPEDANCE:

Electrochemical impedance theory is well-developed branch of ac circuit theory that deals with the response of a circuit to an alternating current (Fig. 2.2) or voltage as a function of frequency. In dc circuits (the frequency equals to 0 Hz) resistance is defined by Ohm's law:

$$E = IR$$

Using Ohm's law, one can apply a dc potential (E) to a circuit, measure the resulting current (I), and compute the resistance (R) or determine any term of the equation if the other two are known. Potential values

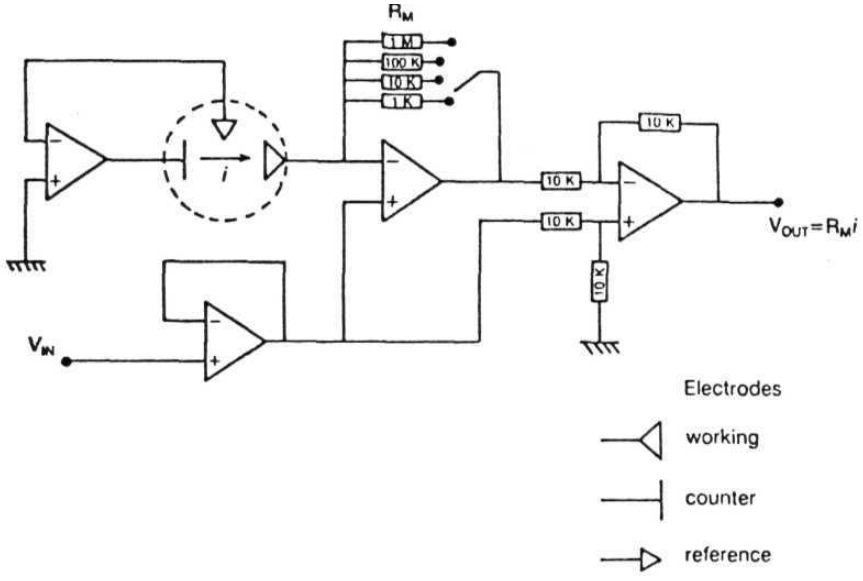

Fig. 2.1. A simple design of potentiostat for three electrode measurements.

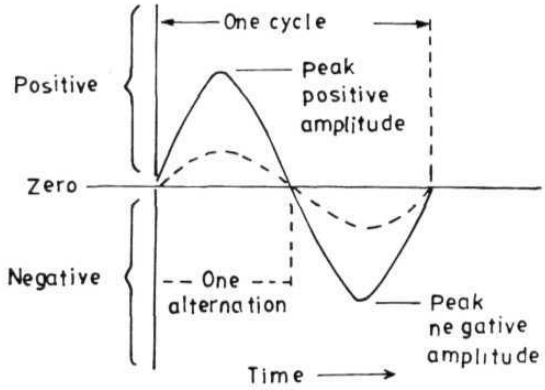

Fig. 2.2. One cycle of alternating current.

are measured in volts (V), current in amperes (A), and resistance in Ohms (Ω). A resistor is the only element that impedes the flow of electrons in a dc circuit. In a similar fashion, we define in an AC circuit the relation:

$$E = IZ$$

In this equation E and I are instantaneous values of potential and current respectively and Z is defined as "impedance", the ac equivalent of resistance.

The terms resistance and impedance both imply an obstruction to current, the flow of electrons. With a direct current (dc) only resistors provide this effect. However, with alternating current (ac) other circuit elements such as capacitors and inductors can also influence the flow of electrons. Systems containing such elements can not only affect the magnitude of an alternating current wave form but also its time dependent characteristics or phase.

In an electrochemical cell, slow electrode kinetics, slow preceding chemical reactions and diffusion can all impede electron flow, and can be considered analogous to the resistors, capacitors, and inductors that impede the flow of electrons in an electrochemical circuit.

Impedance can be expressed as a complex number, where the resistance is the real component and the combined capacitance and inductance (reactance is the imaginary component.

$$V = IZ$$

$$Z = R + iX \qquad X: \text{ reactance}$$

$$= R + iX_C \qquad X_C: \text{ Capacitative reactance only}$$

$$= R - iX_L \qquad X_L: \text{ Inductive reactance only}$$

$$Z = R + i(X_C - X_L) \qquad i = \sqrt{-1}$$

The total impedance in a circuit is the combined effect of all its resistors, capacitors, and inductors to the flow of electrons. The opposition to the flow of current by capacitors and inductors is given by the name reactance, symbolized by X and is measured in ohms (Ω). Since the symbol for capacitance is C, capacitive reactance is symbolized X_C. Similarly, since the symbol for inductance is L, inductive reactance is symbolized by X_L.

Capacitors and inductors affect not only the magnitude of an alternating current but also its phase. When most of the opposition to current flow comes from its capacitive reactance, a circuit is said to be largely capacitive and the current leads the applied voltage in phase angle. The more capacitive a circuit is , the closer the difference in phase angle approaches 90 degrees. When most of the opposition to current flow comes from its inductive reactance, a circuit is said to be largely inductive and the current lags the applied voltage in phase angle. The more inductive a circuit is, the closer the difference in phase angle approaches -90 degrees.

It is sometimes easier to perform calculations using admittance, the reciprocal of impedance. Admittance is symbolized by Y and measured in Siemens (S). Like impedance, admittance can be expressed as a complex number, where the conductance, (the reciprocal of resistance) is the real component, and the susceptance, (the reciprocal of reactance) is the imaginary component.

$$Y = \mathbf{i}$$

$$Y = C + iG$$

C: conductance
G: Susceptance

2.1. Randies Cell:

One can study an electrical circuit by deriving its impedance equation. However, it is often simpler to perform a measurement on the circuit and analyze the resulting plot, particularly if the distribution of the resistors and capacitors are unknown. One will get a good picture of the real and imaginary impedance components and of the phase shift characteristics as a function of frequency, and based on these results, a satisfactory model can often be built.

The Randies cell is a parallel combination of capacitance and (polarization or charge transfer) resistance. The Randies cell models the electrochemical impedance of an interface and fits many chemical systems. One can easily equate the circuit components in the Randies cell with familiar physical phenomena, such as adsorption or film formation.

In the typical Randies cell Fig. 2.3. RΩ is the ohmic or uncompensated resistance of the solution between the working and reference electrodes, Rp is the polarization resistance or charge-transfer resistance at the electrode/solution interface and Cm. is the double layer capacitance at this interface. If the polarization or charge-transfer resistance, is known, the electrochemical reaction rate can often be calculated. Double-layer capacitance measurements can provide information on adsorption and desorption phenomena. In some systems, a C_{DL} measurement may not represent the double layer capacitance. Rather, it may indicate the degree of film formation or the integrity of an organic coating (2).

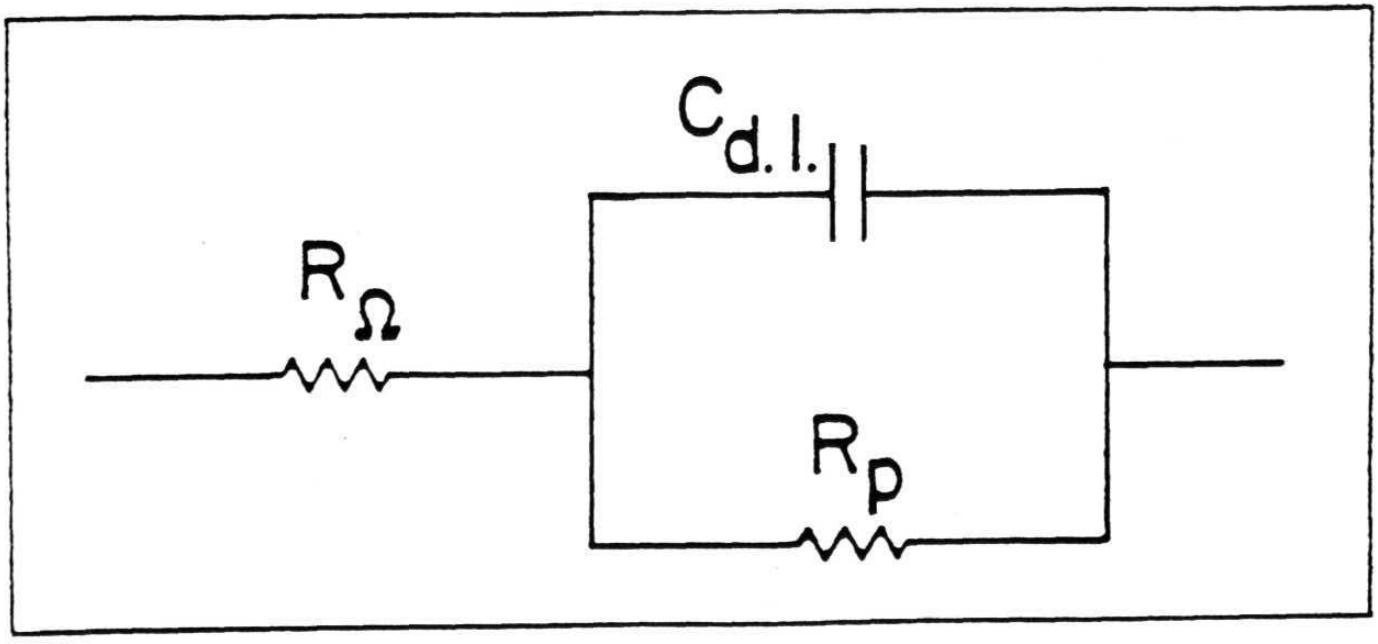

Fig. 2.3. Equivalent electronic circuit for a simple electrochemical cell.

3. LOCK-IN AMPLIFIER

Lock-In Amplifiers have been used for many years to measure extremely small signals. They will measure with high accuracy, a minute signals buried in random noise and surrounded by large discrete interfering frequencies. Lock-ins work by capturing all the signal power available while at the same time rejecting as much noise power as possible.

The lock-in amplifier is an extremely selective ac voltmeter used to measure a single-frequency signal obscured by noise. Operating over a range which may extend from one cycle every 10 seconds (0.1Hz) to two hundred-thousand cycles per second (200KHz), it rejects random noise, transients, incoherent discrete frequency interference and harmonics of the measurement frequency. It produces a dc output proportional to the rms amplitude of the sinusoidal fundamental of the signal.

The lock-in amplifier provides very high sensitivity allowing accurate measurement of ac voltages upto one micro volt with one nanovolt resolution. It also can function as a phase meter and a spectrum analyzer. The lock-in is widely used in low-level optical work, electron spectroscopy, neurological research and in complex impedance studies (3).

A lock-in amplifier in Fig. 2.4 is an instrument which uses phase-sensitive detection, filtering and amplification in order to measure small ac signals. Unlike a conventional ac voltmeter, the lock-in amplifier "locks-in" to the frequency (Fr) of a reference signal and will measure input signals at that frequency only (or to its odd harmonics).

The reference channel can accept a wide variation in frequency, amplitude and wave-shape (i.e. sine-wave, square-wave etc.) of the input signal. The reference channel generates a precision square wave voltage triggered from the applied reference and incorporates a

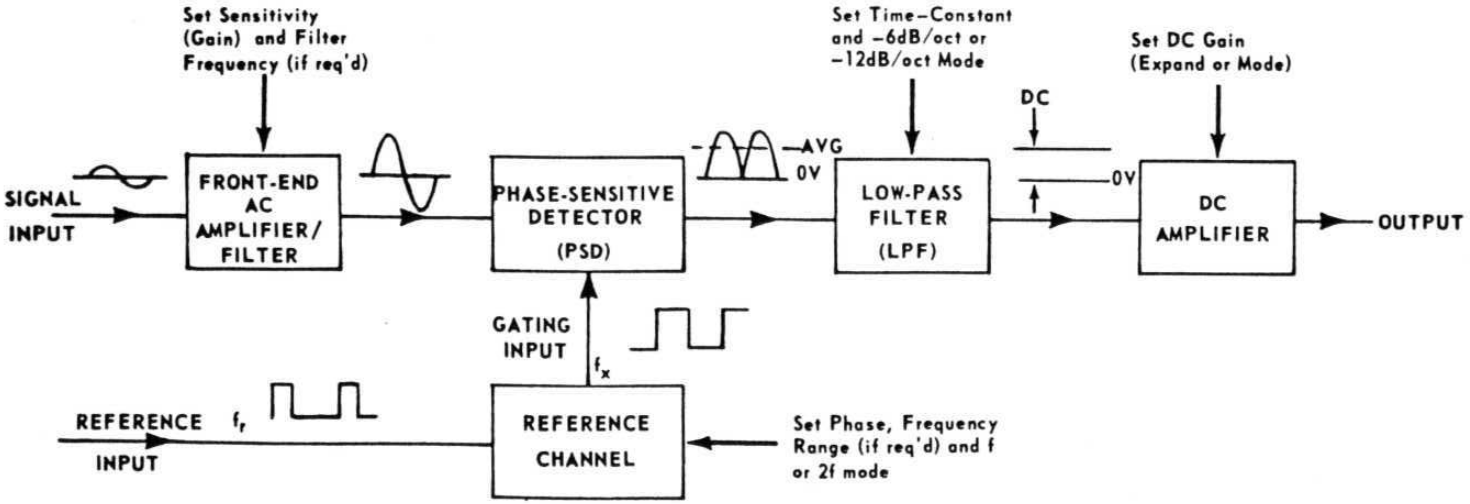

Fig. 2.4. Basic lock-in amplifier.

variable phase shifter. The variable phase shifter is used to adjust the relative phase of the signal and square wave reference voltage appearing at phase-sensitive detector (PSD). Thus lock-in applies the signal and reference to a PSD, which acts as a frequency mixer. The PSD consists of a switching multiplier followed by a low-pass filter and will only detect (or rectify) signal voltage that are synchronous with the reference voltage.

The signal channel of LIA contains a variable gain ac amplifier and uses band pass filtering that prevents any odd harmonics of the reference frequency from getting through the signal channel to the phase sensitive detector where they would produce a dc output of error in the measurement. The signal input to the lock-in is amplified to a level suitable for phase sensitive detection. In many lock-ins the gain of the front-end amplifier is frequency selective (i.e. it is also a filter) in order to improve its overload capability.

The output of the PSD circuits is the product of its two inputs (signal and gating). Any input signal of frequency Fx (i.e. synchronous signals) will produce a dc output, input signals at all other frequencies (i.e. asynchronous signals) will produce an ac output signals.

The low-pass filter (LPF) removes the unwanted ac output signals from the PSD and passes the wanted dc signals to the input of a dc amplifier which amplifies it to a suitable level for display or recording. The dc component which has come through the LPF has a value that depends on the signal amplitude and the phase difference between signal and reference. The user is able to maximize the response to a signal of interest by adjusting the reference phase from the front panel of LIA.

4. FOURIER TRANSFORMATION:

4. 1. Fourier Transformations in impedance measurements

When low frequencies are involved, measurement of impedance using a conventional lock-in-amplifier becomes increasingly difficult as it is not usually designed to work at frequencies much less than 1 Hz. In addition, the time constant has to be considerably increased for reliable phase measurements. Thus, excessive times are required for measurements at each frequency (below 1 Hz). Although other techniques are known and are often used, the most common and direct approach is to use a stored waveform excitation of the sample and analyze the resulting currents by fourier transformation.

4.2. Basics of fourier transformation:

If a function c(t) is given that may have some periodic components, i.e., if it can be expressed as a sum of several periodic functions (like sin ϕ or cos ϕ), then the fourier transformation of the function c(t) is given by the expression:

$$\underline{C}(\omega) = \int_{-\infty}^{+\infty} \exp(i\omega t)\, c(t)\, dt$$

Where 1 is the imaginary constant $\sqrt{-1}$, ω is the variable in the frequency domain and $C(\omega)$ is the fourier transform of c(t). The relationship is reciprocal, i.e., c(t) is the fourier transform of $C(\omega)$. Because of the presence of the $\exp(i\omega t)$ term, $C(\omega)$ is complex, i.e., has real and imaginary components when c(t) is a real function.

In case of experimental data, c(t) is most conveniently given as a series of values in equally spaced time domain. In such cases, the function is not defined in the $-\infty < t < \infty$ but for some finite range, i.e., $0 < t < t$. The mathematical implications of such restrictions is discussed in standard text books and needs no elaboration here (4).

It is desirable that the series of values representing c(t) may contain as many terms as possible but computational requirements (the integration has to be carried out numerically for every value of ω) becomes prohibitively expensive. A new algorithm, fast fourier transformation, developed by Tuckey and Cooley, which is computationally highly efficient, makes routine application of fourier transformation possible even on a small computer. This single development has the greatest impact on a large number of spectroscopic techniques, i.e., NMR, Optical, Mass-spectrometry being the most notable.

4.3. Impedance spectroscopy

Impedance in an electric circuit is defined by analogy with Ohm's law: $V = IZ$, where V is the instantaneous voltage, I is the instantaneous current and Z is the impedance. If V is sinusoidally changing (with time) with a frequency ω, I also will be sinusoidally varying with the same frequency (but may be with a different phase) and Z will be in general a function of ω. This function, $Z(\omega)$ is called the impedance spectrum of the circuit. In general, $Z(\omega)$ will be a complex function and may be represented graphically in several different (but otherwise equivalent) ways.

To obtain experimentally Z at several different frequencies, one does excite the sample with several superimposed sinusoidally varying potential at the same time. In a digital world, this is conveniently done by adding several frequencies (of major interest) and storing the

result as a time series data in a computer. The data is shifted out at a regular interval, converted to the analog from and applied to the sample as a potential. The currents that are generated are converted to the digital form and stored again in a file for future analysis by fast fourier transformation technique.

Once the current data has been transformed into the frequency domain by fourier transformation, the impedance spectrum can be simply obtained by dividing the potential by the current values on a point-by-point basis. The impedance spectra is most conveniently represented by the Bode diagram (Magnitude and Phases separately as a function of log ω) or Nyquist plots.

4.4. Programming details:

1. For studies on low-frequency impedance studies, the following frequencies were chosen: 1,3,7,15,31,63,127,255,511,1003,2007,4015 and 8031 (arbitrary units). These were converted to actual frequencies depending on the actual time taken (data sampling time). Sine waves were simulated using the built-in function in the computer for least two complete cycles totaling 1024 data points for all these frequencies. Phases proportional to the square of the frequencies were introduced in the sine functions so that the excitation power is more or less uniformly distributed over time. The resulting sum is multiplied by 100 and stored as a set of 1024 integer data values in a disk file. Unless the excitation frequencies (so as to avoid overlap due to foldback) need to be altered, this file (named FFT.DAT) is not required to be changed. This was done by a small program in QBASIC.

2. The digital to analog converter facility of the lock-in-amplifier was used for applying the excitation potential. The data file (FFT.DAT) was read, put to the lock-in-amplifier for digital to analog conversion, the analog output was then stepped down to 1:100 using a

resistive network and fed to the modulation input of the polarographic analyzer. This voltage (peak value of 10mV/frequency) was summed to the bias potential and the current value is recorded from current to voltage converter output of the polarographic analyzer. This potential is digitized by the analog to digital converter facility of the lock-in-amplifier and is stored in a file. All the data items (1024 points) were similarly treated and a file containing the 1024 current values were obtained. The total time required for this was also noted. The necessary scale factors for conversion to actual values were also noted.

3. The data series (1024 current values) were next subjected fast fourier transformation. Since this data set is real, two components were obtained after fourier transformation-- real set of data and an imaginary set (component) of data. These data values were phase corrected using an experimental protocol described below.

4. Since the delay in data acquisition cannot be accurately predicted, the phase shifts are variable and frequency dependent. To obtain a table of phase shifts, a standard resistor of 10KΩ was substituted for the experimental cell and the experiment repeated. To improve accuracy, five sets of current values were stored and averaged. The average current values were fourier transformed and phase values were adjusted point-wise to give minimum component of the imaginary impedance. The set of phase values were stored in a file (1024 data points) and used in all experiments for phase correction.

5. After the phase corrections were performed, actual frequencies were computed (from the known time scale of the data acquisition) and impedance values were also converted using known factors. These values were printed out (both real and imaginary components) into a table by the program. Typical frequency range are from 4mHz to 34Hz. The impedance values varied typically from 1Ω to 1KΩ.

All the above programs were written in Microsoft QBasic programming language. the lock-in-amplifier (PAR Model 5210) (used only for its A/D and D/A conversion facilities) was interfaced to the PC using the RS-232C serial port.

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3. BIOELECTROCHEMISTRY OF IMMOBILIZED ALCOHOL DEHYDROGENASE ON PLATINUM ELECTRODE

1. INTRODUCTION

In this chapter we present the results of cyclic voltammetric studies on a platinum electrode containing adsorbed (i) ferrocene, (ii) NAD* and (iii) alcohol dehydrogenase. To prevent adsorbed molecular layers from desorption, the adsorption solution contained a small amount of nitrocellulose. The electrode does not behave reversibly, but the response to alcohol solution is found to be linear in the range of 10-1000μM alcohol concentration. The enzyme was also immobilized by cross-linking with glutaraldehyde and the results are similar. A plot of log [peak current] vs log [alcohol] gave a set of parallel lines for different concentration of immobilized enzyme.

CV studies on the electrode containing (i) ferrocene and (ii) NAD* adsorbed as before shows two prominent peaks. The electrode was used in a solution containing alcohol and enzyme (1, 2 and 5 units/ml) and this also shows clear dependence of the peak current on alcohol concentration, but no clear relation has been observed. Presumably, the solution kinetics is much faster compared to the electrode processes.

Dehydrogenase enzymes belong to the oxidoreductase class. More than 400 different dehydrogenase enzymes are known and have been isolated. Some 250 of them depend on the NAD /NADH couple, and some 150 on NADP*/NADPH. All work on biosensors for NAD* dependent dehydrogenases has utilized the reaction scheme shown in Fig. 3.1 here S is the oxidized and SH the reduced form of the substrate. Most analytical applications with dehydrogenases make use of the detection of NADH formed even though the equilibrium for the reaction often favours the substrate side. The equilibrium of the reaction can be forced to the product side by removal of the product (1).

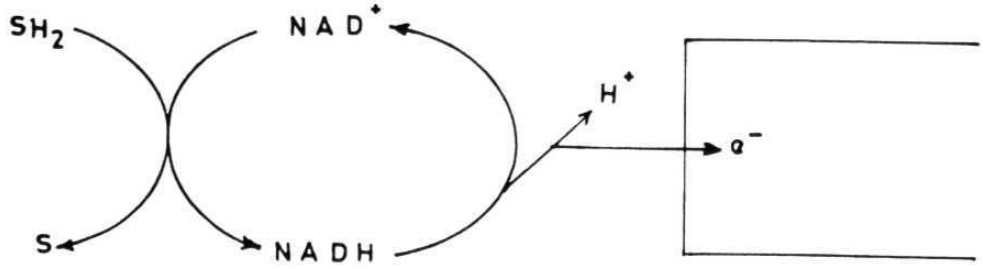

Fig. 3.1. Reaction cycle for dehydrogenases.

2. PREPARATION OF ENZYME ELECTRODE

The bare Pt mesh electrode was cleaned by dipping in concentrated nitric acid followed by burning in an alcohol flame. For cleaning, the same method was used for all the experiments. The thoroughly cleaned electrode was dipped in a solution containing 25 mg of ferrocene and 5 mg nitrocellulose in 2 ml of acetonitrile. The electrode was dipped in the solution for 15-20 minutes and then dried in vacuum. A thin layer of ferrocene was formed on the electrode. The nitrocellulose was also present on the electrode and this prevents falling off of the ferrocene from the electrode. The dried electrode was kept for sometime in buffer (0.1 M, pH 8.6) to remove loosely bound ferrocene. In one set of experiments, this electrode containing only ferrocene was used. This was kept in a H-type cell as show in Fig. 3.2 (2) and a cyclic voltammogram was recorded with a potential range from -350 to +400 mV versus SCE.

Alcohol + NAD^+ ——————> Acetaldehyde + NADH + H*

NADH + 2 Ferrocene (III) ——————> NAD* + 2 Ferrocene (II) + H*

To this solution, NAD* was added to the buffer to a final concentration of 1, 10, 100, 1000 μM and for each concentration the CV was recorded. In this way NAD was adsorbed on to the electrode. Now the electrode contains adsorbed ferrocene and NAD*.

The electrode containing ferrocene and NAD was kept in the buffer solution and the enzyme was added to buffer. The enzyme was added such that the total activity is 1, 2 or 5 units/ml of buffer. After adding the enzyme to the buffer the effect of ethanol was seen. This type of electrode is often refereed to in literature as a pseudoenzyme electrode (enzyme present in solution).

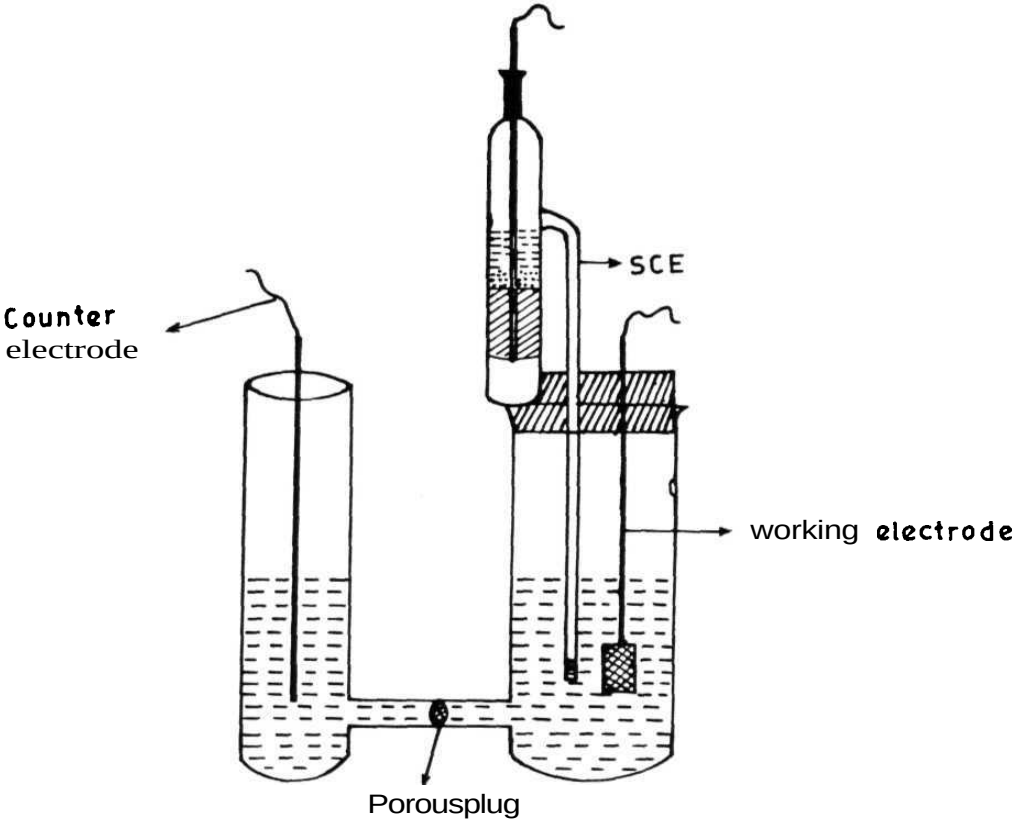

Fig. 3.2. H type cell.

2.1. Enzyme immobilization by adsorption

The same enzyme electrode was cleaned and the adsorption of ferrocene and NAD to the working electrode was carried out as described above. The working enzyme electrode containing ferrocene and NAD was kept in the enzyme solution (5 mg/ml) for 30 minutes and vacuum dried in cold. On the electrode a thin enzyme layer was deposited. The electrode was always kept soaked in the buffer while not in use. The electrode was used in our final experimental setup in a solution containing ethanol and buffer only (no enzyme/coenzyme in solution).

2.2. Enzyme immobilization by glutaraldehyde cross-linking

In this method the enzyme and NAD was immobilized on the working electrode in a BSA glutaraldehyde cross-linked matrix. 100 µl of solution containing phosphate buffer (2.7ml of 0.1M solution at pH 6.8) BSA (17.5%, 1.5 ml) and 50 mg of enzyme was taken and mixed thoroughly. In this solution the electrode was dipped.

20µl of 10% glutaraldehyde solution was added to above electrode by rapid mixing with stirring rod (3). The enzyme electrode was kept dipped in buffer overnight at 4 C before use.

2.3. Recording the cyclic voltammograms

The cyclic voltammograms were recorded through a polarographic analyzer and a X-Y recorder. The solution was gently stirred with a magnetic stirrer. The scan rate was kept fixed at 20 mV/sec. Full scale current range was also kept fixed at 0.5 mA for ease of comparison. The supporting electrolyte used was pyrophosphate buffer (0.1 M, pH 8.6).

3. RESULTS

As we have mentioned earlier, we have recorded the cyclic voltammograms (CVs) at every step of construction of the electrode to monitor the progress of the immobilization process.

3.1. CV of the electrode containing adsorbed ferrocene and NAD (enzyme in solution):

Here the enzyme (1 unit/ml) is in pyrophosphate buffer. As expected, we have used different concentrations of ethanol of 1, 3, 10, 30, 100, 1000μM. As the enzyme catalyzed reaction progresses, the ratio of NAD /NADH on the electrode changes and hence the potential of the electrode is affected. Again, the peak height increases with alcohol concentration, but no direct linear relation was seen.

The experiments was repeated with three different concentrations of enzyme in solution-1, 2 and 5 units/ml. The lowest concentration viz., 1 unit/ml, shows the best results. This is expected because, the lower activity of the enzyme causes slower change in $NAD^+/NADH$ ratio. Since ferrocene is showing irreversible behaviour the lower values are expected to give better results. Ferrocene monocarboxylic acid has been reported to show reversible behavior (4). The relationship for log [current] vs log [EtOH] straight line from 10 μM to 1000 μM ethanol concentration as shown in Fig. 3.3.

3.2. CV of electrode containing ferrocene, NAD and adsorbed enzyme (no free enzyme in solution)

In this enzyme electrode the enzyme is immobilized on the electrode by adsorption. The electrode was kept in H-type of cell containing pyrophosphate buffer of pH 8.6. Small aliquots of alcohol were added to obtain required final concentrations. As before, final

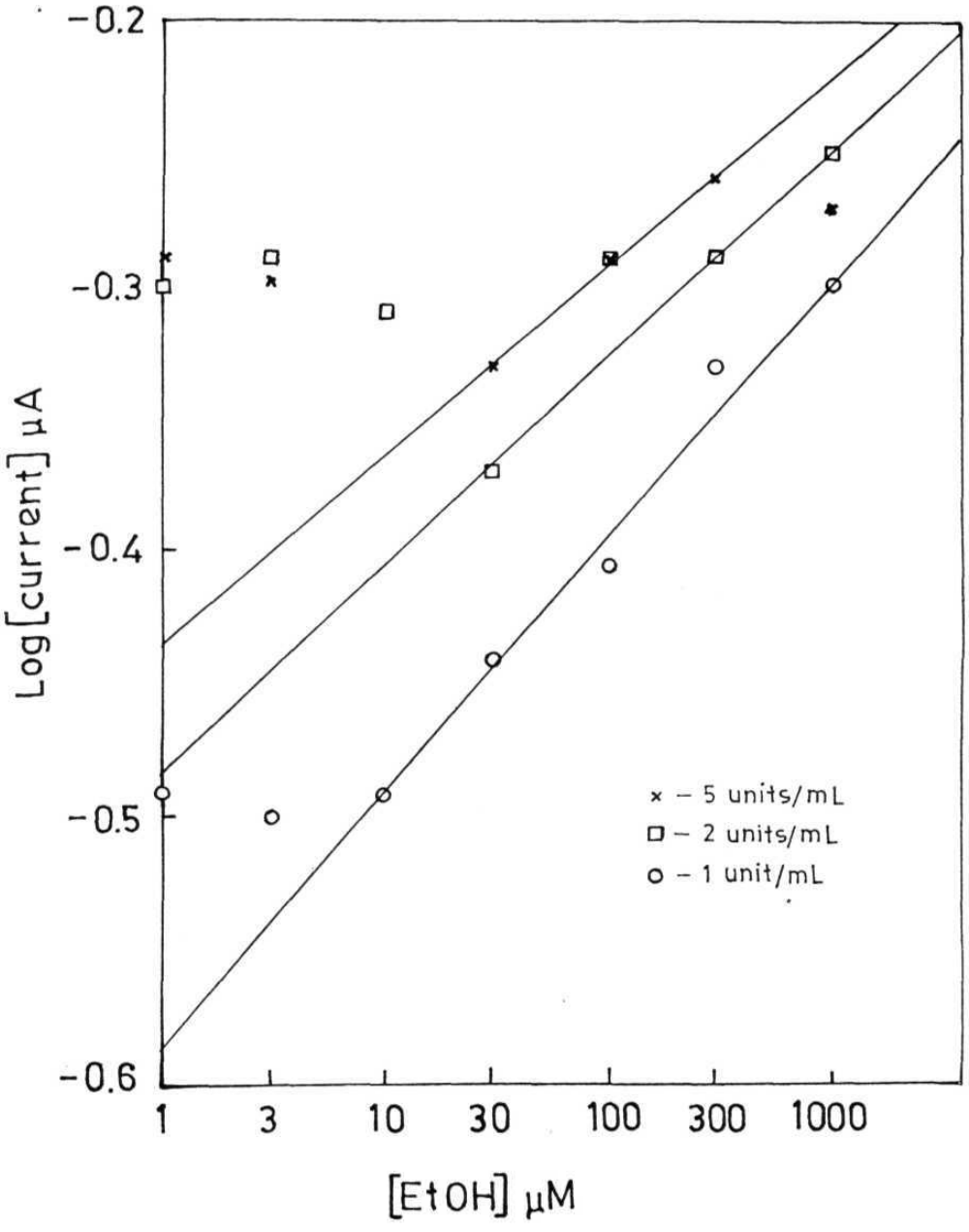

Fig. 3.3. Electrode containing adsorbed ferrocene, NAD* on electrode. Enzyme concentration in buffer x- 5 units/mL; □ - 2 units/mL; o- unit/mL.

concentrations are 1, 3, 10, 100, 300 and 1000 μM. Here also for log [EtOH] vs log [current] shows a linear relationship as shown in Fig. 3.4 from 10 μM to 1000 μM ethanol concentration.

3.3. CV of electrode containing ferrocene, NAD and glutaraldehyde cross-linked enzyme

In this type of enzyme electrode the enzyme is immobilized by cross-linking on BSA matrix as described earlier. The electrode also shows the same behaviour as the other enzyme electrode. This electrode was tested exactly as in previous case. This is also showing linear relationship between log [EtOH] vs log [current) for 10 μM to 1000 μM ethanol.

4. DISCUSSION

All these results point out that the enzyme electrode as constructed above responds to alcohol present in solution. However, the response of the electrode is rather slow and the electrochemistry of the basic mediator, i.e., ferrocene, is irreversible. Suitable derivatives of ferrocene may perform better in this respect. The actual amount of enzyme adsorbed on the electrode is rather uncertain and no attempt has been made to estimate the activity of the enzyme present on the electrode after immobilization. Electrodes prepared at different times shows similar behavior, i.e., a linear response of log [EtOH] vs log [current], but the y-intercepts are different (parallel lines were obtained). This points to the fact that actual amount of enzyme adsorbed onto the electrode is difficult to quantify accurately.

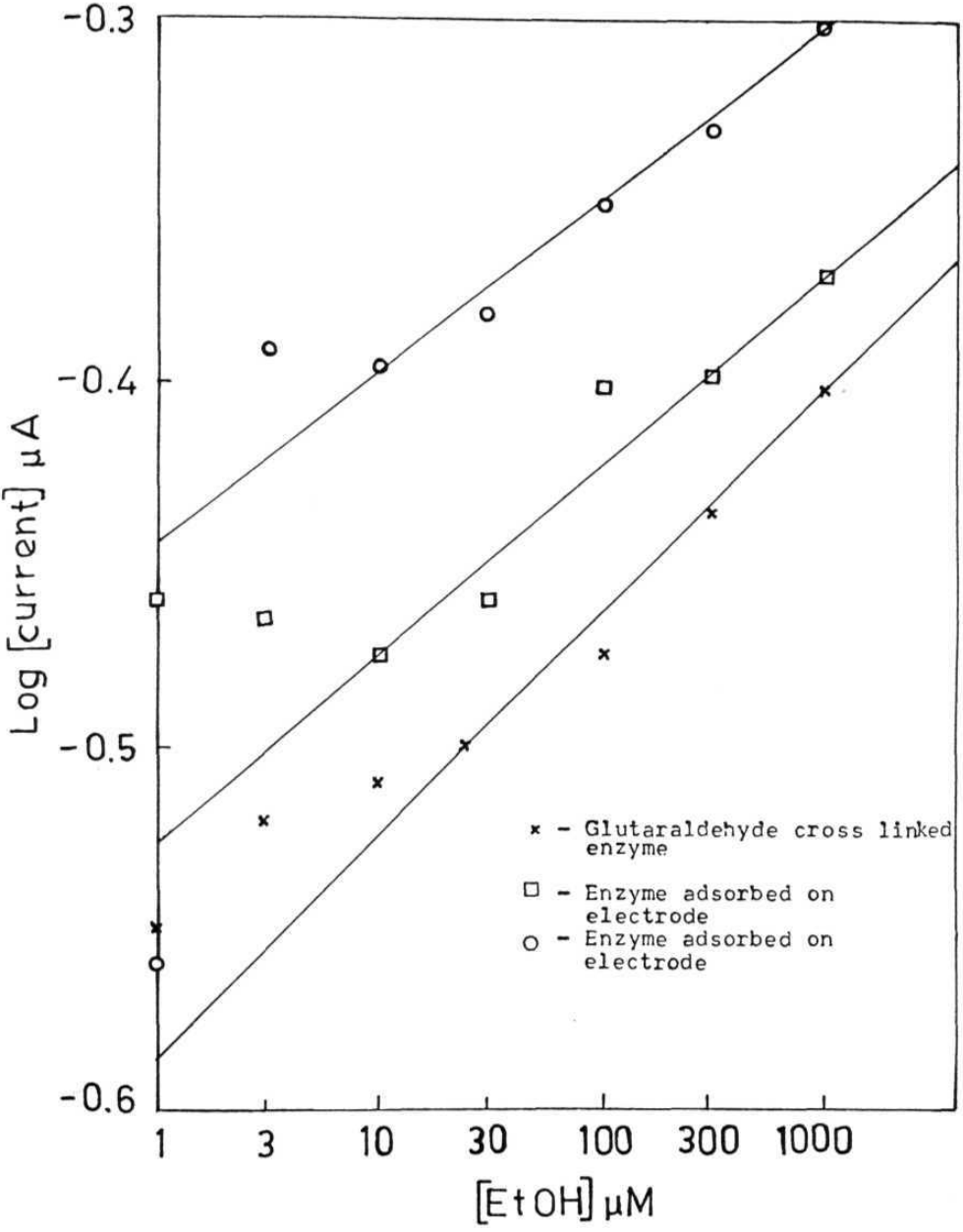

Fig. 3.4. Enzyme adsorbed on to the electrode in two experiments (□ and o) Glutaraldehyde cross-linked enzyme on electrode (x).

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4. AROMATIC AMINOACID MODIFIED ELECTRODES

1. INTRODUCTION

With polymer coatings or electropolymerization of electroactive molecules on an electrode surface a new interesting area has opened leading to the development of chemically modified electrodes (1). The properties of chemically modified electrodes are currently drawing the attention of many researchers because of their catalytic and substrate specific redox characteristics. The modification of the electrode may be done by using different aromatic compounds like pyrrole, thiophene, indole and aniline amongst others (2-5). Some amino acid derivatives (i.e., tyramine) have also been used for electrochemical polymerization (6,7). These are especially interesting because some of them are electronically conducting, such as polypyrrole, which can be prepared as a film on metal substrates by electrochemical polymerization. The preparation and properties of such films were first reported in 1968 by Diaz and coworkers (11).

We report here on the electrochemical properties of polytryptophan/polytyrosine as an electrode modifier and its characterization by voltammetric and impedance measurements. Amino acids were chosen for electrode modification because of their biological compatibility. A polyamino-acid film is more likely to interact favorably with biomolecules compared to other polymers of non-biological origin. However, not all amino acids are suitable for this purpose, because the polymer, if formed at all, may not be electrically conducting. We have therefore focused our attention on the aromatic amino acids, viz., tyrosine, phenylalanine, tryptophan and histidine, which are expected to be electrically conducting after polymerization by charge transfer interaction. However, we find that

phenylalanine and histidine cannot be easily electrochemically polymerized. Tryptophan is also involved in biological charge transfer interactions and is often present at the active site of various enzymes.

2. EXPERIMENTAL

Polytryptophan (PT)/polytyroslne (PTyr) films were formed by electrochemical oxidation of a 100mM tryptophan/tyrosine solution in either (a) 0.2N H_2SO_4 or in (b) 0.1N NaOH. The films formed from H SO and NaOH showed some characteristic differences. The bare substrate was a 1cm x 1cm platinum wire mesh (100 mesh; 0.02mm wire size) welded to a platinum wire 1mm in diameter. The platinum wire in turn was sealed in a glass tube through which electrical contact was made by a mercury pool. For the electrochemical oxidation, and in all other experiments, the standard three electrode setup was used using a saturated calomel (SCE) reference electrode. All solutions were deaerated by passing nitrogen and stirred by a magnetic stirrer; the nitrogen flow and stirring were however, stopped during the actual experiment. Cleaning of the electrode was done as described elsewhere.

2.1. **Film formation:** PT/PTyr films were formed onto the Pt wire mesh electrode either from 0.1N NaOH or 0.2N H_2SO_4 containing 100mM tryptophan/tyrosine. Polytryptophan/Polytyrosine films formed from acidic solution are designated as PT(H SO)/PTyr(H SO) and the films formed from the alkaline solution are designated as PT(NaOH)/PTyr(NaOH). Visible films formed around 1500mV potential but a constant potential was not applied continuously. Instead, the potential was scanned from 0-1500-0mV (vs SCE) at a rate of 20mV/s for one hour (24-25 scans). Films formed this way adhered better to the electrode. A light greenish polymer film could be seen on the

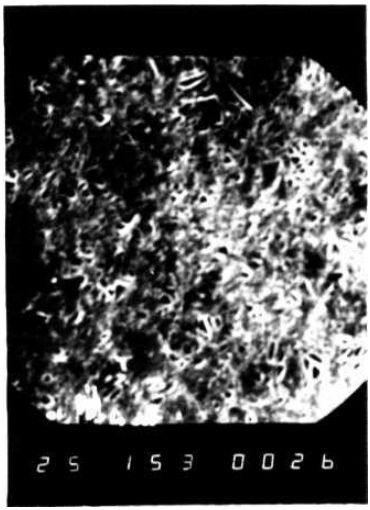
electrode at this stage. This was washed thoroughly with distilled water followed by a final rinse with the supporting electrolyte. This modified electrode was used in all subsequent experiments.

2.2. **Characterization:** We have made preliminary attempts to chemically characterize the film formed by electrochemical oxidation. Since the amount of the available material is quite small, only spectroscopic analysis has been attempted so far. Fluorescence studies suggest that the polymer chain length is probably not very large, as estimated from the shift of the emission peak maxima. The films are formed under acid and alkaline conditions are of comparable chain length. The dimer and the trimer formed remain in solution and shows considerably lower fluorescence. The detailed chemical structure of the polymer has not yet been established. The films are quite stable in acidic and alkaline media for prolonged periods (days) but may be electrochemically inactivated at extreme potentials (greater than 800mV vs SCE). The film formed may have a certain amount of porosity that may interfere with its function. The measurement of porosity has not been done so far but efforts are underway to determine and control the formation of pores. One possible way that is being studied is to use cross-linkers (we have some success with 154 benzidine and spermine) to control pore sizes. Naturally, pore size is a deterministic factor in the permselectivity of the film based on size. A typical SEM of the polymer film is shown in Plate I.

3. ELECTROCHEMICAL MEASUREMENTS

A PAR Model 174A Potentiostat was used in our studies. A conventional three electrode single compartment cell was used in all experiments. A 10 ml electrochemical cell holds the modified or bare Platinum mesh as the working electrode. A thick platinum wire (1.0 mm dia) served as the counter electrode in the voltammetric and impedance measurements. An aqueous saturated calomel electrode (SCE) was used

Plate I. Scanning electron micrograph of the polytryptophan film (153 X).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as the reference electrode. NaClO (100mM) was used as the supporting electrolyte. The impedance studies were carried out by using PAR Model 5210 Lock-in-Amplifier (LIA). The cell was connected to the potentiostat in the usual fashion. A known modulatory voltage (20mV) was applied at a known frequency (80Hz) from the internal oscillator of the LIA to the potentiostat and the output of the potentiostat was connected to the input of the LIA. The output of the LIA was connected to a recorder (Rikadenki digilogue model RY201T). There are two outputs available on the LIA: one in-phase (real part) and the other out-of-phase (imaginary part) components of the current and these two components were recorded separately. The recorder input was also digitized and stored in a PC-XT as a file. The final graphs were plotted from this file using Sigmaplot.

The measured capacitances and resistances were calibrated by replacing the electrochemical cell by a standard capacitor (10μF) or a standard resistor (500Ω) (10). The supporting electrolyte used was 100mM sodium perchlorate which is prepared from double distilled water and adjusted to pH 7. In all cases we have used 100mM sodium perchlorate as the supporting electrolyte unless otherwise mentioned.

4. RESULTS

4.1. Impedance Studies

Figs. 4.1(a,b) and 4.2(a,b,c) show the impedance vs potential curves for the bare Pt electrode, polytryptophan coated and polytyrosine coated electrodes respectively. The potentials were scanned from -800mV to 700mV vs SCE at a rate of 10mV/s. We have applied 20mV modulation amplitude to the potentiostat. The initial potential applied was -800mV, so including the modulation amplitude the total potential applied initially to the electrode will be -800+20mV. During scanning, the potential will change from -800 to 700mV and hence the degree of modulation also will change accordingly.

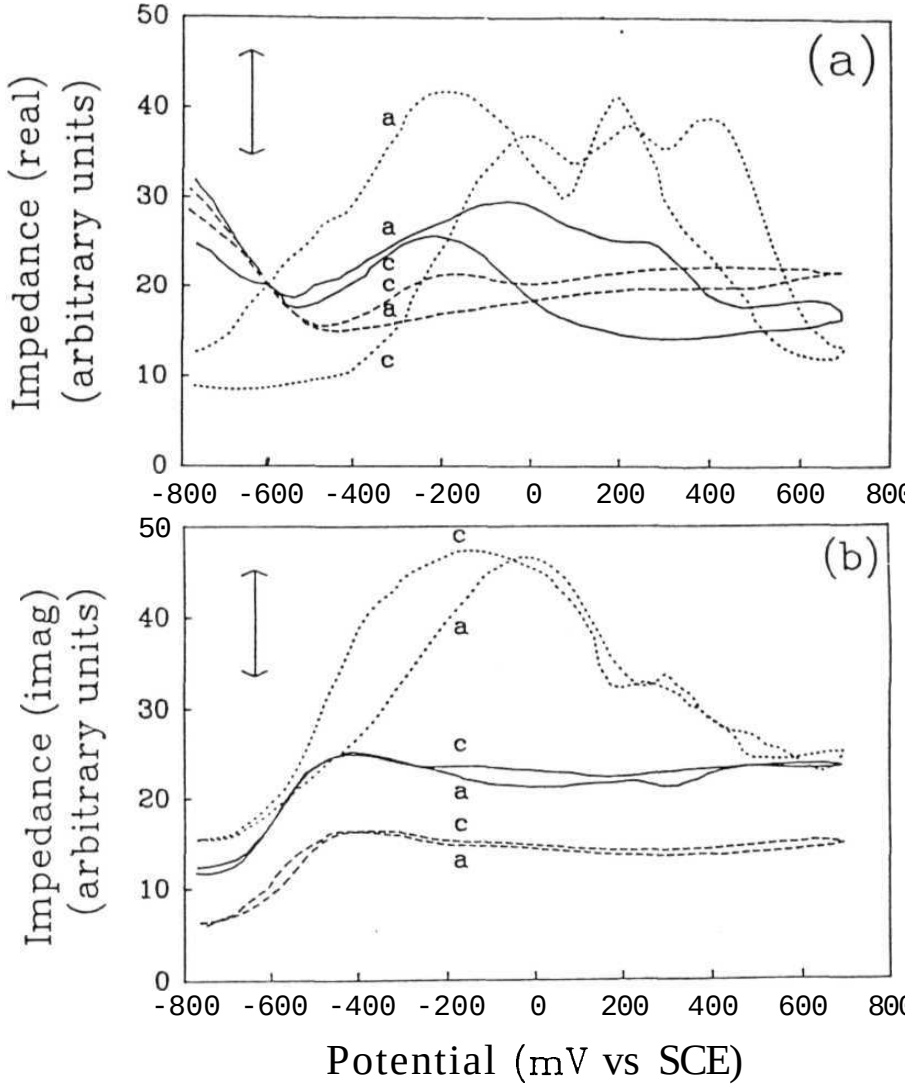

Fig. 4.1(a). Plots for the real components of current for bare Pt mesh (solid line) and PT(H_2SO_4) (dotted line) and PT(NaOH) (dashed line) formed on Pt mesh electrodes. The vertical line corresponds to $2X10^{-3}\Omega^{-1}$ (calibrated by a 500Ω resistor). (b) plots for the imaginary components of current for bare Pt mesh (solid line) and PT(H SO) (dotted line) and PT(NaOH) (dashed line). The vertical line corresponds to $5X10^{-}\ \Omega^{-}$ (calibrated by a 10μF capacitor). a represents the anodic and c represents the cathodic scans respectively. Scan rate is 10mV/sec.

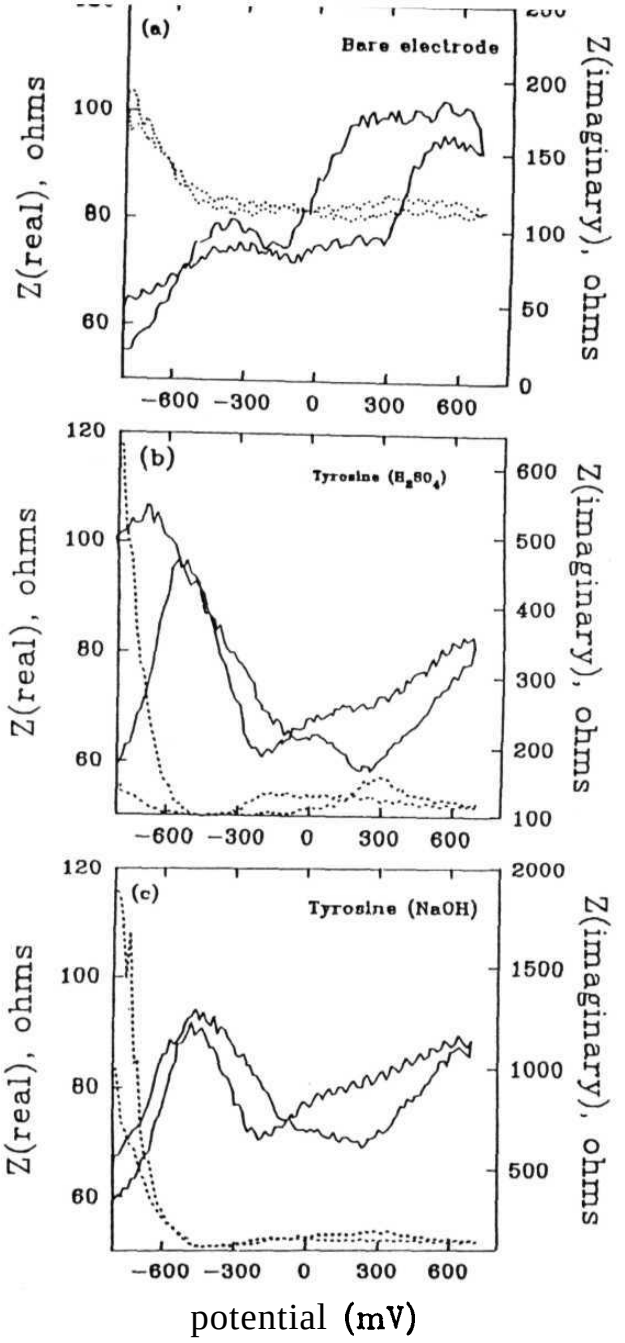

Fig. 4.2. Impedance plots for the real component (solid line) and imaginary component (dotted line) current for bare (a), PTyr(H SO) (b) **and** PTyr(NaOH) (c) modified electrodes

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We have used 80Hz modulation frequency to avoid interference from AC line frequency (50Hz). In addition, the F and 2F line filters on the LIA were also used. These modulation amplitude and frequency will be applied to the working electrode and these will influence the reactions taking place at working electrode. In both Figs 4.1(a) and (b), the Y axis is designated as arbitrary units (same for both) because they refer to the in-phase or out-of-phase components of the complex current flowing through the working electrode. Although it is possible to convert them to actual impedance values from known amplitude factors, it is desirable to use a known resistor and a capacitor to calibrate the observed values. There is a direct reciprocal relationship between the current and the impedance because of the relationship V = IZ (where the symbols have their usual meaning) and in our setup, V has been fixed at 20mV.

In Fig 4.1(a), the resistive or the in-phase component of the current is shown as a function of the bias potential. The PT films formed from H SO showed a considerably larger current in the +400mV potential range (dotted line). The bare electrode, in comparison, showed a relatively flat response (the increase around -800mV may be ascribed to hydrogen evolution). The PT films formed from NaOH showed a very similar, although numerically smaller, characteristic curve compared to that of the bare electrode. It appears that PT(H SO) film can not support significantly higher currents compared to the bare electrode/ PT(NaOH) film.

The capacitative current, or the out-of-phase component of the current is recorded similarly in Fig 4.Kb). Again we note that the PT(H_2SO_4) film shows a significantly higher capacitance in the +400mV range, whereas the features for the bare electrode and the PT(NaOH) electrodes are comparatively similar, with smaller capacitance for the PT(NaOH) film. The reduction in the capacitance below -400mV for all

the three electrodes can be ascribed to increasing ion flux at the double layer, thereby reducing the capacitance. The high capacitance of the PT(H SO) film around OmV is noteworthy.

The impedance studies for the three electrodes bare Pt electrode, polytyrosine (PTyr) formed in H SO and PTyr formed in NaOH solutions are presented in Fig 4.2 (a,b and c) respectively. In all three graphs, the solid lines refer to the real part of the impedance (i.e, in-phase signal) and the dotted lines refer to the imaginary part of the impedance (i.e., the out-of-phase signal). For the out-of-phase component (dotted lines), the right hand side scales are to be used. The graphs are complete scans of impedance as explained earlier. The amplitude of the modulatory signal was rather small (20 mV) and this explains the "noise" seen on the graphs. However, the general features of these three graphs are quite similar. The major source of the measured impedance is the electrode-solution interface and hence the absolute values of the impedance are dependent on the bias potential (and electrode surface area). As mentioned above, only supporting electrolyte (0.1 M NaClO), pH 7.0 was used in all the impedance studies.

For the modified electrodes (both ones) one observes an increase in the parallel resistance (Z real) around -500mV (vs SCE) but this is not pronounced in the bare electrode. Again a sharp decrease in parallel capacitance (Z imaginary is increased) is observed for the modified electrode (particularly for the film prepared from NaOH solution) around -700mV. This suggests the breakdown of the polymer film with attendant decrease of capacitance. Thus we conclude that these electrodes are expected to perform well within the potential range of -300 to 600 mV.

4.2. Electrochemistry of solution redox species

Although the impedance studies provide valuable information about the nature of the electrode surface-solution interface, it cannot predict the behavior of the electrode towards redox properties of various molecules. Towards this end, we have carried out cyclic voltammetric (CV) studies on the modified electrodes using potassium ferrocyanide and p-benzoquinone as typical substrates. These are displayed in Figs 4.3a and 4.3b. In these graphs, the zero currents were not offset to facilitate comparison. All the three electrodes viz., bare Pt, and the PT(NaOH), PT(H SO) modified electrodes exhibit relatively reversible redox behavior. The PT(H SO) electrode curve is essentially similar to the bare electrode except that it is shifted by approximately 0.5 - 0.6mA upwards. This suggests that the background current at both modified electrodes are relatively low when compared to the bare electrode.

For potassium ferrocyanide, which gives a high redox current at the bare electrode, the PT(NaOH) electrode shows considerable improvement. For the PT(H SO) the electron transfer rate is significantly reduced and the CV appears quite flat in comparison. The particular geometry used in our cyclic voltammetry makes accurate and reproducible positioning of the working and the reference electrode somewhat difficult. Hence, considerable iR drop possibly occurs in all our experiments. In Table I, we have recorded the details of the various cyclic voltammetric data as a function of scan rate (from 5 to 200mV/s). For ferrocyanide, it is seen that the peak separation is considerably larger compared to the expected value (60mV). This we attribute to the larger iR drop. In case of p-benzoquinone, the currents are smaller and hence the iR drops are also smaller and at lower scan rates, the peak separation is as expected. The peak currents are also seen to vary linearly as square root of the scan rate, as theoretically expected.

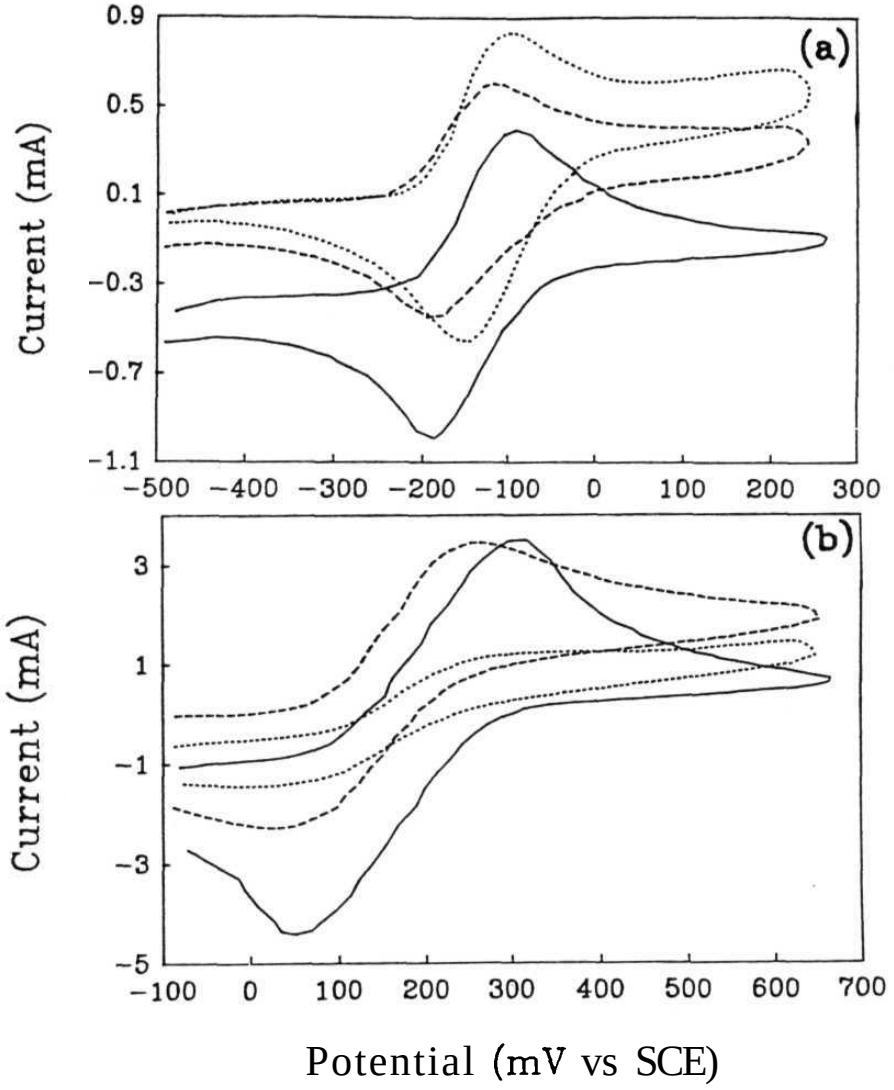

Fig. 4.3(a). Cyclic voltammograms of bare Pt mesh (solid line, PT(H SO) (dotted line) and PT(NaOH) (dashed line) formed on Pt mesh electrodes, in 1mM para benzoquinone containing 100mM sodium perchlorate, pH 7.0. (b) Cyclic voltammograms of bare Pt mesh (solid line), **PT(H** SO) (dotted **line) and PT(NaOH)** (dashed line) formed on Pt mesh electrode, in 10mM K Fe(CN) containing lOOmM sodium perchlorate, pH 7.0. Scan rate is 20mV/sec. in both cases.

TABLE I Cyclic Voltammetric Dnta for Bare and Modified Electrodes

v (V/s)	E_{pa} (V vs. SCE)	E_{pc} (V vs. SCE)	(mV)	i_{pa} (mA)	i_{pa} v	i_{pc} (mA)	v
(a) Bore electrodo In 10 mM potassium ferrocyanide							
0.005	0.230	0.130	100	2.2	31.1	1.5	21.2
0.010	0.240	0.120	120	3 1	31.0	2.5	25.0
0.020	0.255	0.110	145	43	30.4	3.6	25.8
0.050	0.275	0.085	190	6.4	28.6	5.3	23.7
0.100	0.305	0.065	240	8.5	26.8	6.9	21.8
0.200	0.350	0.030	320	11.1	24.8	9.0	20.1
(b) PT(H_2SO_4) modified electrode in 10 mM potassium ferrocyanide							
0.005	0.245	0.120	125	1.46	20.6	0.8	12.4
0.010	0.255	0.115	140	2.00	20.0	1.4	14.5
0.020	0270	0.100	170	2.60	18.4	21	15.2
0.050	0.205	0.090	195	3.60	16.1	2.7	12.3
0.100	0.295	0.0B0	215	4.50	142	3.3	10.4
0.200	0.310	0.075	?35	5.80	12.9	4.0	8.9
(c) PT(NaOH) modiliod electrode In 10 mM potasslurn lerrocyanide							
0.005	0.250	0.130	120	1.63	23.3	1.1	14.8
0010	0.260	0.115	145	2.35	23.5	1.8	18.0
0.020	0.270	0.100	170	3.25	22.9	2.5	18.0
0.050	0.290	0.085	205	4.70	21.0	3.6	16.0
0.100	0.325	0.075	255	6.30	19.9	4.6	14.5
0.200	0.355	0.055	300	8.20	18.3	5.3	11.8
(d) Bare electrode in 1 mM *p*-benzoquinone							
0005	-0.110	-0.170	60	0.22	3.1	05	7.0
0.010	-0.110	-0.170	60	0.42	4.2	0.6	6 6
0.020	-0.110	-0.170	60	0.66	4.6	0.8	6.1
0.050	-0.100	-0.175	75	1.14	5.1	1.2	55
0.100	-0095	−0.185	90	1 65	5.2	1.6	5.2
0.200	-0.085	-0.190	95	2.35	5.3	2.4	5.3
(e) PT(H_2SO_4) modiliod electrode in 1 mM *p*-benzoquinone							
0.005	-0.105	-0.170	65	030	4 2	0 6	9 3
0.010	-0.105	-0.170	65	0.56	56	0.8	BO
0.020	-0.105	-0.175	70	0.88	62	1.0	7.4
0.050	-0.100	-0.175	75	1.52	6.8	1.4	6 6
0.100	-0.090	−0.190	100	2.25	7.1	2.3	7 4
0.200	-0.075	-0.195	120	3.45	7.7	3.0	6.8
(f) PT(NaOH) modiliod electrode in 1 mM p-benzoquinone							
0.005	-0.115	-0.170	65	0.24	3 4	05	7 0
0.010	-0.115	-0.170	65	042	4.2	0 7	7.0
0.020	-0.115	-0.170	65	0.68	4 8	0 8	5 9
0.050	−0.110	-0.190	80	1 20	5.4	1.2	5 5
0.100	-0095	-0.190	9 5	1.65	5.2	1.9	6 2
0.200	-0.090	-0.200	110	2.55	5.7	2.6	5.9

In the present experiment, large currents are seen that cause considerable iR errors. After modification, the peak currents do not decrease considerably. We have still retained the larger electrode mainly because we are planning to couple enzymes onto this electrode and observe the redox processes catalyzed by enzymes. At that stage, the currents are expected to be in the μA range. In addition, a larger electrode area permits us to examine the same film formed on the electrode by spectroscopic techniques.

The same studies repeated for polytyrosine modified electrode in potassium ferrocyanide and p-benzoquinone. Since both are reactive at a bare platinum electrode, the performance of the modified electrode could easily be compared. In Fig. 4.4 the cyclic voltammograms have been presented for ferrocyanide (graph a) and p-benzoquinone (graph b) for the film obtained from acidic and alkaline solutions.

From the CV graphs for ferrocyanide one observes that the relatively sharp peaks seen in bare electrode are reduced significantly in amplitude (for both electrodes) nevertheless, a small hump is still visible suggesting that very fast electron transfer is somewhat inhibited. This is expected because the film is having some resistance which interferes with very fast electron transfer. Thus such modifications are not effective, as expected, when the electron transfer rate is very high in the bare electrode. Interestingly, the peak positions are not altered suggesting that the polymer is not involved in any chemical reactions with the substrate i.e. the electron transfer from the polymer to the metal is not rate determining.

For the p-benzoquinone the three plots (bare electrode, PTyr(H SO and PTyr(NaOH) are practically identical, suggesting that the presence of the film does not significantly alter the electron transfer process. As in the case of ferrocyanide, peak positions /separations are not altered suggesting that the film formed is not acting like a mediator.

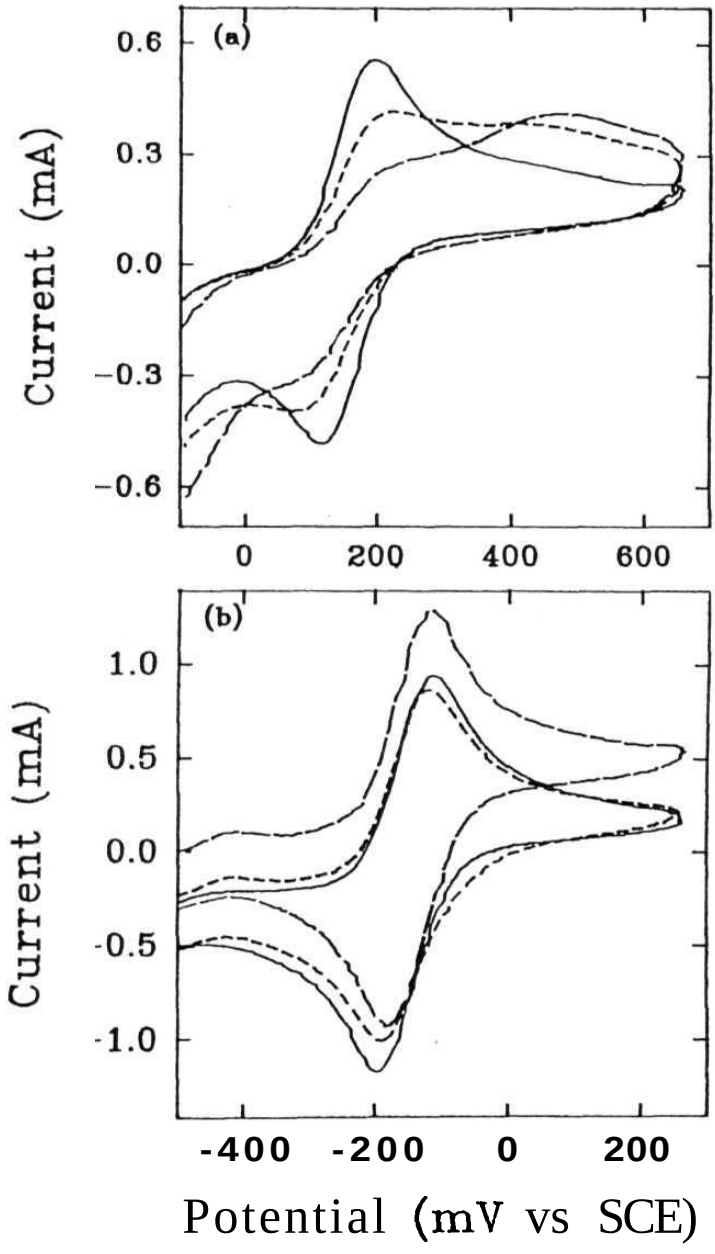

Fig. 4.4. Cyclic voltammograms of bare Pt mesh (solid line). PTyr(H SO) (dashed line) and PTyr(NaOH) (dotted line) (a) is for 1mM K Fe(CN) (b) is in 1mM p-benzoquinone in supporting electrolyte. Scan rate is 20mV/sec in both cases.

5. DISCUSSIONS

PT films made from H_2SO_4 and NaOH solutions show relatively high conductivity in the + 400mV potential range. This is a very important range of potential for various biological systems. The modified electrodes show a smaller AE suggesting that it functions more reversibly compared to the bare electrode. For ferrocyanide (Fig 4.3b), since the current is rather high a larger iR drop makes accurate AE measurement difficult. Nevertheless, a wave is observed suggesting that the high currents cannot be sustained by the PT(H_2SO_4) films. On the other hand, the PT(NaOH) film shows comparable results to that of a bare electrode.

Since the polytryptophan/polytyrosine films formed this way are quite thin, it is difficult to accurately measure its thickness by standard techniques. The actual thickness of the film formed may be estimated by coulometry. Under standard conditions, the thickness may be considered proportional to the number of cycles used during polymerization and we have been able to obtain quite reproducible results. Once the electrode is completely covered, the thickness of the film does not appear to be critical. However, excessively thick films may not be highly conducting.

Since the film itself is colored, optical interference cannot be used to determine its thickness. Measurement of capacitance of the film can be useful, but the dielectric constant of the polymer must be known to determine the thickness. In the early parts of polymerization, optical interference of color is observed and hence the film formed is at least several hundred nanometers in thickness. The literature values of useful polypyrrole films are also in the same range (11).

Although it is not possible at this stage to indicate the nature of the PT/PTyr films formed or the mode of conduction, we suspect the conduction to be electronic in nature. Similar conducting films of this type- viz., polypyrrole, polylndole, polyanillne etc, all have aromatic rings that can conduct by a charge transfer interaction with the electrode. Thus p-benzoquinone may interact with the film by a charge transfer interaction more readily compared to ferrocyanide. This may explain reduced currents for potassium ferrocyanide compared to p-benzoquinone, when compared to the bare electrode.

In all these cases, there is no significant change in the peak positions suggesting that the polymer is not acting as a mediator. This is important in several cases because we do not expect direct oxidation/reduction of the polymer film on the electrode. Further the background current for this electrode also appears to be smaller compared to the bare electrode, suggesting that the redox species (p-benzoquinone) is preferentially reduced. The impedance studies reported here are preliminary in nature as the frequency dependence of the resistance and capacitance has not been carried out. However, we have used a rather low modulation frequency (80Hz) and therefore the results obtained may be correlated well with the DC or static resistance and capacitance. Further, the impedance studies have been carried out in a solution containing supporting electrolyte only and therefore the results obtained are characteristic of the film alone and are free from the effects of the electroactive species. Important kinetic results may be obtained from studies of frequency dependence in presence of an electroactive species. However the results are comparatively difficult to analyze and interpret.

Since tryptophan and tyrosine are naturally occurring biological substances, it is expected to be more suitable compared to several other electrodes currently in use, e.g., polypyrrole. When cross-linked with spermine, there are considerable number of free

amino groups present on the film surface and these can be used for covalent coupling. For example, we have been successful in coupling peroxidase by covalent coupling with glutaraldehyde. It is also possible to have entrapped enzyme (with BSA) using glutaraldehyde. It is however, not possible to trap the enzyme during polymer film formation.. For efficient entrapment, a high enzyme concentration is required and the high potentials used in electropolymerization can inactivate the enzyme. Although covalent coupling shows somewhat lower enzyme loading when compared to the entrapped (cross-linked) enzyme, the sensitivities are considerably higher (12). Other modes of covalent couplings are possible but has not been fully explored. The electrodes so prepared have considerable analytical use as possible biosensors. The SEM picture suggests that the film is porous. Considerable modifications of preparation conditions are required before a pore free homogeneous film may be obtained. The preliminary data are of sufficient importance to merit further studies and development as biosensors.

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5. An enzyme electrode for hydrogen peroxide based on peroxidase immobilized on glassy carbon electrode and impedance studies

1. INTRODUCTION

Hydrogen peroxide is an important compound both in biological and industrial reactions. Development of reliable monitoring techniques has been of great interest in recent years. Among these techniques, amperometric biosensors show significant advantages such as high sensitivity and large linear dynamic range (1). Only in a few cases direct electron transfer between the active site of a redox enzyme and electrode has been reported (2-5). In few cases, a direct electron transfer has been the basis for the detection in amperometric enzyme electrodes. More often, a soluble charge carrier has been used to shuttle the charge between the active site and the electrode. Glucose biosensors are based on a reaction catalyzed by glucose oxidase where the actual electrochemical detection is based on measuring either the decrease in the oxygen tension or the hydrogen peroxide produced. In several instances an organic or organometallic redox mediator has been used as a low molecular weight electron transfer agent between the electrode and the active site of the enzyme (6). Recently it has been shown that when glucose oxidase is immobilized on the electrode surface in the electropolymerization process of N-methylpyrrole, a direct electron transfer takes place (7). By binding the redox mediator covalently to the enzyme a direct electron transfer between the enzyme and an electrode can be obtained (8). These two approaches seem very attractive and may have openings to make further use of redox enzymes in biosensor configurations.

Recently there have been several reports on direct electron transfer from the electrode to the oxidized form of peroxidase. It has been shown to take place with the peroxidase immobilized on carbon electrodes and also to a lesser extent on platinum. A number of different peroxidases viz., horse radish peroxidase, cytochrome c

peroxidase, fungal peroxidase, microperoxidase, and lactoperoxidase have been investigated and have been shown to display this activity, (9-16). Cyclic voltammograms of graphite electrodes modified with adsorbed peroxidase in plain buffer do not reveal any special characteristics distinguishing them from cyclic voltammograms obtained for the basic electrode material. However, in the presence of hydrogen peroxide cyclic voltammograms reveal a very strong reduction wave typical for an electrocatalytic reduction process (11,12,15,16).

2. EXPERIMENTAL

2.1. Instrumentation: The procedure for the impedance measurement was explained in the previous chapter. Impedance measurements were also carried out at different frequencies at zero bias potential (vs SCE) using a PAR model 378 potentiostat and a model 5210EC lock in amplifier. The impedance studies are repeated for the bare electrode as well as modified electrode (polymer coating only; tryptophan in acid solution containing spermine). The standard software supplied by PAR were used for these data.

2.2. **Electrodes:** The modified electrode was studied at several stages of its preparation. The following electrodes were studied.

1. Bare electrode
2. Bare electrode + enzyme (glutaraldehyde cross-linked)
3. Electrode + polytryptophan film
4. Electrode + film + enzyme (glutaraldehyde coupled)
5. Electrode + enzyme (carbodiimide coupled on bare electrode)

Electrode preparation

2.2.1. Bare electrode

The glassy carbon disc (a kind gift from Dr Marc Verhagen, The Netherlands) was cleaned thoroughly by dipping in hot concentrated

nitric acid for one hour and was rinsed with 0.1M phosphate buffer at pH 4.5. The diameter of the glassy carbon electrode disc was 15mm. However, the current densities are low and polarization effects are therefore small. Although no visible corrosion of the surface was observed, it is highly probable that microscopic erosion of the carbon material does take place and new carboxyl functionalities are formed at the electrode when the electrode was cleaned hot concentrated nitric acid. The high background current actually decreases after the film formation. For all the experiments this pretreatment was essential for reproducible results.

2.2.2. Enzyme cross-linked by glutaraldehyde on bare electrode

Horse-radish peroxidase (E.C. 1.11.1.7, Sigma Cat No.P-8375) was dissolved (5mg/mL) in 0.1M phosphate buffer pH 6.0. A stock solution (20mg/mL) of BSA (Sigma Cat No. A-6918) was also made. To a clean eppendorf tube, 50µl of enzyme solution and 10µl of BSA solution were mixed and 2.5µl of 25% glutaraldehyde solution was added. The final concentration of glutaraldehyde was 1%. The solution was thoroughly mixed and poured on to the electrode and left at 4 C for three to four hours. The electrode was washed thoroughly with buffer followed by distilled water and was finally stored in buffer. Polytryptophan film was formed on the glassy carbon electrode as described earlier.

2.2.3. Enzyme coupling to the polytryptophan film by glutaraldehyde

The electrode with the polytryptophan film, as prepared above, was dipped in *2%* glutaraldehyde (GA) solution and left at 4°C with stirring for three to four hours. After GA activation the electrode was thoroughly washed with 0.1M phosphate buffer pH 6.0. and dipped in enzyme solution (5mg/ml) and left at 4°C for three to four hours and washed thoroughly to remove unbound enzyme.

2.2.4. Carbodiimide coupling of enzyme on bare electrode

The pretreated glassy carbon electrode (bare, without polytryptophan) was activated by treating with 1-Ethyl-3-(3-Dimethylamino-propyl) Carbodiimide hydrochloride (EDO. The electrode was dipped in EDC solution at a concentration of 20mg/ml in O.1M phosphate buffer at pH 4.5 for two to three hours at room temperature. Later the electrode was washed thoroughly with O.1M phosphate buffer and was dipped in 5mg/ml enzyme solution and left at 4°C for three to four hours. Finally the uncoupled enzyme was removed by washing and the electrode was stored in buffer.

3. CV STUDIES

Cyclic voltammograms were obtained for the following electrodes:

1. Bare glassy carbon electrode
2. Electrode coated with polytryptophan film
3. Electrode coupled with enzyme by carbodiimide activation
4. Modified electrode with film and covalently coupled enzyme by glutaraldehyde activation

4. IMPEDANCE STUDIES

Two sets of impedance studies were carried out for the above electrodes. In one set of the experiments frequency was kept constant (80Hz) and the bias potential was varied. The following electrodes were studied by this technique.

1. Bare electrode
2. Electrode + film
3. Electrode + enzyme (carbodiimide coupled)
4. Electrode + film + enzyme (glutaraldehyde coupled)
5. Electrochemical Impedance Studies (Bode plots) were done for the bare electrode and electrode modified by polytryptophan coating, at zero bias potential (vs SCE).

5. RESULTS AND DISCUSSION

5.1. General results

Fig 5.1 (a) shows Cyclic Voltammogram (CV) for the bare electrode in phosphate buffer pH 6. Fig 5.1(b) shows CV for the electrode coated with a polytryptophan film in the same buffer in absence of the substrate. Fig 5.1(c) shows the CV for the electrode coupled to the enzyme by carbodiimide modification. The graph is quite similar to Fig 5.1(a). Fig 5.1(d) is similarly the CV for the electrode with a polytryptophan film to which the enzyme is covalently attached by glutaraldehyde coupling. It can be seen that the redox groups on the polytryptophan films have disappeared after enzyme attachment (glutaraldehyde attaching to the free amino groups on the film). Since the chemical structure of the polytryptophan formed by electrochemical oxidation is unknown at present, it is not possible to state definitely the number of amino groups available on the polymer for enzyme attachment. The enzyme was coupled to the film by covalent coupling and the enzyme loading should be as an indication of the available functional groups in the polymer. The enzyme does not attach to the polymer by adsorption because in absence of the coupling agent, the free enzyme can be washed off quite easily. All these voltammograms were recorded in absence of substrate (H_2O_2) and hence no prominent peaks are observed.

Some general conclusions may be drawn on the basis of these voltammograms. The general potential range for the oxidation of the tryptophan is somewhere around 1.1V (vs SCE) and a potential range of -200 to 400mV (vs SCE) was scanned because this range of potential is biologically very important. A decrease In the background current is clearly seen around 0V (vs SCE). The bare electrode and the modified electrode cannot be directly compared because of the presence of redox groups on the film. Although the voltammograms are not very clean, qualitative conclusions can still be made.

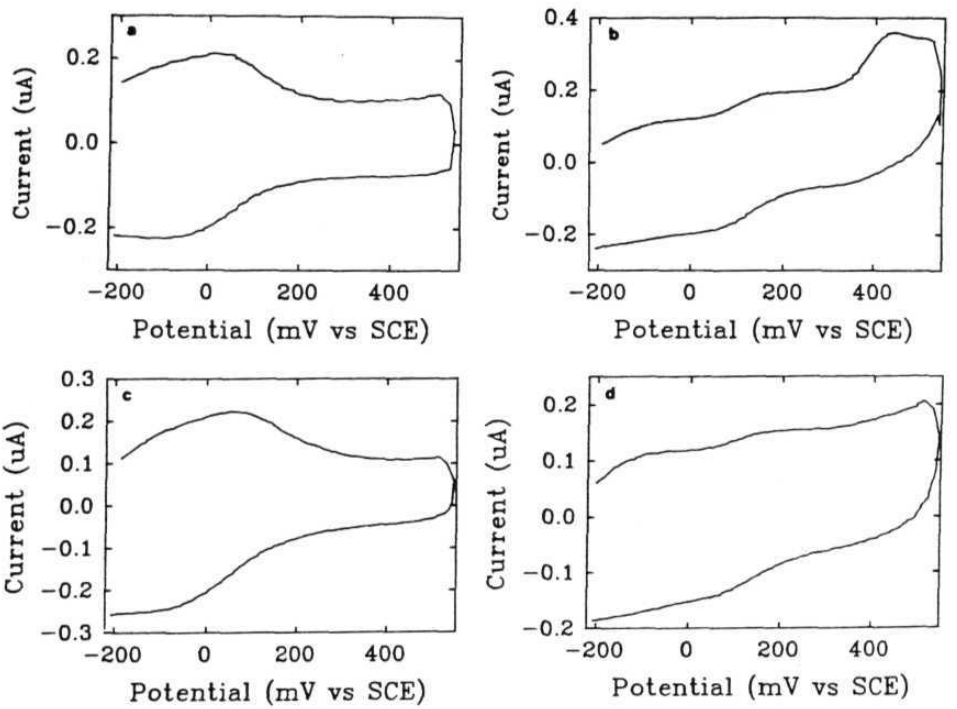

Fig. 5.1(a). Shows CV of bare electrode (b) is CV of polytryptophan film modified electrode (c) CV of carbodiimide coupled enzyme electrode (d) CV of polytryptophan film and glutaraldehyde coupled enzyme electrode. Scan rate 50mV/sec, in sodium phosphate buffer pH 6.0.

For hydrogen peroxide estimation, a potential of -200mV (vs SCE) was used and concentration determined amperometrically. The electrode was usable after 7 days when stored in the refrigerator. No systematic determination of the half-life of the electrode was attempted, because life of the electrode is best determined only when all the other parameters have been optimized. Fig 5.2 shows the concentration dependence of the immobilized enzyme electrodes. The graph with solid circles were obtained in which the enzyme was immobilized by cross-linking with BSA by glutaraldehyde. The polytryptophan film was absent. This technique gives the highest enzyme loading on the electrode and hence relatively high currents are observed. The results obtained with the enzyme immobilized directly on the glassy carbon by carbodiimide are shown by open triangles. This electrode could not be used above 100μM of H_2O_2 concentration for reasons not very clear. The final set, represented by the solid triangles, correspond to the electrode on which the enzyme has been immobilized by covalent coupling using glutaraldehyde to the film. This electrode shows lowest currents (compared to the other two) but relatively higher sensitivity (as indicated by the larger slope).

Impedance studies similarly were carried out at a fixed frequency (80Hz) but at variable bias potential. Fig 5.3(a) shows the two components of impedance as a function of the bias potential for the bare electrode. Fig 5.3(b) shows the impedance plot under identical conditions for the electrode covered with the polytryptophan film. Due to the increase in ohmic resistance and decrease in parallel capacitance (in the terms of Randies cell model), both real and imaginary components are increased in this plot. In Fig 5.3(c) we see the effect of the electrode to which enzyme has been coupled by carbodiimide technique. There is a significant increase in both the series resistance and in the parallel capacitance when compared Fig 5.3(b). The increase in series resistance can be attributed to the blocking of the functional groups (mostly COOH) on the bare electrode.

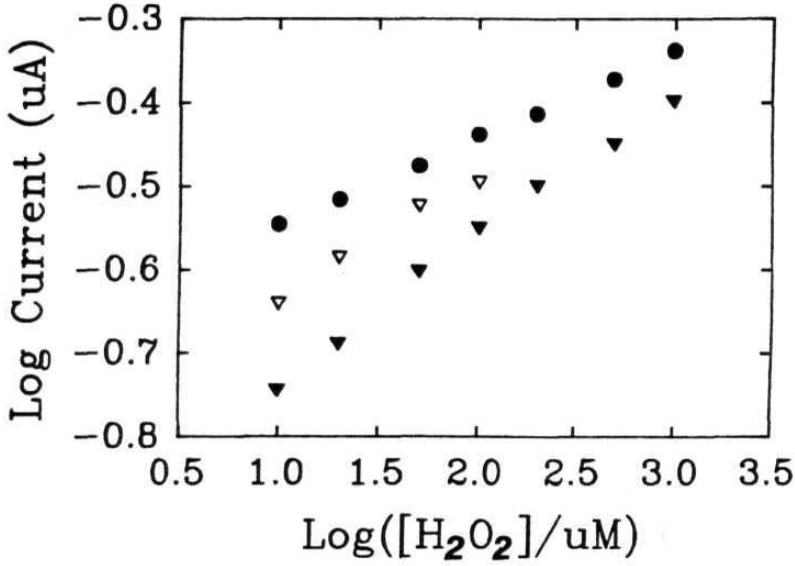

Fig. 5.2. Calibration curves for hydrogen peroxide: solid circles **are** for glutaraldehyde BSA cross-linked enzyme electrode, blank triangles are for carbodiimide coupled enzyme electrode and solid triangles are for polytryptophan glutaraldehyde coupled enzyme electrode in phosphate buffer pH 6.0.

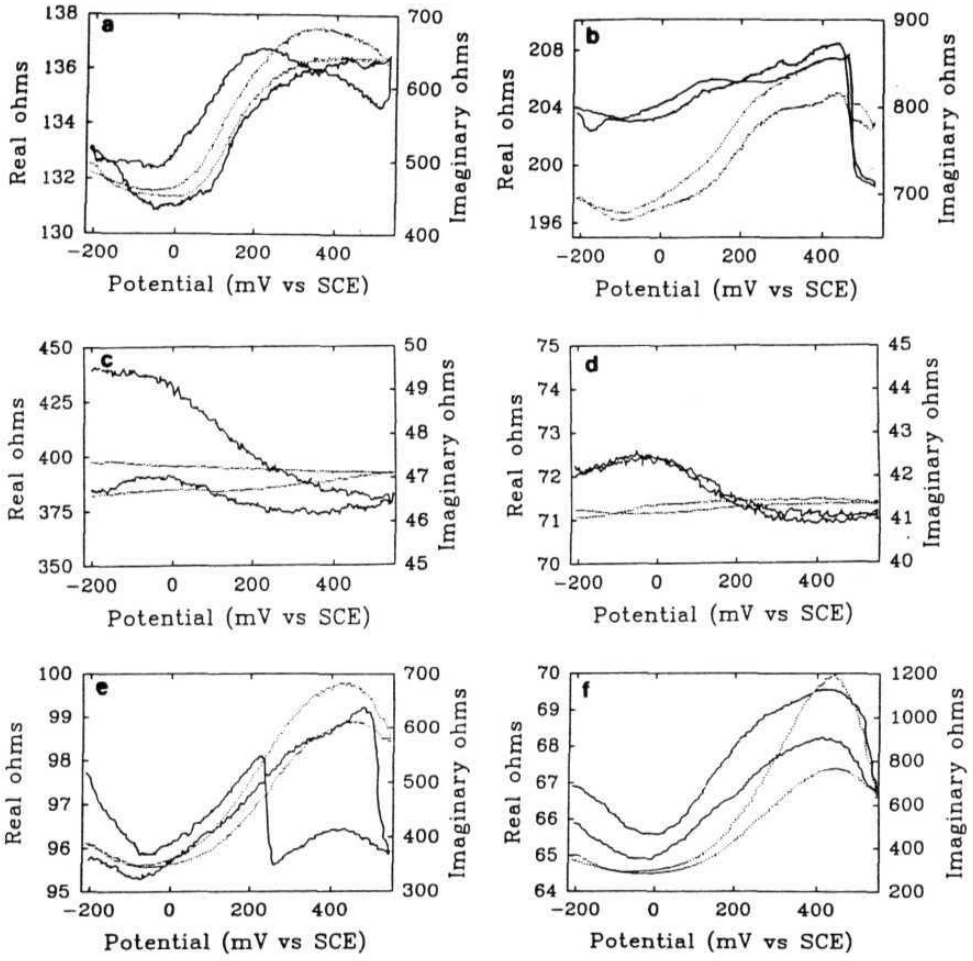

Fig. 5.3. Impedance plots at fixed frequency (80Hz) as a function of potential. (a) is for bare electrode, (b) is for electrode modified with polytryptophan film, (c) is for the carbodiimide coupled enzyme electrode, (d) the same electrode (c) with 1mM substrate, (e) is for the enzyme coupled by glutaraldehyde to the polytryptophan film, (f) the same electrode (e) with 1mM substrate. All the above impedance studies were done in 0.1M phosphate buffer pH 6.0. Scan rate is 10mV/sec. The solid line denotes the real part (left side vertical axis) and dotted line denotes the imaginary part (right side vertical axis).

The increase in parallel capacitance may be due to the non conducting protein (enzyme) layer. In Fig 5.3(d), we present the plots for the same electrode as above but in the presence of 1mM substrate. In presence of the substrate, the series resistance has considerably reduced (due to enzyme catalyzed process) when compared to 5.3(c) leaving the parallel capacitance virtually unaffected. In Fig 5.3(e) shows the same plots for the electrode to which enzyme has been covalently coupled by glutaraldehyde to the film (of 5.3(b)). Fig 5.3(f) shows again the same plot in presence of lmM substrate (of 5.3(e)). Using same arguments as above, we note that the series resistance has slightly reduced in presence of the substrate. It is to be noted that this technique of immobilization gives the lowest enzyme loading and hence the cases for two above enzyme electrodes are not exactly comparable.

In Fig 5.4, we present the impedance plots (Bode) for the same electrode (but prepared at a different time) at 0 bias potential (vs SCE) as a function of frequency. The impedance values in Fig 5.3 and Fig 5.4 are not directly comparable because the electrodes have been prepared at different times and hence are not identical. Nevertheless, the trends seen in Fig 5.3 are clearly seen in this diagram. For example, considering the values at 80Hz (log 80=1.9), We find that the log $Z;\phi$ for the bare and the coated electrode are 2.1, -2° and 1.4, -22 respectively. Inspection of these values shows that capacitance decreases, as described earlier (Fig 5.3).

5.2. Discussion

From the CV and impedance studies, it is clear the polytryptophan film formed as described is a promising medium for enzyme immobilization. Although we have not been able to accurately characterize the chemical nature of the film formed, it appears to be electrically conducting in nature. The impedance studies show that the impedance drops to a low value only when both substrate and enzyme are present and this

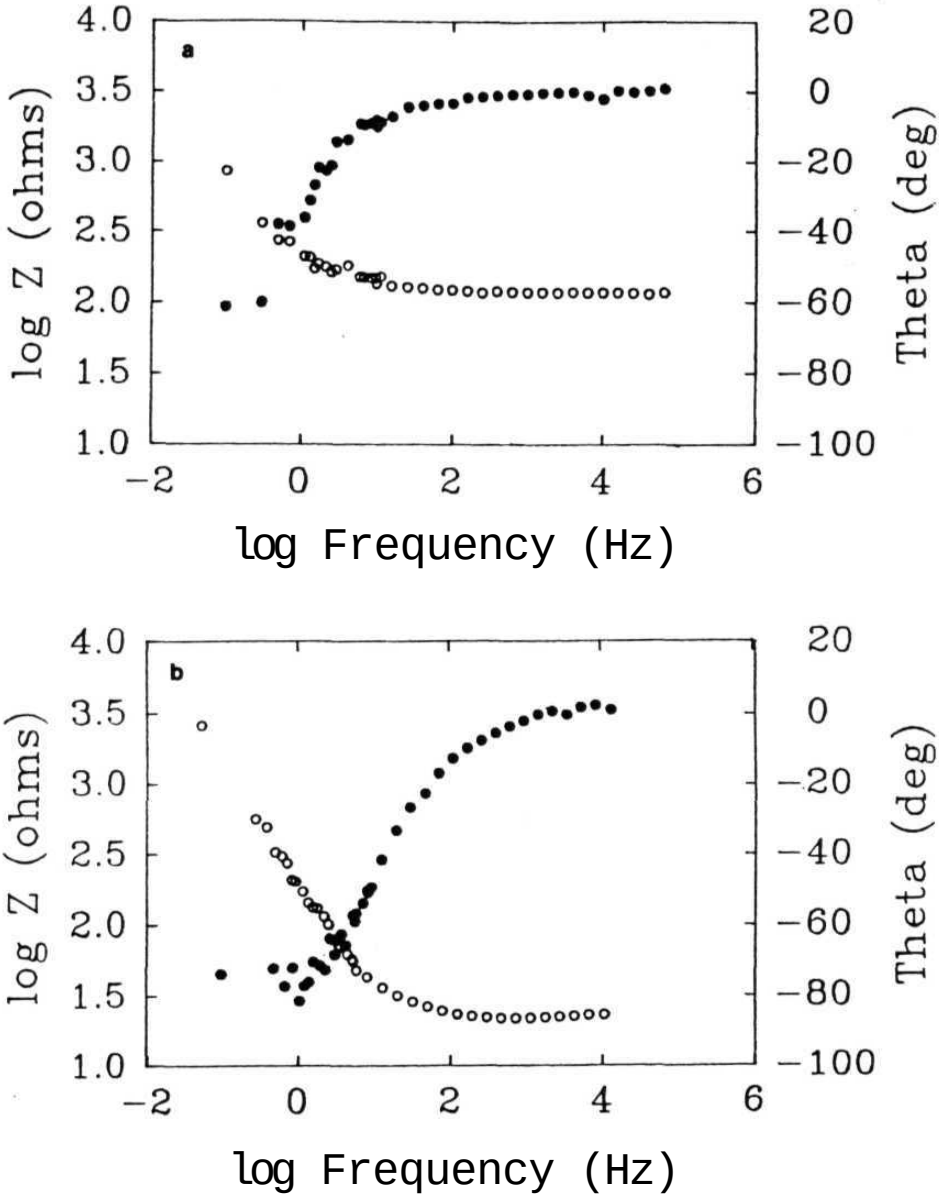

Fig. 5.4. Impedance plots at variable frequency, (a) shows the Bode plot of **the** bare electrode and (b) for **the** polytryptophan modified electrode. Blank circles denotes the impedance (left side vertical axis) and filled circles **are** for the phase angle (right side vertical axis) theta in degrees.

suggests that the film can act in a highly selective fashion. Enzyme coupled to bare electrode by carbodiimide behaves somewhat differently and may not be so useful.

The amount of enzyme actually coupled to the electrode cannot be very accurately determined but it appears that the polytryptophan film coupled to the enzyme by glutaraldehyde has a rather low enzyme activity. It is however possible to increase the enzyme loading by (i) including a larger amount of cross-linker (spermine) or by (11) coupling glutaraldehyde activated film to spermine followed by reaction with glutaraldehyde and finally adding the enzyme. These possibilities have, however, not been explored.

The polytryptophan film when observed under a scanning electron microscope shows a porous structure. This therefore does not completely block the base electrode but increases diffusion resistance to the base material. Also, impedance spectroscopy has not very great use in analyzing kinetic data for which low-frequency impedance dispersion is more suitable. These are currently under investigation.

In conclusion, we add that the polytryptophan film is electrically conducting and enzyme can be easily attached to it. The redox behaviour of the film itself is not important in the potential range studied. As far as the film is concerned, impedance studies show clearly that it is a very important one and worth a more detailed investigation.

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6. Low frequency impedance studies on peroxidase immobilized on a polytryptophan film

1. INTRODUCTION

Although the concept of chemically modified electrodes (CMEs) was first demonstrated in the early seventies, this approach has recently assumed a dominant position in modern electrochemistry. These electrodes, which are made by incorporating specific chemical groups or microstructures on otherwise conventional electrode surfaces, are of special interest because their electrochemical responses have two completely independent components. The usual electrochemical component, determined primarily by the potential at which the electrode is maintained electronically (externally with reference to some standard potential electrode), is supplemented by another chemical component, determined primarily by the reactivity of the groups or structures on the electrode. Thus the electrode is no more an inert system as in conventional electrochemistry, it is a reactive reagent capable of undergoing electrochemical transformation at suitable potentials. Consequently, CMEs offer not only variable characteristics but also the possibility of adjustable physical and more importantly, chemical properties such as charge, polarity, chirality, permeability etc. Therefore, CMEs can be targeted for a specific application or investigation to a much greater level of selectivity or specificity than it was formerly possible with classical or unmodified electrodes. Now CMEs can be rationally designed to provide for an optimal environment for a given task. Analytical biochemistry is certainly one of the foremost areas that are directly affected by this development. Therefore, new techniques to study CMEs have to be developed with this application in mind (1).

The primary aim of the modified electrodes is to design or engineer the surface of the electrode at a molecular level so that it will be recognised by the biological redox species and rapidly exchange electrons with it. Although this approach is fraught with difficulties significant advances have been achieved in this field over the past few years by building on fundamental work in the area of the electrochemistry of modified electrodes (2,4).

2. METHODOLOGY

Classically, an electrode is basically an inert surface on which no significant chemical reaction (except electron transfer) takes place. The electron transfer processes at an electrode has been quite well studied over the last several decades. With functional group modifications of the electrode surface, not only electron transfer but also potential dependent chemical kinetics assumes a major role. A host of electrode surfaces has been developed to select, enhance and stabilize the direct oxidation and reduction of proteins, thus liberating the bioelectrochemist from the obligatory use of mediators when using electrodes to study biological electron transfer. Many researchers have described direct electron transfer to the electrode from the biological molecules (5-9). A simple technique developed by us is of the study of the low frequency impedance measurements on such electrodes.

Impedance spectroscopy of electrodes is not a new phenomena and many studies have been reported on modified electrode with a reversible redox polymer (10,11), at semiconductor electrodes (12), porous electrodes **(13,14)** and conducting polymers like polypyrrole, polyacetylene, polyaniline (15,16), etc. Classically, impedance studies have been conducted on simple electrodes and the results have been variously represented as (i) Bode plots, (ii) Nyquist plots (iii) Admittance plots and various other representations of essentially the same phenomena. The basic limitations in these approaches are that

the electrode is approximated as a simple R-C combination which may be satisfactory at medium to high frequency regions of observations. On the other hand, chemical rate processes cannot be approximated to simple L-C-R circuits because new species are formed and old species are consumed. The chemical reactions take place at characteristic rates depending on the respective specific reaction rates and concentrations of reactive species and these are typically slow processes.

To study chemical processes at an electrode, the first step is to get rid of the Randle's cell model and introduce the concept of sinks and sources. Diffusion is a passive process and can introduce important phase shifts depending on the rate of reaction and the frequency of measurement. Thus low frequency impedance studies assume greater importance in CMEs. Very few low frequency impedance studies of the modified electrodes are available in literature (17,18).

2.1. **Technique:**

Low frequency impedance studies are generally difficult to carry out in simple lock-in-amplifier set ups. Instead, a fast-fourier transformation of the response of a stored waveform on the electrochemical system is generally preferred. A superposition of several sine (or cosine) waves (with suitable phases, as necessary) is stored in a computer. This digitized waveform is applied to the electrode system as a potential and current values are measured. The current values are fourier transformed to separate the various frequency components. These are further phase corrected and the real and imaginary components of the current for various frequencies determined. The major advantage of this technique is its simplicity; the major disadvantage is that it is restricted to low frequency studies only.

Since the frequencies of the stored waveform are already known, "folding" of the spectra does not provide any significant disadvantage and we have therefore deliberately used a number of frequencies that are folded several times. These frequencies are chosen carefully so that they do not overlap with other frequencies after folding.

3. EXPERIMENT:

A simple PC-XT was used in this experiment and the A/D and D/A conversion facilities of our lock-in-amplifier model PAR 5210 was used. The lock-in-amplifier was used only for its A/D and D/A conversion functions provided. The potentiostat 174A (PAR) was used in the AC mode and the modulation potential was applied through the external connection provided on the polarographic analyzer.

A file was created in BASIC using simple programs to generate 1024 points for sine frequencies of 1, 3, 7, 15, 31, 63, 127, 255, 511, 1003, 2007, 4015 and 8031. Each frequency was phase shifted so as to give a quadratic dependence of frequency. This is desirable so that excitation power is rather uniformly distributed in time (19). A plot of this file is shown in Fig. 6.1(a). Each frequency component was conveniently selected to have the same amplitude. These 1024 points are stored permanently in a data file and is used by all excitation programs. A plot of the FFT of the excitation waveform, showing peaks at different frequencies is shown in Fig. 6.1(b). This data file was read, applied to the D/A converter and the output of the D/A is scale down (this is done by a resistor voltage divider so as to provide higher accuracy) and is fed to the polarographic analyzer. The current produced is read, after a constant delay, by the computer through the A/D converter. This constant delay which is required for instrument electronics, introduces a variable phase shift proportional to the frequency which need to be corrected. The 1024 different current values are stored for subsequent fourier transformation.

There are two sources of phase error: one in the original stored waveform and the other due to the lag in the acquiring of current data. The phases can be adjusted manually or automatically in several ways but we have followed a different approach. A separate file was created for a dummy cell of 10KΩ resistance (provided internally in the polarographic analyzer) and the phase was recorded for every point. Since a pure resistance is supposed to give only real current, these phase values therefore reflect the corrections that are required to be applied to any spectra. For a better and more accurate value, we have averaged the phases from 10 scans and these are again stored in a file. After the data acquisition is over, the current values are fast fourier transformed, phase corrected using the above data to give minimum imaginary current values. A plot of the phase corrections required is shown in Fig. 6.1(c). The current values at the appropriate frequencies are used for computation of impedances.

3.1. **The cell:**

A conventional three electrode single compartment cell was used in all the experiments. The electrochemical cell holds modified glassy carbon or bare glassy carbon as the working electrode. A thick platinum wire (1mm dia) served as the counter electrode in the voltammetric and impedance measurements. An aqueous saturated calomel electrode (SCE) was used as the reference electrode. 0.1M sodium phosphate buffer at pH 6.0 was used as the supporting electrolyte.

The glassy carbon disc was cleaned thoroughly by dipping in hot concentrated nitric acid for one hour and was rinsed with 0.1M phosphate buffer at pH 4.5. For all the experiments this pretreatment was essential for reproducible results. Two different preparations of modified electrodes were attempted. In one, the enzyme was immobilized with BSA using glutaraldehyde as the cross-linker. In

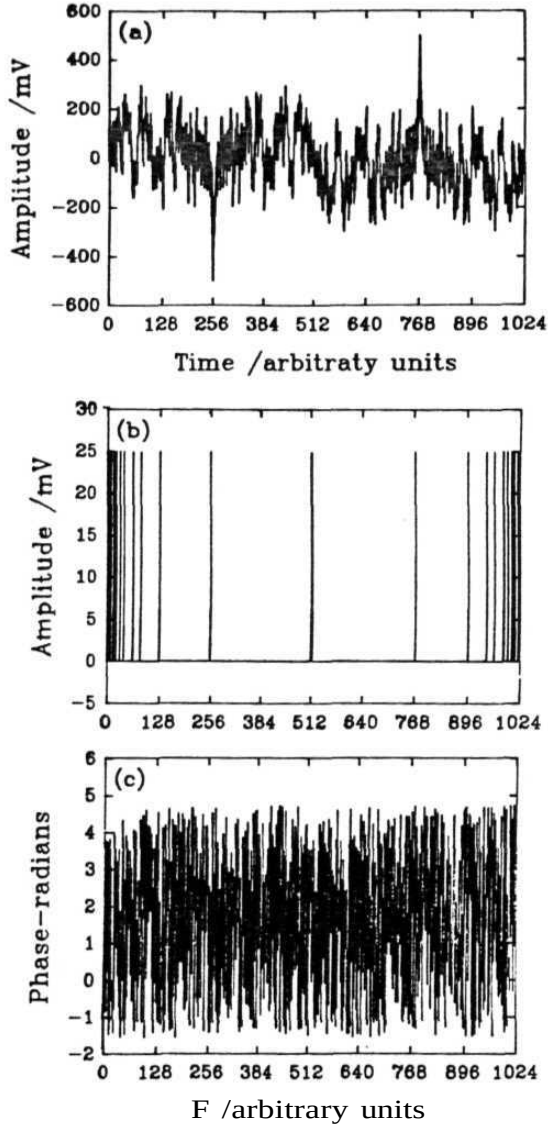


Fig. 6.1. (a) The stored waveform used in the excitation of the cell in the low impedance studies. The amplitudes (Y values) are chosen so that each frequency component gives 10mV (peak to peak) after scaling by a voltage divider. (b) The FFT of the stored waveform plotted in an arbitrary scale. The actual frequencies are dependent on the time taken for the experiment. Also, this spectrum shows multiple folding of the higher frequencies due to slow sampling. The actual frequencies are given in the text. (c) The phase correction required for the dummy cell, averaged for ten scans. The phase corrections appear to be noisy due to multiple folding of the higher frequencies.

another preparation, a polytryptophan film was formed by anodic oxidation and the enzyme was covalently coupled to this polymer film by glutaraldehyde.

3.2. Electrode modification:

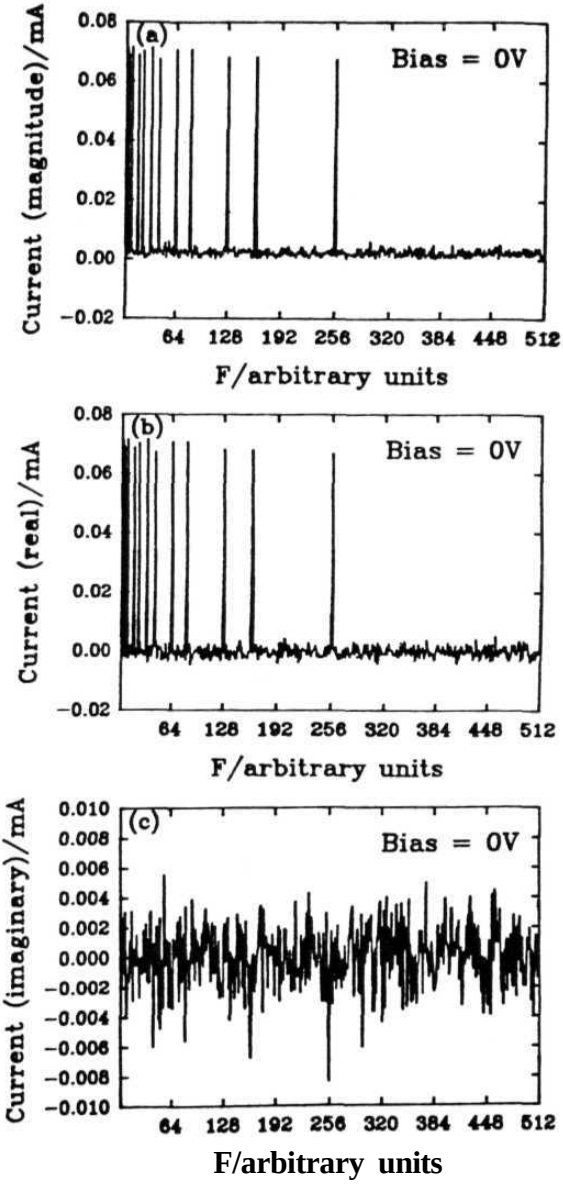
Horse-radish peroxidase (E.C. 1.11.1.7, Sigma Cat No.P-8375) was dissolved (5mg/mL) in 0.1M phosphate buffer pH 6.0. A stock solution (20mg/mL) of BSA (Sigma Cat No. A-6918) was also made. To a clean eppendorf tube, 50µl of enzyme solution and 10µl of BSA solution were mixed and 2.5µl of 25% glutaraldehyde solution was added. The final concentration of glutaraldehyde was *V/..* The solution was thoroughly mixed and poured on to the electrode and left at 4°C for three to four hours. The electrode was washed thoroughly with buffer followed by distilled water and was finally stored in buffer.

Polytryptophan films were formed on to the pretreated glassy carbon electrode from 0.2N sulphuric acid containing 100mM tryptophan. Visible films formed at 1500mV potential but a constant potential was not applied. Instead, the potential was scanned from 0-1500-0mV (vs SCE) at a rate of 20mV/sec for one hour (24-25 scans). Films formed this way adhered better to the electrode. A light-bluish polymer film could be seen on the electrode at this stage. This was washed thoroughly with distilled water followed by a final rinse with the supporting electrolyte. The electrode with the polytryptophan film, as prepared above, was dipped in 2% glutaraldehyde (GA) solution and left at 4 C with gentle stirring for three to four hours. After GA activation the electrode was thoroughly washed with 0.1M phosphate buffer pH 6.0. and dipped in enzyme solution (5mg/ml) and left at 4 C for three to four hours and washed thoroughly to remove unbound enzyme.

4. RESULTS **AND** DISCUSSION:

To establish the quality of the software developed and the hardware used, we have run an experiment with a standard cell made up with with a 100μF capacitor in parallel with a 120n resistor. A small series resistor (20Ω) was also used. This cell, made up of purely passive elements, was connected instead of the working cell as usual. The results obtained with this configuration are shown in Fig 6.2. It is to be noted that at the low frequencies used, the capacitor hardly plays any significant role and the impedance is 140Ω throughout the spectra. The imaginary component of the current appears very noisy because of the low frequencies used. For all practical purposes the real part of the spectra is virtually identical with magnitude spectra, as expected.

In Fig. 6.3, we have plotted the currents after fourier transformations for the covalently coupled enzyme for two substrate concentrations (10 and 1000μM of H 0) at two different bias potentials. It is to be noted due to that multiple folding, high frequency peaks are shifted to different locations. The peaks are clear and strong at the bias potential of zero mV (w.r.t. a SCE). At 160mV bias, the peaks are rather low in intensity, as expected. The electrochemical process at the electrode clearly acts in a manner similar to an electronic notch filter at the characteristic frequency, energy is absorbed and hence less current transmitted. At all other frequencies, however, the full current is allowed to flow freely. Although the differences are not dramatic, they are never the less significant. Fig. 6.3 (a) is for real component at zero potential bias (b) is for magnitude at zero bias (c) is for real component at 160mV bias and (d) is for magnitude at 160mV bias all these are at 10μM substrate concentration. The same measurements were taken for 1000μM substrate also they are e, f, g, and h.



Fif. 6.2. Current spectrum Tor the standard impedance cell, showing the (a) magnitude (b) real and (c) imaginary components. Modulation amplitude used was 10mV. The standard cell was made of a 120Ω resistor in parallel with a 100μF capacitor and a 20Ω series resistor.

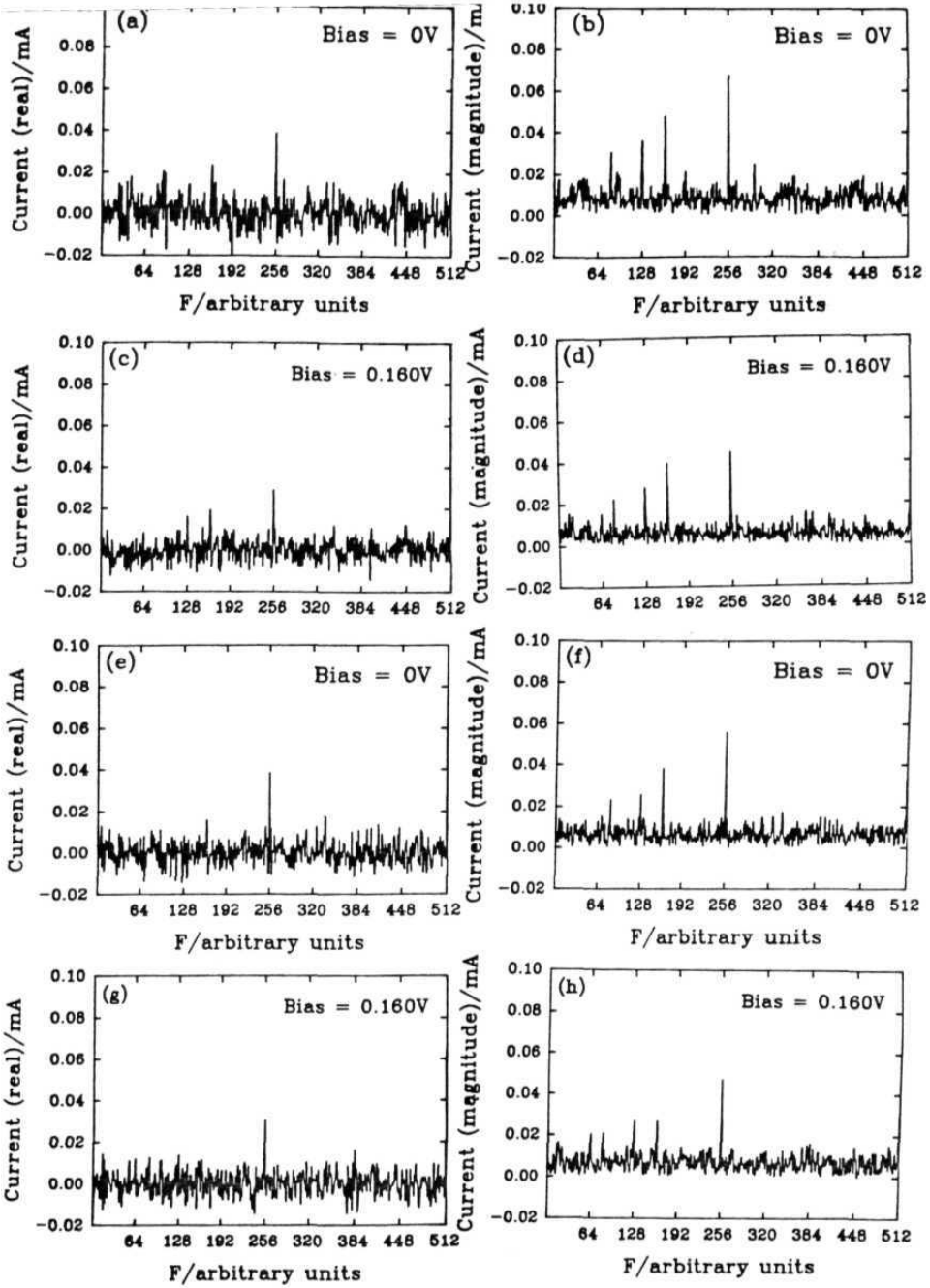


Fig. 6.3. Currents for the covalently coupled enzyme to the polytryptophan film coated electrode. (a), (b) are for 10μM substrate concentration and zero bias potential (c), (d) are same as (a), (b) but for 160mV bias potential. (e), (f) are for 1000μM substrate concentration and zero bias potential (g), (h) are same as (e), (f) but for 160mV bias potential.

Similar experiments carried out with enzyme cross-linked with glutaraldehyde BSA, at two different bias potentials and substrate concentrations, were carried out.

In Fig. 6.4, we have converted the currents values at different frequencies and converted them to impedance values. Plots of the impedance (Z) vs log frequency (f) are reported for both electrodes at two different bias potentials and for two different substrate concentrations. We observe two prominent peaks (high impedance) at approximately 0.05 and 5 Hz in all the cases. These peaks are absent for the clean electrode (no PT film present) in Figs. 6.4(a) and 6.4(b). In Figs. 6.4(c) and 6.4(d), the blank electrode contains the polytryptophan films and hence these peaks are also present in these two figures.

In an AC impedance experiment, a modulatory voltage (say 10mV) is superimposed on the electrode (along with the bias potential). This causes a small oscillatory voltage to be applied to the electrode and also the diffusion of reactive species. There is a particular low frequency at which the specific reaction rate constant (heterogeneous rate constant at the surface of the electrode) matches the oscillatory frequency and current decreases and hence impedance increases sharply. This we observe in our experiments at two major frequencies-- one around 0.05Hz and the other around 5Hz. These two frequencies are seen for the polytryptophan coated electrode also and hence are most probably linked with the electron transfer processes at the PT film. It is important to note that in our experiments, higher substrate concentrations give rise to greater impedances (lower currents) at these frequencies. Computer simulation of impedance plots arising due to chemical processes at an electrode has been treated by Diard et al (20). Since the exact nature of the electrochemical process is not known in our case, a complete analysis has not been possible.

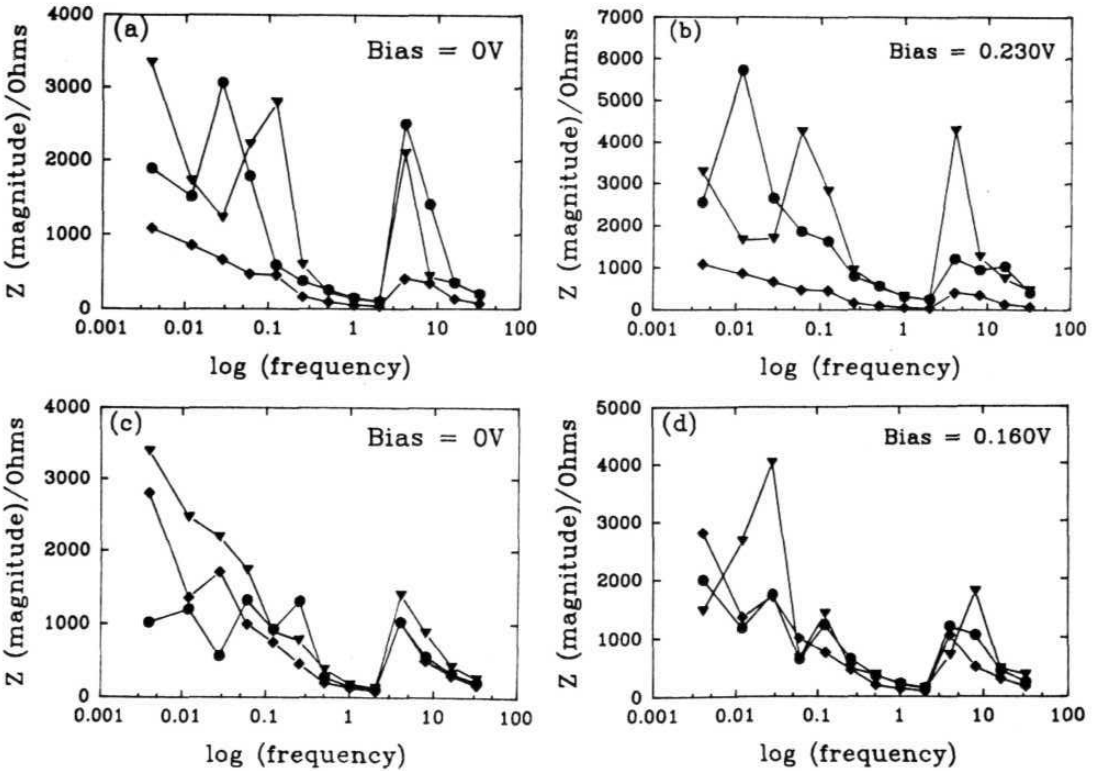


Fig. 6.4. Plots (a) & (b) are for the BSA Glutaraldehyde cross-linked enzyme electrode at zero and 230mV bias potentials (◆ are for blank without polytryptophan film electrode, ● are for 10μM substrate and ▼ are for 1000μM substrate concentration). 4(c) & (d) are for the covalently coupled enzyme to polytryptophan coated electrode by glutaraldehyde at zero bias and 160mV bias potential (◆, ●, ▼, are for the polytryptophan coated electrode, 10μM substrate concentration and 1000μM substrate concentration respectively using the same electrode).

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7. AMPEROMETRIC BIOSENSOR BASED ON REDOX-ENZYME IN CARBON PASTE ELECTRODE

1. INTRODUCTION

Carbon paste electrodes (CPE's) for voltammetry consist of a carbon powder combined with a pasting liquid. The CPE is characterized by a decreased residual current and a better reproducibility of currents compared to the pure carbon material and this improved detection limits and reproducibility of analysis is obtained. The properties of the CPE depend on the specific components employed the manner of preparation and maintenance.

The main shortcomings of the CPE are the solubility of the pasting liquid in organic solvents and the fragile surface. Therefore, several carbon composite electrodes based on carbon and a solid matrix have been evaluated. Due to its insulating nature efficiency in the presence of a solid matrix decreases electrode reaction rates more than a pasting liquid does unless an active carbon surface is exposed (1).

Amperometric biosensors have been at the focus of electroanalytical research since the first "enzyme electrode" for the detection of glucose was reported by Updike and Hicks in 1967 (2). More than 1000 papers have been published since then on biosensors for glucose and for a series of other analytes one of the major obstacles to be solved in the construction of enzyme based biosensors is now to optimize the electron transfer reaction between the cofactor of the redox enzyme used and the electrode. All redox enzymes rely on a cofactor as the redox active compound for activity. In all classes of redox enzymes, except the nicotinamide dependent dehydrogenases, the cofactor is

strongly bound within the enzyme structure causing sterlc hindrances for a direct electron transfer between the active site of the enzyme and the electrode.

2. FLOW INJECTION ANALYSIS (FIA)

The determination of chemical parameters is of great interest in all kinds of bioprocesses. In industry, on-line measurements of nutrients, metabolites and products to be used for process control are most important. For the solution of such problems on-line analytical methods are in demand and the use of either FIA or biosensors (3)

FIA was introduced by Ruzicka and Hansen (4) and Steward (5) in the mid 70s and has since become almost universally adopted as an important analytical tool. Samples can be introduced either continuously or as a well defined plug in a flowing stream for transport to a detection device where the concentration of the analyte is measured. The sample can be processed in a number of ways in the flow system (e.g., dilution, Chemical reaction, removal of interferents). The operation can be performed automatically and reproducibly as the sample is transported from the place of introduction to the place of detection. The carrier may be a homogeneous liquid, or it may be segmented by gas-bubbles. The introduction of gas-bubbles decreases the dispersion of the sample drastically and consequently it decreases the time for an analysis. The technique is the most exploited and it is used in many commercial auto analyzer systems.

Usually, changing the injected sample volume is a powerful way to change dispersion and the effects of the sample volume on the FIA response have been discussed in detail elsewhere (6). Small sample volumes give low peak heights whereas large sample volumes produce broad peaks, although increasing sample volumes may also increase the

sensitivity. Dispersion can be minimized with a proper system design. Lines connecting the injector port and the detector should be as close as possible and flow rates should be low.

The detection system is an important component with regard to the dispersion. Flow cell detectors, e.g., amperometric should have low dead volume to avoid peak broadening. Flow electrodes used in FIA are generally of the wall-jet, thin layer or tubular type. The main difference between them is the position of the working electrode in relation to the flowing stream. The wall-jet electrode is a well defined hydrodynamic electrode in which the flow is due to jet of fluid which impinges perpendicularly onto a planar electrode surface and spreads out rapidly over the surface. Wall-jet electrodes were first described by Glauert in 1956. Efficient mass transport, short residence time on the electrode surface, low dead volume, and insignificant surface fouling make the wall-Jet electrodes very suitable to use in FIA systems (7).

Enzymes have been used in conjunction with various electrodes for the construction of enzyme electrodes. The use of amperometric enzyme electrodes is rationalized by the proposed use of the inherent selectivity shown by the enzyme to promote a selective detection of the enzyme substrate. However, in most instances the necessary applied potential of the enzyme electrode is either too low or too high to allow the electrochemical reaction to occur without interfering reactions or results in excessive background currents. The optimal potential range for an amperometric biosensor to promote sensitive and selective detection should be between -200 and 0 mV vs SCE where the background current switches signs and thus takes its lowest value, electrochemical reduction of molecular oxygen and oxidation of easily oxidizable species (e.g., ascorbate, urate, paracetamol, catecholamine, etc) are negligible and not contributing to the response signal.

Oxidoreductases or "redox-enzymes" are of particular interest for the construction of amperometric enzyme electrodes since an electron transfer reaction takes place in the enzymatic conversion of the substrate. Many investigations have been performed on trying to obtain a direct electron transfer between the redox-cofactor of the oxidoreductase and an electrode at a low overpotential (8,9). In most instances, however, a direct electron transfer is hindered either because steric or kinetic barriers prevail. To circumvent these effects, small molecules acting as charge transfer mediators can be used to shuttle the charge to/from the active site of the redox-enzyme from/to the electrode. The great interest shown in the construction and studies of chemically modified electrodes (CME) had a great impetus on recent developments in the immobilization of both active mediators and enzymes on electrodes (10-13). The use of mediators and mediator modified electrodes for amperometric biosensors was reviewed by Bartlett et al. (14).

Immobilization of the enzyme in close conjunction, directly on the electrode surface, or in a composite electrode are recent ways to promote fast overall reaction kinetics in contrast to immobilize the enzyme in a separate membrane, which is after fabrication put on the electrode surface. In most cases, however, these electrodes suffer from lack of long-term stability. Some attempts have been made to stabilize the immobilized enzyme in amperometric biosensor configurations (15,16). However, it seems as though only recently systematic research been focused on how to stabilize the immobilized enzyme (17,18). Similarly, little attention has been paid on how to further speed up the overall reaction kinetics of the enzyme probe.

The last decade has seen unprecedented interest in the development of probes for the qualitative and quantitative monitoring of biological samples of analytical interest. Many of these are based on enzymes and a number of different procedures are available for enzyme

immobilization. Whereas much enzyme-sensor development is devoted to glucose oxidase, because of its recognized importance in glucose assay as a diagnostic monitor diabetes, there is also a growing interest in the development and application of other sensors.

One of the major enzymes that has attracted a considerable degree of attention is lactate oxidase (LOD). Blood lactate concentration is indicative of certain pathological states such as shock, respiratory insufficiencies and heart diseases. Measurement of blood lactate is of importance in critical care, sports medicine, and in neonatology. The lactate level in cerebrosplnal fluid is a sensitive determination of serum lactate dehydrogenase (LDH) and is useful in the diagnosis of many diseases including myocardial infraction, hepatitis and many kinds of carcinoma (19).

The lactate concentration in the blood rises rapidly as a result of anaerobic glucose metabolism when delivery of oxygen to body tissues is insufficient. The fact that the hydrogen ions produced from dissociation of lactic acid are effectively buffered by bicarbonate makes pH measurements less useful than direct lactate determination.

In sports medicine, the lactate concentration is a very useful indicator for assessing the general physical condition of an athlete or racing animal. Highly trained individuals produce less lactate than unconditioned ones for a given exercise.

The involvement of lactate in the metabolism of glucose posses more demand on the use of a lactate sensor in conjunction with a glucose sensor for diabetic patients, the applicability of such a combination has ben demonstrated in the evaluation of an endocrine artificial pancreas.

In the food industry, measurement of lactate in dairy products and for the control of additives (in wines etc.) is also of considerable

importance. The lactic acid content of food products influences their flavor, stability and storage quality.

Several different methods are available for the electrochemical determination of lactic acid by use of immobilized lactate oxidase, but the below three have been most widely used.

Lactate oxidase from Mycobacterium smegatis which acquires flavin mononucleotide as a cofactor in the consumption of oxygen, converts lactate into acetate and carbon dioxide. The measurements of oxygen depletion is used as an indirect means of estimating the lactate level.

Lactate oxidase from Pediococcus species, an enzyme dependent on flavin adenine dinucleotide (FAD) catalyses the conversion of lactate into pyruvate and hydrogen peroxide by consumption of oxygen. In this case the production of hydrogen peroxide can be measured as an indicator of the lactate concentration.

In the third approach, the catalytic activities of LOD and lactate dehydrogenase (LDH) are coupled. The LOD being enzymatically regenerated by the LDH. This enzymatic cycling reaction greatly amplifies the response. Although this system is conceptually attractive and yields low detection limits, it suffers from slow response time (4-9 min). Also, the preparation of the enzyme support involves some complicated steps and takes several days (20).

3. EXPERIMENTAL:

3.1. Carbon paste electrodes:

Plain graphite-paraffin oil paste was prepared by thorough mixing of 40µl of paraffin oil (Fluka, cat. no. 76235) with 100 mg of graphite powder (Fluka, cat. no. 50870). Plastic syringes (Fig. 7.1) 1.0 ml syringe, Brunswick 81/79J03, with a tip of 7.0 mm OD and 1.8 mm ID)

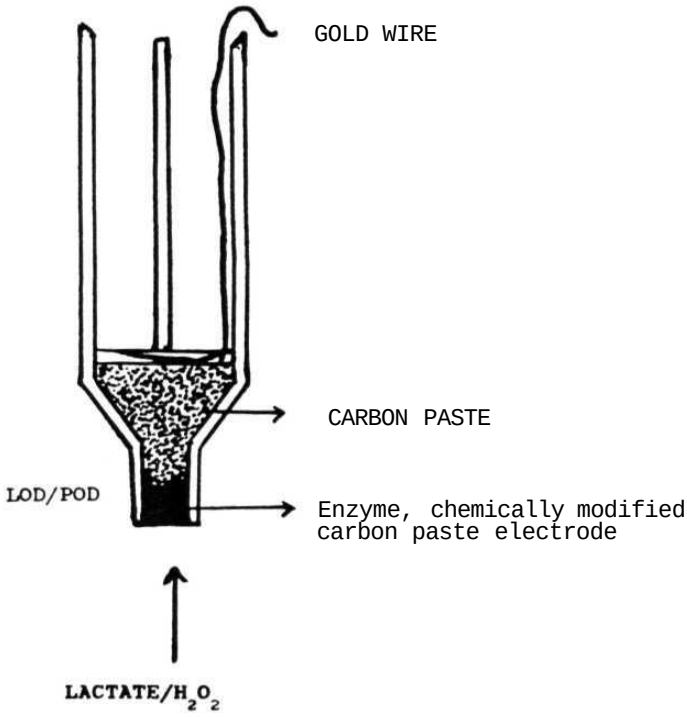

Fig. 7.1. Lactate enzyme electrode

were filled with the graphite-paraffin oil paste leaving about 3 to 4 mm empty in the top to be filled with enzyme modified by inserting a gold thread into the paste. After aliquots of enzyme modified pastes were filled into the end of the syringes, the end was gently rubbed on glass to produce a flat shining electrode surface with an area of about 0.024 cm . The final electrodes were mounted in a flow-through amperometric cell of the wall-Jet type (21) under three electrode potentiostatic control using a potentiostat (Zata Electronics, Lund, Sweden). When not in use the enzyme electrodes were stored in a dry state at 4 C. A platinum wire and a saturated calomel electrode (SCE) served as the counter and reference electrodes respectively. The cell was connected to a single line flow injection (FI) system using either an Automated Sample Injection Analyzer (Ismatec, SA, Glattburg-Zurich, Switzerland) or an in-lab made system described earlier (21). Connections between the various part of the FI system were made with Teflon tubings, ID 0.5 mm, and Altex screw couplings. All solutions were degassed before use to prevent microbubules to appear in the flow system.

3.2. Lactate sensor based on co-immobilized l-lactate oxidase and fungal peroxidase:

1 mg of L-Lactate oxidase (LOD, EC 1.1.3.2, from pedlococcus sp., Sigma cat no. 10638, obtained as a lyophilized powder with an activity of 30U mg") and 1 mg of fungal peroxidase (ARP, from Arthromyces ramosus, EC 1.11.1.7, kindly provided as a gift from Suntroy Ltd., Japan, lot no. 900511, obtained as a lyophilized powder with an activity of 250U mg^{-1}) were dissolved into 200µl of 0.1 M phosphate buffer at pH 7.0. 200µl of a 0.32% solution of PEI were also added to the enzyme solution before letting the mixture immobilize on 100 mg of heat treated (700°C, 1.5 s) graphite for 16 hours at 4^{o}C. After that, the graphite-enzyme mixture was allowed to dry in vacuum before addition of the pasting liquid, 40µl of phenylmethylsilicon oil (silicone DC 710, Alltech Associates, Arlington Heights, Il, USA) (16).

4. RESULTS AND DISCUSSION

All oxidases depend on a cofactor strongly bound within the enzyme structure. The nature of the cofactor may be of different chemical structures (e.g., flavins or copper containing structures). What they all share and in contrast to the dehydrogenases is that they make use of molecular oxygen as the natural reoxidation agent in the enzyme catalyzed reaction. Depending on the class of oxidase, molecular oxygen is either reduced to form hydrogen peroxide or water. Well-known hydrogen peroxide producing oxidases are e.g., glucose, L- and D-amino acid, galactose, alcohol, and choline oxidases and water producing oxidases e.g., tyrosinase and ascorbate oxidase (7). As molecular oxygen is strong oxidizing agent all oxidase catalyzed reactions can be regarded as chemically irreversible. A general reaction for an oxidase catalyzed reaction can thus be written:

$$\text{Substrate} + 0 \xrightarrow{\text{oxidase}} \text{product} + H\,0 \qquad (1)$$

From reaction (1) it is obvious that the reaction can be followed electrochemical either by sensing the decrease in oxygen tension or the increase in hydrogen peroxide concentration. The necessary applied potentials to either of the electrochemical detection reactions (reduction of molecular oxygen at -600 mV or oxidation of hydrogen peroxide at +600 mV) are, however, far too low or too high to allow very sensitive and/or interference free detection of the substrate of the oxidase. Much research has therefore been focused on trying to be able to amperometrically follow oxidase based reactions at more optimal potentials (18). This can achieved e.g., by the use of chemically modified electrodes with electrocatalytic activity for hydrogen peroxide oxidation (13,16) or with mediating functionalities acting as alternative electron accepters to molecular oxygen (14,16).

Horse radish peroxidase (HRP) and other peroxldase have often been used in conjunction with oxidase based reactions to promote selective and sensitive detection of the substrate of the oxidase, be it spectrophotometrically or electrochemically (16). Recently it was found that an apparent direct and a very efficient electron transfer could be obtained primarily various carbon electrode materials and immobilized peroxidases in the presence of hydrogen peroxide (16,22-30). In the presence of hydrogen peroxide a bioelectrocatalytic reduction current of hydrogen peroxide starts to appear at about +600 mV vs SCE at pH 7.0 (22-30). As the E_{appl} is made more negative the response current is increased and reaches a steady-state plateau at about -200 mV. The mechanism for this behaviour, a truly direct electron transfer or a mediated one brought about by oxygen containing functionalities (quinones), is still debated (16,22-30). Peroxidases contain a protoferriheme group, most often ferriprotoporphyrin IX, as the redox active prosthetic group contained in the active site. When the native form of the peroxidase reacts with hydrogen peroxide, the prosthetic group oxidized in a single 2e step, transforming the native form into compound-I :

native form + H 0 > compound-I

The rereduction of compound-I to the native form occurs in two separate one electron steps with the intermediate formation of compound-II :

compound-I + 1 e~ ——> compound-II (2)

compound-II + 1 e^- ——> native form (3)

Obviously reactions (2) and (3) can occur at the electrode surface without the deliberate attachment of mediating functionalities. In the presence of high concentrations of hydrogen peroxide there is a

risk for an irreversible deactivation of the peroxide due to the transformation of the prosthetic group into a higher oxidation stage. This form of the peroxidase is denoted compound-III.

Peroxidase modified electrodes were shown to work successfully as electrochemical sensors for hydrogen peroxide monitoring within the optimal potential range for an amperometric sensor. Several papers have appeared on the construction of such sensors based on solid (24-28), carbon fibers (16), carbon paste (16,22,29), or composite electrodes (29). A number of papers also report on the co-immobilization of a hydrogen peroxide producing oxidase with a peroxidase on solid (26,28,31) or carbon paste electrodes (16). Fig. 7.2 shows the reaction cycle for a carbon paste electrode containing a hydrogen producing oxidase co-immobilized with HRP. Fig 7.3 shows a hydrodynamic voltammogram obtained for the LOD/ARP electrode. As is seen, the response current for hydrogen peroxide (0.1 mM) is relatively high already at +300 mV and is increased as the potential is made more negative, reaching a constant response plateau between 0 and -200 mV. In the positive potential range the response to L-lactate (0.1 mM) essentially follows that of hydrogen peroxide. For potentials more negative than -50mV, the response, however, drastically decreases, probably reflecting a deactivation of LOD within this potential range. An applied potential of -50mV was considered as the optimum one for further investigations. Fig. 7.4 shows the calibration curves (log-log plot) for both substrates. As is clearly seen strictly linear response ranges for both substrates are obtained for almost three orders of magnitude with the response to L-lactate being about one tenth of that for hydrogen peroxide. The detection limit was in the range of about 1 μM. The slopes of the log-log plots differ, however, and was found to be 0.87 and 0.63 for hydrogen peroxide and L-lactate, respectively. Concentrations higher than 1 mM of hydrogen peroxide were not tested because of the risk for irreversible deactivation of the ARP due to an expected formation of compound-III.

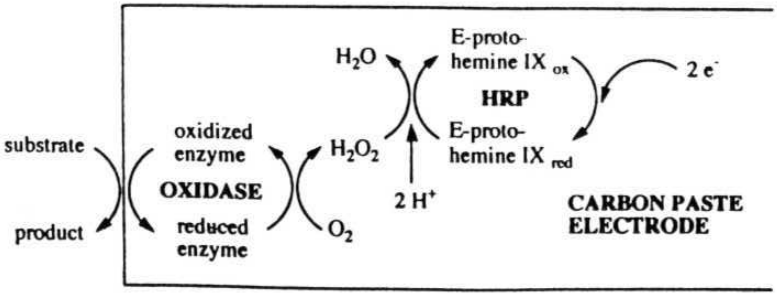

Fig. 7.2. Schematic representation of the reaction sequence of a sensor based on an oxidase co-immobilized with a peroxidase.

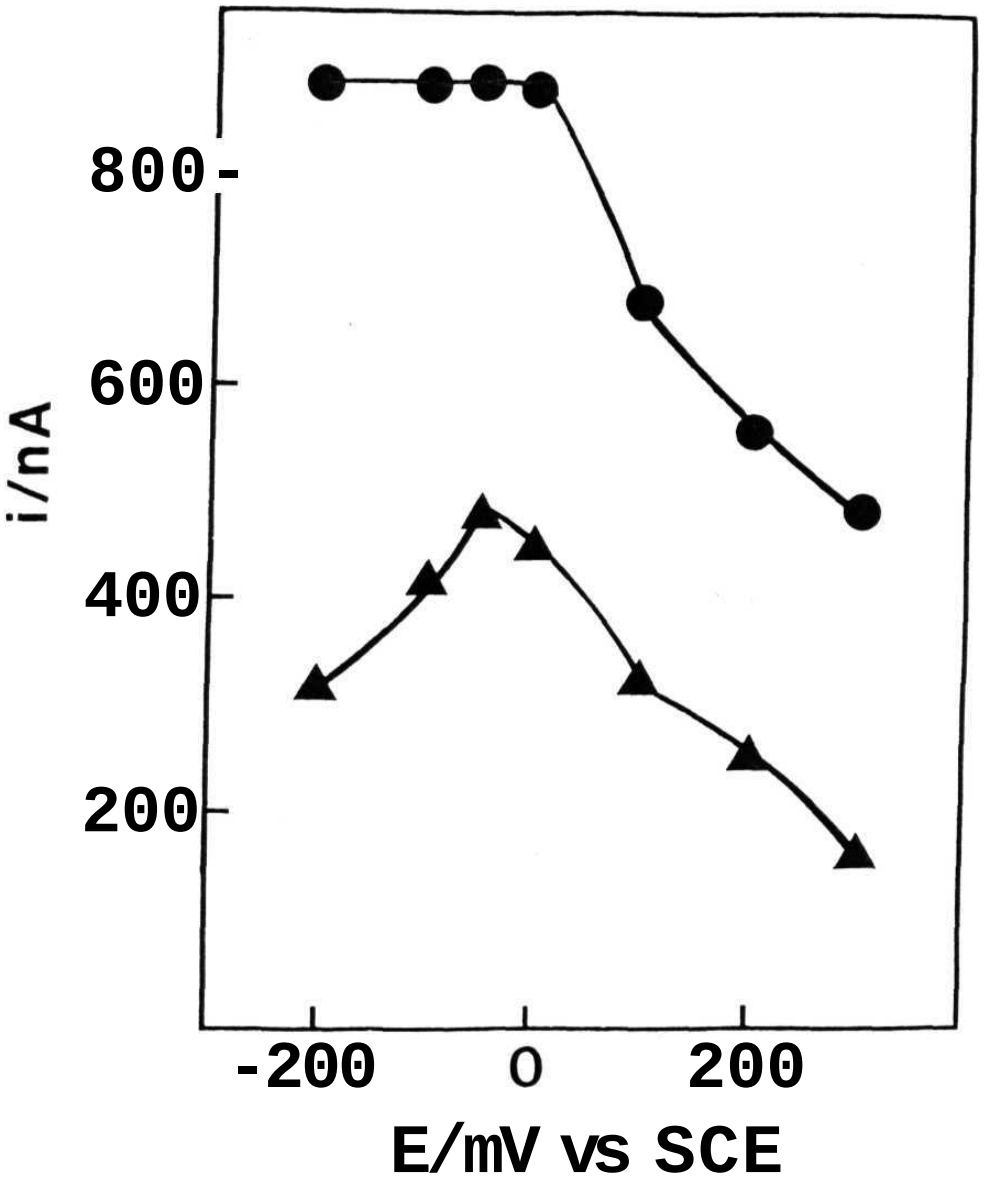

Fig. 7.3. Hydrodynamic voltammograms for (•) 0.1mM hydrogen peroxide and (▲) 0.1mM L-lactate of a LOD/ARP-PEI modified carbon paste electrode. The following conditions were chosen; injection volume 50µl, carrier 0.1M phosphate buffer at pH 7.0 (0.8 ml min).

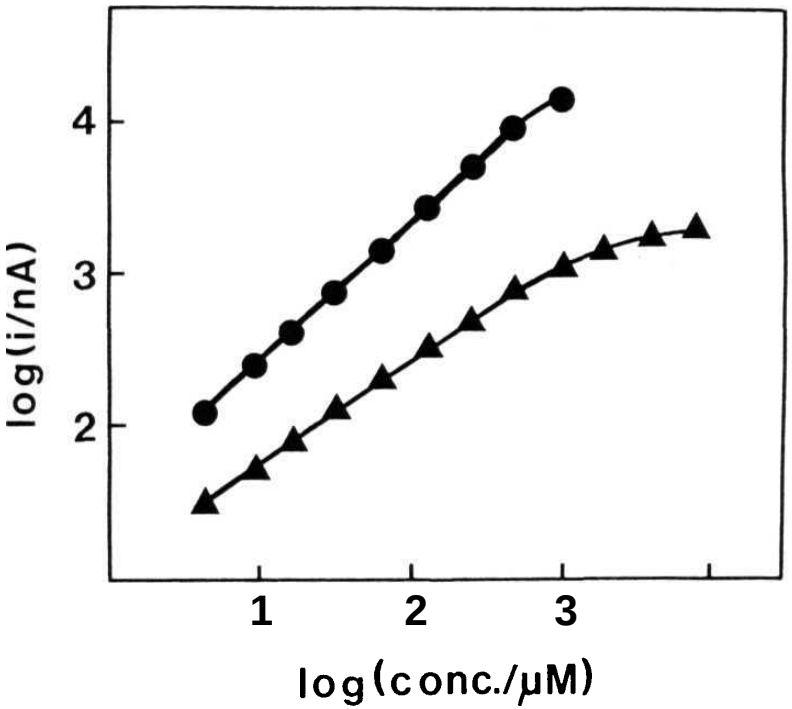

Fig. 7.4. Log-log plots of calibration curves for (●) hydrogen peroxide and (▲) L-lactate obtained with a LOD/ARP-PEI modified carbon paste electrode.

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8. SUMMARY AND CONCLUSIONS

Several different enzyme electrodes were studied by both cyclic voltammetry (CV) and impedance measurements. polytryptophan (PT) and polytyroslne (PTyr) modified electrodes were prepared by electrochemical oxidation. The polymer films so formed are studied by CV and impedance measurements. These films can be activated by glutaraldehyde or carbodiimide because they have free amino and carboxyl groups. My studies on enzyme electrodes are divided in four following parts.

(A) In first part of the study yeast alcohol dehydrogenase was immobilized on Pt wire-mesh. Cyclic voltammetric studies on a platinum electrode containing adsorbed ferrocene, NAD , and alcohol dehydrogenase were carried out. To prevent adsorbed molecular layers from desorption, the adsorption solution contained a small amount of nitrocellulose as binder. The electrode does not behave reversibly but the response to alcohol solution is found to be approximately linear in the range of 10-1000μM alcohol concentration. The enzyme was also immobilized by cross-linking with glutaraldehyde and the results are similar. A plot of a log(peak current) vs log[alcohol] gave a set of a parallel lines for different concentrations of immobilized enzymes.

CV studies on electrode containing ferrocene and NAD adsorbed as before shows prominent peaks. The electrode was used in a solution containing alcohol and enzyme (1,2, and 5 units/ml) and also shows clear dependence of the peak current on alcohol concentration, but no clear relation has been observed. Presumably, the solution kinetics is much faster compared to the electrode processes.

(B) In the second part of the experiments the polymerization of tyrosine and tryptophan and their impedance behaviour, were studied.

Poly tyrosine/tryptophan films were prepared by electrochemical oxidation of a solution 50mM tyrosine/tryptophan in H SO or NaOH. The base electrode substrate was a Pt-mesh electrode. A standard three electrode setup was used in all the experiments. The Pt-mesh was cleaned by dipping in concentrated HNO . These films were deposited onto the Pt-mesh electrode from the solution at 1500mV potential with respect to SCE as reference electrode. Instead of applying a constant potential, the potential was scanned from 0 to 1500mV to 0 at a rate of 20mV/sec for 10 cycles. A light bluish-red polymer film could be seen after 10 cycles. This was washed with buffer and used in all cyclic voltammetric and impedance studies.

The impedance studies were carried out using PAR model 5210 Lock-in-Amplifier (LIA). The cell was connected to the potentiostat as usual. A known amount (20mV) of modulatory voltage at a known frequency (80Hz) were applied to the potentiostat and the output of the potentiostat was connected to the input of the LIA. The output of the LIA was connected to a digital/analog recorder. There are two outputs available on the LIA: one in-phase (real part) and the out-of-phase (imaginary part) and these were recorded separately. After film formation impedance studies were done by scanning from -800 to 700mV.

For the polytyrosine (PTyr) modified electrode one can observe an increase in the parallel resistance (Z real) around -500mV (vs SCE) but this is not pronounced in the bare electrode. Again a sharp decrease in parallel capacitance (Z imaginary is increased) is observed for the modified electrode (particularly for the film prepared from NaOH solution) around -700mV. This suggests the break

down of the polymer film with attendant decrease of capacitance. Thus we conclude that the electrodes are expected to perform well within the potential range of -300 to 600 mV.

CV studies of the modified electrodes are checked for their redox behavior using potassium ferrocyanide and p-benzoquinone. From the CV graphs for ferrocyanide, one observes that the relatively sharp peaks seen in the bare electrode are reduced significantly in amplitude for both the modified electrodes. Nevertheless, a small hump is still visible suggesting that fast electron transfer is somewhat inhibited. This is expected because the film is having some resistance which interferes with fast electron transfer. Thus, such modifications are not effective, as expected, when the electron transfer rate is very high in the bare electrode. Interestingly the peak positions are not altered suggesting that the polymer is not involved in any chemical reactions with substrate, i.e, the electron transfer from the polymer to the metal is not rate determining. However, a reduction in background current was clearly seen. Similar results were obtained for p-benzoquinone.

Polytryptophan (PT) films were formed from acid/alkali as described in previous experiment. The PT films formed from H SO (denoted as PT(H SO)) showed a considerably larger current in the + 400mV potential range in the impedance studies. The bare electrode in comparison showed a relatively flat response. The PT films formed from NaOH (denoted as PT(NaOH)) showed a very similar although numerically smaller characteristic curve compared to that of the bare electrode. It appears that PT(H SO) film cannot support significantly higher currents compared to the bare electrode/PT(NaOH) film.

PT(H SO) film shows a significantly higher capacitance in the + 400mV range whereas the features for the bare electrode and the PT(NaOH) electrodes are comparatively similar, with somewhat smaller

capacitance for the PT(NaOH) film. The modified electrodes were also checked towards redox properties of ferrocyanide and p-benzoquinone by CV studies. These are showing the similar behavior like PTyr modified electrodes. Here again the background current is reduced significantly when compared to the bare electrode.

(C) In the third part of the experiments, Horse-radish peroxidase was immobilized on a polytryptophan film by covalent coupling and glutaraldehyde cross-linking. Here the working electrode used was a glassy carbon disk. Covalent coupling was done by carbodiimide activated bare electrode and also by glutaraldehyde coupling to a polytryptophan film. The polytryptophan film was formed by electropolymerization of tryptophan from acid solution (H_2SO_4) with a cross-linker (spermine). The enzyme was coupled to this film by covalent coupling with glutaraldehyde.

Enzyme electrodes prepared by glutaraldehyde coupling, cross-linking and carbodiimide coupling were shown to give a linear response from 10μM to 1000μM H_2O_2. Impedance studies of the polytryptophan coated electrode shows increased parallel resistance and decreased parallel capacitance, as expected. In presence of the substrate, the parallel resistance was considerably reduced (due to enzyme catalyzed process) leaving the parallel capacitance virtually unaffected. The polytryptophan modified electrodes were also studied by varying the frequency. Bode plots shows no significant difference for bare and PT coated electrode. Low frequency studies may be useful to study this type of studies.

The same glassy carbon electrode was modified by polytryptophan coating and horse-radish peroxidase was immobilized by covalent coupling using glutaraldehyde. Low frequency impedance was studied by stored waveform excitation of the electrode and the current values were stored in PC-XT. The current values were transformed by fourier

transformation and the impedance values were determined. Low frequency impedance measurements were done in 10 and 1000 μM H_2O_2 concentration. In brief, the results obtained are:

(1) Graphs suggest that current values saturate for 1000 μM hydrogen peroxide concentration. In other words 1000μM H_2O_2 concentration does not show 100 times larger peak. Thus the chemical and enzymatic rates are rate limiting.

(2) For zero and 160mV bias potentials the peak heights are different as expected.

(3) From the point of 2 magnitude vs log frequency, we see two prominent peaks. One around 10Hz (0.1 sec rate constant) and another around 0.01 Hz (100 sec rate constant). Thus these are two process associated with the electrode reactions different time values.

(D) In the last part of the the experiments a lactate biosensor was constructed by co-immobilizing lactate oxidase and fungal peroxidase in carbon paste electrodes. The calibration curves for both substrates is clearly seen to give a linear response ranges for both substrates are obtained for almost three orders of magnitude with the response to lactate being about one tenth of that for hydrogen peroxide. The detection limit was in the range of about 1μM.

In brief the highlights of my work can be concluded as follows.

1. Polytyrosine and polytryptophan films are formed by electrochemical oxidation at suitable potentials. In both the cases the conduction may be electronic in nature.

2. These modified electrodes can be activated by glutaraldehyde and carbodiimide and coupled to enzymes.

3. Impedance studies show that both the films give higher specificity and lower noise, and CV studies indicate that although the response decreases (peak current) actual sensitivity increases (lower background current) for both the films. These films are expected to be biocompatible.

4. Lactate biosensor was prepared by co-immobilizing lactate oxidase and fungal peroxidase- shows linear response over a wide range of lactate.

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