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# DEVELOPMENT OF AN AMPEROMETRIC BIOSENSOR FOR THE DETECTION OF ALCOHOL

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS IN SCIENCE IN BIOCHEMISTRY AT MASSEY UNIVERSITY

RUTH LARGE

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## ABSTRACT


The aim of the following work was to design a biosensor for the detection of ethanol. A biosensor is an analytical device in which a biological sensing element is connected to or integrated with a physical transducing element. Amperometric enzyme biosensors utilise one or more enzymes to convert a substance which cannot be measured electrochemically to one which can be. In the case of an alcohol biosensor one of two enzymes (alcohol dehydrogenase and alcohol oxidase) can be used to convert electrochemically stable alcohol to either hydrogen peroxide or NADH which can be oxidised.

In the design of an alcohol biosensor there are three major variables to consider, these are; enzyme type, electrode material, and immobilisation technique. The goal was to select optimum conditions for the formulation of the desired sensor. In the present work the electrode materials used were platinum, carbon (foil and paste) and the conducting organic salt N-methyl phenazinium.Tetracyanoquinodimethane (NMP.TCNQ). The immobilisation techniques used were; adsorption, cross-linking to a protein matrix and covalent binding.

Of the biosensors produced from a selected combination or these variables each was tested by one or more of the following; cyclic voltammetry, enzyme assay, and amperometry. The most promising approach appears to be that of conjugating enzyme to haemin and allowing the conjugate to bind irreversibly to platinum via the haemin group. An electrode made with the organic salt NMP.TCNQ looked promising also but because the salt is readily oxidised it is unstable and therefore not an ideal electrode material.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADH | Alcohol dehydrogenase |
| AO | Alcohol oxidase |
| BSA | Bovine serum albumin |
| CMC | 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate |
| DMF | Dimethyl formamide |
| DCC | Dicyclohexylcarbodiimide. |
| EDC | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). |
| GA | Glutaraldehyde |
| GO | Glucose oxidase |
| HS | Hydroxysuccinimide |
| NAD(P) | ß-nicotinimide adenine dinucleotide (3′ phosphate) |
| NAD(P)H | ß-nicotinimide adenine dinucleotide (3′ phosphate) reduced form |
| NMP.TCNQ | N-methyl phenazinium. tetracyanoquinodimethane |
| PMS | Phenazine methosulphonate |
| TBApTS | tetrabutyl ammonium p toluene sulphonate. |

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# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.1 Biosensor applications

Much of the science of analytical chemistry is concerned with the measurement of ions and fluids (Walt 1992). The ideal analytical instrument measures an analyte specifically and sensitively whilst avoiding the need to process the sample. An instrument which has these qualities and is also cheap, portable and robust would be the epitome of analytical devices. The recent development of electrochemical sensors for analytical applications has made a major contribution towards this goal (Schulz 1991, Roe 1992).

Electrochemical sensors are devices whose output reflects their chemical environment. Sensors in this category are as diverse as high-temperature oxide sensors, fuel cells, ion-selective electrodes and, more recently, enzyme electrodes and CHEMFETS (chemically-sensitive field-effect transistors). The final output of such sensors is either a voltage (potentiometric sensors) or an electrical current (amperometric sensors) (Hall 1988). Clark in the mid-1950s may be credited with the creation of the first electrochemical sensor: an electrode to measure dissolved oxygen in blood, utilizing a plastic membrane permeable to gases.

Biosensors are a group of electrochemical sensors defined as devices in which a biological sensing element (biointerface) is connected to or integrated with a physical transducer (Scheller et al). This addition capitalises on the high specificity of biological systems to add selectivity to the response of the physical sensor to a particular analyte. In this way, the sensing element, biointerface and the method for coupling the two may be optimised for any particular analytical problem (Byfield and Abuknesha 1991).

The potential commercial and scientific applications of biosensors are many and diverse, encompassing clinical medicine (assay of blood or urine concentrations), industry (monitoring of fermentation processes) (Luong et al 1991) and environmental monitoring for public health and safety purposes. The major advantage of biosensors is the ability to provide "on-line", real-time data, eliminating the need to take samples to the analytical laboratory. They are usually simple, rapid and dependable devices which do not require pre-processing of the sample.

## 1.2 Biosensor Classification

Biosensors consist of three different elements; a sensing element, a biological element and a transducing element to couple the two. The sensor portion of a biosensor may measure light emission or absorbtion, fluorescence, temperature change, optical change, electrical voltage or electrical current. The latter two sensing elements may be defined as electrochemical sensing elements.

The biological element may be either:

(a) a direct bioaffinity component utilising a binding event to detect substances (for example antigen and antibody interactions), or

(b) enzymatic or metabolic systems where recognition of the substrate by an immobilised receiver (enzyme) is followed by rapid chemical conversion of the substrate to a corresponding product which is detectable by the sensor.

Biosensors may also be categorised according to the method of immobilisation of the biological element on the sensor:

(a) First generation, involving membrane-entrapped or membrane-bound biological components

(b) Second generation, adding covalent coupling of the co-substrate

(c) Third generation, involving immobilisation of the biological component directly on an electronic element, such as the open gate of a field-effect transistor (FET).

These generations have evolved from the need to have the biological element in close proximity to the sensing element.(Guilbault 1988, Vadgama 1990).

### 1.3 Amperometric sensors

This subset of sensors is based on the exchange of electrons between an electrode poised at a fixed potential and an electroactive compound or ion in its environment. This "working" electrode may be either a cathode or an anode, depending on the expected direction of electron transfer, i.e. whether the electroactive material is to be oxidised or reduced.

Selection of an appropriate potential at which to poise the working electrode is commonly aided by the technique of cyclic voltammetry, in which the applied voltage is swept up and down a selected range and the redox current measured. A plot of current versus applied potential is a cyclic voltammogram and shows a characteristic increase in current at the potential where oxidation or reduction of an electroactive species occurs. In biosensor applications, the potential used is normally high enough to reduce the concentration of the electroactive species at the electrode surface to zero, so the current flowing in the circuit is limited by the rate of diffusion of this material to the

electrode. Factors affecting this rate include temperature, degree of solution mixing and the thickness of the biological membrane as well as the concentration of the electroactive species in bulk solution (Higgens 1988, Kemp 1985).

Amperometric sensors commonly use an interface containing an enzyme or enzymes to effect the conversion of an analyte which is not itself electroactive to one which can be measured amperometrically. In this case, sensor response also depends on the rate of conversion of the analyte to the electroactive species, i.e. the enzyme activity (Allen et al 1990).

The classical apparatus for applying a predetermined potential difference across two electrodes is a potentiometer. As shown in figure 1.1 (page 5), a potential (E) is applied between the counter and working electrodes and the current flowing through the working electrode is monitored. In theory any point in this system could be grounded but it is common practice to ground the current meter return to minimise stray noise pickup.

This "two-electrode" system is adequate when currents are small relative to the sizes of the electrodes. However if the solution resistance increases, the current at constant applied potential will decrease. This problem is overcome by replacing the counter electrode with two others: a reference electrode which monitors the potential of the solution near the working electrode and drives a potentiostat circuit whose output is connected to an auxiliary electrode which completes the circuit (figure 1.1).

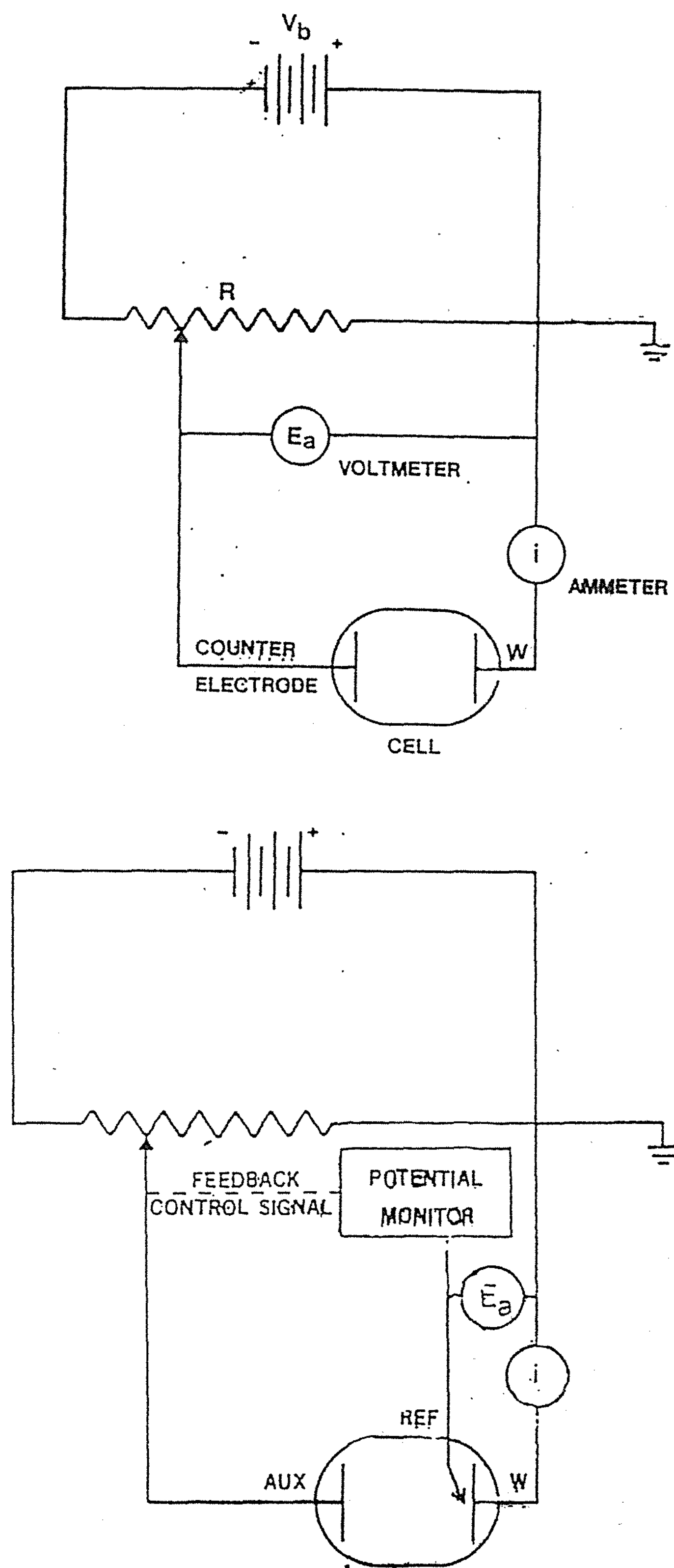



Figure 1.1 Electronic circuit for applying a potential difference to a working electrode. Upper: Two electrode system. Lower: Three electrode system.

This circuit allows the potential applied to the auxiliary electrode to vary so that the voltage across the working electrode/solution interface is maintained constant. In this way, all changes in current flowing in the working electrode circuit are due to changes in the transfer of electrons between the electroactive material and the electrode. With small electrodes, measurement of currents in the nanoampere range is found to be necessary.

### 1.4 Enzyme immobilisation

A sensitive and highly selective method for the determination of a given substrate utilises an immobilised enzyme in conjunction with either the two-electrode, or three-electrode system above, providing that (Kulys 1987):

(a) the compound to be measured is (or can be converted to) a substrate for the enzyme, and

(b) one product of the reaction is electroactive and can be oxidised or reduced at an appropriate "poised potential" on the working electrode.

The first practical electrode of this kind was reported by Updike and Hicks in 1967 who used glucose oxidase immobilised in a gel on a Clark-type amperometric oxygen electrode to measure glucose concentrations in biological fluids.

The most common materials in use for the design of working electrodes are platinum, gold and carbon. Of these carbon has the advantage of being cheap and readily available, while platinum has the advantage of a lower background current but is expensive.

Carbon as an electrode material is also very versatile ranging from carbon paste to spectroscopic graphite

(Dryhurst and McAllister 1984). Spectroscopic graphite is available in rods and is used in arc or spark emission spectroscopy. Carbon paste electrodes consist of an electrical contact at the bottom of an insulating well: the well is then filled with a paste of graphite in a suitable mulling liquid. Pyrolytic graphite is crystalline in structure and is anisotropic, rods can be cut from this material and the surface polished. Pyrolytic graphite has a variety of oxides and other functional groups on the surface making it a useful electrode for derivitisation. Carbon foil electrodes are made from compressed graphite and may be modified with mediators, adsorb enzyme or modified to covalently  Glassy carbon electrodes are isotropic, impermeable to gases, electrically conductive and highly resistant to chemical attack, glassy carbon also has functional groups on its surface (Wring and Hart 1992).

An important aspect of sensor development is finding reaction conditions that effectively immobilise a sufficient concentration of the enzyme in an active, accessible and stable form to provide adequate sensor performance (Gibson and Woodward 1991). There are four main approaches to enzyme immobilisation; physical adsorption to a solid surface, entrapment in a polymeric gel or within microcapsules, crosslinking by means of bifunctional reagents often in combination with the aforementioned, and covalent binding to a reactive insoluble support.(Beh et al 1989,Sternberg et al 1988,Abdulla et al 1989, Weethall 1976, Lee et al 1989).

### 1.4.1 Adsorption

Substances such as alumina, charcoal, clay, cellulose, silica gel,glass, hydroxy-apatite and collagen are known to adsorb enzyme non-specifically. Ion-exchangers such as DEAE cellulose, CM cellulose can also be added to the list (Beh et al). The advantage of adsorption is the avoidance of complex reagents for electrode modification, and a minimum

of preparation and clean-up steps. Adsorption also tends to be less disruptive of the enzyme protein than chemical methods. However binding is more susceptible to environmental changes in pH, temperature and ionic strength.

### 1.4.2 Entrapment

Preparing a polymeric gel in a solution containing an enzyme results in entrapment of the enzyme. Polyacrylamide, starch, nylon and silastic gels can be employed for this purpose. However the relatively large thickness of gel layers presents a diffusional barrier to the analyte and gives a slow response to the sensor. Moreover, there is a continual loss of enzyme activity by surface leaching from the gel. Crosslinking with glutaraldehyde may overcome the latter (Guilbault and Neto 1985). Enzyme may also be entrapped in thin layers of polypyrrole and polyanaline.

### 1.4.3 Crosslinking

Bifunctional agents, such as glutaraldehyde, that induce non-specific intermolecular crosslinking between molecules of the enzyme protein can also bind enzymes to appropriate solid supports. Crosslinking of the enzyme itself yields an insoluble product but is expensive and inefficient since much activity is often lost. Diffusional limitations may result from this method of deposition and the lack of rigidity or mechanical strength is a disadvantage.

### 1.4.4 Covalent binding

This may be accomplished through functional groups in the enzyme protein which are not essential for its catalytic activity. Use is often made of nucleophilic functional groups present in amino acid side chains of proteins for coupling. It is important when utilising this technique to choose a support matrix which also has available functional

groups which can be activated by an appropriate reagent. Covalent bonding has the great advantage that the enzyme is unlikely to be released from the support matrix during use (Wingard 1985, Axen et al 1967).

## 1.5 Oxidase based electrodes

All enzymes catalysing oxido-reductions are termed oxidoreductases, which includes dehydrogenases and oxidases. An oxidase functions via a multiple redox electron carrier system using where oxygen acts as the terminal acceptor. All oxidases feature a flavin group and the enzyme itself exists in both oxidised and reduced forms depending on the oxidation state of the flavin group (Tatsuma et al 1991). Oxidases commonly produce the electroactive species hydrogen peroxide; these include xanthine oxidase, cholesterol oxidase, galactose oxidase, lactate oxidase, pyruvate oxidase, glucose oxidase, and alcohol oxidase. For an electrode-based system an oxidase enzyme can be immobilised at the electrode surface where exposure to substrate will lead to production of hydrogen peroxide which can then be measured amperometrically.

It would be ideal to couple the transfer of electrons from a redox centre couple inherent in the enzyme directly to the surface of the electrode rather than detecting the production of hydrogen peroxide. Unfortunately redox couples in enzymes such as glucose oxidase and alcohol oxidase do not undergo redox reactions with an electrode at sufficiently fast rates to make practical devices. However, addition of an external electron mediator can speed up this process. A mediator, in this context, is a low molecular weight redox couple which can transfer electrons from the active site of the enzyme to the surface of the electrode, thereby establishing contact between the two.(Cardosi and Turner 1991)

In order to be a suitable mediator a substance must:

(a) be chemically stable in the reduced and oxidised state, as it must be able to cycle constantly between the two states.

(b) be able to be reduced/oxidised within the pH range of the biosensor (usually between pH 5-9).

(c) be a specific electron acceptor for the enzyme and not participate in any side reactions.

(d) exhibit fast reaction kinetics.

Use of mediators to speed the rate of electron transfer offer distinct advantages in terms of improved sensitivity and the ability to use lower polarising voltages, which increases sensor specificity. However soluble mediators have the disadvantage of having to be added to the sample; a more practical approach would be to have the mediator firmly anchored to the surface of the electrode along with the enzyme.

**1.6 Dehydrogenase based electrodes**

The dehydrogenases are a group of oxidoreductase enzymes that are dependent on the nicotinamide cofactors $NAD^+$ or $NADP^+$ as electron acceptors. Dehydrogenases catalyse the oxidation of specific substrates with the concomitant production of the reduced cofactor NAD(P)H. The reduction of $NAD(P)^+$ to NAD(P)H can be utilised in biosensor systems by amperometric reoxidation of the reduced form at the working electrode (Tatsuma et al 1991).

At clean metal electrodes oxidation of NAD(P)H only occurs at large over-potentials and proceeds through radical

intermediates which can lead to interference and/or electrode fouling. The use of mediators to receive electrons from NAD(P)H and pass them on at a lower potential would again be desirable. Examples of such species are polymers containing orthoquinone and redox dyes adsorbed to graphite. Conducting organic salts can also be used, for example N-methyl phenazinium tetracyanoquinodimethanide (NMP.TCNQ), can be used in conjunction with alcohol dehydrogenase. Being electrically conducting this organic salt can be formed into pellets and used to make the working electrode (Bartlett 1990).

The need to add soluble nucleotide coenzymes to the system is another disadvantage to the use of dehydrogenases in biosensors. Attempts to immobilise the nucleotide on the sensor have generally resulted in large decreases in activity and loss of sensitivity.

## 1.7 An alcohol biosensor

Interest in a device to measure alcohol concentration is two-fold. Alcohol is a common analyte in the brewing industry and the clinical field requiring a portable measuring device. Monitoring of alcoholic fermentation has obvious applications in the brewing industry and medico-legal applications of an alcohol sensor for breath/blood levels would find wide use by law-enforcement and medical agencies. There is also a local interest by Massey University's Alcohol Research group, who require a device to continuously monitor ethanol concentration in metabolic experiments without taking samples.

It is possible to construct a microbial sensor for alcohol using alcohol-utilising micro-organisms and this was the basis for an electrode designed to be used in the measurement of fermentation processes, the electrode is robust but is not very selective or stable. A simple,

sensitive and accurate alcohol electrode has been obtained using a Clark-type oxygen electrode on which alcohol oxidase and catalase are immobilised (Verduyn et al 1983). The electrode is essentially an oxygen sensor which measures the decrease in oxygen tension in a layer of immobilized protein due to the action of alcohol oxidase after an addition of alcohol. Electrode responses were small and the electrode is not especially stable. There is an industrial analyzer for alcohol which employs alcohol oxidase sandwiched between a polycarbonate and a cellulose acetate membrane.

Alcohol oxidase systems are much the same as those used for glucose oxidase; alcohol response may be monitored by direct anodic detection of peroxide or with the use of a mediator. There are many publications on glucose sensors utilising glucose oxidase and some of the methods that have been reported have been repeated experimentally with alcohol oxidase as well as glucose oxidase in this thesis. Enzyme electrodes of alcohol dehydrogenase have been prepared using the conducting organic salt NMP.TCNQ (Zhao and Buck 1990), this electrode proved highly successful, giving responses to ethanol at a poised voltage of 0 V. Electrodes utilising membrane entrapped alcohol dehydrogenase with the mediator phenazine methosulpate have been reported . The use of phenazine methosulphate to lower the potential at which oxidation of NADH occurs is a continuing theme in the use of alcohol dehydrogenase as the basis for an alcohol biosensor as NADH oxidation occurs only at high potentials. Amperometric biosensors employing alcohol dehydrogenase (Suzuki et al 1975, Gestrelius et al 1975) have not been successfully applied to ethanol analysis in "real " samples.

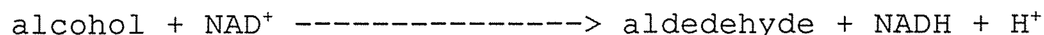
### 1.7.1 Available enzymes

To achieve the aim of a working enzyme-based amperometric sensor two enzymes can be utilised: alcohol oxidase and alcohol dehydrogenase.

Alcohol oxidase catalyses the reaction;

alcohol + $O_2$ ----------> aldehyde + $H_2O_2$

Alcohol Dehydrogenase catalyses the reaction;



alcohol + $NAD^+$ ---------------> aldedehyde + NADH + $H^+$

Glucose oxidase for reasons of economy, and availability, was used as a reference enzyme. Glucose oxidase was used to test techniques which could be adapted to alcohol oxidase because of the similarities between the enzymes.

Electrochemical sensors utilising these enzymes must be designed to detect hydrogen peroxide in the case of the oxidases and NADH in the case of alcohol dehydrogenase. The oxidase enzymes are perhaps the more promising enzymes to work with as they require no addition of cofactor. NAD must be added to the substrate or co-immobilised with the enzyme in the case of alcohol dehydrogenase.

Alcohol dehydrogenase is available at a high degree of purity, but has a low turnover number which limits the sensitivity in biosensor applications. On the other hand, the activity of commercially available alcohol oxidase is relatively low and it is less substrate specific: methanol is oxidised at higher rates than ethanol.

Utilising these two enzymes experiments were carried out to determine which combination of electrode material and immobilisation technique will render a good working electrode. Platinum, carbon and organic metals are good conductors and can be used in conjunction with either adsorption, entrapment, crosslinking, or covalent binding to construct a working electrode.

### 1.7.2 Aim of the present work

Thus the aim of this project was to determine the optimum combination of;

(a) enzyme

(b) electrode material, and

(c) immobilisation method

which would yield an alcohol biosensor of adequate sensitivity and stability.