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Characterisation and applications of AC impedance biosensors

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Characterisation and Applications of AC Impedance Biosensors

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Abstract

The aim of the work described in this thesis is the characterisation and development of ac impedance biosensors systems through the evaluation of the electrical, physical and chemical processes involved, in order to provide a better understanding of the complex performance of these devices.

The investigations of ac impedance biosensors included here cover equivalent circuit analysis, the characterisation and optimisation of an ac impedance model urease electrode and the design and proof-of-feasibility of an ac impedance biosensor for the detection of creatinine in urine, important for its medical applications.

The activity of urease is assessed in solution and immobilised forms and the dependence of the urea biosensor performance on enzyme loading, immobilisation method, temperature, pH and electrolyte concentration reported. We show that Nafion-immobilised urea sensors offer optimum performance in tests on serum samples, combining high sensitivity and long shelf-life with giving no sign of electrode polarisation effects. High intra-batch reproducibility is also reported.

Following the interest of British Nuclear Fuels Ltd. in the detection of creatinine in urine, creatinine biosensors constructed with two different enzymatic systems are investigated. A single-enzyme creatinine deiminase electrode and a multi-enzyme creatininase / creatinase / urease electrode are characterised with similar results. Based on our previous work using Nafion and its useful electrical properties at high ionic strengths, this polymer was employed to immobilise the enzymes on interdigitated gold electrodes. The performance of these creatinine biosensors in urine samples is assessed and shown to offer a viable alternative to the standard spectrophotometric creatinine assay.

The equivalent circuit modelling of the biosensor system demonstrates that, depending on the immobilisation process, the ionic product generated at the enzyme electrode either diffuses towards the electrolyte or remains trapped in the enzyme phase. Relatively low sensitivity was recorded when the ions diffused towards the bulk phase, and high sensitivity, but low reproducibility was observed when the ions stayed trapped within the enzyme layer.

Index of Contents

Chapter 1 Introduction to Biosensors

A biosensor is an analytical device which uses biologically-sensitive material to detect biological or chemical species directly without the need of complex sample processing. By combining the selectivity of biology and the processing power of the transducer, it is able to provide either qualitative or quantitative results, offering powerful analytical tools with major applications in medicine, environmental diagnostics and the food processing industry.

Biosensors are constructed by attaching a biologically-sensitive material, which is responsible for the recognition of the test species and provides the selectivity and sensitivity, to a suitable transducing system which converts the biochemical response into a quantifiable and processable electrical signal. The two most important properties of any proposed biosensor are its specificity and its sensitivity towards the target analyte. The specificity of a biosensor is entirely dependent on the properties of the biological component because this is where the analyte interacts with the sensor. However, the sensitivity of the integrated device is given by both the biological component and the transducer because there must be a significant biomolecule-analyte interaction and a high efficiency of subsequent detection of this reaction by the transducer.

Figure 1.1 shows a schematic representation of a biosensor.

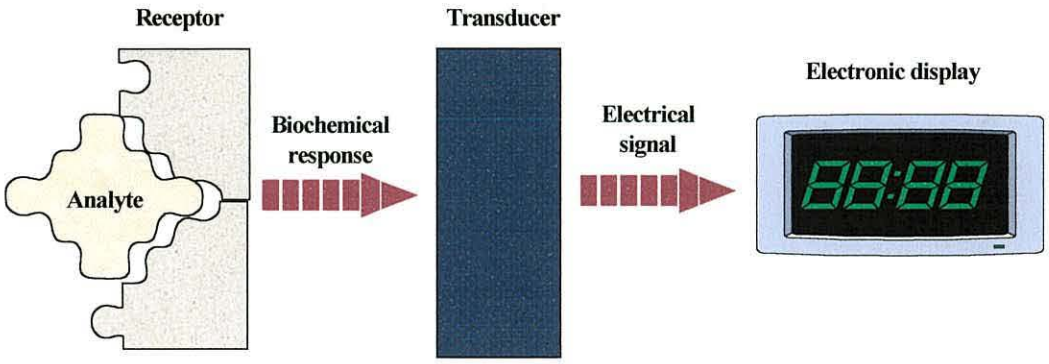


Fig 1.1. Schematic configuration of a biosensor.

The molecular recognition is achieved by a simple lock-key principle of the receptor and the analyte to be recognised. When such biological molecules interact specifically and reversibly, a change takes place in one or more physico-chemical parameters associated with the interaction, producing ions, electrons, gases, heat, mass or light. Any of these quantities can be converted into electrical signals by a suitable transducer, amplified, processed and displayed as required.

The nature of the transducer and the transduced parameter will depend on the type of bioanalytical event concerned with the detection of the analyte. However, since several parameters can often be altered during an analytical reaction pathway, the choice of the device is not necessarily restricted to a single transducer. Some of the transducers commonly employed in biosensor technology as well as their measurement mode and typical applications are included in table 1.1 (Sethi, 1994).

Transducer System	Measurement mode	Typical applications
Electrochemical: Conductimetric	Conductance	Enzyme-catalysed reactions
Electrochemical: Enzyme electrode	Amperometric	Enzyme substrates, immunological systems
Electrochemical: Field Effect Transistors	Potentiometric	Ions, gases, enzyme substrates, immunological analytes
Electrochemical: Ion-selective electrodes	Potentiometric	Ions in biological media, enzyme electrodes, immunoelectrodes
Electrochemical: Gas-sensing electrodes	Potentiometric	Gas-, enzyme-, organelle-, cell-tissue-electrodes, enzyme immunoelectrodes
Electrochemical: Impedimetric	Impedance	Enzyme immunosensors
Piezo-electric crystals, surface acoustic devices	Mass change	Volatile gases, vapours, immunological analytes
Optoelectronic, fibre optics and waveguide devices	Optical	pH, enzyme substrates, immunological analytes
Thermistors, diodes	Thermometric/calorimetric	Enzyme, organelle, whole cell or tissue sensors for substrates, gases, pollutants, antibiotics, vitamins, immunological analytes

Table 1.1. Types of transducers exploited in biosensors (Sethi, 1994).

A review of chemical biosensors in terms of the biological components employed to recognise the analyte, of the transducer systems which translate the chemical response into an electrical quantifiable system and of the immobilising agent that attach both the biologically-sensitive material and the electrical transducer together follows below.

1.1. Biological components employed in biosensors technology

The biomolecules and biological systems employed in biosensor technology can be divided into the following main groups:

(i) Enzymes: proteins which catalyse specific biochemical transformations.

(ii) Antibodies: globular serum binding proteins which bind the corresponding antigen with high specificity and affinity.

(iii) Whole cells: intact biological structural units which contain a wide range of biomolecules and which respond to many types of chemicals.

(iv) Receptors: protein systems which interact with specific chemicals such as hormones resulting in conformational changes.

All of these are described below, with special emphasis on enzymes, not only because they have been the biological component employed in our investigations, but also for the outstanding role they play in biosensor technology. Other biological components employed such as organelles, tissue or membrane systems, have found so far only limited application in the area of biosensors and will not be discussed in detail.

1.1.1. Enzymes

Enzymes are proteins (polypeptide structures) which catalyse specific chemical reactions, that is they accelerate the rate of reaction of a particular chemical reactant without being consumed or altered in the process. Enzymes are found in all types of cells; any given cell typically contains many hundreds of enzymes, although the exact distribution and quantity of enzymes present varies considerably according to the natural function of the cell. Enzymes are highly specific both in the reaction catalysed and in their choice of reactants, called substrates. An enzyme usually catalyses a single chemical reaction or a set of closely related reactions. The degree of specificity for a given substrate is usually high and sometimes virtually absolute.

The structure of individual enzymes varies widely within the basic framework of a polypeptide structure and determines the catalytic role of the molecule. The 3-dimensional structure of an enzyme is formed by the complex and highly precise folding of a single polypeptide chain of typical molecular weight ranging from 13 kDa to 50 kDa (1 Da is equivalent to 0.9997 at-g). A small portion within this structure, typically defined by a group of 5 to 10 amino acids is spatially arranged in a specific relative conformation, which interacts with the substrate specific to the enzyme during the transition state when an enzyme / substrate complex is formed. Such a region of the enzyme molecule is referred to as the active or catalytic site.

The active site of many enzymes is located in the interior of the protein structure to promote specificity towards the substrate and to reduce non-specific interaction and access to inhibiting molecules. Certain enzymes also display one or more additional active areas defined by specific amino acid arrangements capable of binding subsequent reactants or co-factors. A co-factor is a non-protein organic chemical required to achieve maximum efficiency in the enzymatic catalysis. Moreover, activators such as metal ions may similarly interact with the enzyme or the enzyme / substrate complex to facilitate the reaction in certain enzymatic systems. The active site of an enzyme is designed by evolution to facilitate chemical transformation of the substrate by promoting redistribution of electron density within the substrate molecule through the formation of ionic or hydrogen bonding interactions. Hydrophobic forces are also believed to play a role in the formation of the enzyme / substrate complex.

The energy required to achieve the intermediate transition state in the reaction is substantially lowered as a result of these interactions which, in turn, results in a more rapid rate of reaction. Conversion of the enzyme / substrate complex to product is followed by dissociation of products from the active site to regenerate reactive enzyme. The rate at which the enzyme converts substrate into product is reflected in the enzymatic activity, which is defined as the amount of substrate converted to product per unit weight of enzyme per unit time under defined conditions of pH and temperature. Another important parameter in enzyme catalysis is the turnover number, defined as the number of substrate molecules transformed per molecule of enzyme per second.

Enzyme specificity is a major property being exploited in biosensor technology. Compared with other chemical catalysts, enzymes demonstrate a significantly greater level of substrate specificity because of the constraints placed on the substrate molecule by the active site environment involving factors such as molecular size, stereochemistry, polarity, functional groups and relative bond energies. A further important parameter for biosensor performance is sensitivity, with respect to detection and measurement of a given analyte. In the case of enzyme-based biosensors the sensitivity is directly dependent on the maximum limiting affinity underlying the formation of the enzyme / substrate complex and the rate of subsequent transformation. A detailed analysis of the kinetics of enzyme catalysis is included in the following chapter.

Since relatively small changes in the conformation of key amino acids at the active site of enzymes can cause dramatic changes in enzymatic activity and substrate specificity, it follows that factors which influence protein structure and conformation will also influence the stability of enzymatic action. Denaturation can be caused by changes in pH, temperature and pressure, or exposure to UV radiation, detergents, organic solvents or certain chemicals such as urea or guanidium salts.

Isolation of enzymes and subsequent incorporation into an in vitro operating environment will generally result in a loss of some of the enzymatic activity, although such a decrease can be minimised by recreating suitable pH, temperature and co-factor conditions.

1.1.1.1. Enzyme sensors

A major proportion of the research and development work carried out on biosensor technology has been concerned with enzymes. As enzymes catalyse the conversion of specific substrates into products without being consumed in the process, they are particularly suitable for biosensors that function as continuous monitors of analyte concentration. The basis for the majority of enzyme sensors is the conversion of the analyte of interest to a detectable product by an immobilised enzyme. Such a product will have quantifiable electro-activity (e.g. redox activity), or optical properties (e.g. fluorescence, absorbance).

Three other approaches that have been reported are: enzyme thermistors, where the heat absorbed or radiated by the enzyme catalysed reaction of the analyte is measured and related to analyte concentration; inhibited enzyme sensors, where the inhibiting analyte blocks a specific enzyme reaction, altering the electrochemical or optical signal, and therefore the analyte is not the natural substrate of the enzyme; optical enzyme sensors in which the signal originates from the enzyme itself (or an associated molecule such as a co-factor) rather than from a product of the enzymatic reaction.

The main aim of enzyme-based sensor research is to make use of the high specificity and high sensitivity which typify an enzyme-substrate interaction as the basis for a detection and measurement system. The interaction between an enzyme and its substrate needs consumption of the substrate and formation of products. This effectively comprises an intrinsic signal-generating process if transduction of a suitable property of the enzymatic reaction product is carried out.

The first generation of enzyme sensors were mediator-free enzyme electrodes. This type of sensor consisted of a suitable enzyme immobilised close to the surface of an ion-selective electrode. From this first approach carried out by Clark and his collaborators in 1962, enzymes have received different treatments and have been incorporated into a wide variety of immobilisation methods and transducer systems, making them the most employed biological recognition system in sensor technology.

1.1.2. Antibodies

Antibodies are serum proteins which are produced by two types of blood cells, B-lymphocytes and plasma cells, in response to a foreign substance. Such a non-self substance is called immunogen because it provokes an immune response, i.e. the production of many antibodies. In most instances, an individual antibody will recognise one substance only, which is called the antigen. In biosensors based on antibodies, the analyte is either the corresponding antigen of the antibody used or part of it (hapten).

An antibody-antigen interaction is characterised by two major properties that may be exploited for sensing or detection purposes: its high activity, up to 10^{-18} M of antigen can be detected in certain laboratory assays based on antibodies (immunoassay), and low cross-reactivity, relatively minor changes in the structure of a given antigen can make it almost invisible to an antibody produced in response to the original unmodified structure. The antigen-antibody reaction may therefore be regarded as highly specific.

Antibodies consist of four polypeptide sub-units comprising two identical large or heavy chains and two identical small or light chains, held together by non-covalent forces and covalent interchain disulphide bonds. The approximate molecular weights of each heavy and light chain are 50 kDa and 22 kDa respectively. The key portions of the antibody molecule containing the antigen binding sites are termed the Fab fragments, each of which comprises an entire light chain and a segment of the heavy chain. Fab fragments are often used for biosensor applications instead of whole antibody molecules to reduce non-specific binding (antibody molecules contain carbohydrate portions which are likely to bind non-antigenic components) and prevent the formation of antigen-bridged complexes which distort conventional quantification methods in immunoassays. At the same time, greater control over the stereochemistry of the immobilisation process is gained when using Fab fragments, through use of –SH groups produced from cleavage of disulphide (S-S) bonds during Fab fragment preparation.

Antibodies have been used very widely in laboratory-based immunoassays for detection of an extensive range of individual single analytes, each one of which must be the corresponding antigen of the antibody used in the test. Antibodies cannot be synthesised by chemical means, they can only be obtained by inducing the generation of the required antibody using an animal species with a well developed immune-system. Several factors influence the immunogenicity of a particular molecule:

(i) Foreignness. A healthy immune-system will not raise antibodies against a molecule that is naturally present in the animals blood stream. Antibodies can however be raised in one animal against proteins or other macromolecules (even antibodies) derived from a sufficiently dissimilar organism. This is because of differences in the protein sequences and hence three-dimensional structures of proteins from the two organisms.

(ii) Molecular size. The most highly immunogenic chemicals are large macromolecules (>100 kDa). Molecules of molecular weight under 10 kDa are weakly or non-immunogenic. Many target analytes of interest, drug molecules for instance, fall into this last category. However, they can be covalently attached to a large carrier molecule such as bovine serum albumin (BSA) or key-hole limpet haemocyanin (KLH) which renders the conjugated drug molecule immunogenic, that is, the drug molecule becomes an antigenic determinant within the drug-protein conjugate.

(iii) Chemical structure. A certain degree of chemical complexity is necessary to ensure an efficient immune response. Amino acid homopolymers such as poly-L-lysine have a high molecular weight but present a poor immune response because of the regularity of the structure. A small protein of the same molecular weight would have a structural complexity several orders of magnitude higher and would give a strong immune response.

An antigen (Ag) interacts with an antibody (Ab) raised to one of its antigenic determinants (portion of structure to which antibodies are produced) with a high binding affinity. The strength of the interaction is dependent on the complementarity of the fit of the antigenic determinant to the binding site of the antibody. The binding forces present in the Ag-Ab complex are non-covalent, normally electrostatic, hydrogen bonding, hydrophobic and Van der Waals forces.

In order to discuss the specificity of antibodies which is extremely high in general, it is necessary to define the heterogeneity of immune response. Immunisation of a single antigenic determinant will produce a range of Abs which recognise and bind different sites on the antigenic unit with different binding affinities. This phenomenon is termed the heterogeneity of the immune response. Cross-reactivity of antibodies occurs as a

result of the interaction of some of those antibodies with substances of chemically similar structure; in general it is the low affinity antibodies which are the ones that show the greatest cross-reactivity, since they tend to recognise a smaller portion of the antigenic determinant than those of high affinity.

For biosensor applications, it is usually necessary to isolate the biological component and put it to use in a non-ideal in vitro operating environment. When considered purely in terms of ease of manipulation, flexibility of use and operation in sensing applications, antibodies offer significantly more advantageous properties than enzymes. However, this advantage must be tempered by the greater difficulties associated with signal generation in an Ab-based test compared with an enzymatic one.

1.1.3. Whole cells

Biological cells are small membrane-bounded structures which contain a high concentration of chemicals including enzymes, nucleic acids, ions, many different types of protein, small organic molecules and many others. The simplest living cellular systems are unicellular algae or bacteria where the entire organism consists of a single cellular unit. In higher organisms many billions of cells are present, each of which has a specific function governed by the genetic code of the organism.

The structural and chemical complexity of a cell confers sensitivity to an extremely wide range of compounds, although individual types of cells can show particular physico-chemical or biological changes to a specific type or group of chemicals. A major challenge in exploiting intact cells in biosensor research is that of achieving selective transduction of a specific biochemical event or process within the complex cellular chemistry.

The sensitivity and specificity of a particular cell for a given chemical varies depending on the type, source and environment of the cell, and the exact nature of the chemical itself. It is not possible to present any general guidelines to their behaviour towards the presence of a particular substance.

The stability of biological cells is of critical importance for biosensor applications. In general, cells derived from higher organisms are more difficult to isolate and maintain in a viable form for the extended periods of time required in most sensor formats. The most biologically robust cells are derived from less complex organisms, such as yeast, bacteria and algae.

1.1.4. Molecular receptors

Molecular receptors are cellular proteins which bind specific chemicals in a way which results in a conformational change in the protein structure. Such a change triggers a cellular response, like opening an ion channel or secreting an enzyme. In general, molecular receptors are distinct from receptors on the basis of their composition, which is a single protein structure, though possibly containing more than one subunit. Among the important receptors found to date are receptors for hormones, amino acids, insulin and neurochemical transmitters, in addition to those which are able to bind synthetic bio-active chemicals such as drugs.

Molecular receptors show intrinsic signal amplification properties and high specificity, two relevant features to their incorporation into biosensor devices. However, the relatively difficult experimental procedures necessary to isolate the receptor proteins, the present inability to obtain more than very small amounts of pure receptor and the rapid loss of biological function which follow isolation, are important limitations to the study and use of molecular receptors.

1.2. Immobilisation of the biological component

In addition to the specific properties of the biological component and the transducer system forming the biosensor, the reliability of its performance will also depend on the binding of the biological component to the sensor surface and its ability to remain there during use.

Several methods of immobilisation have been applied to biological materials to stick them on, or in close proximity to, the sensing element. Simple adsorption on a surface of alumina, clay, glass or ion exchange resins can attach biological materials for a single, non-repeatable measurement. Chemical groups of the biological component which are non-essential for activity can alternatively be covalently attached to chemically activated supports, such as glass, synthetic polymers or cellulose. Bifunctional agents that induce inter-molecular chemical cross-linking can also be introduced to bind biological materials to solid supports.

The next section describes in depth the immobilisation methods most widely employed in enzyme sensors. Although they are intended to be used with enzymes as the biological component, most of them can also be applied to antibodies, cells and other recognition agents.

1.2.1. Enzyme immobilisation

Immobilised enzymes are defined as enzymes which are physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Cabral et al, 1983). The term immobilised enzyme includes:

(i) Enzymes modified to a water–insoluble form by suitable techniques.

(ii) Soluble enzymes used in reactors equipped with a semipermeable ultrafiltration membrane, allowing the passage of reaction products

resulting from the hydrolysis of high molecular weight substrates but retaining the enzyme molecules inside the reactor.

(iii) Enzymes whose mobility has been restricted by attachment to another macromolecule, with the composite resultant molecules being water-soluble.

A large number of methods have been developed to immobilise enzymes on solid matrices over the last few years, some of them are summarised in table 1.2 and discussed further below.

1.2.1.1. Cross-linking method of immobilisation

The cross-linking immobilisation method is based on the formation of covalent bonds between the enzyme molecules by means of bifunctional or multifunctional reagents. It leads to three-dimensional, cross-linked enzyme aggregates that are completely insoluble in water, without the use of water-insoluble carriers. This method involves the addition of an appropriate amount of cross-linking agent to an enzyme solution in conditions that give the desired insoluble derivative. At the moment, optimum conditions for achieving good insolubility while retaining considerable enzyme activity must be determined experimentally, until enough information about the primary, secondary and tertiary structures of enzymes is available to allow prediction of the best immobilisation chemistry that will provoke minimal distortion of the enzyme and its derivatives.

Some of the reagents that have been used for the preparation of immobilised enzymes are included in table 1.2.

Multifunctional Reagents for the preparation of immobilised enzymes by intermolecular cross-linking		
Diazobenzidine	Diazobenzidine-2,2'-disulfonic acid	
Diazobenzidine-3,3'-dianisidine	Diazobenzidine-3,3'-dicarboxylic acid	
4,4'-Di-isothiocynato biphenyl-2,2'-disulfonic acid	4,4'-Difluoro-3,3'-dinitrophenyl sulfone	
1,5-Difluoro-2,4-dinitrobenzene	Trichloro-s-triazine	Toluene-2,4-di-isothiocyanato
Glutaraldehyde	N,N'-Hexamethylene-bi-siodoacetamide	Hexamethylene di-isocyanate

Table 1.2. Multifunctional reagents for the preparation of immobilised enzymes by intermolecular cross-linking (Cabral et al, 1983).

These reagents possess two identical functional groups (homobifunctional reagents). Heterobifunctional or multifunctional agents have been more often used for the binding of enzymes to carriers than for intermolecular cross-linking.

Among the considerable number of cross-linking agents found in the literature, glutaraldehyde has found widespread use for enzyme immobilisation and is by far the most commonly used cross-linking reagent. The linkages formed between the enzyme and glutaraldehyde are irreversible and survive extremes of pH and temperature. Studies of the preparation of immobilised enzyme with this method (Broun, 1976) have shown that insolubilisation of the protein is critically dependent on a delicate balance of factors such as the concentrations of the enzyme and cross-linking reagent, the pH and ionic strength of the solution, the temperature and the time of the reaction.

The most important advantage of the cross-linking method is that one bi- or multi-functional reagent can be utilised to prepare, in a simple manner, immobilised enzymes, that are almost pure protein. However, immobilisation of enzymes solely by their

intermolecular cross-linking into large aggregates has found rather limited application because of the difficulties encountered in controlling such reactions, the need for a large amount of enzyme, the often unavoidable inactivation of the enzyme caused by the participation of the active centre on the cross-linkage, and the mechanical properties of the gelatinous nature of these enzyme derivatives. This immobilisation method has been extensively employed in conjunction with other methods, to overcome the shortcomings pointed out above.

1.2.1.2. Carrier binding method of immobilisation

The carrier binding method, which consists of the binding of enzymes to solid supports, is the most commonly used method of immobilisation of enzymes. It can be further divided into four categories according to the binding mode of the enzyme: physical adsorption, ionic binding, metal binding and covalent binding.

When enzymes are immobilised in this way, care must be taken in the selection of carriers, as well as the binding procedures. Although there is no universal carrier, a number of requirements should be addressed in any material considered for immobilising enzymes. It should be stable in solution and should not deteriorate under operational solution conditions of pH and composition. Although both organic and inorganic carriers have been suggested for immobilising enzymes, it appears that inorganic supports can best meet the requirements for industrial use due to their stability against physical, chemical, thermal, and microbial degradation, their mechanical strength, and their structural stability. On the other hand, the most important advantage of organic supports is the ease with which enzymes can be attached yielding enzymatic preparations with good activity and operational stability.

1.2.1.2.1. Physical Adsorption

This method is the oldest and probably simplest way of preparing immobilised enzymes. It consists of bringing an aqueous solution of the enzyme into contact with the adsorbent surface. Because no reactive species are involved, there are little or no conformational

changes in the enzyme on immobilisation, so derivatives with specific activities similar to that of the soluble enzyme might be expected. The adsorption is dependent on experimental variables such as pH, nature of the solvent, ionic strength, quantity of enzyme and adsorbent, time and temperature.

A major influence on the quantity of enzyme adsorbed to a solid support is enzyme concentration exposed to the surface of the carrier during the immobilisation process. The activity increases with increasing enzyme concentration, approaching a saturation value asymptotically at higher enzyme concentrations.

The main disadvantage of immobilising the enzyme by physical adsorption is the risk of desorption of the protein from the carrier, which occurs during utilisation due to the weakness of the binding forces, with subsequent loss of catalytic activity and contamination of products. Therefore, some enzymes might stay active only for a short period of time, limiting the reliability of adsorption techniques when absolute immobilisation of an enzyme is desired.

1.2.1.2.2. Ionic binding

The ionic binding immobilisation method is based mainly on ionic binding of enzyme protein to solid supports containing ion-exchange residues. The main difference between physical adsorption and ionic binding is the strength of the linkage of the enzyme to the carrier. In fact, in some cases physical adsorption might also take place in the ionic binding process.

As in the physical adsorption process, the immobilisation of enzymes is easily carried out, using the same procedures. The resultant binding forces (ion-ion interactions) are stronger than those in the case of the physical adsorption, although less strong that in the covalent binding. Nevertheless, leakage of the enzyme from the carrier might occur in some situations, as with physical adsorption, with variation of pH or when high substrate or high ionic strength solutions are used. Thanks to the ionic character of the linkage between the enzyme and the support and the mild conditions for immobilisation,

little conformational changes occur with the enzyme, leading to immobilised forms with high enzymatic activities.

Ion exchangers are used as the carriers for ionic binding. Most frequently they are organic supports with ion exchange residues, although there are also inorganic forms (especially silica) with the same or similar ion exchange residues. The organic polymers are derivatives of polysaccharides, mainly cellulose and Dextran, and the synthetic polymers are mainly polystyrene based. According to the type of ion-exchange residues, these carriers may be classified as anion or cation exchangers, based on the properties of exchanging anions (Cl^-, OH^-, etc) of the carrier with anionic residues of the enzyme or cations (H^+, Na^+, etc) respectively.

1.2.1.2.3. Chelation or metal binding

Active immobilised enzymes have been prepared using transition metal compounds for the activation of the surface of organic and inorganic carriers by the formation of chelates between the enzyme and the activated carrier.

Although this method is very simple in its application to immobilising enzymes, only one step for the activation of the support and another for the binding of the substrate, the operational activities obtained with high molecular substrates are poor, which yields to deactivation of the enzymes. Desorption can also occur during long term storage, showing that weak interactions between the enzyme and the activated support are an important feature of this technique.

1.2.1.2.4. Covalent binding

The covalent binding method is based on the covalent attachment of enzymes to water-insoluble matrices. This method has been the most widely spread and one of the most thoroughly investigated approaches to enzyme immobilisation.

As with cross-linking, covalent binding is strong, so that stable immobilised enzyme preparations have been obtained that do not lose enzyme into the solution, even in the presence of substrate or high ionic strength solutions. The immobilisation of an enzyme by covalent attachment to a support matrix should involve only functional groups of the enzyme that are not essential for its catalytic action, and therefore the active centre of the enzyme must be unaffected by the various reagents used.

The wide variety of binding reactions and of matrices with functional groups capable of covalent coupling makes this a generally applicable method of immobilisation. Nevertheless, the compositional and structural complexity of proteins has not allowed, except in a very limited number of cases, the application of general rules by means of which the method best suited for a specific task could be predicted.

1.2.1.3. Entrapment method of immobilisation

The entrapment method is based on the occlusion of an enzyme within a constraining structure (lattice of a polymer matrix or membrane) tight enough to prevent the release of the protein while allowing penetration of substrate. Consequently, only reactions involving relatively small reactants may be carried out successfully by using entrapped enzyme preparations. The entrapment technique can be carried out using a gel matrix, a fibre matrix or through micro-encapsulation.

The gel entrapment method involves entrapping the enzyme within the interstitial spaces of cross-linked water-insoluble polymer gels. It is easy to perform and non-invasive, allowing the enzyme to retain intact most of its characteristics. These gels, however, are quite weak from a mechanical point of view and have an open network with a broad distribution of pore sizes that often allows the enzyme to leak out.

As an alternative to gels, enzyme can be entrapped within the micro-cavities of synthetic fibres. With this technique, the physical entrapment of an enzyme is achieved by dissolving a fibre-forming polymer in an organic solvent immiscible in water and

emulsifying this solution with the aqueous solution of the enzyme. Fibre entrapped enzymes are applied industrially in the pharmaceutical and food processing industry.

A way to overcome the leaking problem of the immobilisation of enzymes with gels and fibres is to enclose the protein molecules within semipermeable polymer membranes. Such micro-encapsulated enzymes are physically contained within the semipermeable membrane and can not leak out, while external substrates can diffuse across the membrane to be processed by the immobilised enzymes.

Advantages of this method of immobilisation include the extremely large surface area for contact of substrate and enzyme within a relatively small volume, and the real possibility of simultaneous immobilisation of multiple enzymes in a single step. Nevertheless, the method can only be applied to low molecular weight substrates and occasionally inactivation of the enzyme might take place.

One of the most employed immobilisation techniques is the entrapment of the biological component within the pores of a polymer matrix or membrane. Such pores must be large enough to allow diffusion of the test species and product, and small enough to prevent loss of the active, high molecular weight biological agent.

1.3. Electrochemical biosensors

The modern concept of a biosensor is derived form the early work by Clark and his co-workers (1962). They proposed that enzymes could be immobilised at electrochemical detectors to form intelligent enzyme electrode sensors which would enhance the versatility of the base detector. The use of electrochemical detection in biosensors has become increasingly extensive since then, forming the basis for the most successful commercially available biosensors produced to date.

Electrochemical sensors usually employ a three-electrode configuration (working electrode, counter electrode and reference electrode) and measure the change in the relevant electrical parameter at the electrode surface. Depending on the electrical

parameter monitored, three main classes of electrochemical biosensors can be distinguished: potentiometric (detecting the electrical potential), amperometric (measuring the intensity of the electric current) and impedimetric (monitoring the impedance, admittance or any of their components

1.3.1. Potentiometric biosensors

Potentiometric measurements involve non-faradaic electrode processes, with no net current flow, and operate on the principle of an accumulation of charge density at an electrode surface, which results in the development of a significant potential at the electrodes. This potential is proportional to the logarithm of the analyte present in the sample and is measured relative to an inert reference electrode, also in contact with the sample.

Potentiometric measurements have been most frequently developed around pH sensitive electrodes and the same analytical reagents have been used in pH-FETs, with any of the enzyme pathways resulting in a change in proton concentration being liable to be applied here.

Potentiometric sensors and FET devices have also been applied to immunoassay techniques involving antibody-antigen complex formation.

1.3.1.1. Enzyme modified ion-selective electrodes

A potentiometric enzyme modified ion-selective electrode is formed by immobilising an enzyme onto an ion-selective electrode, ISE, which results in a highly selective and sensitive method for the determination of a given substrate. An ion-selective electrode is a device that develops an electrical potential proportional to the logarithm of the activity of an ion in solution, the element responsible for its selectivity and sensitivity being the sensing membrane.

The ion-selectivity and sensitivity of an ion-selective membrane results from a very small energy barrier for the transport of one particular ion across the solution / membrane interface while a rather large energy barrier is maintained for the transport of all other ions. When the solution concentration of the selected ion increases, the ions will tend to diffuse through the solution and across the interface creating a charge imbalance and a variation in the potential across the interface. This interfacial potential change is then measured by the electrode and related to the concentration of the selected ion.

ISEs can be divided into several categories according to the composition of their sensor membranes:

(i) Glass electrodes where the sensing membrane is a very thin membrane of glass and in which the composition of the glass determines the selectivity of the membrane. Glass electrodes sensitive to H^+, and to cations in the order $Ag^+ > H^+ > K^+ > NH_4^+ > Na^+ > Li^+$, Ca^{++}, Mg^{++} are available.

(ii) Solid-state electrodes, where the sensor is a thin layer of a single mixed crystal or precipitate which is an ion conductor. Solid-state electrodes can be homogeneous (the membrane is a pellet prepared from a precipitate, mixture of precipitates or a single crystal) or heterogeneous (the membrane is a precipitate or mixture of precipitates dispersed in an inert supporting matrix, such as silicone rubber or PVC).

(iii) Liquid ion-exchange electrodes, prepared by dissolving an organic ion exchanger in an appropriate solvent and holding the solution in an inert matrix, such as PVC or poly(methyl methacrylate).

(iv) Special electrodes, which employ a coating over the membrane of an ion-selective electrode. This coating is usually a gas-permeable membrane, as for electrodes sensitive to CO_2 or NH_3. The gas diffuses through the membrane and alters the pH of an internal filling solution. The pH change is measured with a glass electrode and is proportional to the concentration of gas which enters the membrane. Enzyme modified ion-selective electrodes also fall into this category, the coating containing an enzyme which converts a substrate to an ion which is detected by the ISE.

Some of the ion-selective electrodes useful in the construction of enzyme electrodes are included in table 1.3.

Potentiometric Ion-Selective Electrodes	**Detection**
NH_3	urea, amino acids, glutamine, glutamic acid, nitrate, nitrite, creatinine, lyase and deiminase enzymes
CO_2	urea, amino acids, decarboxylative enzyme systems
pH	penicillin, RNA, DNA, glucose, enzyme reactions giving pH change
I^-	glucose, amino acids, cholesterol, alcohols
CN^-	amygdalin

Table 1.3. ISE sensors useful in the construction of enzyme electrodes (Kuan and Guibault, 1987).

Figure 1.2 shows a diagram of a typical enzyme modified ion-selective electrode.

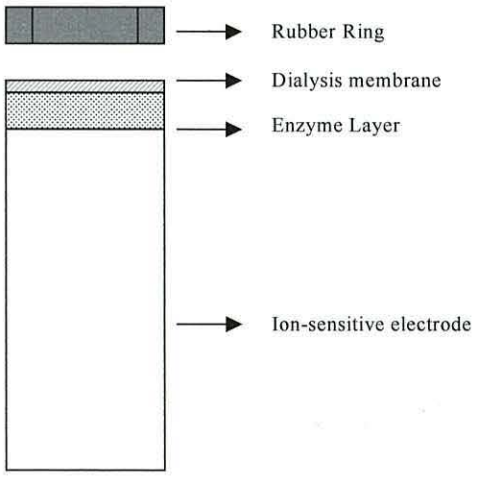


Fig 1.2 Enzyme modified ISE probe (Kuan and Guibault, 1987).

In enzyme modified ion-selective electrodes, the enzyme is immobilised on the sensing membrane of the electrode, covered with a dialysis membrane to protect it from scratching and avoid any leakage, and finally secured with a rubber ring that ensures the enzyme forms a uniform layer on top of the selective membrane. Depending whether the sensor is designed for gas detection or measurement of ions in solution, the enzyme modified electrode must be placed in close proximity to or in contact with the solution under test.

The enzyme electrode is employed as any other ion-selective electrode. The potentiometric probe must be plugged directly into a digital volt-meter and the potential readings for each concentration tested are then plotted against the concentration in a linear-log plot. A reference unmodified electrode is used together with the enzyme electrode or, alternatively, it can be combined as an integral part of it. The potential difference generated by the change in ionic concentration across the sensing membrane is measured relative to the reference electrode under conditions of zero current flow.

The operation of an enzyme electrode can be divided in five stages: (1) substrate transport to the surface of the electrode, (2) substrate diffusion through the enzyme layer towards the active site, (3) catalysed generation of product, (4) transport of product molecules through the enzyme layer to the surface of the electrode, and (5) measurement of product at the electrode surface. If rapid stirring of the solution is possible, reducing dramatically the transport time of the substrate molecules towards the enzyme, and the enzyme layer is kept very thin, so that diffusion time through it is almost nonexistent, the response time of the enzyme electrode will theoretically approach that of the bare sensor.

The activity of the sensed ion S and the membrane potential are logarithmically related according to,

$$E = E_0 + \frac{R \cdot T}{n \cdot F} \cdot \ln\{a_S + \kappa_{SP} \cdot (a_P)^{Z_S \cdot Z_P}\} \qquad [1.1]$$

where E_0 is a constant baseline potential, R is the gas constant, T is the absolute temperature, a_S is the activity of S (the activity is approximately equal to the concentration in diluted solutions), n is the valency of the ion, F is the Faraday constant ($1F = N_A \cdot q_e^-$) and κ_{SP} is a selectivity coefficient introduced to account for the influence of any disturbing ions P of activity a_P. Equation [1.1] is a modification of the Nernst equation under thermodynamic equilibrium conditions,

$$E = E_0 + \frac{R \cdot T}{n \cdot F} \ln a_s \qquad [1.2]$$

where all parameters have been previously defined.

Potentiometric methods have the advantage that there is no net consumption of material and hence mass transport is not important. However, such sensors suffer from two main disadvantages. Local thermodynamic equilibrium is necessary at the electrode / membrane interface in order to obtain accurate information about the concentration of the analyte in the solution. This requires the electrode kinetics to be fast. Moreover, the exponential dependence of the analyte activity on the electrode potential introduces higher errors in the calculation of the concentration.

Nevertheless, practical advantages of such potentiometric sensors like simplicity of instrumentation, low cost and the easy availability of a large number of different and reliable ion-selective electrodes, still make them a common choice in urea monitoring.

1.3.1.2. Enzymatic Field Effect Transistors (ENFETs)

Enzymatic field effect transistors (ENFETs) have been directly derived from enzyme electrodes by substituting the ion-selective electrode with an ion-sensitive field effect transistor (ISFET) and applying a thin enzyme layer directly over the ion-selective membrane (Bergveld, 1970; Caras and Janata, 1980, 1985). One of the inherent problems of ISEs is that the output signal from the sensors is typically very noisy as a consequence of the high electrical impedance of the ion-selective membrane; the wires

connecting the high impedance electrode to the amplifier serve as antennae, responding to any change in the local electromagnetic field. To reduce this problem and keep electrical disturbances to a minimum, wires are normally fabricated from shielded cable and their length kept to a minimum. ISFETs integrate the impedance transformer into the electrode body by placing the ion-selective membrane directly on the gate of the transistor. The low impedance output of the FET can then be connected to the electronics through wires of any convenient length without the need for shielding. In addition, the small size and well-defined geometry of the ENFET requires only minimal quantities of enzyme, greatly reducing the production costs.

Figure 1.3 shows a diagram of a typical sensor.

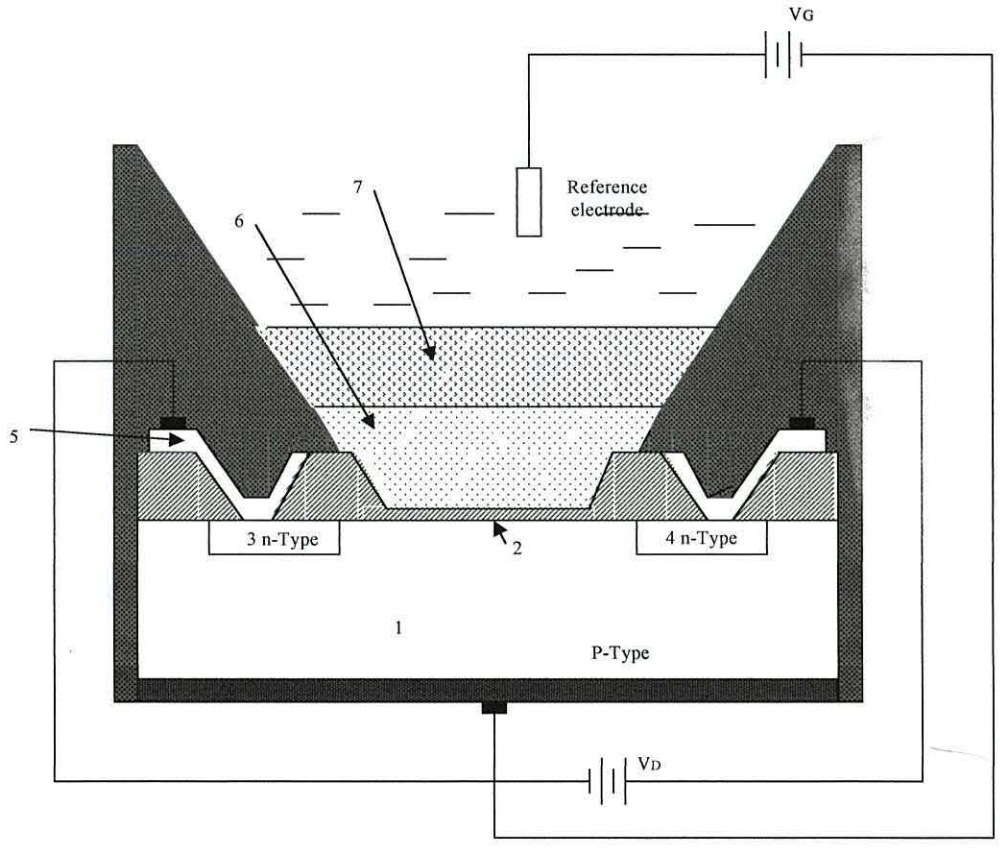


Fig 1.3. Diagram of a typical ENFET sensor: (1) p-type Si substrate, (2) insulator, (3) n-type source, (4) n-type drain, (5) metal contacts to source and drain, (6) ion-selective membrane, (7) immobilised enzyme layer. (Blackburn, 1987).

When the enzyme substrate is added to the solution, it diffuses into the enzyme layer where the catalysed reaction takes place. Either the consumed reactant or generated product is then monitored by the underlying ISFET. In the case where the created products are monitored, the newly formed species in the enzyme layer diffuse both into the bulk solution and towards the ion-selective membrane, causing an increase in concentration at the surface of that membrane. A steady-state condition is reached where the ionic product concentration remains constant at the interface between the enzyme layer and the ion-selective membrane. Since the reaction rate depends upon the substrate concentration, this steady-state concentration of products is determined by the solution concentration of the substrate. As explained for ion-selective electrodes, this variation of charge density generates a variation in potential, which acts as the gate potential V_G in a standard FET. The magnitude of V_G controls the passing of current from the drain to the source, I_D, which forms the basis of the transistor action of the FET. By measuring I_D, the potential difference at the interface between the ion-selective membrane and the enzyme layer can be deduced, and therefore the concentration of substrate present in solution.

1.3.2. Amperometric biosensors

Amperometric techniques were the first electrochemical methods to be applied to biosensor development and, at the early stages in biosensor research major effort was concerned with amperometric systems utilising redox enzymes.

Amperometric biosensors in general represent the most developed branch of biospecific electrodes. They combine the advantages of faradaic electrode processes (high sensitivity, linear concentration dependence, selectivity and independence of the sample buffer capacity) with the high specificity of enzymes or other biocatalytic systems such as organelles, micro-organisms and tissue slices.

The most interesting feature in amperometric detection is its ability to provide high sensitivity tests (10^{-7} M – 10^{-8} M) for the analysis of electroactive species present in biological test samples. Although many molecules of biological interest are not intrinsically electroactive and can not be directly detected amperometrically, the use of

enzymes which catalyse specific redox reactions can facilitate the production of electroactive species which can then be determined electrochemically. Based on the principle of detecting a concentration gradient of the electroactive product, amperometric sensors are only able to detect the formation or consumption of reaction partners, and are not suitable to measure changes in the electron density which do not result from a chemical conversion. Furthermore, since redox reactions are based on the transfer of electrons between two substances, amperometric sensors are generally restricted to two-substrate reactions catalysed by oxido-reductases. Therefore the concentrations of both the substrate and the cofactor influence the reaction rate. Considerable effort has been dedicated to eliminate the dependence on the cofactor concentration, resulting in its elimination as a reagent in analysis using amperometric biosensors.

This method of detection involves applying a constant potential across a sensing or working electrode and a counter or auxiliary one in the test sample and measuring the resultant steady-state or limiting current. Such a current is the result of an electrode reaction taking place by electron transfer and will be proportional to the concentration of electroactive species. It is important to identify and control the conditions which define the determining step of the overall electrolytic process. The rate of the electron transfer process occurring directly at the electrode can be controlled by variation of the applied potential so that the current is not limited by electron transfer, but controlled by mass diffusion, adsorption or the kinetics of the relevant chemical process.

The overall sensor current i_T an be described by the following generalised reaction,

$$\frac{1}{i_T} = \frac{1}{i_{ID}} + \frac{1}{i_{ED}} + \frac{1}{i_{CT}} + \frac{1}{i_{AD}} + \frac{1}{i_K} + \ldots \qquad [1.3]$$

There are two diffusional terms, i_{ID} and i_{ED}, which define the rates of internal and external diffusion respectively. External diffusion relates to diffusion in bulk solution up to the electrode or a membrane / solution interface, and internal diffusion involves movement within a membrane or reaction layer. i_{CT} represents charge transfer, i_{AD} the adsorption of reactants on a membrane or on the electrode surface and i_K relates to the

catalysed chemical reaction proceeding at a finite rate. It should be emphasised, however, that the terms in [1.3] are not completely independent of each other.

Amperometric sensors are frequently covered with a protective membrane which is permselective to the analyte of interest. The membrane isolates the electrode from the biological fluid and at the same time may contain a thin reagent layer, such as an enzyme layer, essential to the detection system. The membrane presents two other important functions: it enables the species to partition across the membrane / solution interface producing an enhancement or hindering of the response, and it creates a diffusional barrier so that, although a slow response might result, the response of the sensor remains unaffected by the stirring of the solution and therefore, the external diffusion term in [1.3] becomes irrelevant.

1.3.2.1. Amperometric enzyme electrodes

Amperometric enzyme electrodes benefit from the specificity of enzymes for recognising target molecules and the direct transduction of the rate of reaction into a current.

The first generation of amperometric enzyme electrodes was centred around the oxidation of glucose (Clark and Lyons, 1962; Guibault and Lubrano, 1973) and relied on the natural enzymatic reaction:

$$\text{Glucose} + O_2 \xrightarrow{GOD} \text{Gluconolactone} + H_2O_2 \qquad [1.4]$$

where GOD is glucose oxidase (EC 1.1.3.4). The electrode was merely used to measure the concentration of either the natural substrate O_2 or the product H_2O_2. This was a relatively complicated device with two membranes, and the accuracy of the response of the sensor was affected by the ambient concentration of oxygen.

Second generation systems have been developed (Cass et al, 1984a,b) in which the enzyme performs the first redox reaction with its substrate but is then re-oxidised by a mediator as opposed to oxygen. The mediator is then oxidised by the electrode,

$$\text{Glucose} + \text{GOD/FAD} \longrightarrow \text{Gluconolactone} + \text{GOD/FADH}_2 \qquad [1.5a]$$

$$\text{GOD/FADH}_2 + 2\text{M} \longrightarrow \text{GOD/FAD} + 2\text{M}' + 2\text{H}^+ \qquad [1.5b]$$

$$2\text{M}' \longrightarrow 2\text{M} + 2\text{e} \qquad [1.5c]$$

FAD represents a flavin redox centre in glucose oxidase and the mediator M/M' is assumed to be a one electron couple. Cass and his collaborators showed that various ferrocene ferrocinium couples can act as efficient mediators, among other species such as $[Fe(CN)_6]^{3-}$ and N-methylphenazinium (NMP^+).

An even simpler and more direct method reported was to have no mediator but to find an electrode material on which the reduced enzyme $GOD/FADH_2$ could be directly oxidised (Albery and Bartlett, 1985; Albery et al, 1985),

$$\text{Glucose} + \text{GOD/FAD} \longrightarrow \text{Gluconolactone} + \text{GOD/FADH}_2 \qquad [1.6a]$$

$$\text{GOD/FADH}_2 \longrightarrow \text{GOD/FAD} + 2\text{H}^+ + 2\text{e} \qquad [1.6b]$$

Figure 1.4 illustrates the kinetic scheme for a third generation enzyme electrode assuming the following model of enzyme catalysed reaction (Albery and Craston, 1987),

$$\text{S} + \text{E} \leftarrow \overset{k_1/k_{-1}}{\longrightarrow} \text{ES} \leftarrow \overset{k_2/k_{-2}}{\longrightarrow} \text{E'P} \leftarrow \overset{k_3/k_{-3}}{\longrightarrow} \text{E'} + \text{P} \qquad [1.7]$$

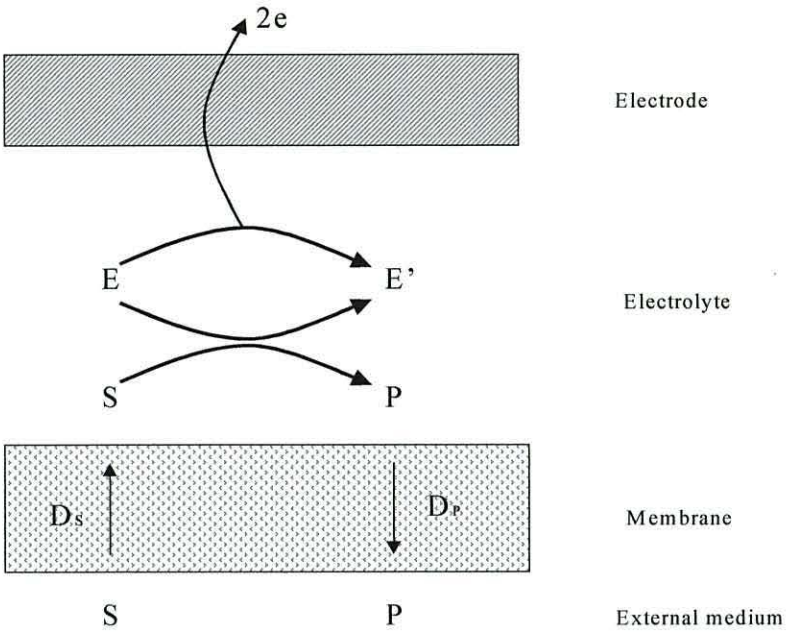


Fig. 1.4. Kinetic scheme of a third generation amperometric enzyme electrode (Albery and Craston, 1987).

In developing enzyme electrodes it is important to be able to identify the rate limiting step that determines the overall performance of the device, in order to deduce correctly the concentration of substrate from the measurement of the current. This step could be external transport of the substrate towards the membrane, diffusion through the membrane, reaction of the substrate with the enzyme, transport of the product back through the membrane or regeneration of the enzyme.

If the catalytic activity of the enzyme is high, the overall rate of the reaction will be limited by mass transfer to the catalytic layer and the intensity of the current will be proportional to the concentration of analyte present in the solution. On the other hand, if the enzyme electrode operates under kinetically controlled conditions, the relationship between the current and the concentration will be non-linear. However, such sensors are operated with a membrane between the enzyme layer and the solution, which provides a barrier and a response proportional to the diffusional flux which is not limited by enzyme kinetics.

1.3.3. Impedimetric biosensors

The impedimetric principle of measurement is applicable to a wide range of chemical systems as many chemical reactions produce or consume ionic species and therefore alter the overall electrical impedance of the solution. However, their most beneficial feature is also their main drawback, as the resistance of a solution is determined by the migration of all ions present, therefore making impedance measurements generally non-specific. Nevertheless, such lack of specificity might be overcome by monitoring the changes in conductance produced by the catalytic action of enzymes immobilised on, or in close proximity to the impedimetric transducer device.

The principle of operation of impedimetric biosensors involves the application of an electric field across a pair of electrodes surrounding an electrolyte solution which contains an enzyme and the species to be detected. The electric field is generated by the application of a sinusoidal voltage waveform across the electrodes which minimises faradaic processes, double layer charging and excessive interfacial polarisation on the electrode surface.

Although potentiometric and amperometric techniques have been widely applied to the development of analytical devices for the detection, quantification and monitoring of biological analytes, an increasing interest exists towards the exploitation of solution impedance as an alternative measuring principle. The monitoring of solution impedance was originally developed as a method of determining chemical reaction rates but, since the beginning of the seventies (Hanss and Rey, 1971; Lawrence, 1971; Lawrence and Moores, 1972), it has been applied to enzyme catalysed reactions. Notably, the urease catalysed hydrolysis of urea has been one of the main objects of study.

Impedance-based sensors have often been criticised because of their lack of selectivity. In order to obtain specificity towards a certain analyte, a differential method has to be applied. Since most of the physiological solutions of interest have relatively high ion content, an accurate measurement relies upon the efficiency of the differential sensor to

extract the relatively low analyte-induced impedance changes from the high background conductivity.

Impedimetric biosensors are further described on chapter 4.

1.4. Other types of biosensors

Other sensing systems, such as those based on piezoelectric or optic measurements will only be briefly outlined below. Impedance sensors based on dielectric measurements, as the ones investigated in our research work, are described further in other parts of this thesis.

1.4.1. Impedance dielectric biosensors

Impedance dielectric sensors measure the change in the impedance, admittance or any of their components (capacitance, conductance, resistance or susceptance) in a solution arising from a chemical reaction due, for example, to he production or removal of ionic or dipolar species. Such variation is directly related to the concentration of substrate initially present in the solution under test. Impedance dielectric biosensors employ two electrodes and are sensitive to variations in the electrical properties of all phases between those two electrodes. Figure 1.5 shows a diagram of the phases conforming an impedance dielectric biosensor.

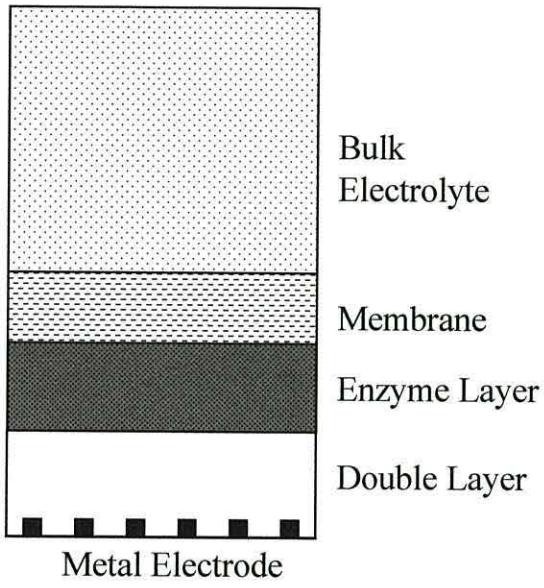


Fig. 1.5. Multiphase system representing an impedance dielectric biosensor.

The substrate dissolved in an electrolyte solution diffuses through the dialysis membrane towards the enzyme phase where is chemically transformed. The product species affect the electrical properties of one or more of the phases and this change can be measured.

When an ac signal of varying frequency (usually from 10 Hz to several hundred kHz) is applied to the electrode, the conductance and capacitance characteristics show the electrical profile of all the phases, each of them being dominant over a specific frequency interval (Taylor and Macdonald, 1987). A change in the ionic or dipolar nature of the system produced by the products generated in an enzyme catalysed reaction originate a variation in the capacitance and conductance (susceptance and resistance) of each of the phases, which can be directly related to the initial substrate concentration present in the sample. The work included in this thesis was performed using this sensing configuration. More information on impedance dielectric biosensors is included in chapters 4 and 7.

1.4.2. Piezoelectric biosensors

Piezoelectric biosensors are based on the measurement of change in resonant frequency of a piezoelectric crystal as a result of mass changes on its surface. These are caused by the interaction of test species or analyte with a biospecific agent immobilised on the crystal surface. The frequency of vibration of the oscillating crystal normally decreases as the analyte binds to the receptor coating the surface. Such sensors generally operate by the propagation of acousto-electric waves, either along the surface of the crystal or through a combination of bulk and surface, and are generally called surface acoustic wave devices.

1.4.3. Optical biosensors

Optical biosensors make use of fibre optics and integrated optical or optoelectronic transducers for measuring biological reactions. Enzyme reactions alter the optical properties of some substrates. Light emission from a biological element or its response to illumination may be conveniently monitored via fibre or other optical waveguide devices.

Chapter 2 The Enzymes – Characteristics and Enzyme Kinetics

The experimental work included in this thesis was based on the quantifiable detection of urea and creatinine by monitoring the changes in conductance generated through the relevant catalysed reaction. Urease was employed to catalyse the hydrolysis of urea and creatinine deiminase and a multi-enzyme configuration formed by creatininase, creatinase and urease were used to investigate the chemical transformation of creatinine.

Both the characteristics of the enzymes and the kinetics of the catalysed reaction have to be taken into account in the design and analysis of biosensors based on enzymatic recognition. A brief description of the enzymes employed and a review on enzyme kinetics is presented in this chapter.

2.1. Characteristics of urease

Urease (urea amidohydrolase EC 3.5.1.5) is widely distributed throughout nature in bacteria, algae and plants (Fahmy et al, 1998; Zerner, 1991; Mobley and Hausinger, 1989) but the most common source of urease for laboratory and clinical use are Jack bean meals. Native Jack bean urease is an hexamer of 590 ± 300 kDa molecular weight (Scheller and Schubert, 1992). Each approximately 98,300 Da subunit contains two nickel ions of different valency which are involved in substrate binding and conversion. The active centre of urease contains a SH group essential for the stability of the enzyme. The isoelectric point of the enzyme is at pH 5 and the optimum temperature of catalysis at 60 °C (Scheller and Schubert, 1992). Its optimum pH is 6 and the reported Michaelis constant falls between 4 mM and 6 mM (Lawton et al, 1989). Heavy metal ions, Ag^{+} and Hg^{++} especially, inhibit its catalytic activity.

Urease catalyses the hydrolysis of urea by cleaving the amido bonds of the molecule according to the following reaction

$$\text{Urea} + 3H_2O \xleftarrow{\text{urease}} 2NH_4^+ + HCO_3^- + OH^- \qquad [2.1]$$

In aqueous solution, ammonium and carbonate ions are involved in two pH dependent side reactions

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad [2.2a]$$

$$HCO_3^- \leftrightarrow CO_2 + OH^- \qquad [2.2b]$$

At pH below 8, ammonium ions and carbon dioxide are the favoured products in the above reactions.

Urease is a commonly employed enzyme in biosensor investigations. Its key role in routine determinations of urea in blood and urine (Thavarungkul et al, 1991), as well as its involvement in the theoretical analysis of enzyme kinetics (Fahmy et al, 1998; Leszko et al, 1996) and biosensors optimisation as a model enzyme (Lee at al, 2000; Sheppard et al 1996; Mikkelsen and Rechnitz, 1989), have made it the object of extensive study. Moreover, some additional applications of urease are: as an enzyme label in potentiometric immunoassays (Green, 1987), monitoring the effects of fertilizers on the soil (Qin and Cabral, 1994), determination of heavy metal ions in wastewaters (Volotovsky et al, 1997), diagnosis and treatment of human and animal pathogenic states induced by bacterial urease infections such as urinary stones, pyelonephritis, gastric ulceration and hepatic coma (Zerner, 1991), and control of circulation of nitrogen (Mobley and Hausinger, 1989).

2.2. Characteristics of creatinine deiminase

Creatinine deiminase (creatinine iminohydrolase EC 3.5.4.21) is usually purified from micro-organisms. It is formed by six subunits and has an approximate molecular weight of 260 kDa. It shows optimum catalytic activity around 70 °C and pH 9. The isoelectric point of creatinine deiminase is at pH 4.4 and can be inhibited by heavy metal ions (Ag^{+} and Hg^{++}), o-phenanthroline and monoiodoacetate. The Michaelis constant for the hydrolysis of creatinine catalysed by creatinine deiminase is reported to be approximately 30 mM (Barman, 1974).

Creatinine deiminase is commonly employed in the clinical determination of creatinine. The catalysed hydrolysis of creatinine take place following the reaction below,

$$\text{Creatinine} + H_2O \xleftarrow{\text{creatinine deiminase}} \rightarrow 1\text{-Methylhydantion} + NH_3 \qquad [2.3]$$

The ammonia generated is usually assayed by coupling creatinine deiminase with creatinine kinase, pyruvate kinase and, most commonly, glutamate dehydrogenase (GLDH) which catalyses the action of ammonia on α-oxoglutarate and NADPH according to the following scheme,

$$NH_3 + \alpha\text{-Oxoglutarate} + NADPH + H^{+} \xleftarrow{\text{GLDH}} \rightarrow \text{L-Glutamate} + NADP^{+} + H_2O \qquad [2.4]$$

The reduction of the absorbance of NADHP is measured and proportionally related to the concentration of the ammonia released in the hydrolysis of creatinine.

This clinical assay needs rather pure enzymes and, since the physiological level of creatinine is very low, the values have to be corrected for pyruvate and creatine. All these factors make the procedure laborious, costly and time consuming. An alternative method of measuring creatinine which does not require enzyme coupling makes use of impedance techniques. As shown in equation [2.2a] ammonia molecules exist ionised as ammonium ions in aqueous solution at pH below 8 and the concentration of creatinine can be calculated from the variation of the conductance of the solution. Ammonia gas

can be calculated from the variation of the conductance of the solution. Ammonia gas sensors and pH-sensitive electrodes can also be constructed based on the enzymatic action of creatinine deiminase only.

2.3. Characteristics of creatininase and creatinase

Creatininase (creatinine amidohydrolase EC 3.5.2.10) and creatinase (creatine amidinohydrolase EC 3.5.3.3) are usually purified from micro-organisms.

Creatininase is formed by eight subunits, each of them presenting a zinc ion involved in substrate binding and conversion. Each molecule has an approximate weight of 175 kDa and shows optimum catalytic activity around 70 °C and pH 7. The isoelectric point of creatininase is at pH 4.7 and can be inhibited by heavy metal ions (Ag^{+} and Hg^{++}), N-bromosuccinimide and EDTA. The Michaelis constant for the hydrolysis of creatinine catalysed by creatininase is reported to be approximately 32 mM (Barman, 1974).

Creatinase is formed by two subunits and has an approximate molecular weight of 67 kDa. Its catalytic activity is optimum around 45 °C and pH 7. The isoelectric point of creatinase is at pH 3.5 and can be inhibited by heavy metal ions (Ag^{+}, Cu^{++} and Hg^{++}) (Barman, 1974).

Creatininase is employed for enzymatic determination of creatinine when coupled with creatine amidinohydrolase and sarcosine oxidase in clinical analysis, where the oxidation of sarcosine can be monitored using amperometric methods. The reaction sequence is shown below,

$$\text{creatinine} + H_2O \xleftarrow{\text{creatininase}} \text{creatine} \qquad [2.5a]$$

$$\text{creatine} + H_2O \xleftarrow{\text{creatinase}} \text{urea} + \text{sarcosine} \qquad [2.5b]$$

$$\text{sarcosine} + O_2 + H_2O \xleftarrow{\text{sarcosine oxidase}} \text{methanol} + \text{glycine} + H_2O_2 \qquad [2.5c]$$

Alternatively, creatininase and creatinase can be co-immobilised with urease to detect creatinine impedimetrically according to the following scheme,

$$\text{creatinine} + H_2O \leftarrow \overset{\text{creatininase}}{\longrightarrow} \text{creatine} \qquad [2.6a]$$

$$\text{creatine} + H_2O \leftarrow \overset{\text{creatinase}}{\longrightarrow} \text{urea} + \text{sarcosine} \qquad [2.6b]$$

$$\text{urea} + 3H_2O \xleftrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- + OH^- \qquad [2.6c]$$

Gas electrodes and pH-sensitive electrodes can also be employed when creatininase, creatinase and urease are immobilized together.

2.4. Enzyme kinetics

In general, a chemical reaction can be defined as the transformation of a group of reactants into a group of products with no net loss of matter. Therefore, a reaction would involve the breakdown of some chemical bonds and the formation of others, and so there will be a change in the energy between the species and the surroundings.

The Transition State Theory is a well-known general theory, which describes the thermodynamic changes that accompany any chemical reaction. According to it, any chemical reaction goes through a transition state that has a higher energy than either the substrate(s) or the product(s). The rate of the forwards reaction depends on the temperature and on the difference in free energy between that of the reactants and the transition state, which is called the free energy of activation ΔG. Figure 2.1 shows the energetic pathway of a catalysed reaction according to the Transition State Theory.

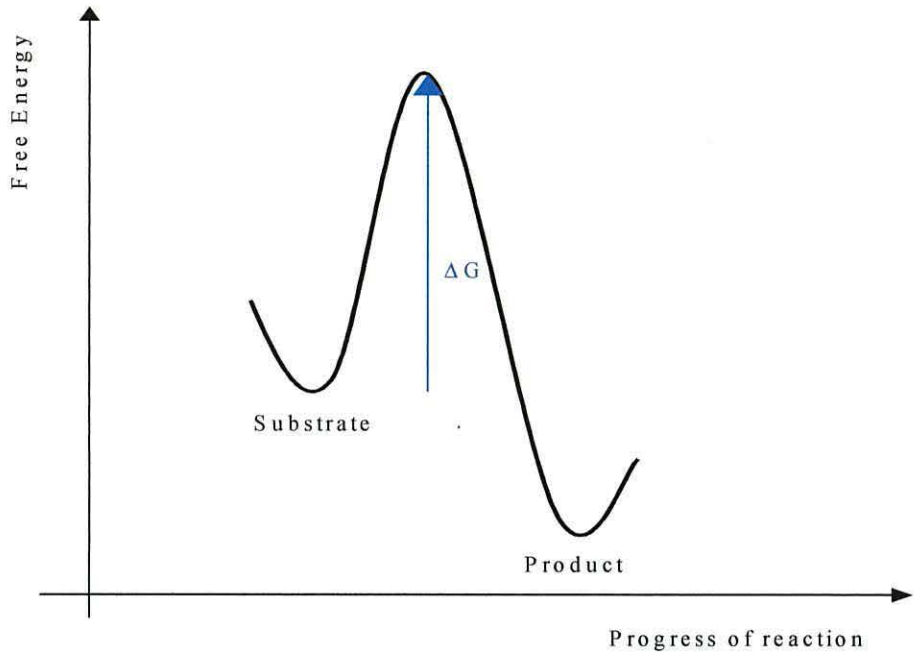


Fig 2.1. Energy scheme of the progress of a chemical reaction according to the Transition State Theory. Enzymes catalyse the reaction by reducing ΔG.

The reaction rate is proportional to the fraction of molecules that have free energy equal or greater than ΔG and such proportion increases with temperature. Enzymes accelerate reactions by reducing the activation barrier ΔG. The combination of substrate with enzyme creates a new reaction pathway that has a transition state of lower energy than in the absence of enzyme. The making and breaking of chemical bonds by an enzyme are preceded by the formation of an enzyme / substrate complex in which the substrate is bound to the active site of the enzyme. This enzyme / substrate complex is energetically metastable and is characterised by a higher energy level than that of the initial reactants and the final products.

The presence of a catalyst accelerates the chemical reaction with no overall thermodynamic effect: the change in free energy produced in the reaction is independent of the action of the catalyst. Enzyme-catalysed reactions are characterised by the reduction of the energy of activation. The formation of the enzyme / substrate activated complex generates an alternative path for the reaction where the potential barrier is diminished considerably. The reduction of the potential barrier facilitates the reaction by increasing the probability of reactant molecules having kinetic energies high enough to

break the bonds in the reactant molecules and allow the formation of new bonds and new product molecules.

Before the reaction, enzyme and substrate molecules are continuously moving, with the only restriction being from the friction forces with other molecules. In energy terms, most of the energy of the molecules will be kinetic energy. When an enzyme and a substrate molecule get sufficiently close to reach a distance at which electrostatic interaction becomes relevant, the molecules will start losing kinetic energy and gaining potential energy. A maximum in potential energy (and therefore a minimum in kinetic energy) is reached when the bonds of the initial molecules are breaking down and the bonds of the enzyme / substrate complex are being formed. Such an energy level represents the energy of the activated compound (enzyme / substrate complex) or the energy of the transition state ΔG. Once the substrate is transformed into products, the system will lose potential energy from the total breakdown of the enzyme / substrate complex bonds and the formation of the product bonds. Such potential energy is transformed into kinetic energy, allowing the product and enzyme molecules to be free to move again.

In order to overcome the potential barrier originated by the transition state energy, the kinetic energy of the reactant molecules needs to be equal or greater than the activation energy ΔG. If all the reactant molecules had kinetic energy equal or greater than ΔG, the reaction would be immediate and would only be retarded by diffusion. But experimentally the change in the product concentration with time is described as an exponential growth: product molecules are being created at a maximum rate at the beginning of the reaction and the concentration then tends to a constant value.

This behaviour is explained by the assumption that not all the reactant molecules have the same kinetic energy and in fact only a few of them show kinetic energies above ΔG. The distribution of the kinetic energies of the molecules in a sample is described for ideal gases by the Boltzmann function centred about the average kinetic energy of the system. According to the Boltzmann factor, the probability of a molecule having energy above ΔG per mole at absolute temperature T is given by $e^{-\Delta G/RT}$. This energy factor can be interpreted as the fraction of collisions produced with sufficient energy to make it efficient and therefore produce a reaction. So only molecules with kinetic energies

above the activation energy ΔG will be able to produce efficient collisions capable of forming activated complexes.

The velocity factor K of a particular reaction will then be given by the Boltzmann factor modulated by a frequency factor A which describes the number of collisions and by a steric factor P related to the probability of a collision taking place with the right orientation of the reactant molecules.

$$K = PAe^{-\Delta G/RT} \qquad [2.7]$$

As the temperature of the sample increases, the number of molecules with energies above the activation energy will increase and so will the velocity factor and, as it describes the probability of a successful collision taking place, so will the velocity itself of the reaction.

At the first stages of the reaction the probability that a molecule has energy above ΔG is very high and therefore the velocity is at its maximum value. As the reaction goes on, the most energetic molecules will have been already turned into products and the peak of the Boltzmann distribution would be shifted to lower energies, so the number of molecules available with energies high enough to overcome the potential barrier will decrease considerably. At the same time the molecules will be losing energy as friction due to collisions that have not resulted, because of low energies or wrong orientations of the molecules, in an activated complex. In this situation the increase in rate of production of product is diminished down to zero, when no more molecules reach the minimum energy required for the reaction or when equilibrium is reached between direct and inverse processes.

The mathematical description of the chemical reaction in macroscopical terms is very simple and can be derived from the definition of the velocity of a reaction. For first order reactions,

$$S \xrightarrow{k} P \qquad [2.8]$$

where S represents the substrate, P the product and k is the equilibrium constant of the reaction, the change in concentration of substrate with time will be given by

$$\frac{d[P]}{dt} = -\frac{d[S]}{dt} = k \cdot [S] = k \cdot ([S]_0 - [P]) \qquad [2.9]$$

Integrating,

$$\int \frac{d[P]}{[S]_0 - [P]} = \int k \cdot dt \qquad [2.10]$$

$$-\ln\{[S]_0 - [P]\} = kt + c \qquad [2.11]$$

When $t = t_0$ ([P]=0).

$$[P] = [S]_0(1 - e^{-kt}) \qquad [2.12]$$

where k is given by [2.8]. So the product concentration at any given time will depend exponentially on time, and linearly on the initial substrate concentration. If instead of a first order, we were dealing with a second order reaction, the product concentration would be proportional to $[S]^2$, if only one species of substrate molecules is present, or $[S] \cdot [S']$ if there were two species of substrate molecules, and so on.

The first problem to face when studying enzyme reactions is the impossibility of defining the reaction order of the whole process. Most enzyme reactions do not show a constant dependence of the velocity with the substrate concentration that can be described as first, second or third order, but the dependence is modified depending on the substrate concentration itself. In fact, the normal shape of v([S]) experimentally observed is a rectangular hyperbola which passes through the origin, with asymptotes $[S] = K_M$ and $v = v_{max}$. The low [S] region can be approximated to a straight line (direct proportion between v and [S]) bending towards a maximum velocity where the velocity is independent of the substrate concentration.

Assuming a known number of active sites per enzyme molecule, substrate molecules collide and interact with enzyme molecules and bind to them occupying their active sites. Once the enzyme / substrate complex is formed, and the reaction is completed, the product molecules dissociate from the enzyme so that new substrate molecules can bind to the same active site. When the concentration of substrate is low enough and there are always free active sites to be filled in the enzyme molecules, the velocity of the reaction will be proportional to the substrate concentration. But when the number of substrate molecules exceeds that of enzyme binding sites, the catalyst will not be able to keep pace, and not all substrate molecules will be turned into product, even if they overcome the barrier potential and collide with the right orientation. In such a situation the enzyme achieves its saturation point and the velocity of the reaction will only be proportional to the concentration of enzyme itself (that is, to the number of active sites) and to the rate of dissociation of the enzyme-substrate complex.

The general scheme of a simple substrate enzyme reaction is given by

$$E + S \xleftrightarrow{k_1/k_{-1}} ES \xrightarrow{k_2} E + P \qquad [2.13]$$

where E represents the enzyme, S and P the substrate and product respectively of the enzyme reaction; k_1 and k_{-1} are the equilibrium constants of the forward and reverse formation of the enzyme / substrate complex, and k_2 is the equilibrium constant of product formation. If steady-state conditions are assumed (d[ES]/dt = 0), the velocity of the enzyme reaction is described by Michaelis-Menten kinetics. If the rates of association and dissociation of the enzyme / substrate complex are kept the same,

$$\frac{d[ES]}{dt} = k_1 \cdot [E][S] - (k_{-1} + k_2) \cdot [ES] = 0 \qquad [2.14]$$

If $[E]_0 = [E] - [ES]$ at any time, then [2.14] can be rearranged as

$$[ES] = \frac{[E]_0[S]}{[S] + K_M} \qquad [2.15]$$

where K_M is the Michaelis-Menten constant, defined as

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad [2.16]$$

Therefore, the velocity of formation of products is given by

$$v = k_2 \frac{[E]_0[S]}{[S] + K_M} = \frac{v_{max} \cdot [S]}{[S] + K_M} \qquad [2.17]$$

Normally, the concentration of substrate is much larger than that of the enzyme, so the depletion of substrate can be considered negligible during the course of the reaction, and the velocity can be approximated to

$$v = \frac{v_{max} \cdot [S]_0}{[S]_0 + K_M} \qquad [2.18]$$

As previously mentioned, the dependence of the velocity on the substrate concentration is a rectangular hyperbola with asymptotes in v_{max} and v_{max}/K_M. v_{max} varies with the concentration of enzyme present but K_M is independent of enzyme concentration and is a characteristic of the particular enzyme investigated. In most cases, K_M values lie between 10^{-2} to 10^{-6} mol/l. In addition, since the catalytic step is normally the rate-limiting one, k_1 and k_{-1} are much larger than k_2, and K_M describes the equilibrium of the first step of the reaction. In general, K_M gives an indication of the affinity of the enzyme for the substrate: a low K_M indicates high affinity whereas a high K_M indicates low affinity.

The catalytic constant is another important parameter in enzyme kinetics. For simple two step reactions: $k_{cat} = k_2$, but it can be a function of the enzyme / product complex as well. k_{cat} is known as the turnover number of the enzyme because it represents the maximum number of substrate molecules converted to products per active site per unit time.

2.4.1. Mass transport - diffusion

When the chemical reaction takes place in homogeneous solution, it proceeds at the same rate uniformly throughout the medium and it is therefore necessary to consider only the way in which concentrations of the various components change with time. On the other hand, if the reaction takes place at a surface, the concentrations of the reactants and products will change locally at that surface, which leads to concentration polarisation, and transport from areas of high concentration to areas of low concentration may become an important factor affecting the rate of the overall reaction. From the three mechanisms of mass transport that can occur in solution, diffusion, convection and migration, only the first one is relevant to our study.

Diffusion is dependent on the random motion of species which, when concentration differences appear in a solution, will lead to a homogeneous and uniform distribution of the species in the solution. Therefore, chemical species will move from regions of high to low concentration at a rate that is dependent upon the difference in concentration between the two regions, until a stable state is reached, where no concentration difference exists. This behaviour is described by Fick's laws of diffusion,

$$j = -D\left(\frac{\delta c}{\delta x}\right) \qquad [2.19]$$

$$\frac{\partial c}{\partial t} = D\left(\frac{\partial^2 c}{\partial x^2}\right) \qquad [2.20]$$

where j is the net flux of material, D is the diffusion coefficient and $\delta c/\delta x$ is the concentration gradient.

In the case of chemical reactions taking place at surfaces and where no product is consumed, as for enzyme layers immobilised in potentiometric and impedimetric biosensors, all product molecules will be lost from the sensor surface by mass transport alone. In such a system, and assuming Michaelis-Menten kinetics for the enzyme catalysed reaction, three cases can be distinguished: uniform concentration of species throughout the enzyme layer so that mass transport across the enzyme layer is neglected; concentration polarisation in the enzyme layer, so that mass transport not only occurs

through the bulk solution but also in the enzyme layer, and concentration polarisation in the bulk solution. Each of these situations is described by the following equations, respectively,

$$\frac{lk_2[E][S]_l}{K_M + [S]_l} = k_D([S]_\infty - [S]_l) \qquad [2.21]$$

$$D_s\left(\frac{d^2[S]}{dx^2}\right) - \frac{k_2[E][S]}{K_M + [S]} = 0 \qquad [2.22a]$$

$$D_P\left(\frac{d^2[P]}{dx^2}\right) - \frac{k_2[E][S]}{K_M + [S]} = 0 \qquad [2.22b]$$

$$D_S\left(\frac{d[S]}{dx}\right)_{x=l} = k_D([S]_\infty - [S]_l) \qquad [2.22c]$$

where all variables have been previously introduced and the sub-indexes refer to the location within the biosensor: l refers to the interface between the enzyme layer and the bulk, and ∞ to a region of the bulk phase far from the enzyme layer. Unfortunately only the first equation can be solved analytically, and the other two sets can only be approximated by numerical methods.

If a complete study of the kinetics of an enzyme sensor is attempted, then all mass transport effects have to be taken into account, and only in the case of substrate saturation can these be neglected, deriving the total rate of product generation from that of the chemical catalysed reaction itself.

Chapter 3 Impedance Detection Techniques – Dielectric Theory and Equivalent Circuit Analysis

Although the concept of employing the changes in electrical impedance to monitor relevant properties of a biological / biochemical system has known several important successes (discovery of the molecular thickness of biological membranes, voltage-gated conductivity of nerve axons) the application of impedimetric techniques in biosensing still remains underexploited. The impedance approach can be applied to a broad range of biochemical studies, provided a good understanding exists of the biochemical / biological processes and their electrical analogous.

Many electrochemical techniques are based on the application of a dc potential to a working electrode in order to measure the resultant current that flows in a circuit completed by a counter electrode (Bard and Faulkner, 1980; Kissinger and Heineman, 1984). However, increasing exploitation of sinusoidal exciting voltages in the study of electrode processes in aqueous media has occurred over the last three decades (Schwan, 1966; Macdonald, 1977; Archer and Armstrong, 1980; Bard and Faulkner, 1980; Bond, 1980; Gabrielli, 1980; Buck, 1982; Macdonald and McKubre, 1982; Taylor and Macdonald, 1982). This approach presents two main advantages: the sinusoid offers convenient technical and mathematical features together with an excellent signal:noise ratio based on the use of a steady-state analysis; and not only the voltage but the frequency as well of the exciting wave-form may be altered, so that we may consider this technique as a particular form of spectroscopy, referred to as dielectric spectroscopy.

Therefore, the familiar idea of employing the frequency-dependent absorption of ultra-violet, visible and infrared light for material analysis can be extended to other forms of electromagnetic radiation of lower frequency, at least in the study of bio-analytical

devices. In such cases, and below 30 MHz, an electrode is required to act as an interface between the exciting electric field and the sample so that, in a pure electrochemical case, it would be possible to study the frequency-dependent, passive electrical properties of the system consisting of the electrodes plus the biological sample. In other words we would be considering the frequency-dependent impedance or admittance of the system.

Dielectric properties relate to the ability of a material to polarise under the influence of an electromagnetic field. As the polarisability of a material depends on the structure and on its molecular and atomic properties, dielectric measurements have been broadly employed by researchers in order to elucidate the intrinsic physical configuration of dielectric materials.

In such a way, dielectric spectroscopy has increasingly been employed for materials characterisation as a fast and non-invasive technique, which subjects the sample to low stress. Its advantages over other physicochemical measurements (simplicity of sample preparation, easy variation of sample sizes and measurements conditions, broad frequency band applicable) also make of it a useful tool in the investigation of biological systems and interfaces.

3.1. Dielectric materials

Dielectric materials have the unique property of being able to store electrostatic charge. If an electric field is applied to a conductor, its charges will freely move in response to the field until the electric field within the material is null. On the other hand, dielectrics are materials whose electrons are strongly bound to its atomic structure, not being free to move through the material under the influence of an applied electric field. The electric field will produce no charge carriers, but electrical dipoles will appear when the bound charges in the atoms of the dielectric are displaced from their normal charge centres by the action of the field.

In order to understand the principles of the dielectric phenomena, a review of the concepts of electrostatic interaction between charges acting at a distance from the applied electric field is included below.

3.1.1. Electric Potential and Charge Densities

Figure 3.1 shows a polarised dielectric of volume v'.

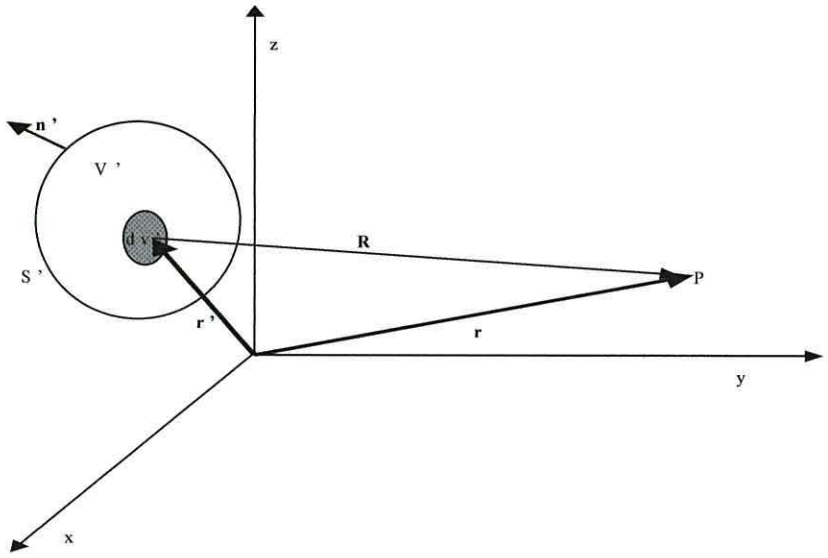


Fig 3.1. Electrostatic potential outside a polarised dielectric.

The total dipole moment of the differential volume dv' is **dp'**. Therefore, the electric polarisation vector **P'**, defined as the dipole moment per unit volume is given by

$$d\vec{p} = \vec{P}(\vec{r})dv \qquad [3.1]$$

where **p** is defined as

$$\vec{p} = q\vec{d} \qquad [3.2]$$

q represents the charges of the dipole and **d** the distance between them. Dipole moments are traditionally measured in Debyes, 1Cm=3 10^{-29} D.

The electrostatic potential V at any point P due to the dipole moment of any differential of volume dv' is given by

$$dV(\vec{r}) = \frac{d\vec{p}'\cdot\hat{R}}{4\pi\varepsilon_0 R^2} = \frac{\vec{P}(\vec{r}')\cdot\hat{R}dv'}{4\pi\varepsilon_0 R^2} \qquad [3.3]$$

where **dp'** is the dipolar moment associated with dv', R is the distance between the observation point (where the electric potential is being calculated) and the polarised dielectric, **r'** is the distance from the origin to dv' and ε_0 is the permittivity of free space (ε_0 = 8.854 pF/m). The potential produced by the entire dielectric is therefore

$$V(\vec{r}) = \int_{V'} \frac{\vec{P}(\vec{r}')\cdot\hat{R}dv'}{4\pi\varepsilon_0 R^2} = \frac{1}{4\pi\varepsilon_0}\int \vec{P}(\vec{r}')\cdot\nabla'(\frac{1}{R})dv` \qquad [3.4]$$

Using properties of the divergence operator we can finally write

$$V(\vec{r}) = \frac{1}{4\pi\varepsilon_0}\int_{V'}\frac{(-\nabla'\cdot\vec{P})dv'}{R} + \frac{1}{4\pi\varepsilon_0}\int_{V'}\nabla'\cdot(\frac{\vec{P}}{R})dv`$$

$$= \frac{1}{4\pi\varepsilon_0}\int_{V'}\frac{(-\nabla'\cdot\vec{P})dv'}{R} + \frac{1}{4\pi\varepsilon_0}\oint_{S'}\frac{\vec{P}\cdot\hat{n}'da'}{R} \qquad [3.5]$$

This expression corresponds to the potential V produced by a volume charge distribution ρ_b throughout the interior of the volume of the dielectric and a surface charge distribution σ_b around its surface, where ρ_b and σ_b are defined as,

$$\rho_b = -\nabla'\cdot\vec{P} \qquad [3.6a]$$

$$\sigma_b = \vec{P}\cdot\hat{n}' \qquad [3.6b]$$

This result can be physically interpreted as follows. Let us consider a volume in the interior of a polarised dielectric. Inside that volume, the charges forming the dipoles will

cancel each other out, giving a zero volume charge density, that is ρ_b=0. However, such cancellation cannot take place on the surface of the dielectric as charge densities of both signs will appear on each surface of the dielectric volume. Overall charge neutrality is conserved as one boundary becomes positively charged and the other negatively. This induced charge, associated with the bound charges in the dielectric, is called the displacement field and is proportional to the electric field and has a direction perpendicular to the surface charge in the direction of the electric field.

3.1.2. Displacement Field

As discussed above, the origin of bound charges is attributed to the constitutive elements of matter, that is, it is not possible to control its distribution. Free charges are, essentially, all the rest. They are called free because it is possible to control their distribution to a certain extent (through physical displacement, etc). The total charge density can then be defined as

$$\rho_{total} = \rho_f + \rho_b \qquad [3.7]$$

From Gauss law and [3.6a]

$$\varepsilon_0 \nabla \cdot \vec{E} = \rho_f - \nabla \cdot \vec{P} \qquad [3.8a]$$

$$\nabla \cdot (\varepsilon_0 \vec{E} + \vec{P}) = \rho_f \qquad [3.8b]$$

It is then convenient to introduce a new vectorial field **D(r)** called dielectric displacement.

$$\nabla \cdot \vec{D} = \rho_f \qquad [3.9a]$$

$$\vec{D} = \varepsilon_0 \vec{E} + \vec{P} \qquad [3.9b]$$

D(r) is continuous throughout the material, except at the boundaries where its normal component is discontinuous if surface free charge density exists.

$$\hat{n}\cdot(\vec{D}_2 - \vec{D}_1) = \sigma_f \qquad [3.10]$$

Even though only free charges appear in [3.10], it does not mean the only sources of **D** are free charges. According to Helmholtz's theorem only scalar sources have been found and $\nabla \times \vec{D}$ will give us the vector sources. From [3.9b] and Maxwell second law,

$$\nabla \times \vec{D} = \nabla \times \vec{P} \qquad [3.11]$$

and the boundary condition for the parallel component of **D**

$$\hat{n}\times(\vec{D}_2 - \vec{D}_1) = \hat{n}\times(\vec{P}_2 - \vec{P}_1) \qquad [3.12]$$

3.2. Classification of dielectric materials

It is possible to classify dielectric materials depending on the relationship between the polarisation field **P** and the electric field **E**.

3.2.1. Permanent polarisation

When the electric field is zero, there are two possibilities for the polarisation field **P(0)**. If **P(0)** is different from zero, the material will be polarised even in the absence of an electric field, showing permanent polarisation. This type of material is called an electret. The case of **P(0)**=0 is much more characteristic and representative when polarisation arises from an applied field. In general, the denomination of dielectric will be kept for this type of material.

3.2.2. Non-linear dielectrics

Even when **P(0)**=0, the relationship between the electric field and the polarisation can appear to be very complicated. However, for most materials, this only happens at extreme conditions such as exceptionally high fields, very low temperature or both. Normally it is possible to express **P** as a series of powers of the components of **E**,

$$P_i = \sum_j \alpha_{ij} E_j + \sum_j \sum_k \beta_{ijk} E_j E_k + \varepsilon(E^3) \qquad [3.13]$$

Where the indexes i, j, k take the values of x, y, z. The specific values for the coefficients α_{ij}, β_{ijk} depend on the particular dielectric material. If second or higher orders in E are needed to describe the material, the dielectric is considered to be non-linear. Although some ceramics fall within this category, our interest is restricted to linear dielectrics.

3.2.3. Linear dielectrics

Materials which only require the first term of [3.13] to describe the dependence of the polarisation with the electric field are called linear dielectrics. The general expressions that relate both vectors components are,

$$P_x = \varepsilon_0(\chi_{xx}E_x + \chi_{xy}E_y + \chi_{xz}E_z) \qquad [3.14a]$$

$$P_y = \varepsilon_0(\chi_{yx}E_x + \chi_{yy}E_y + \chi_{yz}E_z) \qquad [3.14b]$$

$$P_z = \varepsilon_0(\chi_{zx}E_x + \chi_{zy}E_y + \chi_{zz}E_z) \qquad [3.14c]$$

where the proportionality factors χ_{ij} are the components of the electric susceptibility tensor. These components are dimensionless and in general may be dependent on the position of the material, although not on the electric field (non-linear dielectrics). Therefore, in general, neither **P** nor **D** will be parallel to **E** even for linear dielectrics.

Nevertheless and for our description, we can assume that, at a particular given point, all electric properties of the dielectric are isotropic in respect to **E**, that is, they are independent of the direction of the electric field at that specific point. Thus all directions are equivalent and [3.14a], [3.14b] and [3.14c] can be written as a function of one proportionality factor only.

$$\vec{P} = \chi_e \varepsilon_0 \vec{E} \qquad [3.15]$$

where χ_e is the electric susceptibility. Combining [3.15] and [3.9b],

$$\vec{D} = (1 + \chi_e)\varepsilon_0 \vec{E} = \varepsilon_r \varepsilon_0 \vec{E} = \varepsilon \vec{E} \qquad [3.16]$$

where ε is the dielectric constant or dielectric permittivity. [3.16] can be rewritten in its differential form as

$$\nabla \cdot (\varepsilon \nabla V) = \varepsilon \nabla^2 V + \nabla V \cdot \nabla \varepsilon = -\rho_f \qquad [3.17]$$

As most liquid, gases and highly symmetrical solids are electrically homogenous, the electrical susceptibility and dielectric permittivity are constant and [3.17] simplifies to

$$\nabla^2 V = -\frac{\rho_f}{\varepsilon} \qquad [3.18]$$

which is known as Gauss law.

3.3. Microscopic polarisation

3.3.1. Electronic polarisation

In the absence of an electric field, the electronic clouds corresponding to each molecule of a non-polar material are symmetrically distributed around the nuclei, so each molecule has a dipole moment equal to zero. If an electric field is applied to the dielectric, the electric forces tend to move them apart in the direction of the external field, causing a small displacement (10^{-10}m – 10^{-11}m) between the negative and positive centres of gravity. Such a molecule is said to be polarised and presents an induced dipole moment different from zero (probably multipole moments of higher orders as well) as long as the field is being applied. The magnitude of the dipole moment depends both on the atom charge and the strength of the existent electric field.

Considering an atom of positive nucleus of charge Ze surrounded by an electron cloud of charge –Ze and assuming that this negative charge is distributed with uniform density throughout a sphere of radius r_0, an electric field **E** would generate a force |Ze**E**| in the positive nucleus and an equal in magnitude and opposite in direction force on the electron cloud, displacing the centres of charges in opposite directions by a quantity x. Substituting the electrostatic force and the charge values in Coulomb's law,

$$Ze\left|\vec{E}\right| = \frac{Ze\dfrac{Zex^3}{r_0^3}}{4\pi\varepsilon_0 x^2} \qquad [3.19a]$$

where Z is the atomic number, e is the charge of the electron in coulombs, r_0 is the distance of the electronic cloud to the centre of the atom and x is the displacement originated by the application of the electric field. Simplifying [3.19a],

$$Zex = 4\pi\varepsilon_0 r_0^3\left|\vec{E}\right| \qquad [3.19b]$$

By definition Zex is the induced dipole moment of the atom (see equation [3.2]), so

$$\vec{p}_{ind} = \alpha_e \vec{E} \qquad [3.20]$$

where the proportionality factor is the electronic polarisability of the atom, α_e.

3.3.2. Ionic polarisation

Ionic or atomic polarisation is characteristic of ionic substances, such as sodium chloride, and can only be found in substances of high degree of order in the atomic lattice structure whose molecules are formed from atoms having an excess charge of opposite polarities. When an electric field is applied, the relative position of positive and negative ions will end to be shifted, inducing a dipole moment distinct from that of the displacement of the electronic clouds around each individual atom. Both polarisation mechanisms are related to the applied electric field in a similar manner, therefore, the ionic polarisation is given by

$$\vec{P}_i = \alpha_i \vec{E} \qquad [3.21]$$

where α_i is the ionic polarisability of the molecules.

3.3.3. Orientational polarisation

Molecules in polar materials present asymmetric charge distributions so that there is a permanent dipole moment associated with them even in the absence of an electric field. Orientational polarisation is associated with dipolar substances that show such an intrinsic dipole moment, even though it is not generally observed at no-field conditions.

In equilibrium these dipole moments are randomly oriented owing to thermal agitation and the dielectric material presents no overall dipole moment. However, in the presence of an external field, the electric force will exert a torque on each individual dipole making it rotate in the direction of the field in such a manner that each elementary volume of the dielectric presents a net dipole moment in that same direction. Higher

this discussion because of their minimum contribution to the potential at relatively long distances.

The calculation for the orientational polarisation was first carried out by Debye using the method previously developed by Langevin to explain the behaviour of paramagnetic substances in terms of permanent magnetic moments. In order to simplify the calculation, it is assumed that the permanent electric moments are independent of the temperature and applied field, that they are free to rotate and that the interaction energy between the dipoles is small relative to the thermal energy kT of each molecules. These restrictions do not apply to all solids but gases and liquids are included in such requirements.

The potential energy of a dipole of moment **p** in an electric field **E** can be written as

$$U = \int_{\pi/2}^{\theta} pE \sin\theta d\theta = -pE\cos\theta \qquad [3.22]$$

where θ is the angle between the dipole **p** and the field **E**.

In the absence of an electric field, the number of dipoles with orientation direction lying between arbitrary angles θ and θ+dθ will be proportional to the solid angle dΩ subtended by the elemental area on a sphere of unit radius. Moreover, when an electric field is applied along the *z*-direction, the molecules will tend to line up in the direction of the acting field, occupying minimum energy positions with the final orientation depending on their thermal energy. The influence of thermal motion modifies the number of dipoles included in any solid angle dΩ by the Boltzmann factor exp(U/kT) so that

$$N(\theta) = A2\pi \sin\theta d\theta \exp\left(\frac{pE\cos\theta}{kT}\right) \qquad [3.23]$$

where A is a proportionality factor determined by the total number of dipole molecules per unit volume.

The average moment of each molecule in the direction of the field is therefore given by

$$\vec{p}_{av} = \frac{\int_0^{\pi} A2\pi \sin\theta \exp(\frac{pE\cos\theta}{kT})\vec{p}\cos\theta d\theta}{\int_0^{\pi} A2\pi \sin\theta \exp(\frac{pE\cos\theta}{kT})d\theta} \qquad [3.24a]$$

Integrating

$$\vec{p}_{av} = \vec{p}[\coth a - \frac{1}{a}] = \vec{p}L(a) \qquad [3.24b]$$

Where a = pE/kT and the function L(a) is known as the Langevin function. From a practical point of view, a<<1 even for highly polar molecules and fields of the order of 10^3 V/cm, so the Langevin function can be approximated to a/3 so that

$$\vec{p}_{av} = \frac{p^2\vec{E}}{3kT} = \alpha_0\vec{E} \qquad [3.25]$$

where α_0 is the orientational polarisability.

Finally, and by adding the three polarisability factors mentioned, it is possible to obtain the total dipole moment per molecule

$$\vec{p} = (\alpha_e + \alpha_i + \frac{p^2}{3kT})\vec{E} \qquad [3.26]$$

This equation is known as the Langevin-Debye formula.

3.3.4. Interfacial polarisation

Interfacial polarisation arises from the presence of electrons or highly mobile small ions, which are able to migrate over distances of macroscopic magnitude. If the dielectric is heterogeneous or if it is formed by different phases, each of them with a distinctive conductivity, such charge carriers will move through the more conductive phases and accumulate on the boundaries that separate these from the more resistive phases. This ionic build-up takes place commonly on electrode surfaces in liquids and lattice defects, voids in the material structure and strains in solid dielectrics. Effectively each conducting region will become polarised so that there is an apparent increase in the average moment of the molecules given by

$$\vec{p}_s = \alpha_s \vec{E} \qquad [3.27]$$

Therefore the average dipole moment per molecules is given by

$$\vec{p} = \alpha \vec{E} \qquad [3.28]$$

and the electric polarisation by

$$\vec{P} = N\alpha \vec{E}_{local} \qquad [3.29]$$

where N is the number of molecules per unit volume and α is the total polarisability, given by the sum of electronic, ionic, orientational and interfacial terms. $\mathbf{E}_{local}$ is the electric field intensity acting on each molecules and is referred to as the internal or local field, which consists partly of the applied field and partly of the field resulting from the mutual interaction of the other molecules polarised under the influence of the external field.

Following the model developed by Lorentz, which assumes that beyond short distances from a molecule the medium can be treated as macroscopically continuous, and

considering a simplified model of an isotropic dielectric medium where only the long-range dipolar forces are considered, the local field can be written as

$$\vec{E}_{local} = \vec{E} + \frac{1}{3}\vec{E}(\varepsilon_r - 1) = \vec{E}\frac{(\varepsilon_r + 2)}{3} \qquad [3.30]$$

3.4. Dielectrics in alternating fields

When an alternating field is applied to a dielectric material the orientation of the dipoles, and therefore the polarisation, will tend to reverse every time the polarity of the field changes. The polarisation follows the alternations of the field without any significant lag at low frequencies, so the permittivity remains independent of the frequency and keeps the same magnitude as in a static field. As the frequency is increased the dipoles can no longer rotate fast enough so that their oscillations will start to lag behind those of the field. If the frequency is raised further, any permanent dipole present in the medium will be unable to follow the field and the contribution to the static permittivity from this molecular process ceases, which normally occurs in the radio-frequency range (10^4 Hz – 10^6 Hz) of the spectrum. At higher frequencies, usually in the infrared range, relatively heavy ions start being also unable to follow the variations of the field, so that the contribution from the atomic or ionic polarisation also ceases and only electronic polarisation remains.

In an alternating electric field, the displacement polarisation leads to electric oscillations. This is a resonant process with resonant frequencies of 10^{12} Hz to 10^{13} Hz for the ionic polarisation and 10^{14} Hz to 10^{15} Hz for the electronic polarisation.

All of these processes lead to a fall in the permittivity of the dielectric material with increasing frequency, a phenomenon which is usually referred to as dielectric dispersion. An anomalous dielectric dispersion occurs when the various loss processes merge into one another. Dispersions taking place from complete orientational polarisation at low frequencies to negligible orientational polarisation at high radio frequencies or higher

(e.g. the dielectric relaxation for water centred at 17 GHz), where no resonance absorption appears are referred to as dielectric relaxation processes.

3.4.1. Dielectric Relaxation

As opposed to atomic and electronic polarisation, orientational polarisation is not a resonant process because the molecular dipoles have inertia. The response of the orientational polarisation to a change of the electric field strength is therefore always retarded.

Each dielectric relaxation process is defined by a characteristic relaxation time τ required to reach a new equilibrium state after changing the excitation. It depends strongly on temperature because it is closely related to the viscosity of the material: the dipoles cannot follow the field variations without a measurable lag because of internal friction forces and these increase with the viscosity of the medium. Characteristic relaxation times can reach any value, from a few seconds to days or years, depending on the material, the temperature and the mechanism involved.

Real dielectrics also contain charge carriers which can be moved by electric forces between potential walls, formed by non-ohmic blocking contacts or internal boundaries. As mentioned above, this leads to a space charge and interfacial polarisation referred to as electrode polarisation and Maxwell-Wagner polarisation respectively. Because of the close dependence of the interfacial polarisation processes and the conductivity, these charge-carrier relaxation processes are also named conductivity relaxations. The relaxation time τ_c of a conductivity relaxation is given by

$$\tau_c = \frac{\varepsilon_0 \varepsilon_r}{\sigma_{dc}} \qquad [3.31]$$

where σ_{dc} is the dc conductivity of the material.

Inferred from [3.31], the relaxation time of a charge-carrier relaxation is generally larger than the relaxation times of the dipolar relaxations in the same material and for a given temperature.

Following from [3.9b] and [3.16],

$$\vec{D} = \varepsilon_0 \varepsilon_r \vec{E} = \varepsilon_0 \vec{E} + \varepsilon_0(\varepsilon_\infty - 1)\vec{E} + \varepsilon_0(\varepsilon_r - \varepsilon_\infty)\vec{E} = \vec{D}_{Vc} + \vec{P}_{Rs} + \vec{P}_{Rl} \qquad [3.32]$$

ε_∞ is the relative permittivity measured immediately after introducing the electric field or at sufficiently high frequencies to unable all relaxation processes to follow the field changes. $\mathbf{D}_{Vc}$, $\mathbf{P}_{Rs}$ and $\mathbf{P}_{Rl}$ are the vacuum, resonance and relaxation contributions respectively to the dielectric displacement.

The limit low frequency permittivity (ε_r ($t \rightarrow \infty$)) is called the static permittivity ε_s. Thus, the static polarisation is given by

$$\vec{P}_{Rl,s} = \varepsilon_0(\varepsilon_s - \varepsilon_\infty)\vec{E} = \varepsilon_0 \Delta\varepsilon \vec{E} \qquad [3.33]$$

where $\Delta\varepsilon$ is called the relaxation strength and ε_s, ε_∞, and $\Delta\varepsilon$ are defined for a single relaxation process. Generally, several relaxation processes associated with different components co-exist in a given heterogeneous material, in which case all these quantities should be specified separately for each different process.

3.4.2. Debye Relaxation

Let us assume a sinusoidal excitation voltage is applied to a dielectric, the electric field being

$$E(t) = E_m e^{j\omega t} \qquad [3.34]$$

The stationary polarisation will oscillate with the same angular frequency ω but retarded by an angle δ.

$$P_{Rl}(t) = P_{Rl,m} e^{j(\omega t + \delta)} \qquad [3.35]$$

If the time dependence of the polarisation after applying the field is given by simple first-order kinetics,

$$\dot{P}_{Rl}(t) = \frac{dP_{Rl}(t)}{dt} = \frac{P_{Rl,s} - P_{Rl}(t)}{\tau} \qquad [3.36]$$

and we substitute [3.35] in [3.36], the relaxation polarisation results in

$$P_{Rl}(t) = \frac{P_{Rl,s}}{1 + j\omega\tau} \qquad [3.37]$$

The resonant part of the polarisation and the vacuum contribution to the electric displacement are always in phase with the electric field. Taking this into account, the electric field can be written as

$$D(t) = P_{Rl,m} e^{j(\omega t + \delta)} + (P_{Rs,m} + D_{Vc,m}) e^{j\omega t} \qquad [3.38a]$$

with

$$P_{Rs,m} + D_{Vc,m} = \varepsilon_0 \varepsilon_\infty E_m \qquad [3.38b]$$

From [3.37], and [3.33],

$$D(t) = \frac{\varepsilon_0(\varepsilon_s - \varepsilon_\infty)}{1 + j\omega\tau} E + \varepsilon_0 \varepsilon_\infty E \qquad [3.39]$$

and finally, using [3.16]

$$\varepsilon_r = \frac{\varepsilon_s - \varepsilon_\infty}{1 + j\omega\tau} + \varepsilon_\infty \qquad [3.40]$$

ε_r is complex and, by separating its real and imaginary parts, the Debye equations can be obtained

$$\varepsilon_r = \varepsilon' - j\varepsilon'' \qquad [3.41a]$$

$$\varepsilon' = \frac{\varepsilon_s - \varepsilon_\infty}{1 + \omega^2\tau^2} + \varepsilon_\infty \qquad [3.41b]$$

$$\varepsilon'' = \frac{\varepsilon_s - \varepsilon_\infty}{1 + \omega^2\tau^2}\omega\tau \qquad [3.41c]$$

Although ε' is only the real part of the relative permittivity, it is usually called the permittivity and it can be physically identified with a measure of the energy stored in the oscillations of the dipolar units. ε'' is called the dielectric loss and it is related to the energy dissipation in the material due to internal friction. These Debye equations describe the dielectric spectrum of a single Debye relaxation.

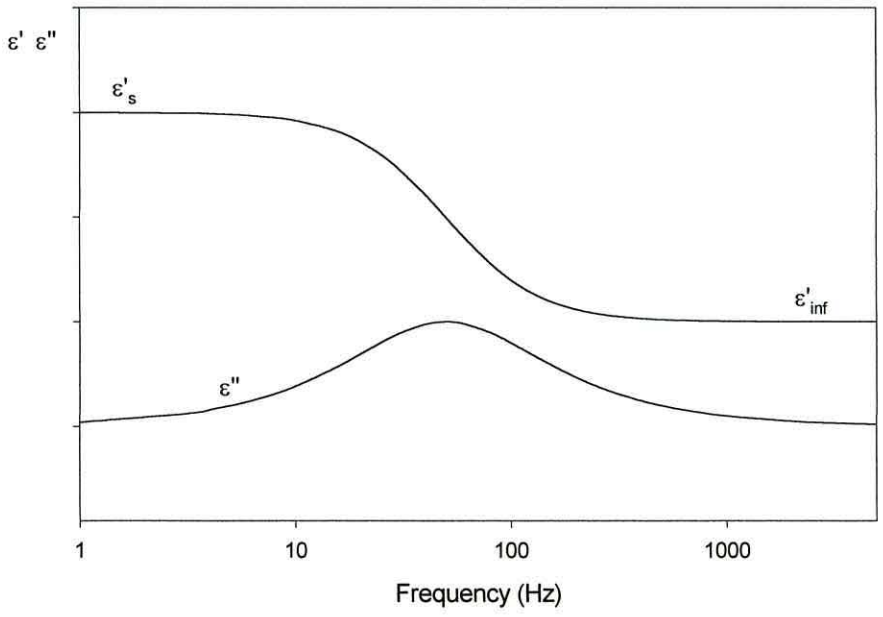


Fig 3.2. Real and imaginary part of the permittivity representing a single Debye dielectric relaxation process

The relaxation strength and relaxation time of a single Debye relaxation can be determined from both the permittivity and dielectric loss characteristics in the frequency domain. τ can be determined from the position of the loss maximum or the turning point of the permittivity curve, respectively. $\Delta\varepsilon$, on the other hand, is the difference between the limits of $\varepsilon'(\omega)$ for $\omega \rightarrow 0$ and $\omega \rightarrow \infty$ or twice of the maximum value of $\varepsilon''(\omega)$. In practice, however, it is often difficult to determine $\Delta\varepsilon$ and τ because real dielectric spectra are generally broader than the Debye spectrum and their shape is not pre-defined. Furthermore, the frequency range is often limited for technical reasons. Therefore, the measurement of both parts of the complex permittivity gives more reliable information for a proper determination of the relaxation parameters.

A particularly useful way to obtain the relaxation strength and the relaxation time is through the Cole-Cole representation.

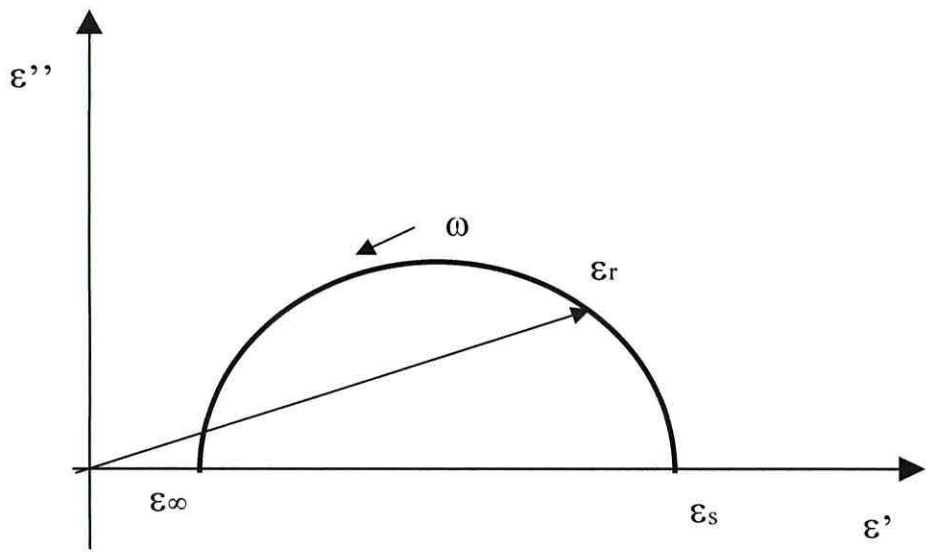


Fig 3.3. Cole-Cole representation of a single dielectric relaxation process

As can be seen in figure 3.3, the locus of the complex number ε_r in the complex plane is a semicircle in the fourth quadrant given by the equation [3.42a]

$$(\varepsilon' - \frac{\varepsilon_s + \varepsilon_\infty}{2})^2 + \varepsilon''^2 = (\frac{\varepsilon_s - \varepsilon_\infty}{2})^2 \qquad [3.42a]$$

In practice, the permittivity and dielectric loss are measured in a middle frequency angle and plotted in the complex plane. Then the semicircle is extrapolated to ε_s and ε_∞.

3.5. Electrical impedance and admittance

A general scheme of an impedimetric experiment is described in figure 3.4.

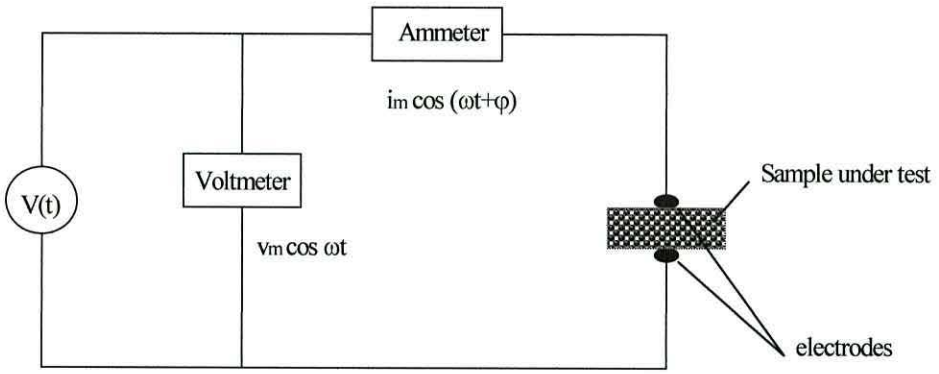


Fig 3.4.Impedimetric experiment in which a sinusoidal voltage is applied to the sample of interest. Such voltage is measured by a high impedance voltmeter and the flowing current by an ammeter.

A small amplitude sinusoidally modulated voltage $v(t)=v_m \cdot \cos\omega t$ is applied across the terminals of a passive circuit, which consists of a biological or chemical sample separating a pair of electrodes. Here, ω is the angular frequency in rad/sec ($\omega=2\pi f$ where f is the frequency in Hz), v_m is the amplitude or maximum (peak to peak) voltage and $v(t_i)$ is the voltage at any given instant t_i. In the quasi- steady state, once any transient signals have decayed, a current flows in the circuit related to the voltage both in magnitude and in phase. Its time dependency is given by $i(t)=i_m \cdot \cos(\omega t + \varphi)$, where φ is called the phase angle. Therefore, although the frequency and sinusoidal nature of the

wave-form remain unchanged after interacting with the system, its characteristics are reflected in the ratio v_m/i_m and in the value of φ.

The system under test may exhibit resistive, capacitive and inductive properties, all of them having a distinct effect on the sinusoidal voltage applied. The resulting currents due to an exciting waveform v(t) for a pure resistor, a pure capacitor and a pure inductor are, respectively, given below.

$$i(t) = (\frac{v_m}{R}) \cdot \cos\omega t \qquad [3.43a]$$

$$i(t) = \omega C v_m \cdot \cos(\omega t + \tfrac{\pi}{2}) \qquad [3.43b]$$

$$i(t) = (\frac{v_m}{\omega L}) \cdot \cos(\omega t - \tfrac{\pi}{2}) \qquad [3.43c]$$

Thus, there is no phase difference between the current and the voltage for a pure resistor, the current leads the voltage by $\pi/2$ rads for a pure capacitor and the current lags the voltage by $\pi/2$ for a pure inductor. A physical way to consider these phase shifts is by taking into account that all energy in the exciting electric field must be either stored (as a capacitive term) or dissipated (as a resistive term) by the system, and that storage takes time whilst dissipation as heat is quasi instantaneous in our time scale. Therefore, the voltage across a pure capacitor would be a function of the current accumulation between its terminals for the time the exciting signal has been applied. Obviously, the intrinsic ability to store charge storage would also have to be taken into account. Considering the voltage has been applied for a long time,

$$v_C(t) = \frac{1}{C}\int_{-\infty}^{t} i_m \cdot \cos(\omega t + \varphi) \qquad [3.44]$$

where $v_C(t)$ is the voltage across the capacitor and the rest of the parameters have been previously defined. Integrating [3.44],

$$v_C(t) = \frac{i_m}{\omega C} \cdot \sin(\omega t + \varphi) = \frac{i_m}{\omega C}\cos(\omega t + \varphi - \frac{\pi}{2}) \qquad [3.45]$$

where we have used the trigonometric identities

$$\sin\alpha = \cos(\frac{\pi}{2} - \alpha) \qquad [3.46a]$$

$$\cos(\frac{\pi}{2} - \alpha) = \cos(\alpha - \frac{\pi}{2}) \qquad [3.46b]$$

Except in active biological systems such as nerve axons (Cole, 1972; Jack et al, 1975; De Felice, 1981) and in certain electrochemical systems which involve corrosion and electro-deposition (Gabrielli, 1980; Macdonald and McKubre, 1982; Gabrielli et al, 1983), inductances are negligible, and we will ignore their effect from now on. Therefore, we may intuitively imagine that for a real system, which possesses both resistive and capacitive properties the phase angle takes a value between 0 and $\pi/2$ rads.

If all electrical analysis takes place under steady-state conditions, that is the system characteristics remain constant over time, it is interesting to change from a time description of the current to the frequency domain situation. For a harmonic exciting signal, that can be characterised by defining phasors that relate each time dependency with a constant parameter. Let us assume y(t) is a pure harmonic exciting signal.

$$y(t) = y_m \cdot \cos(\omega t + \varphi) \qquad [3.47]$$

It is possible to associate y(t) to a time phasor defined by

$$\tilde{y}(t) = y_m \cdot [\cos(\omega t + \varphi) + j \cdot \sin(\omega t + \varphi)] = y_m \cdot e^{j(\omega t + \varphi)} = y_m e^{j\varphi} e^{j\omega t} = Y e^{j\omega t} \qquad [3.48]$$

where Y is the time independent phasor representing y(t) in the frequency domain. From [3.47] and [3.48],

$$y(t) = \mathrm{Re}[Y e^{j(\omega t + \varphi)}] \qquad [3.49]$$

An analogous procedure can be followed for the current and voltage, so that, using complex phasors,

$$V = Z_{total} \cdot I \qquad [3.50]$$

where Z_{total} is the complex impedance of the system as whole. Each element is associated with a characteristic impedance, defined by

$$Z_R = R \qquad [3.51]$$

$$Z_C = \frac{1}{j\omega C} = jX_C \qquad [3.52]$$

Z_R being the resistance impedance, Z_C the capacitor impedance and X_C the capacitor reactance (X_C=-1/ωC). It should be noted that, by definition, the impedance is independent of the voltage across and the current flowing through the system under study, and this linearity property should be taken into account when using these representations. Thus, if the system is represented by a number of different elements in series, the total impedance results from the linear addition of the characteristic impedances of each of them.

For parallel networks, it is easy to describe the system in terms of its admittance, *Y*, which is the inverse of the impedance. Therefore,

$$Y = \frac{1}{Z} \qquad [3.53]$$

If the circuit is formed by a single resistor or a single capacitor only, the admittance associated to it will be given by, respectively,

$$Y_R = G \qquad [3.54a]$$

$$Y_C = j\omega C = jB \qquad [3.54b]$$

where Y_R is the resistance admittance, Y_C the capacitor admittance, G is the conductance and B (B=ωC), the subceptance.

3.6. Impedance analysis

When a dielectric material with two metallic electrodes partially covering the surface at opposite faces is excited, a differential change dV induces a change on the electrodes charge dQ due to polarisation. Figure 3.5 shows a plane parallel capacitor both in vacuum and filled with a dielectric material.

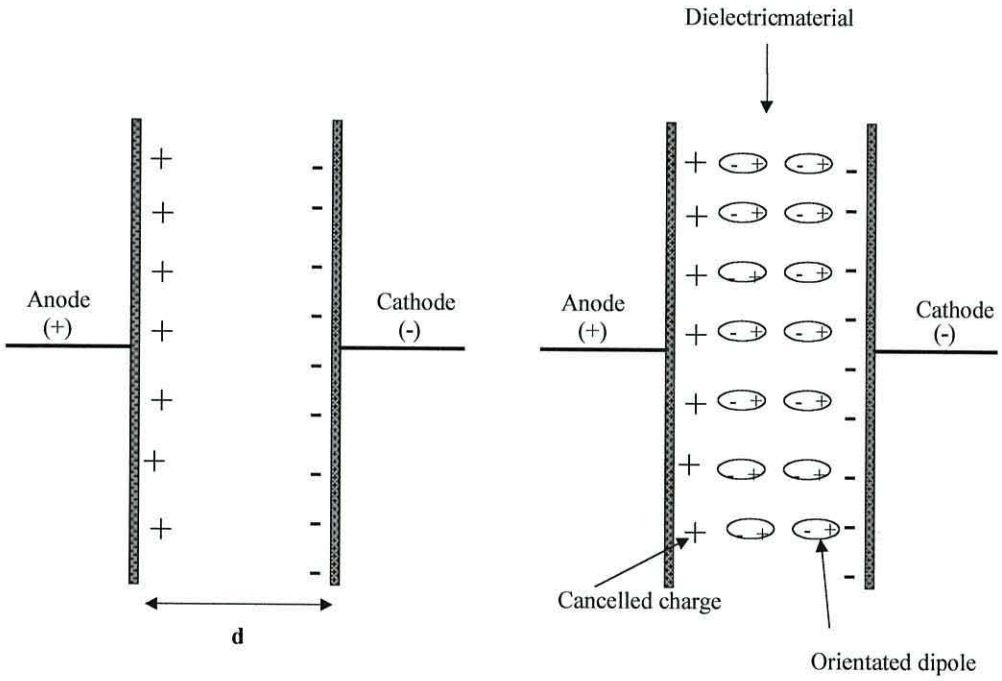


Fig. 3.5. Parallel plate capacitor with plates of area *A* separated by a distance *d* in vacuum and filled with a dielectric material.

When the plates are charged by an external source an electric field **E** is produced, which tends to orient the dipole molecules of the dielectric in the direction of the field, so that the opposite faces of the slab carry bound charges of opposite sign. The effect of these surface charges is to neutralise the bound charges on the plates while the remaining part of the total electrode charge (free charge) is responsible for the electric field in the medium. Therefore, the introduction of the dielectric increases the charge capacity of the system by Q_b.

The capacitance of such a device can be defined as

$$C = \frac{dQ}{dV} \qquad [3.55]$$

This is a general definition valid for interdigitated electrodes covered by a thin dielectric layer, or for an electrolytic capacitor, for a p-n junction in a semi conducting material, etc. However, assuming a parallel-plate capacitor under an homogeneous electric field and a linear and homogeneous dielectric, the capacitance can be easily rewritten as

$$C = \varepsilon_0 \varepsilon_r \frac{A}{d} \qquad [3.56]$$

where A and d are the electrode area and the sample thickness, respectively. It is important to notice that homogeneity of the field is only given for $A >> d^2$, so that fringing fields can be neglected.

Consider an electrical circuit consisting of a sign-wave generator, $v(t) = V_0 \sin(j\omega t)$, a parallel-plate capacitor and an ac current flowing through the circuit given by $i(t) = I_0 \sin j(\omega t + \phi)$. Assuming periodic, sinusoidal signals, the phasor associated with the intensity of the current in the frequency domain will be given by

$$I(\omega) = Y(\omega)V(\omega) \qquad [3.57]$$

Y(ω) is the admittance of the circuit and equation [3.45] is commonly known as Ohm´s law.

The phase shift between I and V is expressed by the loss angle $\delta = \pi/2 - \phi$, from which the loss or dissipation factor is defined

$$\tan\delta = \frac{Y'}{Y''} = \frac{Z''}{Z'} = \frac{\varepsilon''}{\varepsilon'} \qquad [3.58]$$

From equation [3.58] it is possible to state that, physically, the permittivity is a measure of the energy stored in the dielectric whereas the dielectric loss is a measure of the energy dissipated in the dielectric. In spite of not containing more information than ε' and ε'', tan δ is independent of the capacitor geometry and, therefore, it is particularly useful when the geometry is not well defined.

Both the admittance and the impedance are complex numbers characterised by an absolute value and a phase angle or area and imaginary part. For the admittance,

$$I(\omega) = [G(\omega) + jB(\omega)]V(\omega) \qquad [3.59a]$$

where G is the conductance and B is the subceptance. Equation [3.59a] corresponds to a fixed voltage V driving a current I, which real part is in phase with the voltage and which imaginary part precedes the voltage signal by π/2. This situation is given by an equivalent circuit consisting of an ideal capacitor (without loss) B(ω)/ω in parallel with an ideal resistor (which represents the loss in the capacitor) 1/G(ω).

Alternatively, using the impedance,

$$V(\omega) = [R(\omega) - jX_C(\omega)]I(\omega) \qquad [3.59b]$$

where R is the resistance and X_C=ωC is the capacitive reactance. Equation [3.59b] corresponds to a situation given by a constant current I in an equivalent circuit consisting of an ideal resistor R(ω) with a voltage drop in phase with the current in series with an ideal capacitor with a voltage drop delayed by π/2.

Therefore, both equivalent circuits correspond to different physical situations. The parallel equivalent circuit (admittance representation) reflects two physical phenomena existing in parallel, such as conductivity and polarisation in a leaky capacitor. The series equivalent circuit (impedance representation), on the other hand, corresponds to two physical processes occurring in series, such as a capacitor with barrier regions adjacent to a bulk with different conductivities and/or permittivities.

These considerations are particularly important for a proper calculation of the permittivity. Clearly, this is only possible if the admittance representation can be used, because only this representation corresponds to an homogeneous dielectric with a unique permittivity. From [3.59a] and [3.59b],

$$\varepsilon' = \frac{d}{\varepsilon_0 A}\frac{B}{\omega} \qquad [3.60a]$$

$$\varepsilon'' = \frac{d}{\varepsilon_0 A}\frac{G}{\omega} \qquad [3.60b]$$

Equivalent circuits are particularly helpful in recognising and separating processes as a first step of the dielectric analysis. Each process will then have to be considered separately in order to determine the relaxation parameters, because the Debye equations hold only for single relaxations.

Unfortunately, as the Debye model does not include particle interactions, the dielectric response of liquids generally deviates considerably for the Debye type. The loss maxima are generally broader and asymmetric because dipole-dipole interactions lead to a distribution of relaxation times around a most probable value. For the presentation and comparison of experimental data is necessary to modify Debye´s equations so that the parameters $\Delta\varepsilon$ and τ retain their original meaning and also the width and asymmetry of the loss peak are taken into account.

3.7. Equivalent circuit of an experimental impedance cell

In a general sense, an experimental impedance cell is simply two electrodes separated by at least one electrolyte phase. Such a cell will generate an impedance when exposed to a small sinusoidal excitation and therefore we should be able to represent its performance by an equivalent circuit of resistors and capacitors that pass current with the same amplitude and phase angle than the real sample does under a given excitation. A typical circuit is shown in figure 3.6.

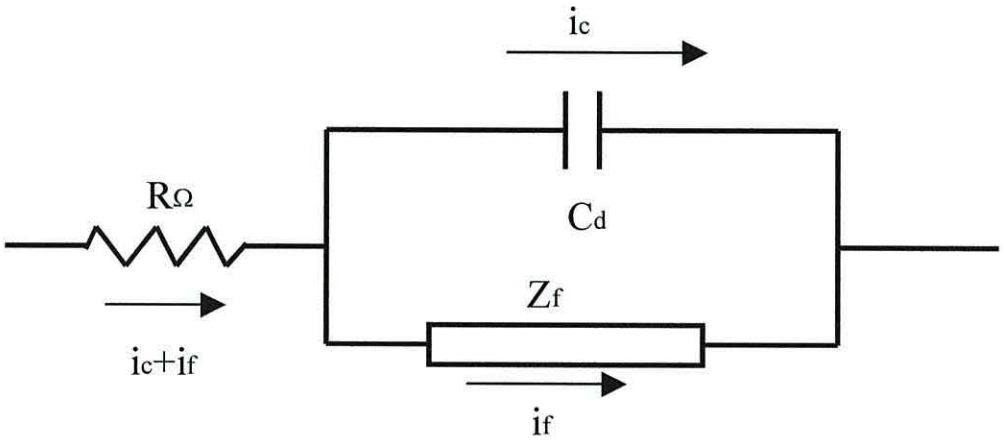


Fig. 3.6. Equivalent circuit of an electrochemical cell
(Bard and Faulkner, 1980)

The parallel elements are introduced because the total current through the working interface is the sum of distinct contributions from the faradaic process and double layer charging. The double layer capacitance closely resembles a pure capacitance, and it is here represented by the element C_d. The faradaic process must be considered as a general impedance Z_f. All current must pass through the uncompensated solution resistance and, therefore, R_Ω is inserted as a series element to represent this effect in the equivalent circuit.

The faradaic impedance has been considered in the literature in various ways, figure 3.7 shows two equivalences that have been made.

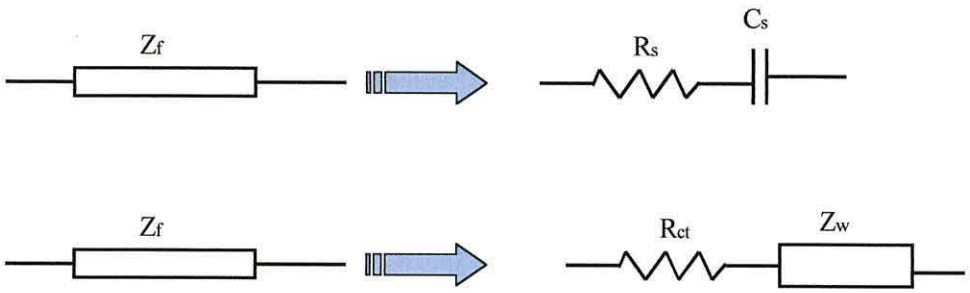


Fig. 3.7. Subdivision of Z_f into R_s and C_s or into R_{ct} and Z_W
(Bard and Faulkner, 1980)

The simplest representation is to take it as a series resistance-capacitance combination comprising the series resistance R_s and the capacitance C_s. An alternative is to separate a pure resistance R_{ct}, the charge transfer resistance from another general impedance Z_W, the Warburg impedance, which represents a resistance to mass transfer (Bard and Faulkner, 1980). In contrast to R_Ω and C_d, which are nearly ideal circuit elements, the components of the faradaic impedance are not ideal, because they change with frequency. A given equivalent circuit represents cell performance at a given frequency but not at other frequencies, in fact a main objective of a faradaic impedance experiment is to discover the frequency dependences of R_s and C_s, in order to transform these functions into chemical information.

The circuits considered in this section are based on the simplest electrode processes. Many others have been devised in order to account for more complex situations, for example those involving adsorption of electroreactants, multistep charge transfer, or homogeneous chemistry. An equivalent circuit designed to represent an impedance biosensor is included in a following chapter, as well as a discussion on the contribution of the constituent phases in the overall response of the enzyme electrode.

Chapter 4. Impedance Biosensors – a Review

Impedance techniques are those which involve the measurement of the impedance, the admittance or any of their components (capacitance, resistance, conductance or subceptance) to describe relevant physical or chemical characteristics of a system. Impedimetry concepts have been widely applied in electrochemical methods such as the faradaic impedance method, ac voltammetry and ac polarography.

Impedance methods used in dielectric spectroscopy and its application to biosensor technology are described in our work. A variety of works have been published covering gas sensors, solvent sensors, immunosensors and other sensors which use conductimetric methods. This chapter reviews the literature on impedance techniques employed for detection and monitoring of different substances.

4.1. General sensing applications of impedimetry

Fouke et al (1988) employed thin-film microelectronic technology to fabricate a miniature, flexible sensor with a two-electrode cell which could be placed directly on the surface of the trachea to assess in vivo the electrical conductance of the fluids present. The measurements were simple with immediate results which could be monitored continuously.

Imbalance in the ratio of ions and therefore fluid tonicity in biological fluids had been used as an index of the altered hydration levels which frequently occur during exercise and also of certain disease states including disorders of transcellular electrolyte transport such as exist in cystic fibrosis (Shwachman and Antonowicz, 1962).

Standard clinical procedures to analyse these fluids required the removal of a sample of fluid from the body, in order to determine the osmolarity of the fluid. Coulometry and photometry could be then used to quantify the activities of the sodium, chloride and potassium ions. Such procedures required multiple transfers and dilutions of the sample, increasing the likelihood of evaporation and thus increasing the apparent concentration of the ions. Moreover, the removal of fluid could alter the surface characteristics of the trachea and actually expose the epithelial surfaces.

Fluid conductivity was a function of osmolarity so, if the concentrations of ions changed, both of them experienced a variation. Fouke et al proposed to use the electrical conductivity of this body fluid as an index of the fluid osmolarity and therefore an index of electrolyte concentration. Such a measurement could be used to monitor altered disease states and a sensor placed directly on the measurement surface would eliminate the intrinsic problems of standard fluid osmolarity determination methods.

In vitro tests of the sensor were carried out prior to its physiological evaluation. Calibration tests in multiple salt solutions, temperature sensitivity assessments and the evaluation of the effects of solution concentration on electrical conductance were used to characterise the performance of the testing sensors.

In vivo tests of the sensor were conducted in the trachea of dogs before and after exposure to ozone, an agent known to alter secretory function. The sensor was mounted on the wall of a latex balloon which was attached to a catheter and lowered into the trachea. When the balloon was blown up, the sensor was in contact with the airway wall. Fluid samples were also collected using a filter paper to be subjected to standard techniques. Osmolarity calculations made from the sensor data were consistently about 25% lower than clinical laboratory osmolarity analysis, perhaps indicating evaporation of the laboratory sample.

Endres and Drost (1991) developed dielectric gas sensors for SO_2 with low cross-sensitivity to other gases and humidity. The sensors consisted of pairs of planar interdigitated electrodes coated with hetero-polysiloxane (HPS) acting as a sensitive layer, which changes its permittivity by gas adsorption. According to investigations by scanning electron microscope, the sensitive film was primarily deposited in the space

between the electrodes. The increase in the dipolar polarisation caused by adsorbed SO_2 molecules was, according to the authors, the main effect for gas detection (Endres et al, 1990).

The interdigitated array electrodes were fabricated following thin-film technology (Al and Ni/Au) with various electrode distances and total widths but maintaining the electrode thickness constant at 0.7 μm. The influence of the electrode geometrical parameters on its performance were later investigated.

The sensors were tested in a temperature-controlled chamber at (30 ± 2) °C with an impedance analyser. The test gases were mixed with dry nitrogen using mass flow controllers and the coated interdigitated electrodes were exposed to various gas concentrations. An electrical saturation point for SO_2 was obtained at about 200 vpm, for CO, CO_2 and CH_4 no impedance change was observed and for NH_4 the electrical effects were very small.

Endres and Drost also showed how the capacitance of the gas-sensitive layer depended on the inter-electrode distance for SO_2 concentrations above 100 vpm. The smallest electrode spacings gave the highest capacitances, whilst the capacitance decreased for increasing separations up to a saturation value of 26.2 μm. Consequently it was advantageous for the design of capacitive gas sensors to keep the distance between the electrodes very small within the technological limitations in order to achieve the highest possible variation in capacitance by the sensitive layer.

One of the many applications of conducting polymers to impedance biosensing was in the design of gas sensors which could be used to mimic the function of the biological chemosensory system. Artificial nose prototypes have been constructed and used in fields ranging from the quality control of foods and beverages to monitoring of specific industrial environment for safety. Normally, they consisted of an array of different sensors coated with appropriate conductive polymers that generated response patterns when exposed to gases. These patterns were subsequently processed by pattern recognition techniques and used to identify the chemical species that form such gases. Gas sensing techniques using ac measurements of various electrical parameters on a

single sensor element have been investigated over the last decade (Amrani et al, 1997; Musio et al, 1995).

Electrically conducting polymers changed their conductivity and relative permittivity when exposed to volatile chemicals, and were therefore suitable materials for their detection, identification and quantification of such analytes. These changes depended on the frequency, the concentration and the type of chemical so, for instance, the frequency dependence could be used to make more selective sensors. A polypyrrole film sensor was designed and constructed by Musio and Ferrara (1997) and its low frequency response to exposure of methanol, acetone, ethyl acetate and ethanol investigated. Impedance response patterns to each vapour were obtained by varying the measurement frequencies.

The sensor designed by Musio and Ferrara consisted of two planar parallel gold plated copper strips on an insulating substrate with a polypyrrole layer in between which consisted of two films deposited using chemical and electrochemical polymerisation. A gas ring was assembled in order to deliver a known flow of volatile chemicals into the gas sensor chamber as described in figure 4.1.

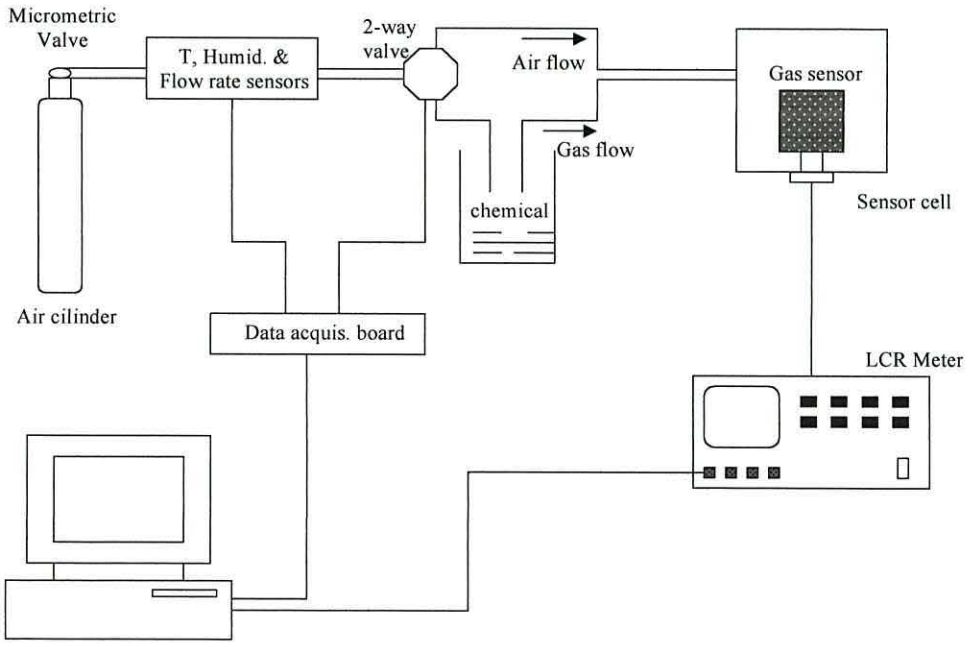


Fig 4.1. Gas sensing device and data acquisition system (Musio and Ferrara, 1997).

The liquid chemical was in a gas container and its vapours were diffused into a stream of dry air which flowed into the gas sensor chamber at a known concentration. The temperature was controlled by a thermostat and the air humidity kept constant throughout all experiments.

The sensor experienced a comparative increase in capacitance when exposed to methanol over the frequency range investigated (20 Hz - 10 kHz), although for acetone exposure, the capacitance was lower than that of dry air at very low frequencies (below 30 Hz) and higher than the reference value above this frequency. The capacitance observed when testing ethyl acetate was generally lower than the reference value. All gases tested (dry air included) showed a continuous capacitance decrease over the frequency range investigated. Musio and Ferrara also measured the sensor resistance in response to all vapours finding it was always higher than the resistance in air and exhibited a very weak frequency dependence. The low frequency response of the polypyrrole layer was rapid and stable, with relative capacitance changes about two orders of magnitude higher than the relative resistance changes.

The characteristic capacitance patterns obtained for the vapours investigated at several frequencies were shown to be very different and typical for each chemical, making possible to distinguish between them. By contrast, no evident difference was reported among the resistance patterns owing to the weak frequency dependence of the sensor resistance. Using capacitance measurements on methanol and ethanol it proved possible to distinguish between two similar alcohols.

A study of time and temperature dependence of the two electrical impedance parameters (capacitance and resistance) was also reported by Musio and Ferrara. The changes due to ageing and temperature of the resistance were found to be frequency independent.

Impedance techniques have also been applied to immuno-sensing devices derived from the direct capacitive immuno-sensor introduced by Newman et al (1986), which was based on the introduction of an additional capacitive component in the response of the sensor that took place when an antibody was immobilised on the surface of the electrode pair, due to the low dielectric permittivity of the immobilised macromolecule. When the pair of antibody-immobilised electrodes were exposed to the specific antigen, an

immuno-complex was formed due to the recognition process taking place, which resulted in an increase in the thickness of the dielectric layer covering the electrode and therefore in a decrease of the sensor capacitance. Such a phenomenon is illustrated in fig 4.2.

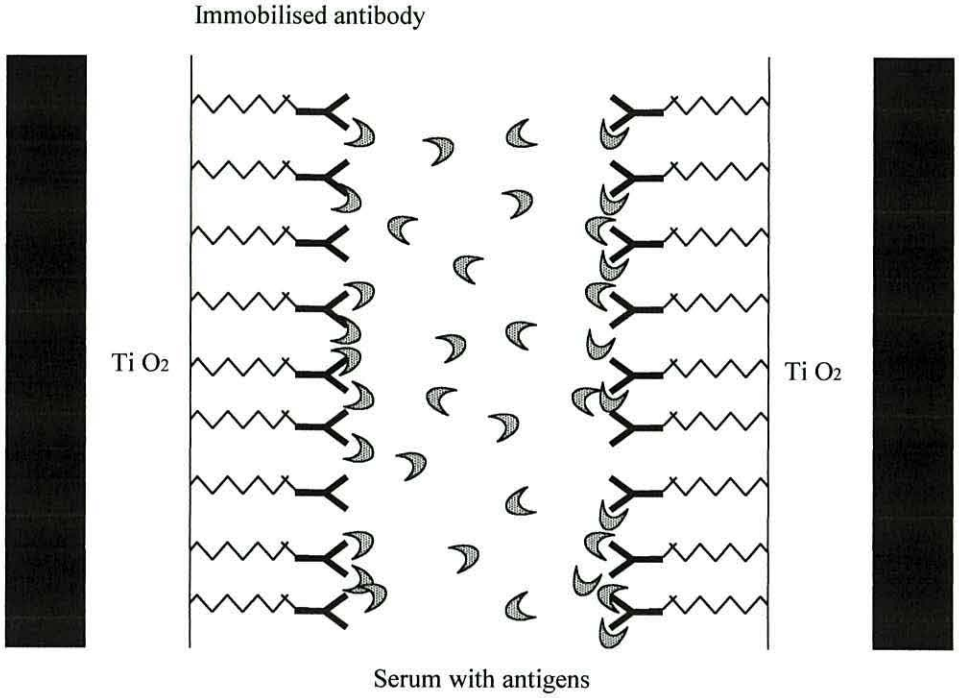


Fig 4.2. Immunoassay by the use of a capacitive sensor in a direct set-up.
(Varlan et al, 1997)

Varlan et al (1997) designed a capacitive sensor for the detection of the allatostatin hormone, involved in insect metabolism, using TiO_2 modified electrodes. The allatostatins are a class of peptides that inhibit the biosynthesis of the juvenile hormone in some insect species. Therefore, they make the juvenile hormone concentration decrease and the insect starts ageing. The allatostatins are released in blood, where they can be measured giving an indication about the developmental stage of the individual insect. In this context, Varlan et al considered the design of an allatostatin capacitive immuno-sensor in order to replace the more expensive and time consuming radio labelled immunoassay (RIA) or enzyme linked immuno-sorbent assay (ELISA).

Various approaches were considered by Varlan et al to immobilise the allatostatin protein on the TiO_2 substrate. Optimum performance was achieved with (3-glycidoxipropyl) dimethylethoxy-silane deposited from an ethanol solution.

The allatostatin-antibody linking process was followed in real time. The antibody was added stepwise, yielding very high concentration values compared to physiological ranges. Despite the high antibody concentration, there still were available sites at the surface of the electrode where the immobilised antigens had not interacted with antibodies after the third addition. The response time of the sensor was limited by the measurement set up and extended up to 15 minutes. The sensor showed good sensitivity characteristics, giving a capacitance decrease of about 1 nF/cm^2 for a 3000 times diluted insect serum sample.

Van Steenkiste et al (1997) investigated the possibility of combining different sensing techniques in order to manufacture a hybrid microsensor array to measure chemical properties of biological liquids, which satisfied the requirements for multi-analyte biochemical analysis. The microsensor array consisted of a flow system board containing the complete liquid handling system, a sensor carrier which contained four sensor chips, interface electronics and a data acquisition mode.

Flow type biosensing devices, such as the one presented by Van Steenkiste et al, take up samples at a constant rate and are able to perform real time monitoring. The development of three-dimensional microstructures (micro-machines) allows for the fabrication of miniaturised flow system biosensors which take up minimum amounts of biological samples and apply to space restricted devices.

An outline of the flow system board employed by Van Steenkiste et al is shown in figure 4.3.

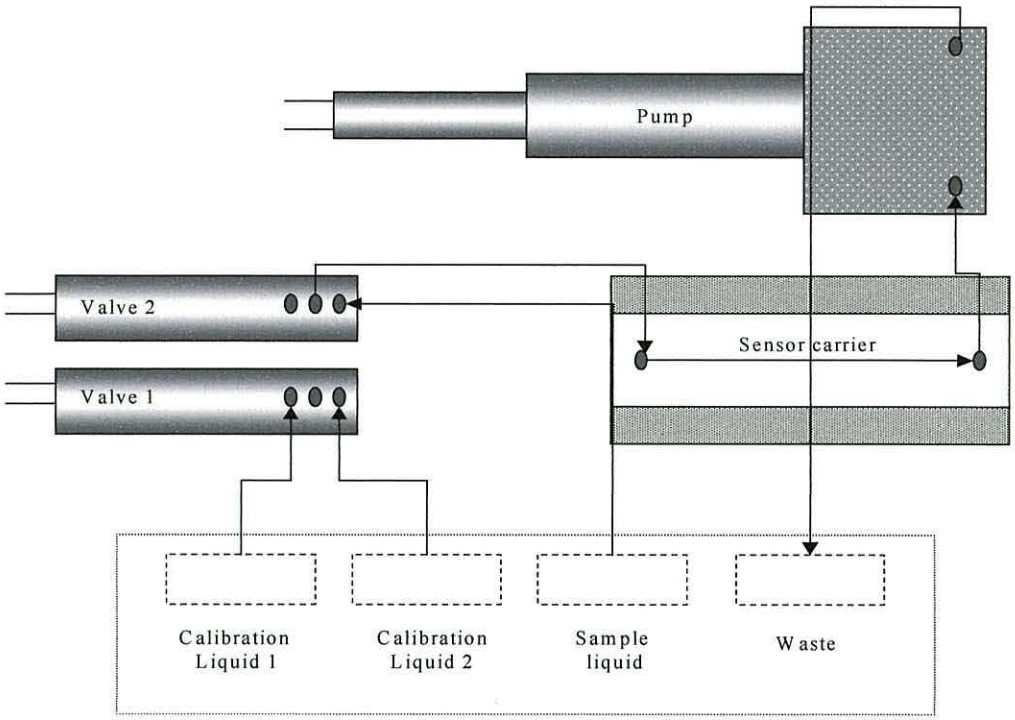


Fig 4.3. Flow system board layout (Van Steenkiste et al, 1997).

The board contained two magnetic valves, a peristaltic pump and a sensor carrier, all interconnected by short tubes. The sample liquid was pumped into the sensor carrier at a constant rate. While the sample in the sensor carrier was continuously renewed, the superfluous liquid was pumped into the waste.

The sensor carrier shown in figure 4.4 was the main element of the flow system and consisted of an internal liquid channel formed by binding a micro-machined glass wafer to a silicon wafer. Four sensor chips were mounted along the liquid channel on the back side of the sensor carrier. Van Steenkiste et al mounted a capacitive pressure sensor, a temperature sensor, a pH-ISFET differential sensor and a pO_2/pCO_2 sensor. The sensors were in contact with the internal liquid channel by through holes in the silicon, and hermetically sealed to the silicon side by epoxy sealing.

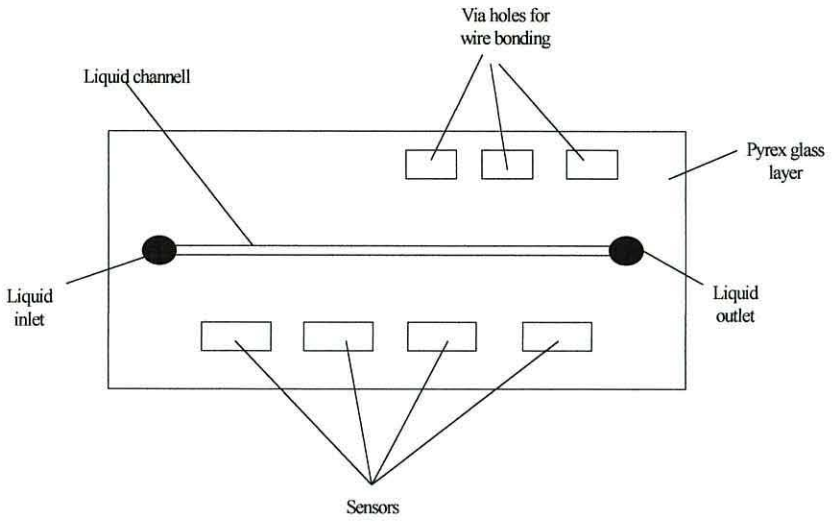


Fig 4.4. Sensor carrier layout (Van Steenkiste et al, 1997).

Van Steenkiste et al performed functional tests in dry environment and in liquid samples and the usability of the sensor array for determining salt and protein concentration and for fermentation in a glucose mineral salt medium with E colis cells was reported.

Apart from monitoring enzyme reactions in biosensors technology, dielectric techniques have been applied for many years to the characterisation of biological materials (amino acids, polypeptides, proteins, cell suspensions, tissues) over the frequency range 1 Hz to a few gigahertz (Foster and Schwan, 1989; Pethig, 1979). For example, impedance techniques have been used to investigate changes in membranes of biological cells in suspension (Pliquett and Wunderlich, 1989; Matanguihan et al, 1994) to measure micromotion of cells on a small electrode (Giaever and Keese, 1993), to detect immunological reactions (DeSilva et al, 1995), to estimate microbial biomass (Harris and Kell, 1985), to detect microbial metabolism (Palmqvist et al, 1994) and to detect protein adsorption to supported lipid bilayers (Stelzle and Sackmann, 1989).

A particular application of impedimetry which gained important attention (Firstenberg-Eden and Eden, 1984; Harris and Kell, 1985) was its exploitation in assessing the number of micro-organisms present in sparse populations, since changes in the electrical properties of microbial culture media were associated with microbial growth.

Conductimetry (Richards et al, 1978; Mackey and Derrick, 1984), impedimetry (Cady, 1978) and capacimetry (Firstenberg-Eden and Zindulis, 1984) have been used.

Given that the low frequency (below Maxwell-Wagner effect dispersion) electrical conductivity of a suspension was found to be lower than that of the fluid in which it was held, it was possible to detect the presence of suspended matter directly by its effects upon an electrical field. Such measurements have been made both in bulk suspension (Irimajiri et al, 1975; Harris and Kell, 1983; Lovitt et al, 1986) and in hydrodynamically focused lowing streams (Dow et al, 1979). Clarke and his collaborators (Blake-Coleman et al, 1984; Clarke et al, 1984, 1985) have also applied impedimetry to the direct assessment of microbial biomass and the studies by Kell and colleagues (Harris and Kell, 1983) have shown that the dielectric properties of cells of a given radius vary monotonically with the volume fraction of the suspended phase.

The feasibility of distinguishing or identifying cells by their frequency-dependent dielectric properties has also been investigated (Harris and Kell, 1985). Although it is true that both size and surface charge density changed from different bacteria, they also depended critically on pH and physiological state. Therefore, simple dielectric spectra were unlikely to contain enough information on their own to be diagnostic.

Other techniques exploiting the bulk conductivity or impedance of cells and tissues, and which have enjoyed a reasonable widespread use, included impedance plethysmography (Nyboer, 1970; Wheeler and Penney, 1982; Anderson, 1984) and pneumography (Pacela, 1966; Henderson and Webster, 1978). Measurements of the dielectric properties of tissue have been used in the testing of freshness (Faure et al, 1972; To et al, 1974; Kent, 1975; Kent and Jason, 1975) and quality of foods (Pfutzner and Fialik, 1982). Regarding tissue measurements, it may also be mentioned that impedimetric techniques have found further application in acupuncture, as significant local decreases of skin impedance exist in the area of the meridian points recognised as significant in this discipline (Becker and Marino, 1982; Jakoubek and Rohlicek, 1982).

Measurements of the conductance of homogeneous solutions have been widely used in environmental monitoring, and have been the method of choice in estimating the salinity of the marine environment (Brown, 1968; Ben-Yaakov, 1981; Wilson, 1981) and soil

treatment (Qin and Cabral, 1994). Similarly, resistivity methods have also been applied to geophysical prospecting (Keller and Frischknecht, 1966), although the physical and mechanistic interpretation of the data has presented a very high level of complexity (Hasted, 1973; Philips, 1984).

It may also be mentioned that using microwave frequencies improved the time resolution of solution conductivity measurements (de Haas and Warman, 1982). Schugerl (1984) proposed a conductimetric method for monitoring bubble size and velocity distribution in microbial fermenters, while the utility of impedimetry in the control of chromatographic elements was discussed by Alder et al (1984).

Ehret et al (1997, 1998) described an effective way of monitoring cellular concentration, growth and physiological state on-line and in real-time based on impedance measurements of adherently growing cells on interdigitated electrode structures. The main effect of cells on the interdigitated electrode structure was due to the insulating property of the cell membrane. The presence of intact cell membranes on the electrodes and their distance to the electrodes determined the current flow and thus the sensor signal. Therefore, concentration, growth and the physiological state of adherent cells could be monitored during cultivation.

Platinum interdigitated electrodes were coated with epoxy resin to allow for growing of cells and impedance spectroscopy was performed by applying an oscillating potential to the sample. The impedance of the system was observed at several frequencies to reflect changes in the behaviour of the cells on the electrodes caused by the undertaken treatments. When a powerful detergent that destroyed the lipid layer of the cell membrane (Triton X-100) was added, the loss of the insulating effect of the cells resulted in a dramatic effect on the sensor signal with a remarkable fall in impedance.

Another set of experiments was carried out by Ehret et al in order to elucidate the influence of proliferating serum components on the sensor signal. When a cell was removed from its original tissue and placed in culture, the surrounding medium had to provide similar environmental conditions to which the cell had been exposed in vivo and had to meet essential requirements for survival and growth of the cell. Serum has been the most widely used biological fluid employed to culture cells outside the body

and the determination of its chemical constituents has been of great significance. Impedance tests were carried out with this aim in samples with and without cells.

For experiments without cells, the sensor signal was insensitive towards different types of medium: with or without foetal bovine serum (FBS), medium changed by cellular metabolism. As for experiments with cells, effects of temperature and fresh medium with foetal bovine serum were clearly distinguishable from effects of fresh medium without FBS. The main reason for the signal change was not only the pure presence of intact cell membranes on the electrodes but also their distance from the electrodes. With this experiment, Ehret et al showed the possibility of detecting the effects of substances influencing the growth, shape and morphology of cells on the interdigitated electrode structures. Such substances could be hormones, special growth factors, cytostatics, antibodies, etc.

The effect of a cytotoxic heavy metal ion such as Cd^{2+} on adherent cells was also studied on-line and in real-time with interdigitated electrodes by Ehret et al. The addition of Cd^{2+} partially destroyed the cell membrane indirectly by interfering with protein synthesis and therefore inhibiting the permanent regeneration of the membrane. The destruction of such a lipid bilayer could be detected by monitoring the drop in admittance in the adherent cells, analogous to that observed with Triton treated samples.

Finally, the long-term stability of the sensor signal was investigated as an important requirement for routine applications of the sensor. Growth and increase in the number of cells were clearly visible in the sensor capacitance. An important loss in capacitance (from 300 nF to 50 nF approximately) was observed in the first day due to a deprivation of serum components (growth factors) produced by the cells themselves. The experiment was continued for 14 days, replenishing the surrounding medium daily. Addition of fresh medium lead to a decrease in capacitance followed by an increase if cells grew very rapidly. The behaviour of the sensor signal between the exchanges of medium was hardly altered.

4.2. Enzyme electrodes based on impedance techniques

In an early paper on conductimetric enzyme assays, Lawrence (1971) listed several properties which allow the application of conductimetric methods to enzymatic reactions. These were: the generation of ionic groups (e.g. amidases catalysis), the separation of unlike charges (e.g. decarboxylases catalysis), proton migration (e.g. esterases catalysis), changes in the degree of association of ionic groups resulting from chelation (e.g. kinases catalysis), and changes in the sizes of charge carrying species (e.g. phosphatases catalysis). Each of the above catalysing agents produce a relative variation in the total conductance of the system under test that can be related to the concentration of substrate present in the sample.

Amidases, such as urease, catalyse the breaking of the bond between the amide group and the rest of the substrate molecule, generating carboxyl and ammonium ions and therefore increasing the overall conductance of the system.

As for decarboxylases such as pyruvate decarboxylase, an overall decarboxylation reaction can be written euther as a hydrolase reaction producing H_2CO_2, or as a lyase reaction producing CO_2. Decarboxylase enzymes catalyse the transformation of the carboxyl group into derivatives by exchanging the hydroxyl group with halogen groups, alcoxides or amides, which increases the concentration of OH^- ions in the solution.

The change in conductance characteristic which appears in kinases catalysis (pyrophosphokinase) is due to the chelation process in which the resulting product binds free radicals, neutralising their charge and decreasing the conductivity of the sample. Kinases catalysis is carried out by transferring a phosphate group from ATP to the relevant substrate.

Phosphatases catalysis (phosphatidylglycerophosphatase) generates charged products of different size than the substrate molecules. The mobility of the ions depends strongly on their size, and therefore a change in size will cause a change in the conductance of the solution.

Although important efforts have been put into the development of chemical sensors, research in the field of impedimetry-based sensing has seemed to fall into the background, compared with investigations on amperometric and potentiometric methods. Nevertheless, the basic technique is straightforward and has been stated as a good alternative to other electrochemical principles for a range of analytes, amongst which urea has been the most frequently studied (Watson et al, 1987; Thavarungkul et al, 1991).

Lowe (1984, 1985) and Ballot et al (1984) took the lead in the application of impedimetry to biosensor design, with the firm conviction that many of the chemical and biochemical reactions previously exploited in potentiometric and amperometric enzyme electrodes could be equally or better assessed conductimetrically. Similarly, Arwin (1982) made use of enzyme-reaction dependent changes in the double layer capacitance of symmetrical metal electrodes as a measure of enzyme or substrate activities. We can also mention the use of conductimetry to improve the response time (Powley et al, 1980) and selectivity (Powley and Nieman, 1983) of ion-selective electrodes that are normally used in a potentiometric mode.

As impedimetry-based biosensors detect the total impedance of a sample, their main drawback has been their relative lack of selectivity in distinguishing the impedance associated with an analyte of interest from the rest of the contributing species. Nonetheless, this problem can be overcome, obtaining specificity towards a certain analyte, by applying differential methods through auxiliary reference electrodes or analogous approaches.

The first conductimetric transducer was introduced by Lowe and his research group (Watson et al, 1987). It consisted of an oxidised silicon wafer with serpentined, interdigitated gold electrode pairs on one surface in a planar configuration (see section 5.1.3.1). The close proximity between the electrodes in each pair allowed a small-amplitude sine wave to induce a measurable alternating current (ac) response. The resulting signal was linearly related to solution conductance. The transducer was tested with urease immobilised on the sensing surface in a cross-linked albumin-glutaraldehyde gel layer to detect urea in diluted human serum samples. The results

correlated well with clinical results and measurements in the 0.1 mM to 10 mM range were feasible in 5 mM buffer.

The same group also tested the enzymes penicillinase and aryl acyl amylamidohydrolase (Lowe, 1985) immobilised on film of cellulose acetate and cellulose nitrate respectively, obtaining calibration curves of root mean square current against substrate concentration for the range 0.05 mM to 10 mM.

Mikkelsen and Rechnitz (1989) reported the design and characterisation of model conductimetric biosensors for urea and D-amino acids using the enzymes urease and D-amino acid oxidase (EC 1.4.3.3), respectively. The urea report is included in section 2.1.3. D-amino acid oxidase oxidises D-amino acid following the reaction below,

$$\text{D-amino acid} + O_2 + H_2O \xrightarrow{\text{D-amino acid oxidase}} \text{2-keto acid}^- + H_2O_2 + NH_4^+ \qquad [4.1]$$

Because the hydrogen peroxide produced by the enzyme reaction acts as an inhibitor, it is necessary to co-immobilise it with catalase, in order to convert the peroxide to water and oxygen.

The design of the conductimetric transducer employed by Mikkelsen and Rechnitz is included in figure 4.5.

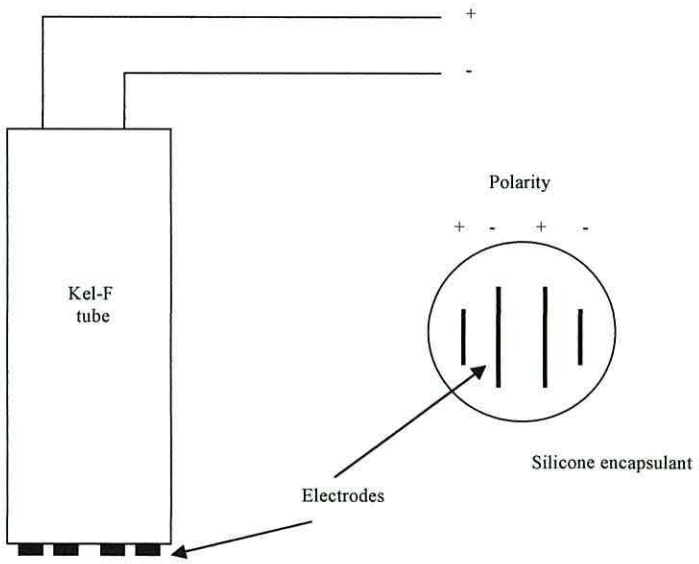


Fig 4.5. Side and end views of the conductimetric transducer design employed by Mikkelsen and Rechnitz (1989).

The electrodes were made of platinum wire and embedded in a silicon rubber plug sealing one end of a plastic tube. The sensing surface consists of four parallel electrodes connected to the two external leads so that the polarity alternated across the sensor surface in order to maximise the resulting cell constant for this configuration.

D-amino acid oxidase and catalase were co-immobilised using covalent binding to a collagen membrane, which was then held tightly over the surface of the conductimetric transducer with an O-ring. A detection limit of 1 μM and linear range extending for two orders of magnitude were obtained for the assay of D-methionine with the D-amino acid oxidase electrode. The optimum pH for the immobilised enzyme system was determined through its response to D-methionine solutions between 5 μM and 35 μM and found to be around 8.6.

The selectivity of the sensor to different amino acids was tested and compared to that of D-methionine, which showed the highest response. Excepting D-proline (0.67 relative response to that of D-methionine) and L-methionine (0.03 relative response), the rest of the amino acids (D-phenylalanine, D-alanine, D-serine) showed relative responses to the response of D-methionine between 0.16 and 0.17).

The response time of the sensor to D-methionine was also measured from 0.2 μM to 2 mM concentration range. Mikkelsen and Reichnitz found a variation in the time for 90% response between 6 and 20 minutes, depending on substrate concentration. The fastest responses took place at the lowest substrate concentrations and increased dramatically up to 14 min at 2 mM. The unusual response time behaviour of this sensor was attributed to the inhibitory effect of the hydrogen peroxide produced in the catalysed reaction.

Finally, the storage stability of the D-amino acid sensor was investigated over 33 days by measuring responses to D-methionine over the 2 μM to 2 mM concentration range in 5 mM glycine buffer, pH 8.6. Results indicated that the membranes provided reproducible responses for 3 weeks following immobilisation.

On the basis of earlier works on the application of conducting polymers such as polypyrrole (Umana and Waller, 1986; Iwakura et al, 1988; Kajiya et al, 1991), polyaniline (Bartlett et al, 1988; Shinohara et al, 1988; Shaolin et al, 1991) and polyindole (Pandey, 1988) to amperometric biosensors , Hoa et al (1992) developed a conductivity sensor modified with electronically conducting polymers. The sensor utilised the change in electronic conductivity of the polymer in response to changes in the microenvironment, such as pH or redox potential of the solution in contact with the polymer. The particular system chosen for investigation was polyaniline-glucose oxidase to determine the concentration of glucose.

Polyaniline was deposited on platinum followed by a thin layer of polyaniline incorporating glucose oxidase. The electrode configuration is shown in figure 4.6.

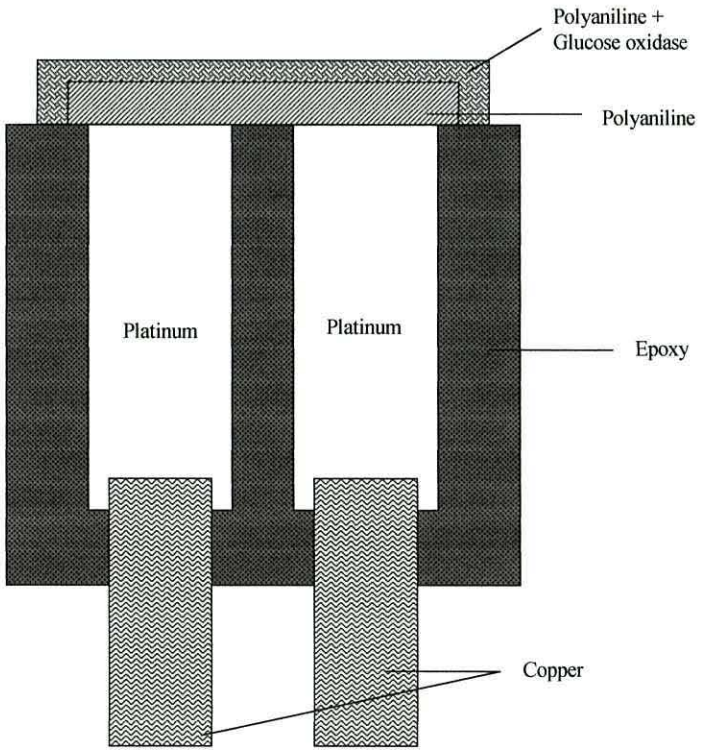


Fig 4.6. Electrode configuration employed by Hoa et al (1992).

The electrode array consisted of two platinum disks embedded in epoxy and separated by a gap. Polyaniline film was deposited onto the platinum surface bridging the gap between the electrodes. Then, a thin polymer-enzyme film was deposited on top.

Therefore, the polymer acts as the immobilisation medium as well as the transducer for converting a biochemical signal to an electronic signal.

The glucose oxidase catalysed reaction produced hydrogen peroxide and D-glucono-δ-lactone which non-enzymatically hydrolyses to gluconic acid, consuming dissolved oxygen during the enzymatic oxidation of β-D-glucose.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\beta\text{-glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \qquad [4.2a]$$

$$\text{D-glucono-}\delta\text{-lactone} \rightarrow \text{gluconic acid} \xrightarrow{H_2O} \text{gluconic acid residue} + H^+ \qquad [4.2b]$$

The consumption / production of any of these species caused a change in the chemical potential of the microenvironment, which subsequently originated a change in electronic potential.

The sensor was immersed in glucose solutions of various concentrations. A reversible and reproducible linear change in the polymer film resistance was found up to 10 mM glucose concentration. The resistance of the polymer film was shown to decrease with increasing concentration of glucose and the selectivity of the device was proven by testing its performance in another sugar, mannose. No variation in the sensor response was perceived to changing concentrations of mannose, demonstrating that the sensor action was a consequence of the enzyme catalysed reaction and hence specific to glucose.

Finally, the performance of the sensor was tested against repeated measurements in a 10mM glucose solution in order to examine its reproducibility. After an initial drop in the response, the sensor output remained stable for more than 14 runs, with a standard deviation below 10%. The initial fall in activity was attributed by Hoa et al to the leaching out or deactivation of loosely bound enzyme.

Shul'ga et al (1994) described the fabrication and characteristics of thin-film conductimetric biosensors for both glucose and urea determination. The details of the urease electrode are included in section 5.1.3.1.2.

Glucose oxidase was immobilised on the surface of the working electrode by chemical cross-linking and the characteristics of the biosensors were measured by means of admittance and impedance changes. The differential signal between the pair of electrodes covered with the immobilised enzyme and those with the blank membrane was recorded for each glucose concentration.

Similar values to those obtained previously by the same research group (Shul´ga et al, 1992) using a glucose field effect transistor were recorded. Therefore, Shul´ga et al assumed that the response of the conductimetric glucose biosensor was meanly due to the highly mobile protons generated by the enzymatic reaction inside the membrane with minor contribution from other ionic species involved The linear range of the calibration curves in 5 mM phosphate buffer extended from 0.1 mM to 2 mM with a sensitivity of 10 μS/mM. A dramatic drop in the response of the sensor was found for increasing buffer concentration. Between 1 mM and 40 mM phosphate buffer, the steady-state response of the glucose biosensor to 0.5 mM substrate decreased about 10 times.

Despite the inapplicability of such sensor to the detection of glucose in blood due to the narrow dynamic range, it was proven to be able to work in diluted serum. Shul´ga et al measured its response by adding known concentrations of glucose to diluted rabbit serum up to a concentration of 0.2 mM. The concentration of glucose in the undiluted sample was established at 3 mM with the biosensor but no comparative data obtained through traditional assay techniques were reported.

Warriner et al (1996) evaluated the use of conductive polypyrrole and non-conductive poly(phenol red) for the construction of enzyme electrodes using ac impedance and spectral reflectance and linear/cyclic voltammetry respectively, and applied their finding to the detection of glucose.

Conducting polymers, especially polypyrrole, have attracted more attention than non-conductive films for their potential application to enzyme electrodes (Bartlett and Cooper, 1993; Bartlett and Birkin, 1993). Glucose oxidase entrapped in polypyrrole has become a key model system for such studies due to its relatively high stability and to the commercial interest of clinical glucose sensors (Reach and Wilson, 1992). The addition

of glucose caused an increase in the resistance of polypyrrole film, an effect that could be reproduced by the sole addition of hydrogen peroxide.

Most glucose sensors have been based on the amperometric detection of hydrogen peroxide, which resulted from the reduction of molecular oxygen acting as electron acceptor. Different theories have been proposed to describe the role of polypyrrole in the transduction mechanism. Koopal et al (1992a, b) and Arai et al (1993) thought that direct electron transfer from enzyme to polymer might be a possible alternative route to transduction, though many workers (Belanger et al, 1989; Schumann et al, 1991) were inclined to believe that polypyrrole acted primarily as a non-conductive matrix. The most widely accepted assumption proposed that a loss of polymer conductivity could be due to an interaction between conducting bipolarons (present on the polymer backbone) and nucleophiles (such as H_2O_2) resulting in the formation of non-conductive products (Pud, 1994).

The working electrode employed by Warriner et al was a platinum disk with a platinum flag serving as the counter electrode. A catalase / polypyrrole / glucose oxidase film was immobilised on the surface of the working electrode.

AC impedance measurements were used to determine the charge transfer resistance of glucose oxidase loaded polypyrrole. This magnitude could be quantified from a plot of Im (Z) and Re(Z) following a modified circuit described by Chang and Huang (1995). Addition of glucose substrate was expected to cause an increase in film resistance due to the products of the glucose oxidising reaction which could be correlated to the concentration of glucose substrate present in the test solution. Tests performed by Warriner et al confirmed that the generation of H_2O_2 could be directly related to the decrease in the conductivity of the film with a linear detection range extending up to 40 mM glucose.

An alternative to catalase in preventing hydrogen peroxide formation also employed by Warriner et al was to replace molecular oxygen with an artificial mediator such as ferri/ferrocyanide. Although ferrocyanide was formed instead of H_2O_2 an increase in film resistance upon addition of glucose was still reported. A reduction in the linear operating range of this sensor was found, with a linear response only up to 15 mM.

Referring to the previously reported work by McNeil et al (1995) and Krause et al (1997) on AC impedance measurements of the capacitance change that electrode polymer coatings undergo during urease-catalysed reaction (see section 4.1.3.2), Saum et al (1998) developed a biosensor for collagenase which also used AC impedance analysis to monitor the degradation of a gelatin layer covering the electrode. The authors described the use of the catalytic activity of the protease to form the basis of an electrochemical sensor which detects the erosion of a surface layer of the protein by the enzyme.

Proteolytic enzymes, employed in detergents fabrication, have had special significance in allergic reactions and protein sensitisation appearing by inhalation or prolonged exposure to hazardous biochemicals (Koochaki et al, 1995). Therefore, the development of a sensing device able to measure the amount of proteases in solution could be very useful for its incorporation into an air sampler or for other process liquid measurements. ELISA methods have been applied with good sensitivity results but their multistage nature makes them slow and time consuming (Cumming et al, 1995). The sensor reported by Saum et al (1998) constitutes an alternative strategy of fabricating a one-step biosensor using impedance measurements for inclusion into aerosols samplers to give a real-time estimate of airborne analyte concentrations.

Interdigitated electrodes were screen printed from a gold paste and gelatin was coated on the surface of the electrodes. Impedance measurements were carried out both on uncoated and protein coated electrodes over the frequency range extending from 1 Hz to 100 kHz. The phase and modulus of the total impedance of the system were recorded and the imaginary and real components were derived from these terms.

Saum et al showed it was possible to detect the presence of a gelatin coating because of the much lower impedance they present compared to uncoated electrodes over the whole frequency range, especially in the high frequency region. Very good correlation was obtained between the responses of different sensors with the same coating, remarkably better than that recorded for uncoated electrodes.

As the protein coating was more conductive than deionised water, its removal by enzymic digestion resulted in an increase in impedance as if the electrode reverted to the impedance of its uncoated state. When gelatin coated electrodes were immersed in collagenase solution, the impedance of the system increased with time in a sigmoidal fashion until reaching a steady state response, which corresponded to the complete stripping of the coating by enzymic digestion. However, this final impedance did not match the impedance of uncoated electrodes in deionised water because the products of the gelatin digestion and the added collagenase itself increased the conductiviy of the solution. The final steady-state impedance was found to be inversely proportional to the collagenase concentration.

Saum et al also found that the ability of the electrodes to detect the presence of a gelatin coating was greatly affected by the electrolyte concentration. Although the range of sodium chloride investigated went from 0.1 mM to 10 mM, the authors only succeeded in detecting the presence of the gelatin coating at very low concentrations (less than 2mM), which would limit the applications of this biosensor to low ionic strength environments.

Chapter 5 Urea and Creatinine Biosensors

5.1. Urea Biosensors

Urea biosensors are based on the hydrolysis of urea by urease according to the equations outlined in chapter 2. Any of the reaction products (ammonium and carbonate ions, ammonia gas) or even pH variations involved can be monitored and correlated to the initial concentration of urea.

The first urea sensor was a membrane potentiometric biosensor developed by Guibault and Montalvo (1969) based on a cation selective glass electrode. Urease was immobilised onto a glass support in which the urea was catalytically converted into ammonium and carbonate ions. The cation electrode then acted as a transducer, transforming the chemical products of the enzymatic reaction into an electric quantifiable signal.

In order to monitor the enzyme products various techniques have been investigated. Among these, electrochemical methods are the most common ones in the literature. Potentiometry using membrane electrodes (pH-sensitive electrodes, ammonium ion-selective electrodes, ammonium ion-selective FETs or pH-sensitive FETs) and enzyme reactors (Jurkiewicz et al, 1998a, b; Gorchkov et al, 1997; Volotovsky et al, 1997; de Gracia et al, 1996; Walcerz et al, 1995; Liu et al, 1995; Vering et al, 1994; Adeloju et al, 1993; Ivnitskii and Rishpon, 1993; Raghavan et al, 1986; Winquist et al, 1984; Gorton and Ögren, 1981; Sundaram and Jayone, 1979; Watson and Keyes, 1976; Guibault and Starklov, 1975) have been thoroughly investigated.

Despite their extensive use in biosensor technology in general, not so many amperometric urease devices have been reported (Cho and Huang, 1998; Trojanowicz et al, 1996; Adeloju et al, 1996; Nikoleis and Siontorou, 1995; Osborne and Girault, 1995;

Rui et al, 1992; Petersson, 1988). As the nature of the hydrolysis of urea generates a variation in the charge density of the sample, other methods such as potentiometry and conductometry have seen more direct application to urea assessment sensors.

Indeed, conductimetric techniques appear as the most growing branch of electrochemical biosensors in the last few years. Their straightforward functioning principle and wide applications have made of them an interesting method to many authors (Lee et al, 2000; Ho et al, 1999;Sangodkar et al, 1996; Sheppard et al, 1996; Shul´ga et al, 1994; Jacobs et al, 1994; Thavarungkul et al, 1991; Mikkelsen and Rechnitz, 1989; Watson et al, 1987).

Other monitoring techniques such as spectrometry (Fahmy et al, 1998; Leszko et al, 1996; Liu et al, 1995; Mascini, 1995; Xie et al, 1991), optical arrays (Arnold, 1987; Opitz and Lübers, 1987; Seifert et al, 1986; Lowe et al, 1983) and thermosensitive systems (Eremeev et al, 1999; Kukhtin et al, 1997; Santhanam et al, 1977) have also been proposed.

5.1.1. Potentiometric urea biosensors

5.1.1.1. Enzyme modified ion-selective electrodes

Different base sensors can be used in the detection of urea through potentiometric ion-selective electrodes. Cation electrodes, measuring NH_4^+; ammonia or carbon dioxide gas electrodes to detect NH_3 or CO_2 generated from side reactions; or pH-sensitive electrodes to monitor pH changes would be applicable.

In the case of pH-sensitive electrodes, variations in the concentration of hydroxide ions are assessed in order to calculate the concentration of urea. In diffusion-controlled potentiometric enzyme electrodes, where the product concentration at the electrode surface depends linearly on the substrate concentration, each mole of urea produces one mole of hydroxide ions. Then, according to the Nernst equation [1.19], the slope of the calibration plot will be a constant equal to 59 mV per urea concentration decade (Blaedel et al, 1972).

5.1.1.1.1. Urease modified ISEs for the detection of ammonium ions

Guibault and Montalvo constructed the first potentiometric enzyme electrode for urea based on a cation selective glass electrode, sensitive to ammonium ions. Further attempts to improve the sensor selectivity led them to change the glass electrode for a silicone rubber-based nonactin ammonium-ion selective electrode as the base sensor (Guibault and Nagy, 1973), and later on to include a three electrode system, (Guibault et al, 1973).

A very successful sensor suitable for urea measurement in blood was developed for Hitachi (Japan) by Tokinaga et al (1984). Two ammonium-sensitive electrodes, one of them covered with immobilised urease, the other acting as reference, were integrated in a Flow Injection Analysis device in a differential circuit. The lifetime of the membrane was reported to be of 2 months and around 60 samples per hour could be analysed.

5.1.1.1.2. Urease modified ISEs for the detection of ammonia or carbonate gas

The above sensors made use of measurements on ammonium ions as the detection technique, but gas sensors for ammonia and carbon dioxide have also been employed to construct urea electrodes. Since these probes were based on gas diffusion through a hydrophobic gas-permeable membrane, no direct interference by sample constituents took place, improving the selectivity of the glass electrode.

A gas sensitive urease ion selective electrode was formed by inclusion of a gas permeable membrane (polyethylene or polytetrafluoroethylene were the most commonly used) between the measuring solution and a flat pH electrode (Stow and Randall, 1973; Anfalt et al, 1973). Under equilibrium conditions the partial pressure of the permeating gas in the measuring solution corresponds to that in the electrode-contacting electrolyte layer. Therefore, a defined relationship exists between the potential of the glass electrode and the concentration of the gas-forming ions, NH_3 and CO_2.

The major drawback of these sensors was their long response time, due to the slow diffusion of the gases. It took several additional minutes to reach a new baseline after

each measurement, so only a few measurements could be processed per hour. Guibault and Tarp (1974) managed to reduce the measuring time to 2-4 minutes per sample by replacing the gas sensitive membrane of the ammonia sensor with an air gap.

The use of polymeric membranes has also been a well- extended practice. Guibault and Mascini (1977) described a highly specific and reproducible enzyme electrode for urea, attaching urease to a Teflon membrane and incorporating it in an ammonia gas electrode. Each sensor was able to perform from 200 to 1000 assays over the concentration range $5 \cdot 10^{-5}$ M - 10^{-2} M.

Kobos et al (1988) described a sensor based on the adsorption of urease on a fluorocarbon membrane, which was then attached to an ammonia-gas sensing electrode. Such a sensor exhibited a sensitivity of 50mV per decade of urea concentration and a response time of 3 minutes.

A further disadvantage of the use of potentiometric gas sensors in the detection of urea was the difference between the optimum pH of the urease as a catalyst and that of the working electrode. While ammonia electrodes operated above pH 8, so that ammonia was favoured as product of side reaction 4.2a, 7 was the optimum pH for the catalysed hydrolysis of urea. High effectiveness in the breaking of urea was needed so that, even though the equilibrium of the conversion of ammonium ions into ammonia was displaced towards the ammonium, enough concentration of ammonia was present in the sample for its measurement.

5.1.1.1.3. Urease modified ISEs for the measurement of pH

As mentioned previously, the increase in pH originated by the hydrolysis of urea could also be monitored as the transduction principle in the design of urea sensors.

Nilsson et al (1973) and Tran-Minh and Brown (1975) described urea sensors based on glass pH-sensitive electrodes in which urease was immobilised around the active sensor tip. Typical response times of the electrodes to the addition of urea was 7-10 minutes and linear ranges of $5 \cdot 10^{-5}$ M – 10^{-2} M were obtained.

Hamann et al (1986) developed another urea sensor based on pH glass electrodes. The sensor reached optimum sensitivity at pH 7.4. Shelf life of 4 - 6 weeks was assured by storing the sensor in a solution containing EDTA.

The mechanically fragile glass electrodes employed in urea sensors have been replaced by antimony (Alexander and Joseph, 1981; Joseph, 1985), iridium (Ianniello and Yacynych, 1983) and miniature palladium electrodes (Szuminsky et al, 1984). Urease has been added to a PVC sheath or cross-linked with glutaraldehyde directly to the sensors surface. Inactivation by heavy metals from the probes could be overcome by addition of EDTA.

A coated wire urea electrode was described by Alexander and Joseph (1981), in which a pH sensing antimony metal wire was covered with a layer of urease. The response time and linear range obtained were 1-2 minutes and $5 \cdot 10^{-4}$ M - 10^{-2} M, respectively. In 1985 Joseph described a similar microsensor which reduced the response time to 30 - 45 seconds.

The signals of the urea sensors working on the principle of ammonia liberation were severely affected by variations in buffer capacity and the pH of the sample. On top of the change in pH due to the hydrolysis of urea, the pH electrode also measured the pH change in the bulk phase generated by sample injection. The implementation of differential measurement techniques eliminated this effect, by introducing a reference electrode with no enzyme on it. Guilbault and Hrabankova (1970) measured urea in blood and plasma by evaluating the difference between the stationary signals of two such sensors and compared their results with those obtained with a spectrophotometric reference method. This principle was further investigated by Vadgama et al (1982) who constructed a measuring device with two high-impedance input amplifiers giving the difference signal directly.

Joseph et al (1985) combined two antimony microelectrodes to form a difference system without a reference electrode, depositing urease on one of the electrodes. The electrode had a response time of only 1 minute and the reported shelf-life was of three months. In this arrangement the response was found to be unaffected by pH.

Kulys et al (1986) also studied urea detection by differential measurements using two antimony electrodes. The urea assay was carried out using a differential amplifier which simultaneously subtracted the potential difference of the enzyme from that of the reference electrode, obtaining a response time of 20 seconds. The linear range of the calibration graph was 0.2 mM – 2 mM, but the sensitivity of the sensor had dropped to 50% after 16 days of operation.

A polymer-modified pH-membrane electrode was developed by Koncki et al (1999) by covalently binding the enzyme directly onto the surface of the polymeric membrane of the pH electrode. The good analytical parameters of this biosensor, mainly its high stability, sensitivity and short response time, allowed its incorporation into a simple double-channel flow injection analysis system, which permitted reproducible urea determination in a millimolar range of concentration (1 mM – 13 mM).

Due to the strong dependence of pH electrodes in general to buffer concentration of the sample, the analysis of blood and serum using this bioanalytical device was reported to be unsuccessful, the main problem connected to the high buffering capacity of blood. Moreover, even though this problem could be overcome by high dilution of the sample, the relatively low concentration of urea in blood would make the sensors useful only for very high levels of urea (over 20 mM).

On the other hand, the bioanalytical system Koncki et al presented was useful for assessment in urine because the high urea content in urine enables dilution of the samples before the test. The reported results of urine analysis using this biosensor-flow injection analysis system were in good agreement to those obtained from an alternative spectrophotometric method.

5.1.1.2. Enzymatic Field Effect Transistors (ENFETs)

With the exception of the urea sensitive ENFET developed by Daniellsson et al (1979), which is based on an ammonia gas-sensitive FET, all ENFETs found in the literature use pH-sensitive ISFETs. Indeed, Danielsson et al (1979) demonstrated the feasibility of

using an ammonia-sensitive PdMOSFET as a transducer in an enzyme transistor using both urease and creatinine deiminase. As for pH-sensitive ISFETs, their operation is based on the consumption of hydrogen ions in the hydrolysis of urease [4.1], previously immobilised in a polymer matrix. Cross-linked albumin, Poly(acrylamide), triacetylcellulose, PVP (poly(4-vinylpiridine-co-styrene) or Nafion are common polymer choices.

5.1.1.2.1. Urease ENFETs for the measurement of pH

Anzai et al (1985) constructed a microsensor for urea analysis by immobilising urease onto the gate of a pH sensitive FET. The sensor showed linear response between 0.5 and 20mM urea, with a slope of 50mV per decade of urea concentration, although the sensitivity of the sensor was drastically reduced in the detection of high urea concentrations, due to the pH rise in the enzyme layer.

Karube and Tamiya (1986) described a similar ENFET which exhibited a response time of 30 seconds and a functional stability of 20 days.

Another urea microbiosensor, based on an iridium oxide electrode, was reported by Suva et al (1986). Under optimum conditions the sensor reached 90% of the steady-state response within 4 seconds after injection of the sample. Despite its detection speed, in 10 mM phosphate buffer the signal for 5 mM urea was very low (3 mV), and the sensor deteriorated rapidly, with a decrease of 20% - 40% in sensitivity after 24 hours.

A dual-gate ISFET chip was normally employed so that one of the FETs could be used as a reference for the ENFET. This auxiliary ISFET, coated with a layer of the immobilising agent without the enzyme, retained the same pH and temperature sensitivity as the ENFET. As both the ENFET and the reference FET responded to fluctuations in the solution potential in the same way, no stable reference electrode was required. Therefore, if the difference between the two drain currents was monitored, the signal would be insensitive to variations in the solution pH, temperature or potential fluctuations, and only pH changes originated within the enzyme membrane by the chemical reaction would be processed.

Nakamoto et al (1987) developed a lift-off photolithographic technique to cover the ENFET gate with the enzyme layer. Extremely fast results were reported, with a response time of only 5 seconds.

Another way of avoiding interferences from variations of pH in the sample solution was by direct feedback of hydronium or hydroxide ions. Van der Schoot and Bergveld (1987a, b) reported enzyme FETs both for urea and glucose, in which the pH within the enzyme layer was kept constant by electrolytic production of H^+ and OH^- ions. This pH-static sensor was also independent of the buffer capacity, showing a extended linear range.

5.1.1.2.2. Urease ENFETs modified with charged polymers

During the last decade substantial research effort has been undertaken to alleviate the main problems associated to pH-sensitive ENFETs. Apart from the dependence of the sensor response on the buffer capacity and pH of the analyte, ENFETs technology has suffered from several other limiting factors: dependence of the sensor response on enzyme immobilisation technology; excessively high value of the detection limit; weak operational and storage stability and a poor control of the membrane thickness due to the individual membrane deposition process. Some of these could be partially solved or at least minimised by applying charged polymeric materials as additional permselective layers on top of the enzymatic membrane, which regulate substrate and product diffusion. Such a practical approach has been employed successfully on urea and glucose-sensitive ENFETs (Volotovsky et al, 1997; Saito et al, 1994; Shul´ga et al, 1992; Soldatkin et al, 1993), obtaining a substantial reduction in the influence of the buffer concentration and an extension in the dynamic range of the sensor response.

Gorchkov et al (1997a, b) cross-linked urease with BSA in saturated glutaraldehyde vapour on the sensor chips and additional Nafion (negatively charged polymer) or PVP (positively charged polymer) membranes were deposited on top of the enzyme membranes by spin coating. Both of them lowered the sensitivity of the sensor response to the buffer and salt concentration and increased that towards urea. The lowest

detection limit was recorded at 0.01 mM urea for Nafion-covered sensors at 10mM phosphate buffer (0.04 mM without additional membrane), and at 0.02 mM urea for PVP at 10 mM TRIS buffer (0.03 mM with no permselective coating).

Another Nafion-covered urease pH-sensitive ENFET was reported by Volotovsky et al (1997) to work as an inhibitor biosensor for heavy metal ion detection. Such sensors measured the concentrations of particular heavy metals by correlating them to the inhibiting effect they produced on an enzyme catalysed reaction. In the case of free urease Ag(I), Hg(II), and Cu(II) were, in that order, the metal ions known to reduce enzyme activity (Preininger and Wolfbeis, 1996). Enzyme immobilisation into a negative charged polymer increased the effect of heavy metal inhibition due to cation accumulation in the polymeric matrix. 50% urease inactivation was observed with 0.2 μM Ag (I), 1.5 μM Hg(II) and 5μM Cu(II).

5.1.1.3. Enzyme Reactors

As mentioned in section 5.1.1.1 a major drawback of potentiometric pH-sensitive devices, both ISEs-based or ISFETs-based, designed to monitor the concentration of converted ammonia in a sample, has been the difference in optimum pH between the optimum pH of the urease as a catalyst and that of the working transducer. Maximum sensitivity of the ion-selective electrode/ISFET was reached when the hydronium concentration in the solution was adequate to ensure maximum conversion of ammonium and carbonate ions into their undissociated forms (NH_3 and CO_2). That is, pH higher than 5 for carbon dioxide and above ten for ammonia was required. On the other hand, urease catalysis of urea was optimum at pH 7. Therefore, for both enzyme electrodes and ENFETs, a compromise had to be found. To obtain optimal conductions for both steps, the enzyme reaction was often separated from the potentiometric indication and a pH change was included between these stages. This set-up was called an enzyme reactor. Three basic types of analytical enzyme reactor have been described (Mottola, 1983): packed bed reactors, open tubular reactors and single bead string reactors.

In packed bed reactors, the enzyme-catalysed reaction was carried out in a column of 100 µl to 10 ml volume. The column was filled with very small particles bearing the immobilised enzyme. The continuous formation of reaction products could be detected either colourimetrically or electrochemically following potentiometric methods. Enzyme carrier materials with advantageous flow behaviour are porous glass with pores of a defined size, organic polymers, like nylon powder, and inorganic polymers.

In open tubular reactors, the enzyme was attached in a monolayer to the inner walls of glass or nylon tubing. Since the loading density was relatively low, for a high degree of substrate conversion a considerable reactor length, up to four meters, may be necessary. Such reactors exhibit better flow characteristics than packed bed reactors and permit a measuring frequency of up to 200 samples per hour.

In single bead string reactors the enzyme was bound both to the reactor wall and to the carrier within the reactor. Compared with open tubular reactors this reactor type provided a higher conversion at lower sample mixing.

Gorton and Ögren (1981) introduced a reactor containing urease bound to porous glass in a flow injection analysis system. The product was detected by means of an ammonia electrode after alkalinisation of the test solution at a rate of sixty samples per hour. No addition of alkali is necessary in a system using a urease reactor and a palladium-iridium-MOS condensator in a flow injection analysis device (Winquist et al, 1985), where the entrapped urease layer was separated from the MOS structure by an air gap. The response time was three minutes and the linear measuring range went from 0.01 mM to 5 mM, but the sensor showed clear signs of degradation after only four days.

Jurkiewicz et al (1998a) designed urease reactors using both open-tube and packed-bed reactors and compared their main characteristics. Measurements were performed with a flow injection analysis system formed by a flow bioreactor and an ammonium-ion sensor represented in figure 5.1.

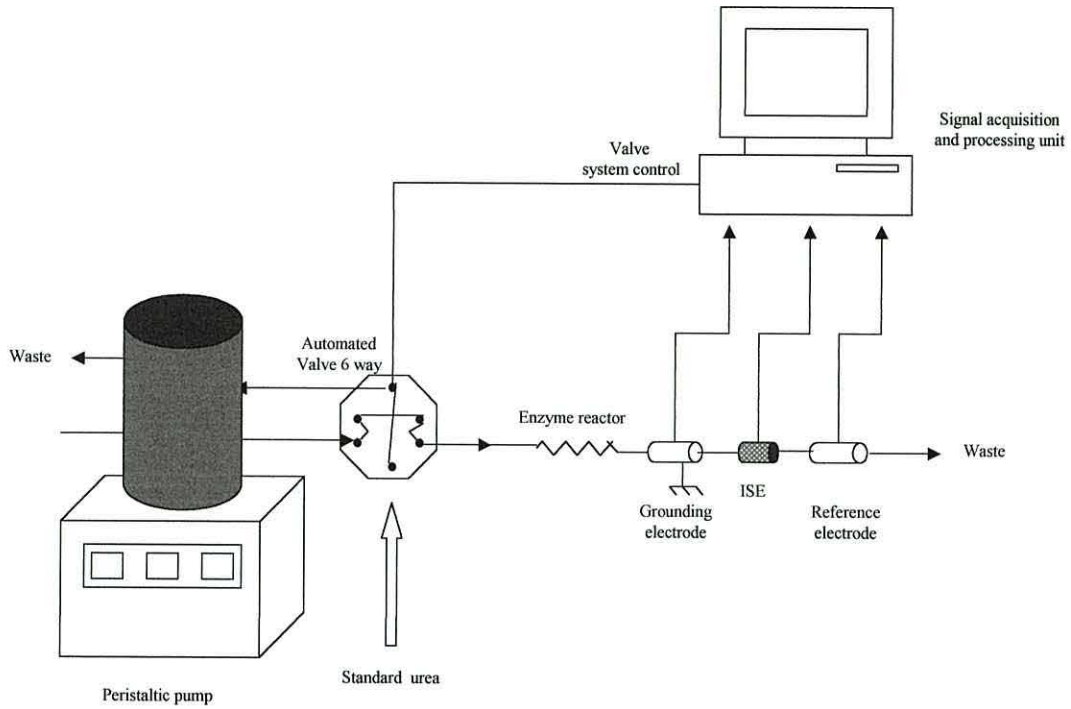


Fig 5.1 Flow injection analysis system (Jurkiewicz et al, 1998).

The carrier buffer solution transported the sample to the enzyme reactor where the substrate was hydrolysed to produce ammonium ions and other non-interfering species. Ammonium ions were then detected by a flow-through solid state ion selective electrode and the potentiometric signal was measured against a reference electrode.

The comparison of the bioreactors working under optimal conditions showed that the packed bed reactors had a higher conversion ratio, a higher sensitivity, a lower detection limit and shorter response times. On the other hand, open-tube reactors exhibited higher reproducibility, higher upper limit of the linearity range, and shorter response times.

Jurkiewicz et al (1998b) also reported a biparametric biosystem for the separate detection of urea and creatinine using flow injection analysis as described above. Urease was covalently immobilised on a nylon open tubular reactor and the resulting ammonium ions detected by means of an ion-selective electrode The analyser was validated off-line by measuring urea from discrete samples from haemodialysis equipment. Results agreed with analyses realised using hospital laboratory methods.

Koncki et al (2000) designed another biosensing system intended to control the haemodialysis progress in analytical clinical processes, allowing monitoring the urea content in real samples. This flow injection analysis system was based on a flow-though ammonium ion-selective electrode and a micro-column containing immobilised urease separated by a gas diffusion module, which prevented potential interfering effects mainly from alkaline cations.

Such biosensors exhibited linear response in the range of urea concentration from 1 mM to 20 mM, being able to analyse around 25 samples per hour. The flow injection analysis system was operating for over three weeks and the results of urea detection in the dialysate were validated using a spectrophotometric reagent kit.

5.1.2. Amperometric urea biosensors

Despite the broad implementation of amperometric techniques in biosensor measuring devices, not many urease amperometric electrodes appear in the literature. Due to the nature of the hydrolysis of urea, potentiometric transducers that monitor the changes in potential due to the generation of ammonium or bicarbonate ions have been the most applied systems in urea detection. But these devices suffer from the inherent limitations of potentiometry: a rather poor response to solutions of low analyte concentration, vulnerability to the interference of other ions present in the sample solution and relatively high detection limit. The advantages of amperometry, such as its greater sensitivity and precision and the lower measurement time, have prompted several research groups to study the adoption of this measuring technique in the assay of urea.

The first amperometric urea electrode developed by Okada et al (1982) consisted of a microbial hybrid sensor where urease and a nitrifying bacteria had been sequentially coupled. Nitrifying bacteria utilize ammonia as an energy source, the respiratory consumption of oxygen being as follows,

$$2 \cdot NH_3 + 3 \cdot O_2 \xrightarrow{Nitrosomonas} 2 \cdot NO_2^- + 2 \cdot H_2O + 2 \cdot H^+ \qquad [5.1a]$$

$$2 \cdot NO_2^- + O_2 \xrightarrow{Nitrobacteria} NO_3^- \qquad [5.1b]$$

Therefore, ammonia liberated by the urease could be oxidized by the bacteria and its consumption of oxygen monitored by an oxygen electrode as described figure 5.2.

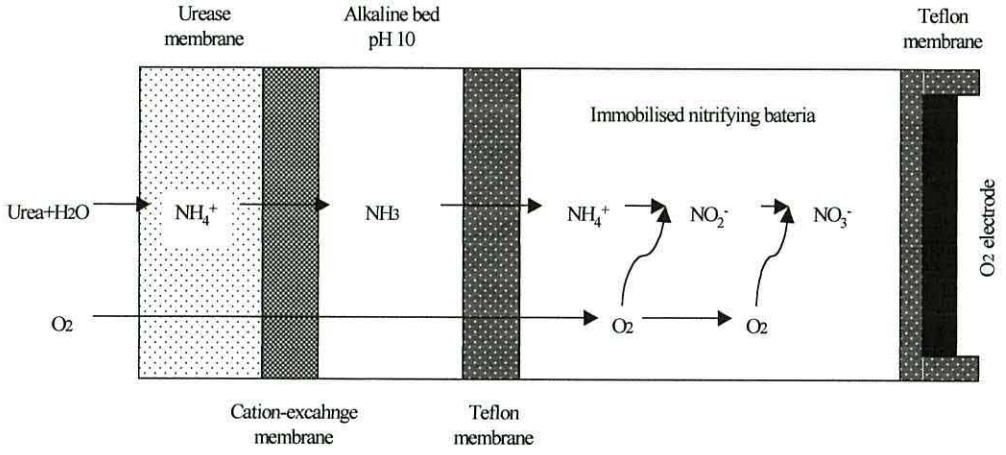


Fig 5.2. Bacterial hybrid amperometric electrode for urea determination (Okada et al 1982).

This multi-membrane configuration ensured that, apart from oxygen and ammonia gas, no substrate was able to diffuse to and be metabolised in the bacterial layer. A linear relationship was observed between the output current decrease and the ammonia concentration for urea concentrations between 2 mM and 200 mM, although it was insensitive to urea concentrations below 2mM and rather slow to respond (7 minutes per measurements). Measurements of ammonia in human urine were performed using the microbial sensor, a biosensor based on optical detection methods and a glass electrode, with good agreement between the three methods. The microbial sensor showed better long term stability.

Another approach to urea detection has been via amperometric indication of a permeant (Ishihara et al, 1985), which determined the change of membrane permeability induced by pH shifts resulting from urea hydrolysis.

Kirstein et al (1985) developed a further method for amperometric urea determination based on the linear dependence of the oxidation current of hydrazine on the concentration of hydroxide ions. Between pH 5 and 9, the anodic oxidation current of hydrazine followed the reaction,

$$N_4H_4 + 4 \cdot OH^- \rightarrow N_2 + 4 \cdot H_2O + 4 \cdot e^- \qquad [5.2]$$

Contrary to potentiometric methods, the calibration curve derived from this technique was linear instead of logarithmic, and the sensitivity of the sensor depended on the initial pH while in potentiometry only the slope of the curve E(log [OH^-]) was constant.

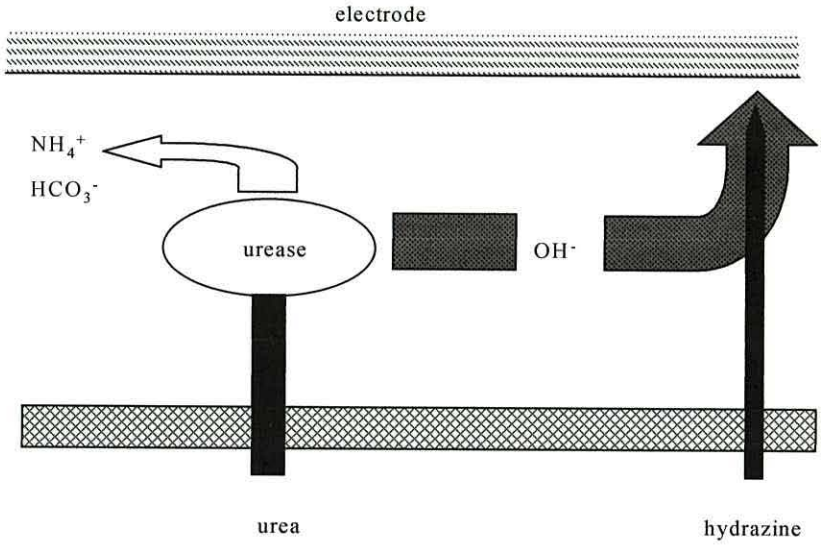


Fig 5.3. Outline of the processes taking place in an amperometric urea electrode based on the pH-dependence of the anodic oxidation of hydrazine (Kirstein et al, 1985).

The response time of this amperometric urea sensor was 7-15 seconds, the sample throughput was 40 per hour using rate assays and the linear measuring range extended from 0.8 mM to 50 mM. Excellent reproducibility was demonstrated by a CV below 1%. However, this pH-sensitive method was, like potentiometric pH electrodes, subject to interference from compounds which may alter the pH and buffer capacity of biological fluids, such as proteins or hydrogen carbonate. In this case, only differential measurements obtained with an auxiliary enzyme-free electrode would give the true urea concentration.

5.1.2.1. Amperometric urea biosensors modified with charged polymers

Cho and Huang (1998) developed a new procedure for urea determination by cross-linking urease onto a polyaniline-Nafion composite electrode which sensed the ammonium ions effectively. Urea molecules diffused towards the electrode surface and underwent the enzymatic reaction to produce ammonium and carbonate ions. The ammonium ions diffused further into the polyaniline-Nafion film and triggered the reduction of polyaniline on the electrode. This process was outlined by the reactions below,

$$\text{urea} + H_2O \xrightarrow{\text{urease}} NH_4^+ + HCO_3^- \qquad [5.4a]$$

$$NH_4^+ + PA^+ \bullet RSO_3^- + e^- \rightarrow PA \bullet NH_4^+ \bullet RSO_3^- \qquad [5.4b]$$

where PA^+ and PA represented the oxidized and reduced forms of polyaniline and RSO_3^- the Nafion skeleton with the immobilised sulphonate groups. Equation [5.4b] described the cation doping process accompanying the reduction of PA at the PA/Nafion electrode. Therefore, whenever an ammonium ion was produced and doped into the PA/Nafion film, the flow of a reduction current would be induced.

Cho and Huang obtained a detection limit of 0.5 μM and a response time of 40 seconds in a flow injection analysis system. The sensors remained in optimal operation conditions for three weeks. No interference of metallic ions (Cu(II), Pb(II), Mg(II) Hg(II)) chlorine, ascorbic acid or uric acid was detected. Both ammonium and urea solutions were tested, obtaining much higher responses for the ammonium solutions. Nevertheless, diluted urine samples were satisfactorily measured, exhibiting results in good agreement with known concentration of urea in urine.

5.1.3. Impedimetric urea biosensors

Although potentiometric and amperometric techniques have been widely applied to the development of analytical devices for the detection, quantification and monitoring of biological analytes, an increasing interest exists in the exploitation of solution impedance as an alternative measuring principle. The monitoring of solution impedance was originally developed as a method of determining chemical reaction rates but, since the beginning of the seventies (Hanss and Rey, 1971; Lawrence, 1971; Lawrence and Moores, 1972), it has been applied to enzyme catalysed reactions. Notably, the urease catalysed hydrolysis of urea has been one of the main objects of study.

Impedance-based sensors have often been criticised because of their lack of selectivity. In order to obtain specificity towards a certain analyte, a differential method has to be applied. Since most of the physiological solutions of interest have relatively high ion content, an accurate measurement relies upon the efficiency of the differential sensor to extract the relatively low analyte-induced impedance changes from the high background conductivity.

5.1.3.1. Impedance urea biosensors based on conductance measurements

The change in conductivity which results from urea hydrolysis was evaluated by Lowe (1986), who prepared conductimetric electrodes by screen-printing techniques. The structure of the electrodes was deposited on a ceramic support by using gold or platinum ink and the printed support was subsequently fired to form metal electrodes. One pair of electrodes were covered by a polypyrrole solution containing urease and the pyrrole was polymerised anodically to give an insoluble film. Extremely small electrode arrays could be produced using this technique. For compensation of non-specific conductivity effects a pair of auxiliary electrodes were covered with pure polypyrrole. The calibration of the device was carried out using appropriately buffered urea solutions to simulate the effect of buffer capacity of the medium.

The same research group described a small-scale electronic urea biosensor based on an urease covered conductivity sensor (Watson et al, 1988). Urease was attached to one

pair of measuring interdigitated or serpentined electrodes in a mixture of BSA and glutaraldehyde.

Conventional ac field was employed as exciting source in order to reduce double layer charging and concentration polarisation at the microelectrode surface. As, for ac signals, the voltage across the terminals of the electrode changes sinusoidally, any build up of charges produced for a positive voltage would be released during the negative part of the cycle and vice versa. For high enough frequencies the alternation of the field would not allow for such accumulation and the ions would only retain traslational motion between the terminals of the electrode.

The output signal was then amplified and taken to a dc converter. A second signal from a reference electrode pair with no urease was taken through analogous steps and the resulting conductivity difference was evaluated.

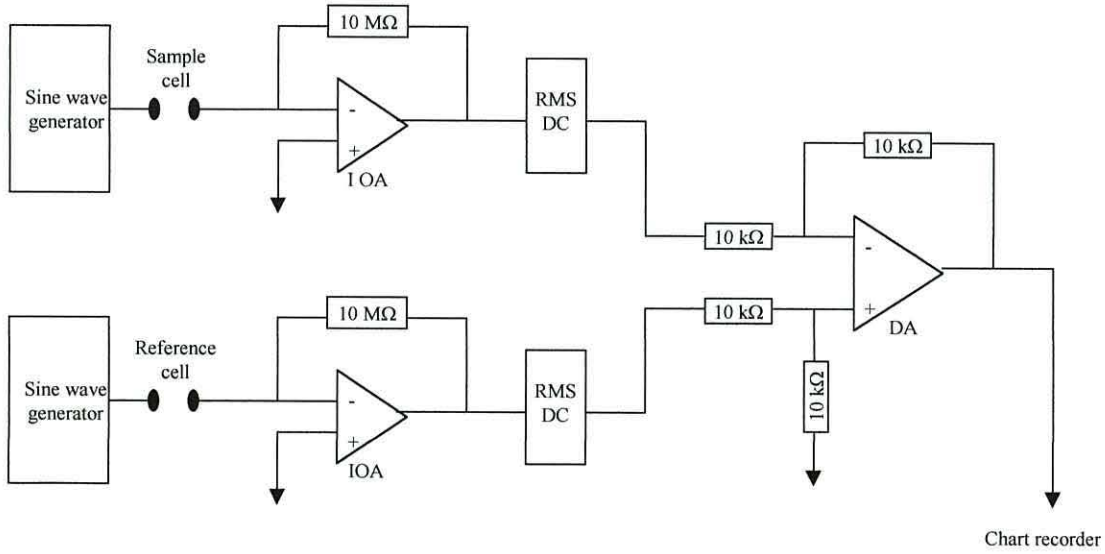


Fig 5.4. Diagram of the differential enzyme conductimeter comprising the sine wave generator, the inverting operational amplifier (IOA), the RMS DC-converter and the differential amplifier (DA) (Watson et al 1988).

The measuring signal obtained showed baseline stability and a linear increase of the output of the instrument over a 5 minutes period, subsequent to the addition of urea. The responses to urea concentrations within the range 0.5 mM to 10 mM were reproducible with a 1% of error. Diluted 25-fold serum samples were also measured showing a linear

relationship between the urea concentrations determined with the microelectronic device and those obtained through standard clinical procedures.

This first reported conductimetric urea biosensor encountered two main sources of error: weak precision in the conductance measurements made and the interference of temperature effects. Errors in conductance measurements were minimised experimentally by tuning the low sine generator to eliminate capacitive effects and by applying a very small amplitude signal to ensure negligible electrochemical interferences.

Mikkelsen and Rechnitz (1989) also developed a urea biosensor based on conductimetric transducers in order to compare it with the one developed by Watson et al (1988) and with potentiometric urea sensors in general. The sensing surface consists of four parallel electrodes, two at the outside and two in the inside of a silicone encapsulant. These were connected to the two external leads so that the polarity alternated across the sensor in order to maximise the resulting cell constant of such configuration.

Urease was immobilised at the surface of the conductimetric transducer by cross-linking it with glutaraldehyde and BSA, or by covalently linking the enzyme to a collagen membrane which was then held tightly over the surface of the transducer with a rubber O-ring. The sensors showed a linear dependence of the response with concentration of urea over the range 0.01 mM to 1.26 mM and the detection limit was established at around 10^{-5} M urea.

The effect of ionic strength on the sensors response was also investigated using pH 8 glycine buffer at 5 mM and 50 mM. The detection limit was about one order of magnitude lower for the 5 mM buffer (0.18 μM - 1.6 μM urea) and the sensitivity about double (19.2 nA/μM - 8.8 nA/μM). However, the response time was shorter for the 50 mM buffer, especially at low urea concentrations.

The model enzyme urease was used in the further development of conductimetric electrodes by Lawton et al (1989). They based their sensor on findings derived from mass-spectrometry studies of the hydrolysis of urea by urease. Under normal pH

conditions, ammonium ions generated during the hydrolysis of urea seem to be retained within the urease molecular structure. Therefore, the reaction could be monitored directly as a result of the increased dielectric polarisability associated with the generation of electroactive ammonium ions within the enzyme structure.

Their conductimetric sensor was constructed by cross linking urease with glutaraldehyde and BSA and depositing the mixture onto the surface of an interdigitated microelectrode array. Such immobilised urease electrodes were immersed in buffer solutions containing different concentrations of urea, and the changes produced in their electrical impedance were monitored. At stable temperature, the sensor could detect down to 0.2 mM urea in solutions of physiological ionic strength. A second system was designed in order to check the selectivity of the conductimetric sensor by depositing urease around, but not covering, the electrodes. No changes in conductance were recorded for such sensors when in contact with urea solutions , indicating that the response of the device was due to electrical changes taking place within the bulk of the immobilised urease film, and was not due to interferential conductivity increases in the surrounding solution.

5.1.3.1.1. Monitoring of urea in blood during dialysis treatments

One of the fields of most direct application of urease-based sensing techniques is the monitoring of urea in blood during dialysis treatments. Each treatment takes several hours during which the concentration of urea in blood decreases from 20 mM - 50mM to below 10 mM with the loss of other low molecular weight compounds. Simple on-line monitoring of urea during dialysis is essential to control its performance and therefore to run the treatment under optimum conditions. Thavarungkul et al (1991) reported the application of a sample handling unit previously used for blood glucose monitoring in combination with a biosensor based on immobilised urease and conductivity measurements. Figure 5.5 shows a diagram of the basic principle of the analytical system employed.

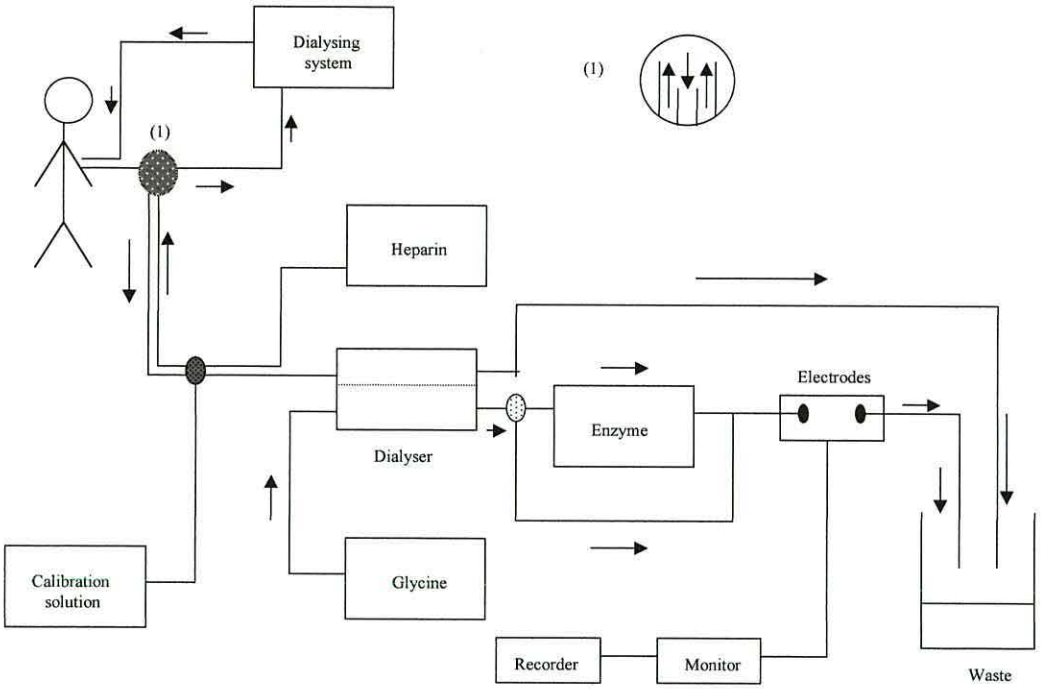


Fig 5.5. Analytical system for continuous monitoring of urea in blood during dialysis (Thavarungkul et al, 1991)

Blood was mixed with heparin in order to prevent blood clotting, following standard clinical procedures. The sample was fed to a dialyser and urea molecules diffused through its membrane into a buffer solution. This solution was either pumped into an enzyme column filled with urease cross-linked onto porous glass, and then to a conductivity electrode, or bypassed the column and directed to the electrode. By comparing the conductivities of both solutions the amplitude of the conductivity change could be quantified.

Thavarungkul et al (1991) described the use of such an analytical system to monitor a patient undergoing dialysis by connecting the sampling probe to the blood circulating tube of the dialysis machine. The responses to urea concentration in blood were compared with off-line analysis carried out by the hospital laboratory and showed excellent agreement. The device showed linear responses from 5 mM to 50 mM urea and very good reproducibility for up to 30 hours of operational time.

The one drawback reported for this device was the slow response time of the system. Since a continuous blood stream was withdrawn the sampling unit had to operate with

very small volumes (around 6 ml of blood per hour) and the slow pumping of blood delayed the sample handling, requiring approximately 6 minutes for full response of the enzyme. Nevertheless, if faster procedures were needed, the system could be modified by changing the pump speed and using appropriate dimensions of the tubing.

5.1.3.1.2. Thin-film conductimetric urea biosensors

Shul'ga et al (1994) described the fabrication and characterisation of a thin-film conductimetric biosensor for urea determination. Each sensor chip contained two pairs of identical gold interdigitated electrodes of sensitive area 1 mm × 1.5 mm. The overall chip lay out and a block diagram of the measurement system are described in figure 5.6.

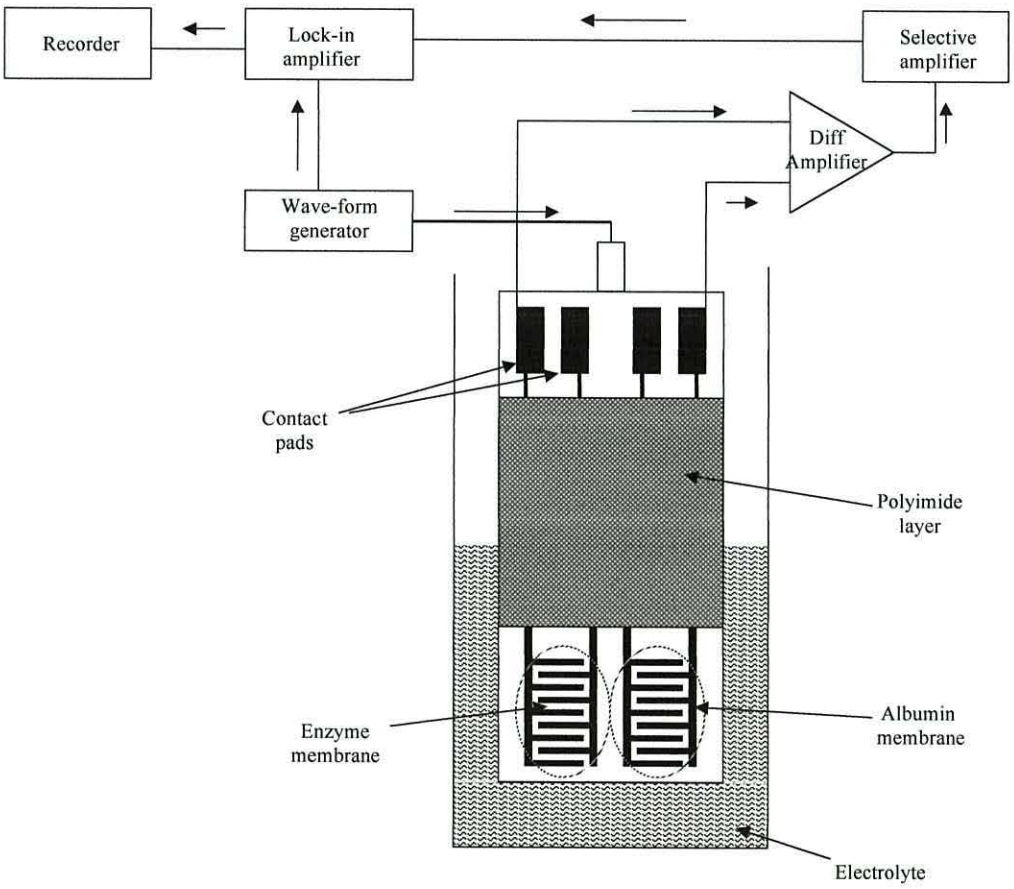


Fig 5.6. Conductimetric chip layout and measurement system for urea determination (Shul'ga et al, 1994).

Urease was cross-linked onto the working pair of electrodes with BSA and glutaraldehyde and a similar membrane with the enzyme substituted by albumin covered the reference pair of electrodes, so that the biosensors characteristics could be recorded by measuring the differential signal of the enzyme electrodes to the reference pair. A 0.1 mM – 5 mM urea linear range was obtained from the calibration curves at 5 mM buffer, in good correlation to previous work of the same authors on urea field effect transistors (Shul´ga et al, 1992).

The magnitude of the steady-state response and the response time of the biosensors appeared to be strongly dependent on the buffer capacity of the solution, which was a problem if the sensors were intended to perform measurements under physiological conditions. The observable decrease in the sensor response was approximately three fold in the interval 1mM to 40 mM buffer concentration.

Another thin-film differential conductivity urea sensor was described by Jacobs et al (1994) using gold electrodes platinised electrochemically. The basic transducer consisted of two identical interdigitated conductivity cells forming a differential pair. The working electrode was covered by a urease membrane which was cross-linked to its surface using a mixture of BSA and glutaraldehyde and the neutral membrane which covered the reference electrode only contained BSA and glutaraldehyde.

The performance of the sensor was characterised by its differential response in terms of a common-mode rejection ratio, which resulted from the extraction of changes between the working electrode signal and the reference one. A conductivity change taking place at the active conductivity cell resulted in a differential output. If, on the other hand, the same conductivity change was applied at both working and reference electrode cells, it produced a common-mode output. The common-mode rejection ratio was therefore defined as the ratio of the differential to the common-mode output for a fixed conductivity.

Urea tests were carried out under temperature controlled conditions and the sensors showed a linear response from 2 mM to 9 mM.

5.1.3.1.3. Model conductimetric urea biosensors

Sheppard et al (1996) developed a model for predicting the response of a conductimetric urea biosensor by combining an analysis of the diffusive transport and enzymatic hydrolysis of urea in the vicinity of the sensor surface with an electric field model for calculating interelectrode impedance. Urea biosensors based on interdigitated electrode arrays were designed and tested in order to validate the model.

The interdigitated electrodes were coated with platinum black electrochemically and masked with polyimide. Figure 5.7 shows an illustration of the impedimetric urea sensor probe.

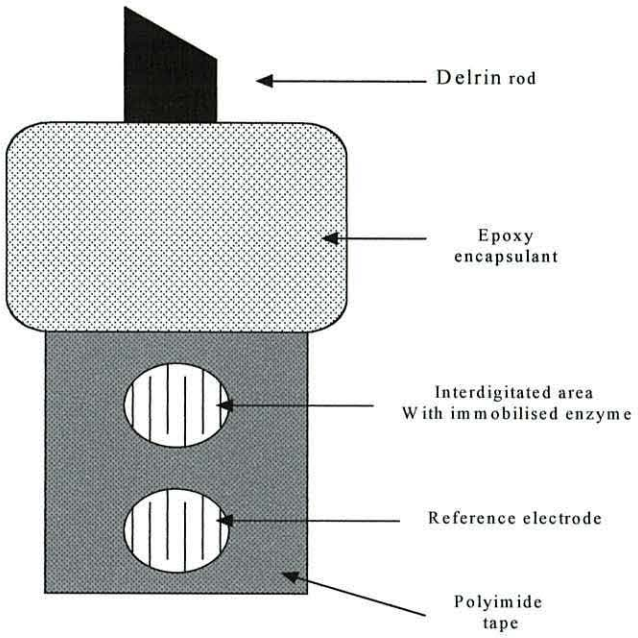


Fig 5.7 Impedimetric urea sensor probe (Sheppard et al, 1996)

Urea tests were carried out through sensor immersion in the urea solution contained in a thermostatted cell at 25 °C. The concentration range studied went from 10 μM to 5 mM urea. At any given concentration, Sheppard et al (1996) reported a logarithmic increase in conductivity with time. That is, the conductivity increased most rapidly upon immersion in the urea solution, and increased less rapidly as time evolved. The

magnitude and speed of the response obtained was directly proportional to the urea concentration.

Whole ranges of urea concentration were consequently repeated up to three times, with no statistical significant variation in responses over the 24 hours period required to perform such experiments. Model predictions fell in good agreement with experimental results.

Lee et al (2000) developed a conductimetric urea biosensor by immobilising urease on a screen printed platinum interdigitated array electrode. Sol-gel chemistry was used as an alternative immobilisation method. This biosensor was designed in a differential format consisting of a working electrode containing urease and a control electrode with bovine serum albumin only. By using the differential signal between the two it was possible to minimise interference effects from other ionic species present in complex biological samples.

Impedance measurements were recorded for urea tests which consisted of the step addition of 1 M urea stock solution to the test solution, until reaching a final concentration of 30 mM urea. A 90% response was obtained in approximately 8.3 minutes and the steady-state response was reached 16.5 minutes after the injection of the urea solution. A relative standard deviation of 4% was reported for electrode-to-electrode reproducibility, and in the performance of a single sensor to successive urea determinations. As for long term storage stability, the urease electrode retained 63% of its original activity after 25 days of intermittent testing of 1 mM urea standard solutions. The authors attributed the activity loss to the denaturation of the immobilised urease and proposed to overcome it by co-immobilising a stabiliser, such as a polyalcohol derivative, in the sol-gel mixture. For the studied urea concentration range (0.03 mM-30 mM), the linear dynamic response interval of the biosensor extended for two orders of magnitude, from 0.03 mM to 2.5 mM urea.

The dependence of the response on buffer concentration and pH was also investigated. Buffer solutions at 1 mM, 5 mM and 50 mM concentration were tested, obtaining a significant decay in sensitivity and an increase in response time and detection limit for the highest buffer concentration. As for the pH effect on the performance of the sensor,

a peak value was obtained between 7 and 7.5 pH units, coinciding with the optimum pH of urease activity.

Finally, Lee et al applied the newly developed biosensor to urea determinations in serum samples, comparing their result with a commercially available blood urea nitrogen test kit based on UV-visible spectrophotometry. Serum samples were diluted 24fold and the differential admittance recorded was converted to urea concentration by reading the value from the calibration graph. The values showed good agreement with those measured with the commercial kit.

5.1.3.2.Impedance urea biosensors based on capacitance measurements

Enzyme-catalysed polymer transformation coupled with electrochemical AC impedance detection has also been employed to design a urea sensor capable of detecting urea in serum samples (McNeil et al, 1995; Krause et al, 1997; Ho et al, 1999). Mc Neil et al (1995) presented an impedance sensor based on the measurement of the change in capacitance of an enzyme-catalysed breakdown of polymer coated screen-printed gold ink electrodes. The action of urease on urea, generating ammonium and hydroxide ions, produced a specific change in pH which resulted in polymer breakdown (commercially available polymer Eudragit S-100 was used) and a four orders of magnitude change in capacitance in the presence of excess of urea.

The same research group presented further advances (Ho et al, 1999) in the type of polymer used and the electrode design, towards a simple, integrated, disposable sensor design for clinical use. The polymer used was a modification of poly(methyl vinyl ether)/maleic anhydride, stable at pH 7.4 and liable to breakdown at more alkaline pH, which had been previously employed as a method to control drug release through polymer dissolution due to an increase in pH (Heller et al 1978).

Ho et al used screen-printed carbon electrodes onto a PVC substrate.

The polymer was then screen-printed onto the interdigitated electrodes covering their entire working area. Finally absorbent pads were soaked in urease solution, allowed to

dry and laid over the surface of the polymer coated electrodes. Figure 4.8 shows the arrangement of the electrode system.

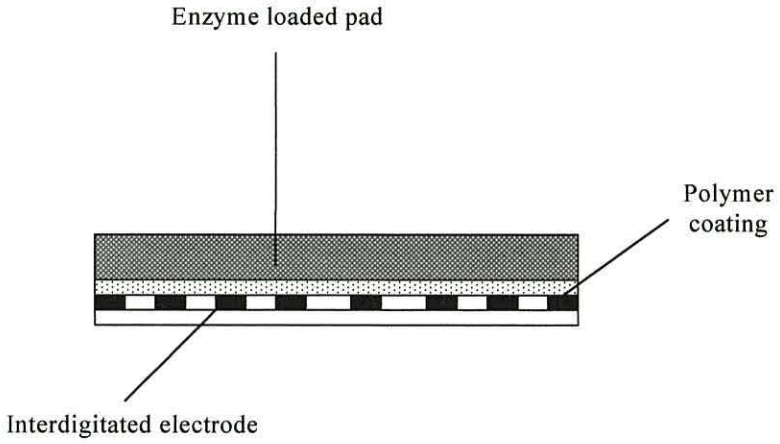


Fig 5.8. Arrangement of the electrode system (Ho et al, 1999).

Urea assays were carried out both in real serum samples and in control serum standard solutions. A calibration plot was produced using the control serum spiked with known concentration of urea from 5 mM to 30 mM.

Each urea assay was characterised by an increase in the capacitance of the polymer coating with time due to its transformation. Equilibrium capacitances were reached within 10 minutes for all urea concentrations employed. The sensor reached its upper detection limit at 30 mM urea, where the final capacitance value approached that of the bare electrode, showing that the polymer had fully broken down. The relationship between the capacitance and the urea concentration remained linear up to 30mM urea concentration.

Additionally, serum samples with and without added urea were tested and their urea concentrations determined by comparison with the calibration plot. These calculations were again compared with the results from a clinical laboratory showing good agreement.

5.1.4. Other urease-based biosensors

Although electrochemical methods are widespread in biosensors technology, a number of alternative transduction techniques have also been reported for urease-based detection of urea. Despite the extensive applications of spectrometric designs to the investigation of the activity of urease and the kinetic performance of the catalysis of urea, optical and calorimetric methods have aroused the most interest in the direct detection of urea.

5.1.4.1. Optical biosensors

5.1.4.1.1. Optical urea biosensors for the measurement of pH

Considerable effort has been devoted to the development of optical pH sensor suitable for continuous in vivo measurement. These pH sensor could subsequently be linked to a urease membrane in order to detect urea by monitoring the change in pH produced by its conversion to ammonium and carbonate ions.

Various indicators have been employed. Sensor based on phenol red (Peterson et al, 1980) measured pH to 0.01 pH units in the range from 7.0 to 7.4. It was stable with respect to calibration for hours and it responded exponentially to changes in pH with a response time of 0.7 minutes to reach 63% of the final value. The trisodium salt of 8-hydroxy-1,3,6-pyrenetrisulfonic acid (HTPS) has been a fluorescent indicator that attracted considerable attention. Wolfbeis et al (1983) reported its outstanding suitability for physiological pH measurements.

Lowe et al (1983) have developed an optoelectronic urea sensor based on the pH indicator bromothymol blue coimmobilised with urease on a transparent cellulose membrane. The measurement signal increased linearly with time within a few minutes after sample addition. The difference between the signals obtained one and two minutes after sample addition was evaluated by using a sample-and-hold differential amplifier. The linear range extended up to 10mM urea.

Optical sensors for pH have a working range of 1 to 2 pH units centred around the pK_a of the indicator. Although there has been considerable effort directed towards finding

indicators that are suitable for the physiological pH range, relatively little work has been performed trying to extend the pH response range of such indicators apart from development of a series of immobilised colorimetric indicators that work at a variety of pH, which has been evaluated for reflectance based pH sensing (Kirkbright et al, 1984).

5.1.4.1.2. Optical urea biosensors for the detection of CO_2

Urease modified optical CO_2 sensors can also be employed as alternative transducers to standard electrochemical methods in urea detection. An optical CO_2 sensor is constructed by placing a pH sensor in contact with a bicarbonate solution and covering it with membrane permeable to carbon dioxide, such as silicone. The carbon dioxide partial pressure determines the concentration of carbonic acid in the internal filling solution therefore establishing the pH of the carbonic acid/bicarbonate buffer system. The range of carbon dioxide partial pressures that can be measured depends on the bicarbonate concentration of the filling solution and on the pH sensitivity of the optical pH sensor. Optical sensors that respond to physiological pHs are suitable as the internal sensing element for sensors that respond to carbon dioxide partial pressures within the range of physiological interest. Therefore, optical pH sensors that have been extensively evaluated for physiological applications have also been used as the internal sensing element in optical carbon dioxide sensors (Vurek et al, 1983; Zhujun an Seitz, 1984; Lübbers and Optiz, 1983).

Despite being based on optical pH sensors, carbon dioxide sensors are actually simpler than these. Because the internal filling solution is isolated from the sample by a hydrophobic gas-permeable membrane, variations in the ionic strength of the sample are no longer a problem. Furthermore, the indicator can be dissolved in the internal filling solution rather than having to be immobilised on a solid substrate. The major problem in developing optical carbon dioxide sensors is to achieve a short response time, since the time to reach equilibrium in the internal filling solution is added to the intrinsic response time of the pH sensor. A solution to this problem could be to reduce the distance that carbon dioxide molecules have to diffuse in solution in order for the device to reach equilibrium.

5.1.4.2. Calorimetric biosensors

Enzyme thermistors and other thermal bio-analysers are based on the measurement of the heat of reaction as a general detection system. Enzyme reactions are accompanied by a considerable heat evolution, generally in the range of 25 kJ/mol to 100 kJ/mol, which makes enzyme calorimetry a very versatile technique. Urea hydrolysis catalysed by urease in phosphate buffer at pH 7.5 is characterised by a molar enthalpy change of –61 kJ/mol, for instance. The lack of specificity due to the general detection principle is compensated by the use of highly specific immobilised biocatalysts, such as enzymes. As the sensing enzymes are located proximal to the thermal transducer, similar advantages to enzyme electrodes appear: repeated enzyme usage, high sensitivity, fast response time and possibility of continuous flow operation.

Several research groups have attempted to develop simple and inexpensive calorimeters for routine use with immobilised enzymes. A small volume calorimeter, in which the enzyme was attached to a thin aluminium foil placed on the surface of a Peltier element as a temperature sensor (Pennington, 1976) was one of the first instruments developed. A drop of the sample was applied on the enzyme layer with the amount of substrate detected as a very small temperature change. The sensitivity, however, was poor and continuous flow operation was not feasible.

A more direct approach was used in the thermal enzyme probes, TEPs (Cooney et al, 1974; Mosbach et al, 1974; Weaver et al, 1976) in which the enzyme was directly attached to a thermistor. Unfortunately the sensitivity was low, as most of the heat evolved in the enzyme reaction was lost to the surrounding solution without being detected by the thermistor.

Considerably more effective detection of the reaction heat was achieved with systems employing small columns with enzyme bound to support particles, as in the enzyme thermistor (Mosbach and Danielsson, 1974; Mosbach et al, 1975) and in the immobilised enzyme flow-enthalpimetric analyser (Bowers et al, 1976). The combination of a flow-enthalpimeter of commercial design with a thermostated, immobilised enzyme column has also been described (Kiba et al, 1984). In these cases

the heat was transported by the liquid passed through the column to the temperature sensor that was mounted at the top of the column or at its outlet.

The possibility of continuous analysis was an additional advantage when using flow-through arrangements. Most of these closely related devices have rather similar performances. As established from reactions of known enthalpy, as much as 80% of heat evolved in such semi-adiabatic instruments could be registered as a temperature change (Danielsson et al, 1979). For a given substrate present at a concentration of 1 mM and with a molar enthalpy change of 80 kJ/mol, a peak height corresponding to 0.01 °C or higher will be expected, and a temperature resolution of 10^{-4} °C is required to give 1% accuracy in the measurement. As most enzymatic reactions are accompanied by considerable heat production (25 kJ/mol – 100 kJ/mol), measurements of concentration as low as 0.1 mM should not present any problems.

The design of the enzyme thermistor was based on a semi-adiabatic capsule which would isolate the active body of the enzyme containing sensor. Figure 5.9 shows such a design of an enzyme thermistor described by Danielsson et al (1981).

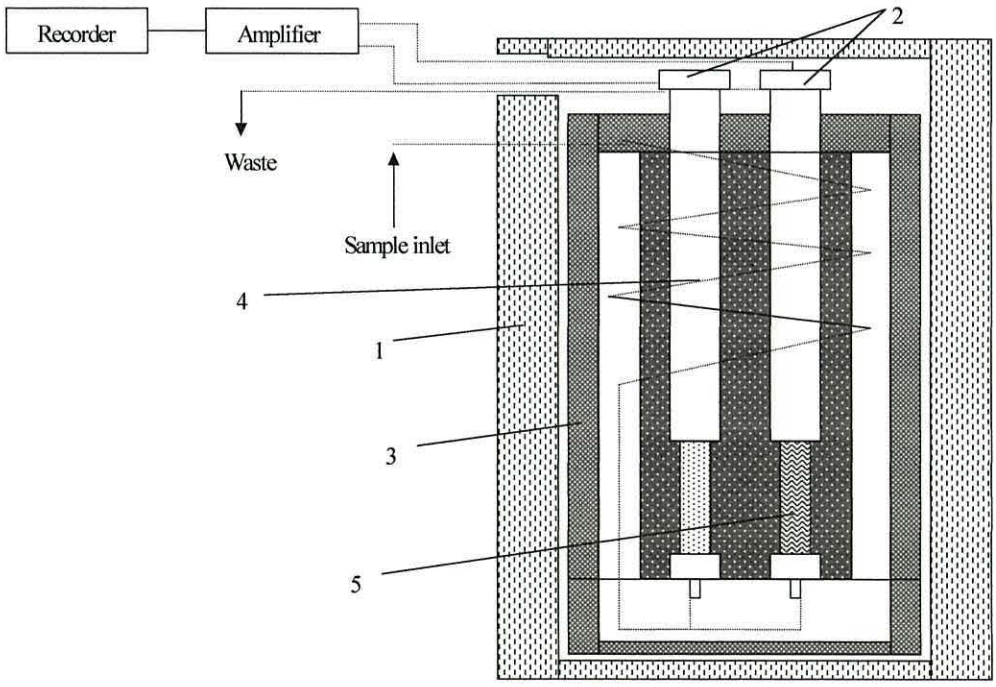


Fig 5.9. Cross –section of an enzyme thermistor in a typical set-up: (1) polyurethane insulation, (2) plexiglas tube/thermistor probe for column insertion, (3) thermostatted aluminium cylinder, (4) heat exchanger, (5) enzyme column with thermistor attached. (Danielsson et al 1981).

Most of the heat produced in the enzyme column was transported out of it by a flow stream and the temperature at the outlet of the column was measured and compared to that recorded by a reference thermistor for differential measurements.

The enzymatic reaction generated a temperature peak, the height of which was taken as a measurement of the substrate concentration. The linear range of the relationship between temperature peak height and substrate concentration was at least 10^{-5} mM to 10^{-1} mM when not limited by reactant concentrations or other factors.

Several routine methods based on immobilised urease for the determination of urea in serum have been described (Tran-Minh and Vallin, 1978; Rich et al, 1979; Bowers et al, 1976; Fulton et al, 1980; Danielsson, 1976). A remarkable wide linearity range was achieved by these authors, typically from 0.01 mM to 200 mM (Danielsson, 1976). Since serum samples were diluted ten times, most samples fell within the 0.3 mM to 10 mM range. Outstanding reusability and time stability was also reported, the same enzyme column could be used for several months for several hundred serum samples. The time required per sample (about two to three minutes) was acceptable for a short series of samples and the precision was characterised with a relative standard deviation of about 1%.

Thermal techniques have also be applied to environmental control analysis. If detection of a specific toxic compound is required, a limiting amount of an enzyme that is inhibited by such compound can be immobilised in the enzyme column of an enzyme thermistor. The concentration of heavy metals, for instance Hg^{2+}, could be determined due to the inhibitory action on immobilised urease (Danielsson et al, 1981). The heavy metal concentration of a sample was determined by comparison of the temperature response to a substrate pulse containing an excess amount of urea before and after introduction of the sample.

5.2. Creatinine biosensors

Creatinine is the final product of creatine metabolism in mammals and has clinical diagnosis importance for being an indicator of muscular, thyroid and renal disfunctions (Kim et al, 1999). Testing its clearance offers an index of renal glomerular filtration rate and a quick and simple biomedical detection of acute myocardial infarction (Khan and Wernet, 1997). For the diagnosis of kidney function, creatinine determinations are increasing in popularity over urea measurements, since the creatinine level is not affected by a high protein diet or metabolic rate, as in the case of urea.

The reference ranges of creatinine concentration in serum/plasma and 24 hour urine are 35 μM to 150 μM and 8.84 mM to 13 mM respectively but during kidney disfunction or muscle disorder the creatinine concentration in serum/plasma could rise to values higher than 1000 μM (Khan and Wernet, 1997; Jurkiewicz et al, 1998a). In clinical practice, there are two significant decision levels: values above 140 μM indicate the necessity of performing other tests for assessment of renal function such as the creatinine clearance test; values above 530 μM are almost invariably associated with severe renal impairment (Madaras and Buck, 1996).

Most of the creatinine determinations in clinical laboratories and almost all the commercially available analysers use spectrophotometric procedures based on the Jaffe reaction (Jaffe, 1986) in which the active methylene group reacts with alkaline sodium picrate to give a red-yellow complex. This method is simple and inexpensive but the reaction is not specific for creatinine and a large number of substances present in physiological fluids may interfere in this assay. Hence samples must be treated in advance to eliminate or minimise interferences. These pre-treatments include adsorption (Hare, 1950; Taussky, 1956), extraction (Taussky, 1956), dialysis (Chasson et al, 1961), use of enzymes (Masson et al, 1981) and kinetic measurements (Araujo et al, 1995; Czervionke et al, 1985; Cruickshank et al, 1988; Hardeman and Backer, 1991). These previous treatments complicate the analytical method and may also reduce the accuracy and increase the cost.

The combination of the creatinine-converting enzymes with sensors indicating primary reaction products, such as ion selective electrodes, ammonia gas sensors or thermistors, is a faster, simpler and a more specific alternative for creatinine determination. Some of the methods reported in the literature are included below.

5.2.1. Potentiometric creatinine sensors

5.2.1.1. Ion selective electrodes

As introduced in section 1.3.1.1, a potentiometric enzyme modified ion-selective electrode is formed by immobilising an enzyme membrane onto an ion-selective electrode, ISE, which results in a highly selective and sensitive method for the determination of a given substrate. A diagram of such a device is shown in figure 1.4.

Their operation principle is based on the variations in charge density and in membrane potential caused by the dissociation of groups contained in the sensitive membrane. Therefore, the sensing membrane generates a potential difference which is measured relative to the reference under conditions of zero current flow. The concentration of the dissociating analyte and the membrane potential are logarithmically related according to the Nernst equation [1.19].

Potentiometric methods require fast electrode kinetics in order to work under local thermodynamic equilibrium when the analyte measurement is taking place. This reduces dramatically the number of transducers that can be employed as indicator electrodes. On the other hand, analyte diffusion is not important and they make use of simple, inexpensive and easily available instruments.

Depending on the creatinine-converting enzymes employed in the assay, different base sensors can be used in the detection of creatinine through potentiometric ion-selective electrodes. Cation electrodes, gas electrodes and pH-sensitive electrodes are very common choices when using creatinine deiminase (creatinine iminohydrolase EC 3.5.4.21), the coupling of creatininase (creatinine amidohydrolase EC 3.5.2.10), creatinase (creatine amidinohydrolase EC 3.5.3.3) and urease (urea amidohydrolase EC

3.5.1.5), or the system formed by creatininase, creatinase and sarcosine oxidase (EC 1.5.3.1).

Thompson and Rechnitz (1974) described the first enzyme modified ion selective electrode for the detection of creatinine as a diagnostic indicator of kidney function. Their rudimentary method of attaching unpurified creatininase to an ammonium probe gave positive but not very accurate results.

In an attempt to improve efficiency, Meyerhoff and Rechnitz (1976) developed a potentiometric creatinine sensor by inclusion of creatinine deiminase between the gas-permeable membrane of an ammonia electrode and a dialysis membrane. The resulting sensor was kinetically controlled and reacted to addition of the enzyme activator tripolyphosphate by an increase in sensitivity from 44 mV to 49 mV per concentration decade and a corresponding decrease of the detection limit.

A few years later, Guibault et al (1980) used creatininase immobilised onto alkylamine glass beads packed into a stirrer and an ammonia electrodes as sensor. The residual ammonia level of blood serum was lowered using several removal techniques.

The same research group (Guibault and Coulet, 1983) achieved the sensitivity required for the assay of physiological creatinine concentrations by using creatinine deiminase. The enzyme was attached to collagen and the resulting membranes were fixed to an ammonia probe. The sensor was stable for 20 days or 100 determinations. The electrode potential was proportional to the logarithmic values of creatinine concentration over the range of 1 mg/l to 100 mg/l.

5.2.1.2. Enzyme reactors

The applicability of potentiometric enzyme modified ion-selective electrodes may be severely restricted by disturbances caused by sample constituents interfering with the binding or enzymatic conversion of the analyte, such as inhibitors or alternative substrates. In particular, for biosensors using coupled enzyme reactions, the substrates of each reaction can interfere, decreasing selectivity.

Such interference can be compensated by using difference measurements, which usually require a reference transducer. An alternative is the use of enzymatic anti-interference systems containing enzymes in front of the sensor that catalyse the conversion of the disturbing agent into innocuous products. Such systems have been applied to analytical enzyme reactors working in conjunction with simple ion selective electrodes and modified field effect transistors.

Mascini et al (1985) employed creatinine deiminase immobilised on the inner wall of nylon tubing for the determination of creatinine in serum. The reaction product, ammonia, liberated in the enzymatic reaction was indicated at an ion selective electrode. Owing to the low sensitivity of the indicator electrode, the linear range was only from 0.01 to 0.2 mM.

In order to avoid signal superposition by endogenous ammonia the sample was first mixed with α-ketoglutarate and pumped through a tube containing immobilised glutamate dehydrogenase. The tube was able to remove 98% of endogenous ammonia present in human blood and urine samples in 50 seconds according to the reaction

$$NH_3 + H^+ + NADH + \alpha - ketoglutarate \rightarrow L - glutamate + NAD^+ \qquad [5.5]$$

The same reaction system, immobilised on porous glass, was used by Winquist et al (1986). The authors introduced a second reactor containing immobilised glutamate dehydrogenase upstream of the creatinine deiminase reactor. The enzyme was coupled in a flow system with an ammonia gas-sensing MOS transistor separated from the aqueous solution by means of a gas-permeable membrane. The detection limit of this system was 0.2 μM, so strongly diluted plasma and urine samples could be tested, and the rate was of fifteen samples per hour. Up to 0.2 mM of endogenous ammonia could be completely removed in the glutamate dehydrogenase reactor.

Jurkiewicz et al (1998a) developed a flow injection analysis system capable of carrying out the continuous and simultaneous determination of urea and creatinine in an automated way. Such a FIA system was an extension of an urea analyser previously reported by the same group to which an additional channel had been implemented. This

channel included a packed-bed reactor containing creatinine deiminase covalently immobilised on controlled-pore glass beads.

The analytical biosystem was initially tested off-line using samples of effluents from haemodialysis equipment. Following this, the analyser was tested in situ and on-line using effluents from haemodialysers measuring initial urea and creatinine concentration in plasma. As the device was aimed at patients with renal dysfunction, the interference of endogenous ammonia was found to be negligible and therefore no system for its elimination or compensation was required.

The analytical biosystem employed is a modification of the one the same authors employed for urea detection and which is described in figure 5.3. Both the uni-parametric urea detector and this bi-parametric device follow the same operation principles. The two channel flow injection system for urea and creatinine is depicted in figure 5.10.

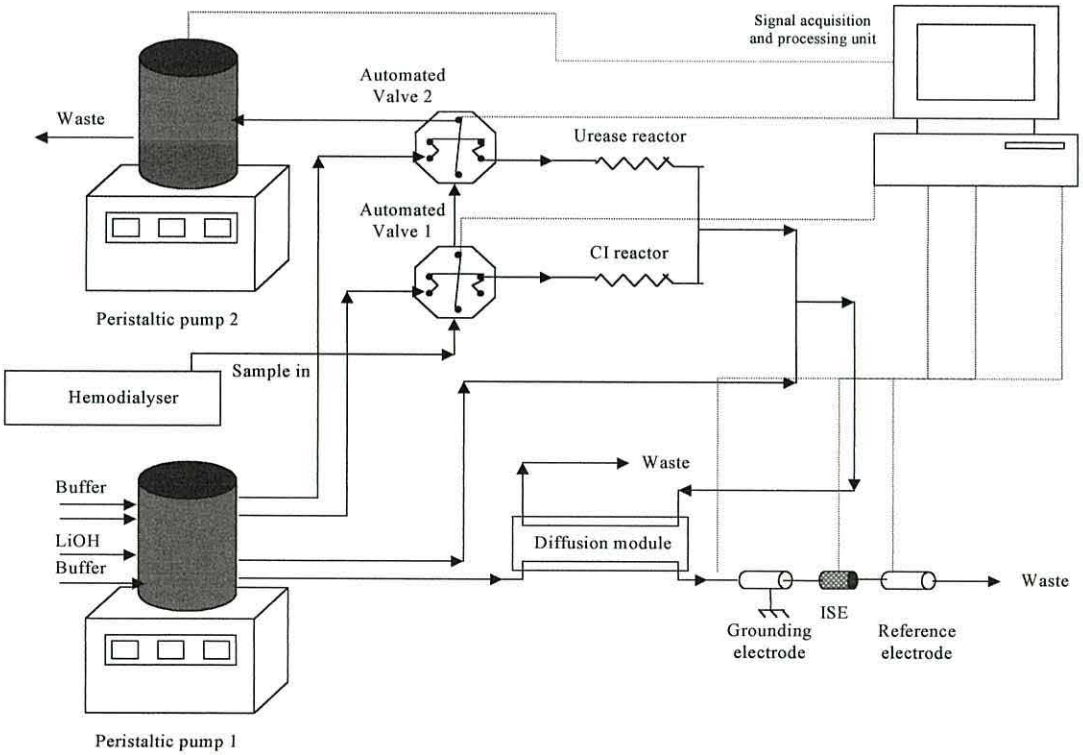


Fig 5.10 Flow injection system used for bi-parametric monitoring of urea and creatinine (Jurkiewicz et al, 1998).

One of the peristaltic pumps continuously impelled the carrier buffer solution and the other impelled the sample towards the injection valves which have the sample connected in series. Samples were injected alternately through two parallel channels. The sample for creatinine determination, which had a lower concentration than that for measuring urea, was injected first. Once the first sample had been injected, a waiting period was required until the signal returned to the baseline and the sample for urea determination was then introduced.

Creatinine deiminase was immobilised covalently on the surface of controlled-pore glass beads filling the reactor. The use of a packed reactor for creatinine determination lead to a greater efficiency of the enzyme reaction, which resulted in greater sensitivity (Jurkiewicz et al, 1998a). The methods employed for the urea reactor were previously described in section 5.1.3.

After passing through the reactors, both urea and creatinine streams merged into a single carrier channel where ammonium ions were converted into ammonia gas which was then detected by means of a solid-state ion selective electrode The potentiometric signal was measured against a reference electrode.

Satisfactory results were reported for the off-line validation of the analyser, both for urea and creatinine. Results agreed with standard analyses carried out using hospital laboratory methods. Moreover the bioanalyser was shown to be capable of reporting the masses of urea and creatinine extracted from a patient connected to artificial kidneys in the course of a haemodialysis session. However no agreement was found for creatinine concentration between the results of the analyser and those of standard methods for on-line validation. The concentration of creatinine in blood plasma was found to differ significantly from the concentration of creatinine in the dry haemodialysis liquid, therefore the analyser was not able to measure the concentration of creatinine in plasma on-line.

5.2.2. Amperometric creatinine biosensors

In contrast to creatinine sensors using the direct indication of creatinine cleavage, amperometric assays require enzyme sequences. Several amperometric sensors for creatinine have been proposed (Kim et al, 1999; Khan and Wernet, 1997; Yamato et al, 1995), all of them based on the enzyme catalytic sequence introduced by Tsuchida and Yoda (1983) where the three enzymes creatininase, creatinase and sarcosine oxidase are coupled to convert creatinine into amperometrically detectable products. A few years ago Nova Biomedical Co. (Waltham, MA, USA) introduced the first commercial creatinine sensor for its clinical benchtop analyser Nova-16, based on these three enzymes and a classical hydrogen peroxide detecting electrode.

Tsuchida and Yoda (1983) and Yoda (1988) immobilised creatininase (creatinine amidohydrolase), creatinase (creatine amidinohydrolase) and sarcosine oxidase together with BSA and glutaraldehyde in a hydrogen peroxide selective membrane. The coupled enzymes catalysed the conversion of creatinine to formaldehyde and glycine with the formation of hydrogen peroxide.

The same authors developed a bi-parametric biosensor capable of assaying creatinine and creatine simultaneously by combining a trienzyme electrode and a bienzyme one. Both electrodes reached a steady signal 2 minutes after sample addition and responded linearly up to 0.76 mM substrate for 25 μl of sample volume. With a sensitivity of 11 nA/mol, the parallel sensors were stable for more than 500 measurements although after 11 days they had lost 20% of activity. The CV of creatinine determination in serum carried out in a single day was reported to be between 1.3 % and 11.7 % and between 4.8 % and 7.6 % for creatine.

Dittmer et al (1988) entrapped the same three enzymes (creatininase, creatinase and sarcosine oxidase) in a gelatin matrix and obtained a functional stability of 15 days for the measurement of creatinine, creatine and sarcosine. The sensitivity for creatinine and creatine gradually decrease after a few days of operation, while that for sarcosine remained almost constant.

Khan et al (1997) developed a trienzyme modified sensor based on platinised shapable electro-conductive film. As the level of creatinine in blood was very low and the three enzymes were coupled together, creatine interfered to creatinine detection. Consequently, the detection limit did not usually cover the minimum necessary physiological range and the sensor had to produce a high response current capable of differentiating small concentration changes, with a very low detection limit. The use of a platinised shapable electro-conductive film covering the working electrode was shown to overcome this problem, producing a high response current and limited interference from the physiological substances.

The base electrodes were fabricated using electrochemical deposition of platinum black and the multi-enzyme system (creatininase, creatinase and sarcosine oxidase in a 2:1:1 proportion) was adsorbed on the platinum black matrix.

The sensitivity of the developed sensors was reported to be dependent on the preparation conditions, the source of the enzymes (and therefore their intrinsic activity), the proportion of the three enzymes in the mixture and the platinum black loading. A sensitivity of 23 $\mu A/mM \cdot cm^2$ and an extended linear range up to 5mM with a detection limit of 1 μM to 2 μM was obtained.

Despite the complexity of co-immobilising three enzymes, the operational stability of the developed creatinine sensor was found to be satisfactory up to one month. Creatinase was the most stable enzyme, maintaining almost 100% of its activity over a period of 20 to 30 days under heavy daily operation conditions. Sarcosine oxidase and creatininase both lost 25% of their activity over this period. As for the operational activity of the response current, it suffered a 35 % drop in three weeks of continuous operation. The linear range for creatinine detection was also significantly reduced in three weeks of continuous operation to 2 mM creatinine.

Another disposable creatinine sensor was reported by Kim et al (1999). They used a thick-film carbon electrode for hydrogen peroxide in combination with creatininase, creatinase and sarcosine oxidase reactions.

Each thick-film sensor strip contained a working electrode and a reference electrode in parallel. Measurements were carried out in a specially constructed microchannel with a total volume of less than 10 μl. A thin multienzyme layer (creatininase, creatinase, sarcosine oxidase) was deposited and the electrode was cut to its final size and covered with a glass film using an adhesive. Capillaries were formed by the epoxy film-glass-film configuration.

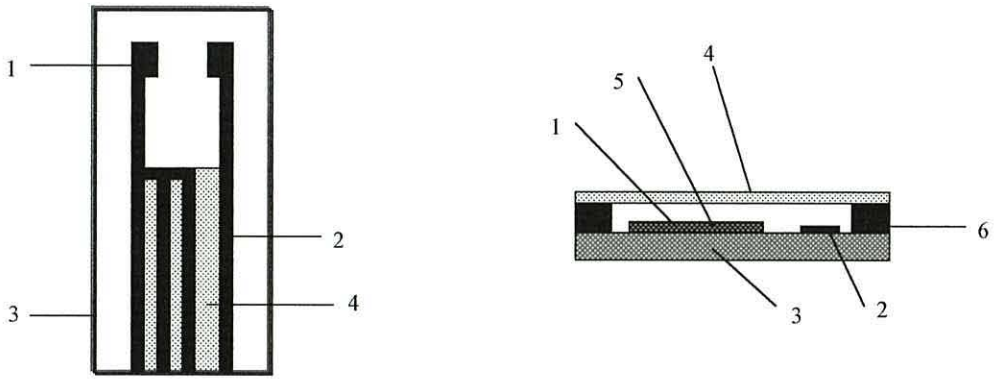


Fig 5.11. Front and cross-section view of a thick-film carbon electrode: (1) working electrode, (2) reference electrode, (3) epoxy film (4) glass film, (5) immobilised enzymes (6) adhesive (Kim et al, 1999).

A drop of test solution ran through the microchannel reaching the enzyme layer and then the enzymes became solubilised and dispersed throughout the volume of the microchannel. The H_2O_2 produced in the catalysed reaction was measured amperometrically by direct oxidation on the surface of the electrode. Creatinine solutions employed produced a linear response between 0.2 mM and 2 mM in a pH 7.5 buffered solution.

5.2.3. Impedance creatinine sensors

Electrochemical impedance measurements have been used mainly to investigate non-biological systems, like protective coatings for metal substrates (Xiao and Mansfeld, 1994; Van Westing et al, 1994). However, new applications for this technique are being found, such as the modelling of biological systems from the impedance response through networks of electrical components (Taylor and Macdonald, 1989; Vandernoot

and Levinkind, 1994). Impedance methods are also beginning to be used for bioanalytical applications. Bataillard et al (1988) and Souteyrand et al (1994) described how a Si/SiO_2 surface could be modified with antibody and used to measure antigen-binding events. Song et al (1993) used electrochemical impedance spectroscopy as an analytical method for the characterisation of cytochrome c adsorbed to alkanethiolate self-assembled monolayers on gold-film electrodes. More recently, Cornell et al (1997) combined electrodes coated with lipid layers containing ion channels with impedance measurements to produce an ion-channel switch biosensor.

Despite the rapid spread of application fields for impedance techniques, few enzyme-based biosensors appear in the literature. Urease and glucose oxidase are the main enzymes used as highly selective biochemical systems in order to characterise and optimise the performance of model biosensors.

Ho et al (1999) applied their investigations on polymer-coated urease modified impedance electrodes (McNeil et al, 1995) to develop a creatinine sensor with similar operation principles (see section 5.1.3.2). This previous impedance sensor was based on measuring a change in capacitance of an enzyme-catalysed breakdown of polymer-coated screen-printed carbon-ink electrodes. Using the commercially available Eudragit S-100, they demonstrated that the specific change in pH originated by the action of urease on urea resulted in polymer breakdown and a subsequent change of four orders of magnitude in capacitance in the presence of excess urea. This showed the potentially higher sensitivity of impedance analysis compared with simple pH measurements. Eudragit S-100 was only tested under restricted laboratory conductions.

Employing a modified poly(methyl vinyl ether)/maleic anhydride polymer, they presented a simple, integrated and disposable sensor design for the clinical detection of creatinine. The modified poly(methyl vinyl ether)/maleic anhydride polymer was found to be stable at pH 7.4 and only broke down at more alkaline pH values.

Screen-printed interdigitated carbon electrodes were coated with the polymer covering their entire working area. Absorbent pads were then soaked in creatinine deiminase solution, allowed to dry and laid over the surface of the polymer coated electrodes. The arrangement of the electrode system is analogous to that showed in figure 5.8.

Creatinine assays were carried out both in control serum and untreated serum spiked with known concentrations of creatinine. The creatinine concentration of the untreated serum samples were determined using the calibration plot previously constructed using control serum-standard creatinine solutions. The results were also compared with reference standard clinical laboratory methods.

Despite their successful previous results in urea detection when employing this method, it suffered from lack of sensitivity for creatinine detection since only 2 mM creatinine could be detected after 20 minutes. Although the use of absorbent pads for enzyme immobilisation had the distinct advantage in that as much enzyme could be applied onto the pad as required, they still found problems for creatinine measurement because the creatinine deiminase commercially available shows a much lower specific activity than urease (11.5 U/mg compared to 479 U/mg). This lack of sensitivity remained a serious problem for measurements of creatinine over the clinical range, as the normal ranges for creatinine in serum are 62 μM – 115 μM (male) and 53 μM – 97 μM (female). In patients with kidney dysfunction or muscle disorder, however, creatinine levels could increase up to 1mM. Therefore, although the system worked at high creatinine levels, which would be found in cases of acute or chronic renal failure, some modifications would be required to obtain the necessary sensitivity for patients with normal levels.

Chapter 6. Urease as a Model Enzyme – Biosensor for Urea Detection

Conductimetric impedance biosensors based on dielectric measurements detect the changes in the conductance of a sample located between two electrodes over a particular frequency range. Such variation in the conductance is proportional to the concentration of product species generated in an enzyme catalysed reaction and therefore, a measurement of the conductance change will also determine the original concentration of substrate present in the sample. This simple approach, which is the basis of all impedance biosensors, is employed here in the description of two impedimetric sensing devices: a urease electrode for the detection of urea included in this chapter and both a single-enzyme creatinine deiminase electrode and a multi-enzyme creatininase / creatinase / urease electrode, for the detection of creatinine, which is described in chapter 8.

Urease was chosen as a model enzyme for its high stability and well-understood catalytic behaviour, which make it an ideal enzyme to use in the construction of a model impedimetric biosensor. In this chapter, the detection of urea by means of an urease impedance electrode is investigated, covering the dependence of its performance on features such as enzyme loading, immobilisation methods, temperature, pH and electrolyte concentration. Measurements of urea in sheep serum are also included.

Following this characterisation study, further investigations were carried out to ascertain the applicability of this biosensor to a medical situation: the measurement of creatinine in urine. In order to do this, two different enzyme electrodes were studied, one based on the single enzyme creatinine deiminase and another on the multienzyme system creatininase / creatinase / urease.

6.1. Materials and Methods

6.1.1. Materials

Gold-coated interdigitated electrodes were manufactured in-house using glass as substrate and following standard photolithographic techniques. The interdigitated sensor square head had a length of 1 cm, with tracks of 0.1 cm width and electrode spacings of 0.1 cm. Figure 6.1 shows a diagram of the interdigitated electrode array.

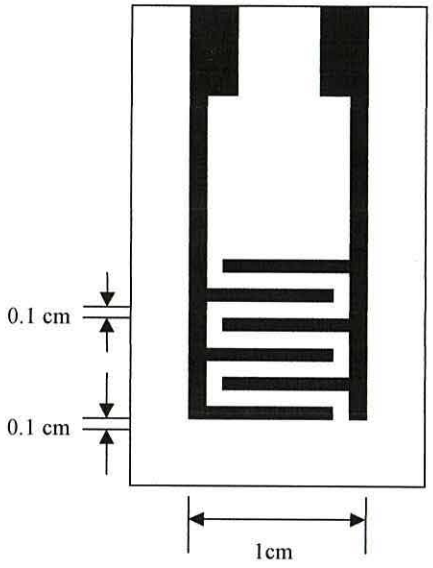


Fig 6.1. Diagram of the interdigitated electrode

All solutions employed were prepared in our laboratory.

All potassium phosphate buffers were prepared dissolving appropriate amounts of dipotassium orthohydrogen phosphate and potassium dihydrogen orthophosphate (both purchased from BDH) in deionised water. The pH of the buffer solutions was adjusted to 7.2 with 10mM solutions of HCl or NaOH when required.

Anhydrous urease (urea aminohydrolase type III from Jack beans, EC 3.5.1.5; 15,000 U/mg – 50,000 U/mg) was purchased from Sigma Chemical Company. Urease solution was employed at a concentration of 40 mg/ml in 10 mM potassium phosphate at pH 7.2.

Three immobilising techniques were followed to fabricate the enzyme electrodes: chemical cross-linking with glutaraldehyde, entrapment in a gelatin matrix and bonding to the electro-active clusters of Nafion polymer.

Glutaraldehyde (Grade 1, 25% aqueous solution) was purchased from Sigma Chemical Company and diluted down to 2.5% using 10 mM potassium phosphate buffer at pH 7.2. Bovine serum albumin (fraction V, 98-99%), necessary to protect the enzyme in the cross-linking process, was also supplied by Sigma Chemical Company and dissolved using 10 mM potassium phosphate buffer at pH 7.2.

Gelatin (type B from bovine skin) was purchased from Sigma Chemical Company and dissolved to 1% also in 10 mM potassium phosphate buffer at pH 7.2.

Nafion (5% perfluorinated solution in alcohol) was purchased from Aldrich and diluted down to 1% using deionised water.

Urea was purchased from BDH. Different concentrations of urea were obtained by dissolution in potassium phosphate buffer.

6.1.2. Immobilisation methods

The performance of urease electrodes was investigated using three different immobilisation methods: physical entrapment in a gelatin matrix, chemical cross-linking using glutaraldehyde and electrostatic interaction with a Nafion polymer.

6.1.2.1. Immobilisation with gelatin

Urease solution at 40 mg/ml concentration was combined with 1% gelatin in 1:1 proportion. Both solutions were prepared in 10 mM potassium phosphate buffer (pH 7.2). 50µl of the enzyme / gel mixture was deposited over the electrode surface. The enzyme-coated electrodes were left at room temperature for around five hours after which a small section of cellulose acetate dialysis membrane was deposited on the surface. A hollow perspex housing was mounted on the electrode surface and covered

with a gold coated copper disc wrapped in Parafine to electrically insulate it from the solution. Such a test cell was then soaked in potassium phosphate buffer (pH 7.2) for approximately twelve hours.

6.1.2.2. Immobilisation with Glutaraldehyde

500 µl of urease solution at a concentration of 40 mg/ml was mixed with 500 µl bovine serum albumin solution (15 mg/ml) and left stirring until no bovine serum albumin was visible in the solution. 10µl of 2.5% glutaraldehyde was then added. All solutions were prepared with 10 mM potassium phosphate buffer (pH 7.2). The deposition and assembly of the enzyme electrodes was performed as for gelatin immobilised urease electrodes.

6.1.2.3. Immobilisation with Nafion

Equal volumes of urease solution enzyme solution and 1% Nafion were combined in 10 mM potassium phosphate buffer (pH 7.2) solution. 50µl of the enzyme / Nafion mixture was deposited on the electrode and left to dry. Once dried, they were covered with dialysis membrane and soaked in potassium phosphate buffer following the same procedure as for the immobilisation with gelatin and glutaraldehyde.

6.1.3. Experimental methods

6.1.3.1. Dielectric Measurements

A diagram of the experimental cell is showed in figure 6.2.

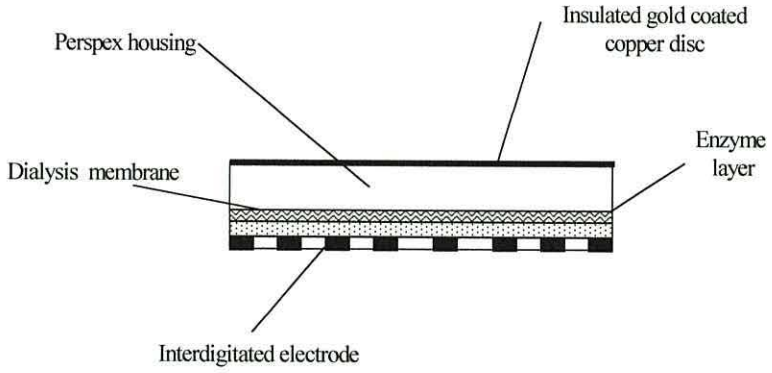


Fig 6.2. Diagram of the experimental cell.

The experimental cell employed a single coated interdigitated gold array mounted facing a hollow perspex housing which was sealed with an insulated gold coated copper disc.

Electrolyte solution was introduced into the cell via an inlet on the top of the perspex cell and a precision ac bridge component analyser (Wayne Kerr 6425) operating over the frequency range 20 Hz to 300kHz was used to measure cell conductance and capacitance. The ac voltage over the specified frequency range from the component analyser (1 V peak to peak, 0 V bias) was applied to the electrodes and capacitance and conductance data were collected by a dedicated PC. A diagram of the experimental set-up is shown in figure 6.3.

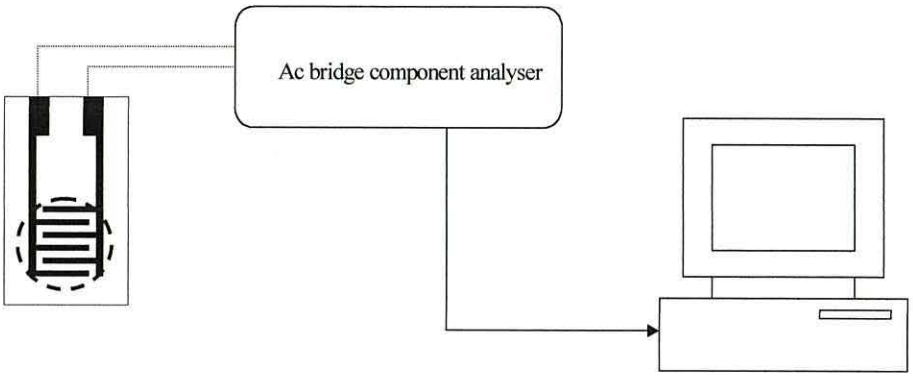


Fig 6.3. Experimental set-up: experimental cell connected to a frequency component analyser and the dedicated PC.

6.1.3.2.Measurement of ammonium ion concentration

Following dielectric measurements, samples of the bulk electrolyte were tested for ammonia product using an ammonia electrode (Russell pH Limited, Ammonia Electrode model 95-5129) in order to determine the concentration of ammonium ions present in the bulk solution and correlate such with the change in conductance measured using impedance techniques.

The gas-sensing electrode was connected to a digital volt-meter and calibrated for each set of measurements. Sodium hydroxide was first added to the test electrolyte to convert ammonium ions to ammonia. The electrode was then placed close to the surface of the test samples so that ammonia vapour emanating from the sample solution diffused through the electrode membrane.

6.2. Results and discussion

Urease (urea amidohydrolase EC 3.5.1.5), as described in chapter 5, is a commonly employed enzyme in biosensor investigation due to its relatively high stability and activity. Urease catalyses the hydrolysis of urea, which produces carbonate and ammonium ions according to the reactions below,

$$NH_2CONH_2 + 3 \cdot H_2O \leftarrow \xrightarrow{urease} \rightarrow 2 \cdot NH_4^+ + HCO_3^- + OH^- \qquad [6.1a]$$

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad [6.1b]$$

$$HCO_3^- \leftrightarrow CO_2 + OH^- \qquad [6.1c]$$

The kinetic constants for the catalysed hydrolysis of urea and other reported enzyme features such as the optimum pH and temperature of the reaction are discussed in chapter 2.

6.2.1. Kinetics of the hydrolysis of urea catalysed by urease

In order to obtain a measure of the activity of the urease in the immobilised state and to ascertain the level of reduction in activity due to the immobilisation process, kinetic data was collected for both the enzyme in solution and as part of the enzyme electrode. A complete study of the kinetics of the hydrolysis of urea catalysed by urease was carried out comparing the behaviour of the free enzyme with that of the enzyme trapped in a gelatin matrix.

The following graphs show the dependence of the velocity of a urea sensor with substrate concentration at steady-state.

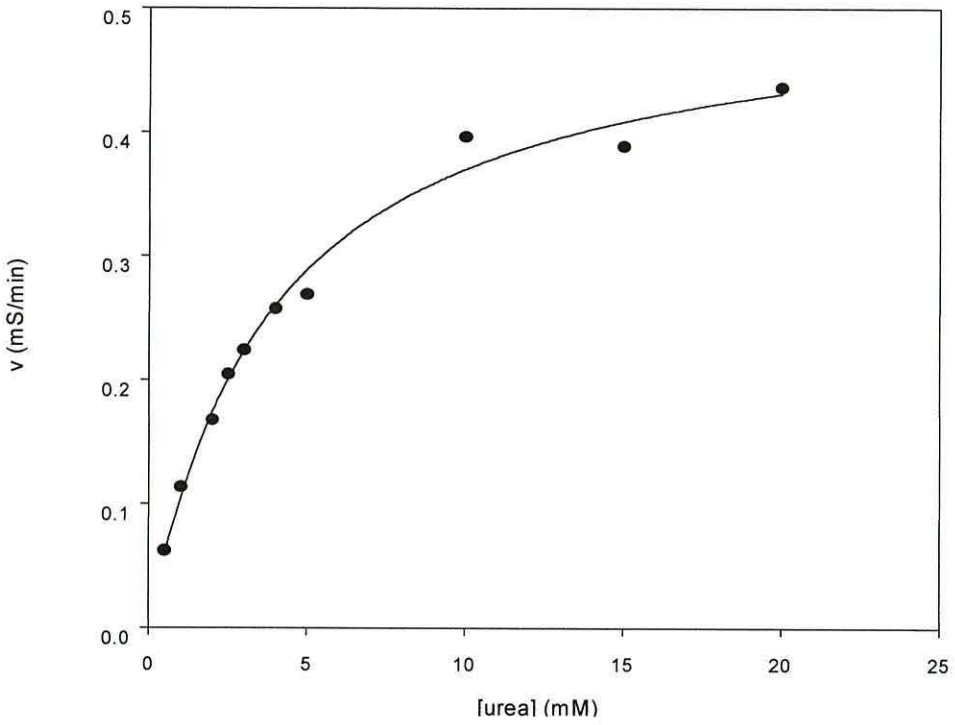


Fig 6.4. Dependence of reaction velocity on urea concentration in solution.

Figure 6.4 corresponds to urease in solution and figure 6.5 to a gelatin immobilised urease sensor. We can extrapolate values for K_M and the maximum velocity by fitting the graphs to an hyperbola given by the following equation.

$$v = \frac{v_{max}[S]}{[S] + K_M} \qquad [6.2]$$

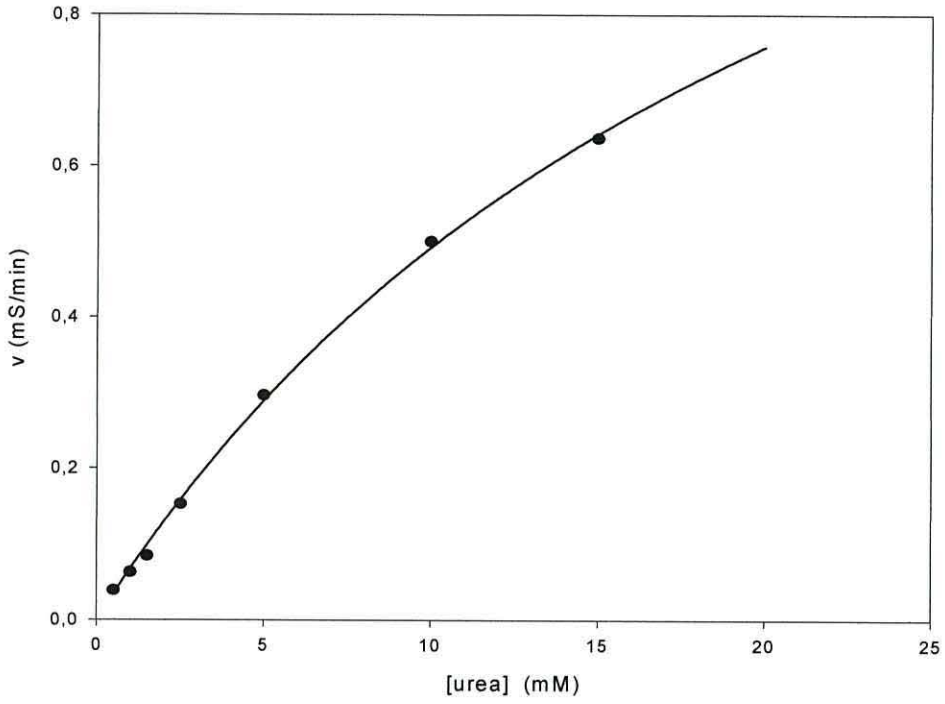


Fig 6.5. Dependence of reaction velocity on urea concentration in typical gelatin immobilised urea biosensor

The experimental results for this study show very different values of K_M and v_{max} for urease in solution and immobilised urease: K_M =(3.9 ± 0.4) mM and v_{max} =(0.50 ± 0.04) mS/min for solution; for solution; K_M =(22 ± 2) mM and v_{max} =(1.59 ± 0.02) mS/min for immobilised urease.

Another way of presenting kinetic data, far more popular than a direct v([S]) plot, is to represent 1/v as a function of 1/[S]. In fact, taking inverses in the direct relationship between the velocity of the reaction and the substrate concentration derived from Michaelis-Menten kinetics and rearranging both terms, produces the Lineweaver-Burk plot as shown below

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_M}{v_{max}} \cdot \frac{1}{[S]} \qquad [6.3]$$

As already stated in chapter 2, the Lineweaver-Burk plot describes the linear relationship between the inverses of both velocity and substrate concentration: v_{max} will be the inverse of the ordinate value where the extrapolated line intercepts the y-axis and

$-1/K_M$ will be given by the intercept of the extrapolated line with the abscissa axis. The Lineweaver-Burk plot of the data is shown below in figure 6.6.

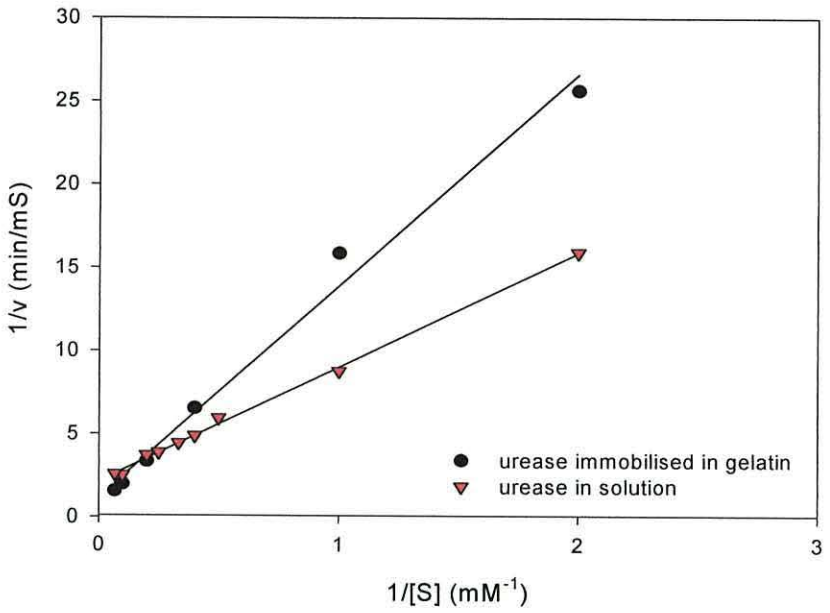


Fig 6.6. Lineweaver-Burk plots for urease in solution (red triangle) and immobilised urease (black circle).

v_{max} and K_M values derived from this plot are: K_M =(3.3 ± 0.3) mM and v_{max} =(0.48 ± 0.03) mS/min for urease in solution; K_M =(11 ± 6) mM and v_{max} =(0.8 ± 0.4) mS/min for the immobilised sensor.

A further way of plotting the data to obtain kinetic parameters is the Woolf plot. This plot also involves rearranging the hyperbola equation that links v_{max} and K_M originally, but in a different way to the Lineweaver-Burk plot, as shown below,

$$\frac{[S]}{v} = \frac{K_M}{v_{max}} + \frac{[S]}{v_{max}} \qquad [6.4]$$

The results are shown in the following graph,

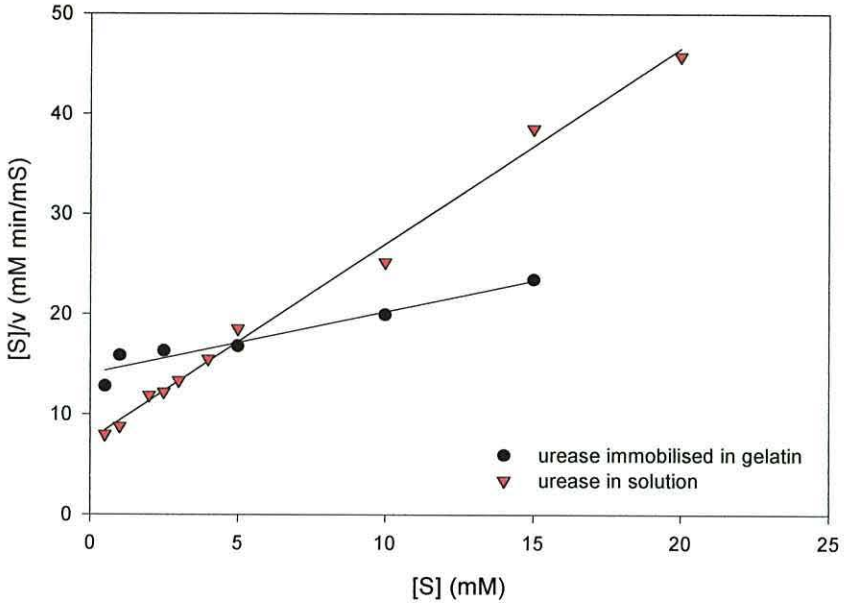


Fig 6.7 Woolf plots for urease in solution (red triangle) and urease immobilised in gelatin (black circle)

The kinetic constants in this case are K_M =(3.8 ± 0.4) mM and v_{max} =(0.510 ± 0.016) mS/min for solution, and K_M =(22 ± 4) mM and v_{max} =(1.6 ± 0.2) mS/min for immobilised urease.

As can be seen in the results stated above, marked differences appear between the kinetic values for immobilised urease and urease in solution as expected. Moreover, the values found for K_M and v_{max} on immobilised urease depend on the analysis method chosen, Lineweaver-Burk plots giving kinetic constants twice as high as those inferred from Woolf plots. Kinetic analysis of urease in solution, on the other hand, gave very close values of K_M and v_{max} for both plots.

The inconsistent results obtained from different methods of analysing the same raw data are a sign of the care needed when investigating immobilised enzyme systems with Michaelis-Menten kinetics. In fact, Michaelis-Menten kinetics represent the kinetics of the enzyme catalysed reaction itself but do not take into account the diffusion dynamics

both of the substrate molecules in the bulk and enzyme phase and of the product molecules out of the enzyme phase towards the electrode and the bulk solution. Therefore, they are not as obviously applicable to immobilised urease systems as they are to urease in solution, and the values derived from them should only be considered as approximate.

In the Lineweaver-Burk plot, 1/v is plotted against 1/[S]. Although the experimental substrate data points lie evenly in the interval between 0 mM and 20 mM, their inverses will tend to cluster near the origin and the highest y values (1/v values) will have a stronger effect on the slope of the straight line. Unfortunately, such data points correspond to those of lower substrate concentrations, where the reaction velocities are more difficult to measure accurately due to the precision limitations of the equipment.

In the case of the Woolf plot, [S]/v is plotted against [S]. Therefore, not only are the experimental data points evenly distributed in the substrate concentration interval studied, but also the velocity data is modulated by its correspondent value of substrate, diminishing the effect of the velocity of the low substrate concentration samples on the final fitting.

The discrepancy between the values derived using the two methods of analysis should not be interpreted as a discussion to favour one against the other, but as a sign of the doubtful reliability of such techniques when applied to immobilised enzymes. As stated before, both methods are directly derived from Michaelis-Menten kinetics, which only take into account the kinetics of the catalysed reaction itself and do not include any dependence on the diffusion of substrate and product species before and after the chemical reaction.

Nevertheless, very positive results have been extrapolated for solution systems, with a K_M value around 7 times lower and a v_{max} value more than two times lower than that found for immobilised urease using the Woolf plot. As mentioned before, K_M can be interpreted as an indication of the affinity of the enzyme / substrate complex: the higher the K_M value, the most likely the complex will occur. So these results seem to indicate that, in solution, the probability of formation of the enzyme / substrate complex is 7 times larger than in an immobilised system. Although it is not simple to predict any

exact values, it is clear that the physical entrapment of the enzyme in a matrix structure will make the bonding between an enzyme and a substrate molecule more difficult than in the case of solution systems.

For an enzyme in solution, the probability of interaction of a substrate molecule and an enzyme molecule depends on the kinetic energy of the reactant molecule, on the number of collisions and on the probability of a collision taking place with the right orientation. This steric factor takes on enormous importance when dealing with immobilised enzymes. The molecules are not free to move but trapped in the holes of a gelatin matrix, which greatly hinders the access of substrate molecules to their active sites reducing the probability of efficient collisions.

As for the maximum velocity, it should be interpreted as an apparent magnitude and not a real parameter of the chemical reaction for immobilised systems, since the speed of the reaction is not enhanced but perturbed by the immobilising process. As discussed above, the entrapment of the enzyme in the gelatin matrix obstructs the free access of substrate molecules to the active site, so the probability of successful bindings will be lower than in the case of free enzyme. This means that, under the same conditions, an immobilised enzyme system will need more substrate molecules to reach its maximum velocity than an enzyme in solution. Mathematically, this could be reflected by a lower slope on the linear part of the quadratic hyperbola that describes the dependence of the product formation velocity with the substrate concentration (see equation 6.2). This would describe a quadratic hyperbola with larger asymptotic values but this would only be the consequence of the displacement of the saturation point of the enzyme towards higher substrate concentrations, as in fact happens in enzyme immobilised sensors.

The influence of diffusion should also be considered when comparing immobilised and solution systems. The larger diffusion constant of the gel matrix compared to that of the solution makes it even more difficult for the substrate to reach the enzyme molecules. The velocity of the substrate molecules diminishes considerably when entering the gel, which causes a reduction in kinetic energy and therefore in the probability of formation of enzyme / substrate complex, expressed by an increase in K_M. Also the polar nature of enzyme, substrate and matrix may perturbate in some way the effective binding of the

enzyme / substrate complex, reducing the apparent affinity between the enzyme and the substrate molecules.

Table 6.1 summarises kinetic parameters for free urease, as well as urease immobilised using gelatin, Nafion and chemical cross-linkage with glutaraldehyde. In all cases, the kinetic parameters were derived from the Lineweaver-Burk equation because it is the most extensively employed in enzyme kinetics.

Parameters	Free Enzyme	Gelatin	Nafion	Glutaraldehyde
K_M (mM)	3.3 ± 0.4	11 ± 4	15 ± 2	32 ± 6
v_{max} (mS/s)	0.5 ± 0.1	1.6 ± 0.2	7 ± 1	11 ± 2

Table 6.1. Kinetic parameters of free (solution) and immobilised urease.

These values are similar to those reported by other workers (4 mM – 5 mM reported by Reithel F. J., 1971; 6 mM reported by Lawton B.A et al, 1989) with larger values of K_M for immobilised urease, indicating a lower enzyme-substrate affinity.

6.2.2. Frequency response and sensitivity

Figure 6.8 shows the frequency response over the range 20 Hz to 300 kHz of the conductance of a typical gelatin immobilized urease electrode for varying urea concentrations. The experimental cell and setup are described in section 6.1.3.

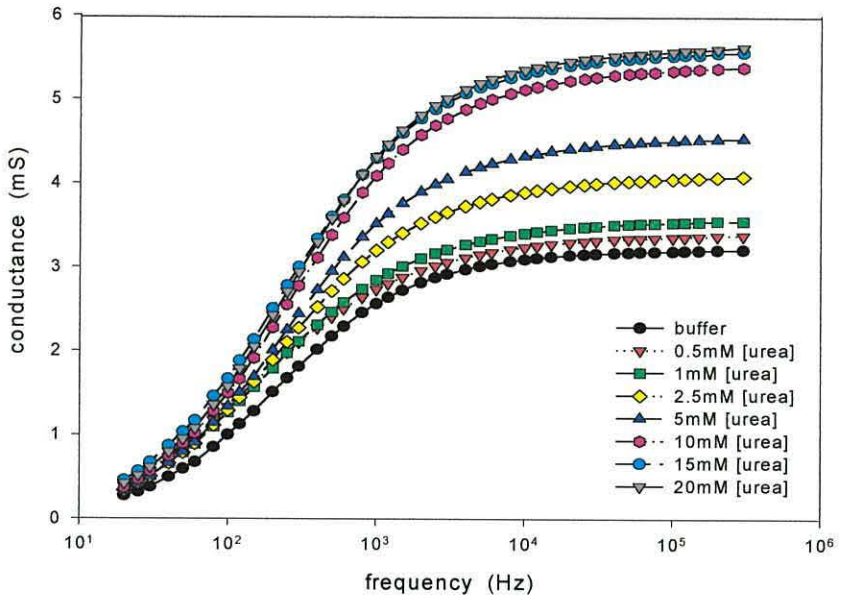


Fig 6.8. Frequency response of the conductance of a typical gel-immobilised urease electrode for varying urea concentration at 10mM phosphate buffer pH 7.2.

It can be seen that the low frequency region (20 Hz to 200 Hz) is relatively insensitive to substrate and the response in this region and at frequencies below 20 Hz has been shown to originate from the formation of an ionic double layer at the electrode surface (Taylor and Macdonald, 1987). In the range between 10 kHz and 300 kHz the measurements predominantly reflect the electrical properties of the enzyme layer and the electrolyte phase, and the results are consistent with a substrate concentration-dependent increase in the conductance of these phases. Therefore, 300 kHz was chosen to measure the conductance values in order to establish urea concentration.

Figure 6.9 describes the change in conductance with urea concentration.

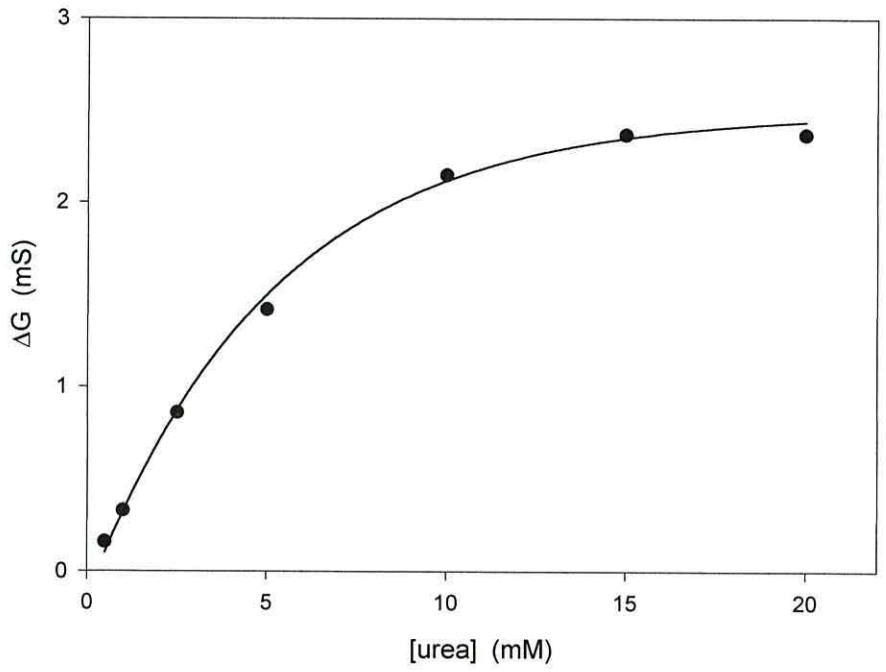


Fig 6.9. Calibration plot describing the change in conductance with urea concentration. Measurements are taken from data shown in figure 6.8 at a frequency of 300kHz

The response of the sensor is the incremental conductance, ΔG, arising from the difference between the steady state conductance for each urea concentration and the conductance of the buffer.

$$\Delta G = G_{urea+buffer} - G_{buffer} \qquad [6.5]$$

The urease electrodes were found to exhibit sensitivities typically of the order 300 μS/mM.

6.2.2.1. Ammonium ion concentration

Ammonium ions are produced in the hydrolysis of urea taking place in the enzyme phase. These ions are free to diffuse from the enzyme layer into the bulk electrolyte and the concentration can be measured independently using an ammonia electrode, so that the concentration of ammonia in the bulk correlates to the conductance changes in the

bulk. As the conductance of both the enzyme phase and bulk phase contribute to the measured conductance of the urease electrode, there is a correlation between the increase in ammonium concentration in the bulk and the rise in total conductance. Figure 6.10 describes how the concentration of ammonium ions in the bulk electrolyte varies as a function of substrate concentration. As expected, this dependence follows that of the increase in conductance of the enzyme phase with urea concentration.

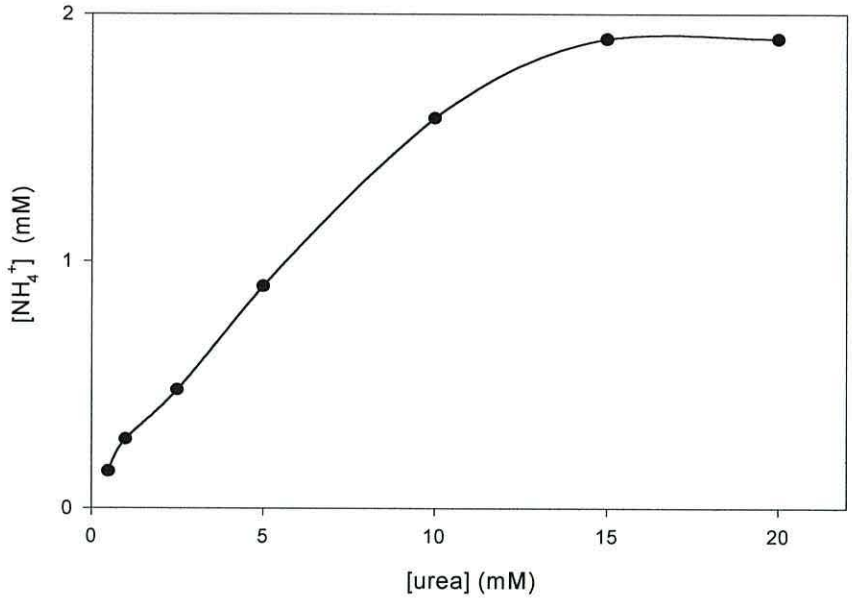


Fig 6.10. Concentration of ammonium ions in the bulk electrolyte as a function of urea concentration.

6.2.3 Enzyme loading

A study of the dependence of the sensitivity and response time with the amount of urease immobilised on the electrode was performed in order to obtain information necessary for biosensor optimisation. Figure 6.11 describes the dependence of the urease electrode sensitivity and the response time, defined as the time to reach 95% of the final value, for a 1mM substrate concentration.

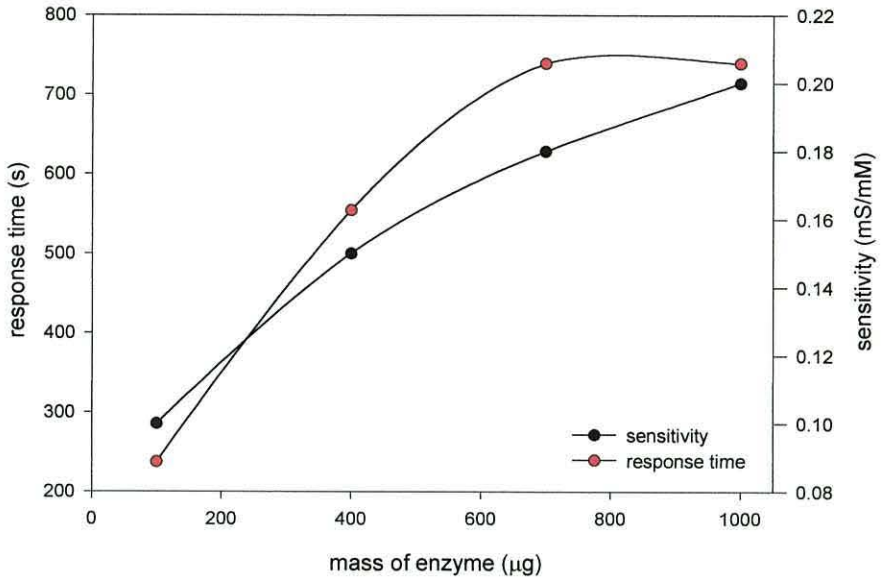


Fig 6.11. Sensitivity and response of a typical gelatin immobilised urease electrode as a function of enzyme loading in 10mM phosphate buffer, pH 7.2.

It can be seen that for the gel-entrapped enzyme sensors over the range of enzyme loadings studied, the sensitivity increases in a roughly linear fashion with increasing amounts of enzyme. From figure 6.11 it can be seen that a tenfold increase in mass of enzyme generates an approximate increase of 2.5 times in sensitivity. As for the response time, it approximates an exponential increase towards an asymptotic maximum with increasing mass of enzyme.

There are a number of factors that can be expected to combine to determine the dependence of the sensors' response on enzyme loading. The activity of the enzyme present in the enzyme phase and its ability to catalyse the hydrolysis of urea is obviously a critical process. Moreover, the diffusion of substrate and product will also be affected by the amount of enzyme entrapped in the immobilising matrix since protein molecules are likely to form larger barriers to diffusion than gelatin for instance. These two factors can be combined in a second order differential equation describing the steady-state system (Eddowes, 1990):-

$$D_s\left(\frac{d^2[S]}{dx^2}\right) - \frac{k_2[E][S]}{K_m + [S]} = 0 \qquad [6.6]$$

where D_s is the diffusion coefficient of the substrate, [E] and [S] are the enzyme and substrate concentrations and k_2 is the rate constant for product formation from the enzyme-substrate complex.

The sensitivity of the interdigitated electrodes with distance from the electrode surface to changes in conductance is also an important factor. Ideally, the product concentration, which determines the magnitude of the sensors' response, should be at a maximum close to the electrode surface where the fringing electric field is strongest.

6.2.4. Immobilisation methods

The stability and reproducibility of sensors fabricated using different immobilisation techniques was investigated.

The enzyme electrodes were fabricated using simple entrapment in the case of gelatin immobilisation, by entrapment in a polar matrix with Nafion and by chemical cross-linkage in the case of treatment with glutaraldehyde. The sensitivities of urease electrodes fabricated employing these methods were measured over a period of 33 days. Urease is a particularly stable enzyme and the shelf life of the sensors appears to reflect this with all sensors exhibiting stable response characteristics for up to 7 days.

The sensors employing gel and Nafion entrapment immobilisations typically produced higher sensitivities than those employing cross-linked immobilisations but the gel-entrapped sensor showed dramatic signs of degradation with decreasing sensitivity as shown in figure 6.12.

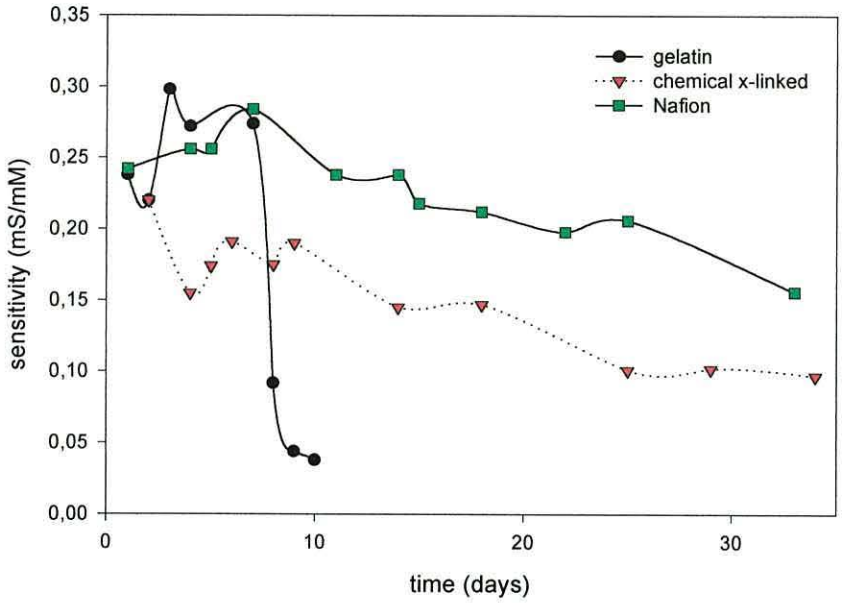


Fig 6.12. Shelf-life of gelatin and Nafion entrapped and chemical cross-linked sensors in 10mM phosphate buffer pH 7.2

Both the Nafion and cross-linked sensors exhibited a gradual reduction in sensitivity from day 10 until the end of the study with the Nafion immobilised urease showing approximately 30% higher sensitivity over the test period. These observations are consistent with the effect of immobilisation on the enzyme being essentially dominated by modification to the flexibility of the enzyme structure. Flexibility has been shown to be crucial in determining the level of activity of many enzymes where even relatively small conformational changes occur during catalysis (Bone, 1987). Protein flexibility in the immobilised state is determined not only by the constraints of chemical bonding but also by the water content of the enzyme in the enzyme phase since in non-solution conditions water acts to electrostatically screen interactions between polar and charged groups within the enzyme structure (Bone and Pethig, 1985).

The gel-entrapped enzyme can be expected to experience relatively little decrease in flexibility compared with that encountered by the protein that has been covalently cross-linked by treatment with glutaraldehyde, because gelatin provides water in the bulk state for hydration of the enzyme. Similarly bulk water is expected to be available in Nafion immobilisation but in this case some decrease in flexibility might be experienced due to

additional electrostatic interactions between the Nafion polymer and the enzyme. Flexibility is also associated with protein denaturation in that more flexible structures are more likely to experience irreversible conformational changes, which result in enzyme inactivation. This may explain the much shorter lifetime of the gel-immobilised urease electrode compared to that of the sensor fabricated using chemical cross-linking. In practice Nafion-immobilised urease appears to produce the optimum enzyme phase, eliciting a sensitivity in the early period equal to that of the gel-entrapped enzyme and in the later region showing a degradation rate comparable to that of the cross-linked enzyme.

6.2.5. Temperature dependence

Figure 6.13 shows the temperature-dependence of the sensitivity of a typical gelatin-immobilised urease electrode.

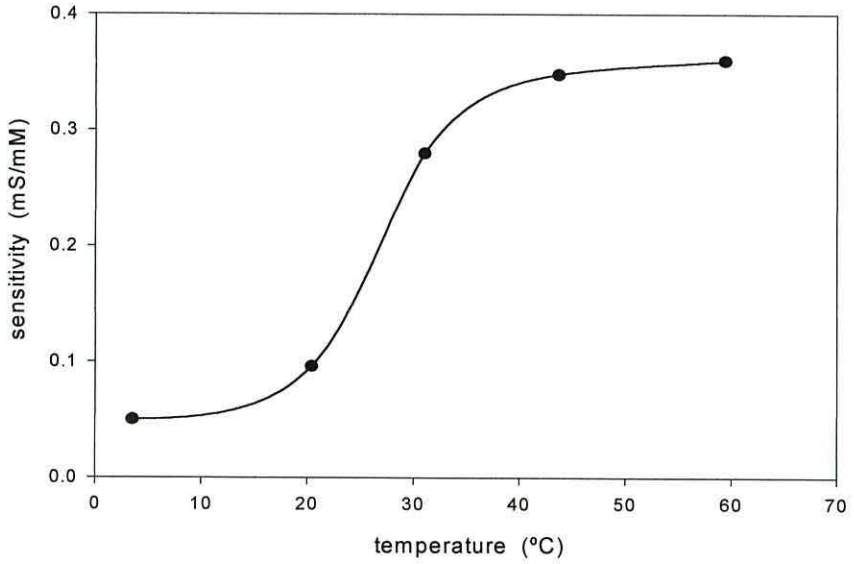


Fig 6.13. Temperature dependence of the sensitivity of a typical gelatin immobilised urease electrode in 10mM phosphate buffer at pH 7.2.

It describes a very low sensitivity (50μS/mM) at temperatures approaching 0°C with a relatively steep rise to a saturation value of 370μS/mM at 60°C. This appears to be

consistent with variation of urease activity with temperature (Reithel, 1959; Scheller and Schubert, 1992).

6.2.6. pH dependence

The dependence of the sensors response with pH is described in figure 6.14.

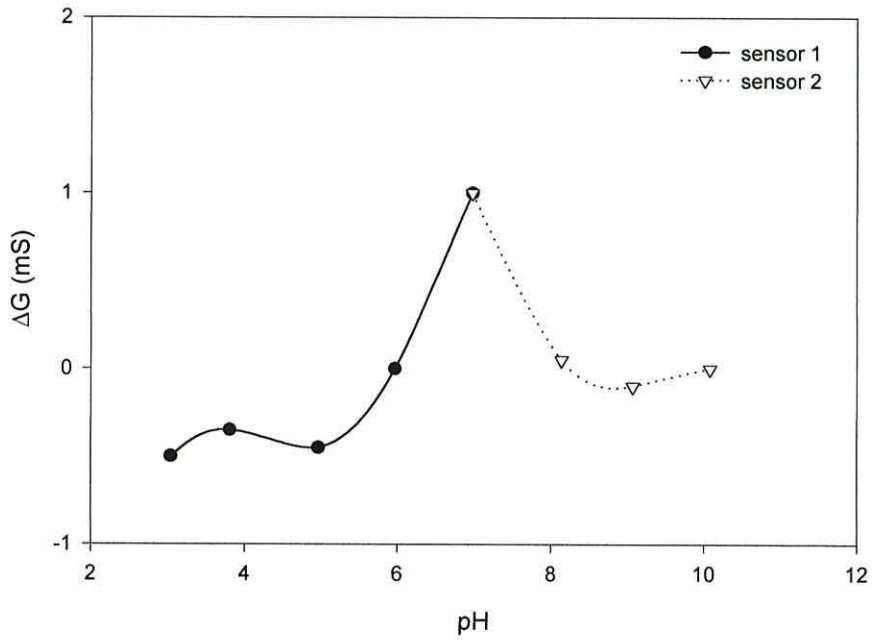


Fig 6.14. pH dependence of the response of a typical gel-immobilised urease electrode to 5mM urea in 10mM phosphate buffer.

pH-dependent changes in the activity of the urease which exhibits a maximum at pH 6.9 in the buffer employed can be invoked to explain in general terms the origin of the characteristic, although the steep decline in sensitivity either side of a relatively narrow peak response is not usually observed.

6.2.7. Electrolyte concentration dependence

There was also a marked variation in the sensitivity of the sensor with electrolyte concentration depending on the electrode treatment and immobilising matrix employed as described in figure 6.15. A common trend for all three sensor types described is a steep increase in sensitivity with electrolyte concentration over the range 1 mM to 30 mM.

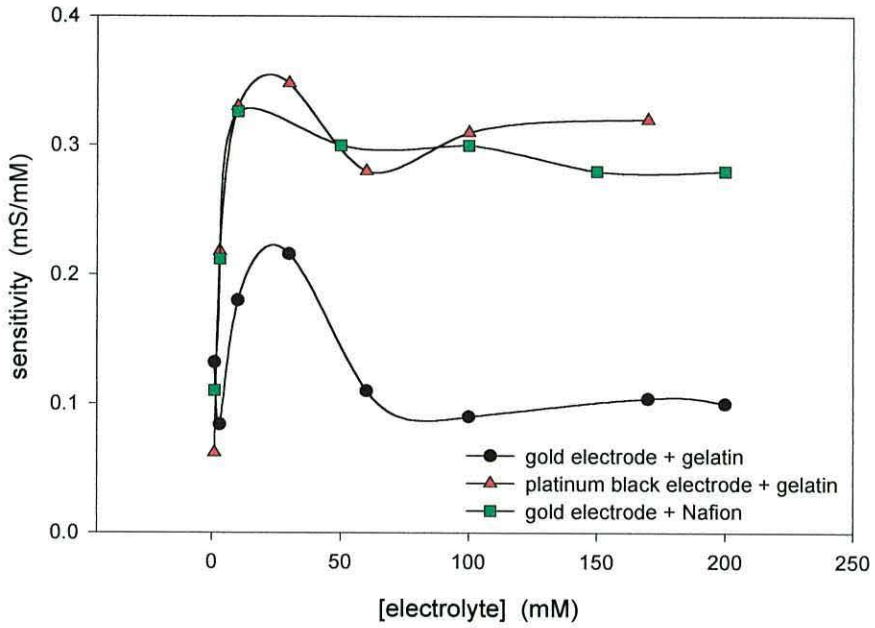


Fig 6.15. Electrolyte concentration-dependence of the sensitivity of urea sensors fabricated using gelatin on gold, gelatin on platinum black and Nafion on gold. Urea concentration = 5mM

This trend is not observed in the response of urease in solution and is therefore unlikely to be associated with an inherent ionic strength-dependent activity of the native enzyme. However, the activity of the immobilised enzyme may still be dependent on electrolyte concentration. Such variation in enzyme activity with ionic strength has been noted in previous work on trypsin and chymotrypsin attached to an immobilising matrix (Goldstein et al, 1964). This work has demonstrated that electrostatic effects result in an increase or decrease in the observed K_M value for enzymatic action in a matrix, which carries like or unlike charges respectively. Engasser and Horvath (1975) have

considered the effect of electrostatic interactions between a charged immobilisation matrix and substrate on the kinetic behaviour of bound enzymes. Their investigation indicates that, for this kind of immobilised system, the value of K_M can be expected to vary with the ionic strength of the supporting electrolyte due to the electrostatic screening effect of the electrolyte.

In the case of the immobilised urease / urea system studied here, although urea is not a charged molecule, it does possess a large dipole moment (4.6 D) and as a result, it is likely that electrostatic interactions with the local microenvironment will be important. Electrostatic screening may therefore be responsible for the type of dependence observed for the urea biosensors at low electrolyte concentrations. The fact that the products are charged might also be important since these are certain to interact electrostatically with the immobilising matrix.

The relatively small sensitivity values observed at low electrolyte concentrations may also be associated with a change in the charge distribution between the enzyme and bulk phases on introduction of urea into the latter phase. It is to be expected that urea molecules, because of their large dipole moments, will be strongly associated with ions in the solution and will modify the hydrated ionic structures. This may result in a lowering of the activity coefficient of the ions in this phase causing a flow of ions from the enzyme phase to re-establish the equilibrium state.

At electrolyte strengths above 30 mM, there is a marked difference in the sensitivity characteristics of the three sensor types. The sensor fabricated using gel-immobilisation of the enzyme onto bare gold electrodes exhibits a dramatic fall in sensitivity to a relatively low level above 100 mM electrolyte. However, gel-immobilised enzyme electrodes where the gold electrodes have been coated by electrodeposition with platinum black exhibit sensitivities which remain approximately constant with electrolyte concentration.

Platinum black electrodes are known to reduce the effects associated with the electric field-induced accumulation of mobile charges at electrode surfaces as a result of the large effective surface areas they possess (Schwan, 1966). The fall in sensitivity observed for gel-immobilisation onto gold electrodes, therefore, is likely to be the result

of this electrode polarisation process which effectively screens the enzyme layer from the applied potential. Sensors produced by immobilising the enzyme onto gold electrodes using Nafion also exhibit sensitivities, which are largely independent of electrolyte concentration over this range. In this case it is likely that electrode polarisation does not occur because of the ion exchange properties of Nafion, which allows compensation of the ions as they approach the electrode surface. These observations are of particular importance for ac conductimetric biosensors design for use in urine or blood where the electrolyte concentration is in the range 150 mM to 250 mM.

6.2.8. Enzyme electrode treatments for measurements at high ionic strength

In order to use a biosensor to measure analytes in human samples, the conditions of urine and blood have to be taken into account. The main difficulty lies in mimicking the ionic strength of those fluids in the laboratory with the use of ionic buffers of molarity similar to that of human urine or blood, i.e. approximately 100 mM to 200mM. At such a high ionic concentration and in the frequency range of our measurements (20 Hz – 300 kHz), undesired phenomena such as electrode polarisation are a problem, especially when using plane gold interdigitated electrodes. The following section summarises the fundamental characteristics of electrode polarisation observed with alternating frequencies as outlined by Schwan (1966).

6.2.8.1. Definition of electrode polarisation impedance

Electrode polarisation phenomena arise at the boundaries between the electrode and the sample under test. It is particularly disturbing for highly conductive materials at low frequencies, which are of predominant interest in physiological research.

When a metal electrode is in contact with electrolytic solution, a dc-boundary potential V_0 appears between the electrode and the fluid. If an alternating current i passes through the electrode, V_0 becomes modulated with an alternating potential V_{ac} whose phase lags that of the current and which is proportional to the alternating current, providing the

current is kept sufficiently small. This can be expressed by the following series development,

$$V_{ac} = a_0 + a_1 i + a_2 i^2 + \ldots \qquad [6.5]$$

where a_0 should be zero by definition of V_0. From [6.5] it derives,

$$V_{ac} = Z_p i \qquad [6.6a]$$

$$Z_p = a_1 \left[1 + \frac{a_2}{a_1} i + \ldots \right] \qquad [6.6b]$$

Therefore the alternating aspect of electrode polarisation can be characterised by a capacitive impedance Z_p independent of the current passing through the electrode. This condition, known as condition of linearity, is only valid for small currents (i.e. V_{ac} sufficiently small compared to V_0). The limiting current that determines the condition of linearity in an electrode / solution system increases with frequency, decreasing to zero as the frequency approaches zero and it depends on the applied voltage and the characteristics of the electrode and solution in contact.

Within the range of linearity, the polarisation impedance measured at a given frequency is independent of the presence of other frequency components of the current passing the electrode. It is then necessary to establish the limit of linearity in testing systems and verify the superposition principle stated above is applicable, so that true concentration values can be derived from the biosensor response, and the detection is not being altered by the effect of electrode polarisation. At low enough voltages, the electrode polarisation impedance can be considered to be in series with the impedance of the sample under test and therefore it simply adds directly to the impedance response of the electrode. Therefore, if Z_p is not frequency dependent, we can eliminate the contribution of the electrode polarisation impedance to the total response by applying differential methods to calculate the real sample impedance.

6.2.8.2. Platinum black and polypyrrole electrodes

Numerous methods have been proposed to overcome electrode polarisation, mainly by covering the electrodes with platinum black and by deposition of different conducting polymers, such as polypyrrole or polyaniline. We followed both lines of work in an attempt to eliminate or at least reduce considerably the screening effect generated by this modulation of the double layer potential that appears at the electrode surface when passing an ac current. Therefore, preliminary tests using the model enzyme urease at 150mM buffer concentration were carried out, in order to select the electrode treatment that most reduces electrode polarisation at high ionic strengths. Further results are included in chapter 7.

Pt black has been widely used by other workers and has been shown to reduce the electrode polarisation impedance of plain gold electrodes by four orders of magnitude (Schwan, 1966). The deposition of a thin layer of platinum black reduces this electrode polarisation phenomenon by increasing the electron exchange rate of the electrode. This increase is due not only to the higher conductivity of platinum compared to that of gold but more importantly, to the dramatic growth of the micro-surface area of the electrode due to the amorphous (non-crystalline) structure of platinum black. Coating the electrodes with semiconducting polymers is also a commonly used method, although quantifiable characterisations of its efficiency have not been reported. Typical calibration plots obtained for urease electrodes coated with platinum black and polypyrrole in 150 mM phosphate buffer are shown in figure 6.16.

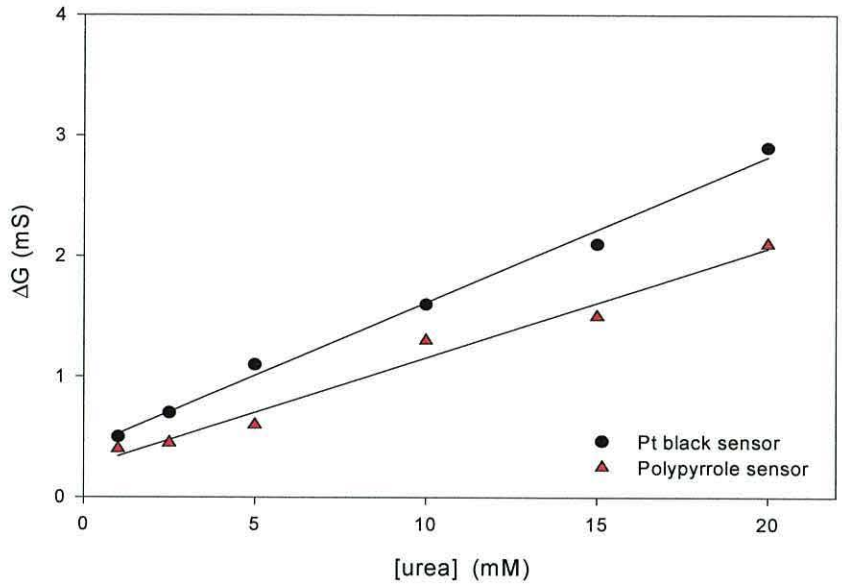


Fig 6.16. Calibration plots for a typical Pt black-gelatin-immobilised urease electrode (black circle) and a typical gold-polypyrrole immobilised urease electrode (red triangle) in 150 mM potassium phosphate buffer at pH 7.2.

Conductimetric responses for enzyme electrodes utilising both types of electrode treatment remain linear within the range of urea concentrations considered, showing no signs of saturation at 20 mM urea concentration and therefore being suitable of utilisation in human plasma.

In spite of the proven suitability of both polypyrrole and platinum black sensors for our purposes, practical reasons made us look for an alternative way of overcoming electrode polarisation problems. The electrochemical difficulties that appear in controlling platinum black electrochemical deposition, as well as the need to age the electrodes before use, make it a crude and not very reproducible system. Covering the electrodes with semiconducting polymers, on the other hand, requires special conditions for deposition, for example clean room facilities, and the resulting films are very delicate.

6.2.8.3. Nafion electrodes

The use of ion-exchange membranes such as Nafion to enhance biosensor performance has been previously reported (Pan et al, 1996; Soldatkin et al, 1997) for use both with uncharged (glutamate) and charged (penicillin) substrates. These works all report improvements in the sensor selectivity either to negatively charged interferants (ascorbate in the catalysis of glutamate) or to the build-up of ions on the electrode surface (ENFETs). The following section includes a summary of the structure and characteristic properties of the Nafion polymer.

6.2.8.3.1. Structure and properties of the Nafion polymer

Nafion^{TM} is a perfluorinated polymer developed by Dupont in the 1960s with useful properties: thermal and chemical resistance, ion-exchange properties, selectivity, mechanical strength, superacidity and insolubility in water. It is formed by etching sulphonic or carboxylic functional groups to poly(tetrafluoroethylene) ionomers in a proportion of 5% to 15%. Figures 6.17a and 6.17b show its general chemical structure.

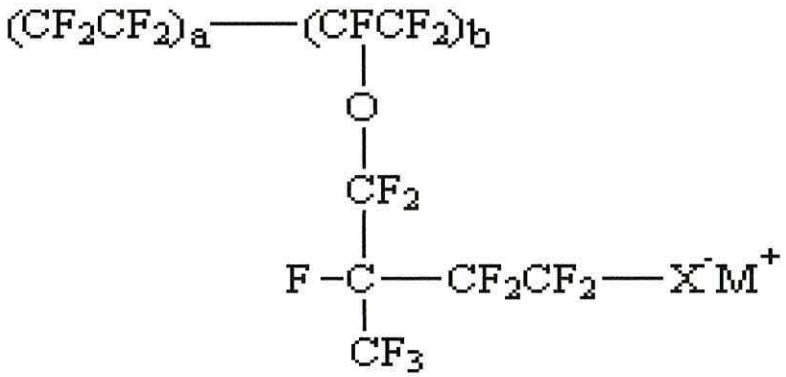


Fig 6.17a Chemical composition of the Nafion polymer

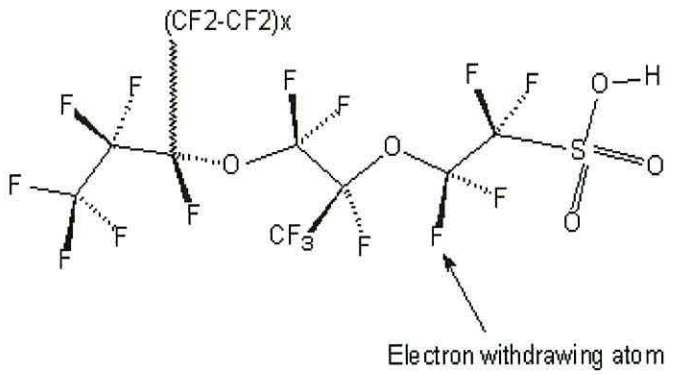


Fig 6.17b. Steric structure of the Nafion polymer

In figure 6.17b, X can be either a sulphonic ($-SO_3H$) or a carboxylic group ($-CO_2H$) and M will be a proton in acid form or a metal cation in the neutralized form. Nafion superacid characteristics, exhibiting acid strengths greater than that of 100% H_2SO_4, are attributed to the electron-withdrawing effect of the perfluorocarbon chain acting on the sulphonic acid group.

In solution, Nafion hydrophobic chains tend to group together, while the ionic groups and their respective counter ions are attracted to each other forming tightly packed clusters. As with other ion-containing polymers, Nafion shows a complex, little-understood structure. Several models describing how ionic groups aggregate within the polymer have been proposed in order to explain both ionic selectivities and ion transport properties of this and other perfluorinated membranes. Among these theories, the Yeager three phase model (figure 6.18) is perhaps the most accepted one (Yeager et al, 1982).

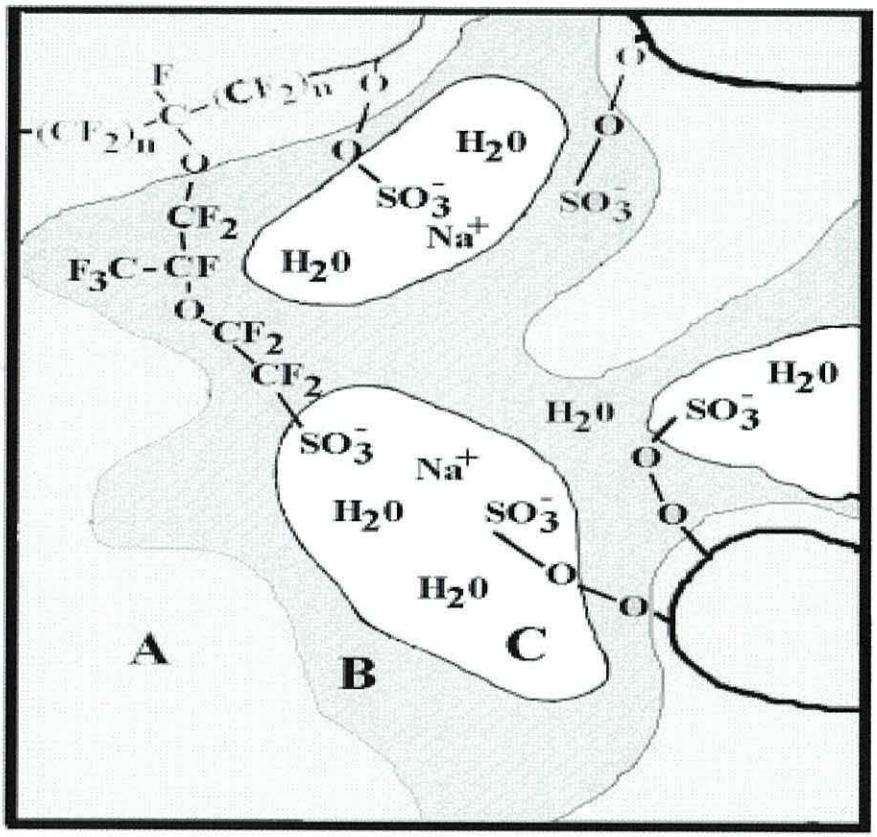


Fig 6.18. The three-phase model for Nafion (Yeager et al, 1982).

It is based on a three-phase clustered system with interconnecting channels within the polymer. These three regions consist of a hydrophobic region formed by the fluorocarbon backbone (A); an interfacial region with relatively large void volumes containing some side chains, water and some of the sulphonic or carboxylic groups which are not in the clusters (B); and the clustered regions themselves, with most of the ionic exchange sites, counter ions and absorbed water. Due to the special ability of Nafion to absorb water, hydration is presumed to entail an increase in cluster diameter as well as in the number of exchange sites, leading to fewer and larger clusters.

6.2.8.3.2. Characterisation of Nafion-coated electrodes and Nafion-based biosensors

Figure 6.19 shows the frequency response of the conductance of a Nafion-coated interdigitated electrode both at 0.1 V and 1 V peak-to-peak. It illustrates how the linearity range of the electrode polarisation impedance at low frequencies decreases with increasing applied voltage.

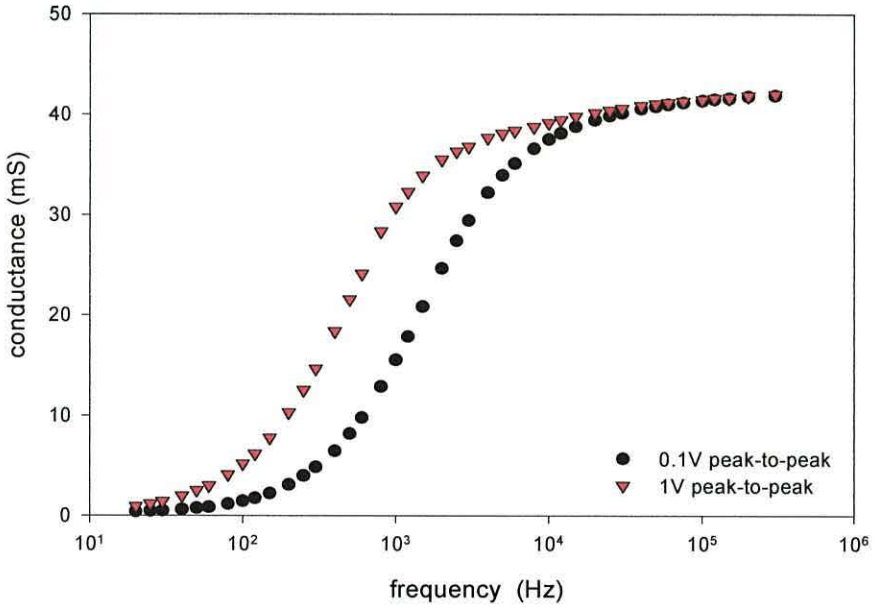


Fig 6.19. Frequency response of the conductance of a Nafion-coated interdigitated electrode in 150mM phosphate buffer at 1 V peak-to-peak.

Figure 6.20 shows the resistance of a Nafion-coated interdigitated gold electrode for varying current at a frequency of 100 kHz, compared to the resistance of a gelatin-coated interdigitated gold electrode and the resistance of an uncoated interdigitated gold electrode. For Nafion-coated electrodes, R remains practically linear in the current interval considered, while it increases dramatically for gelatin-coated and uncoated electrodes at low frequencies, where electrode polarisation phenomena can become dominant.

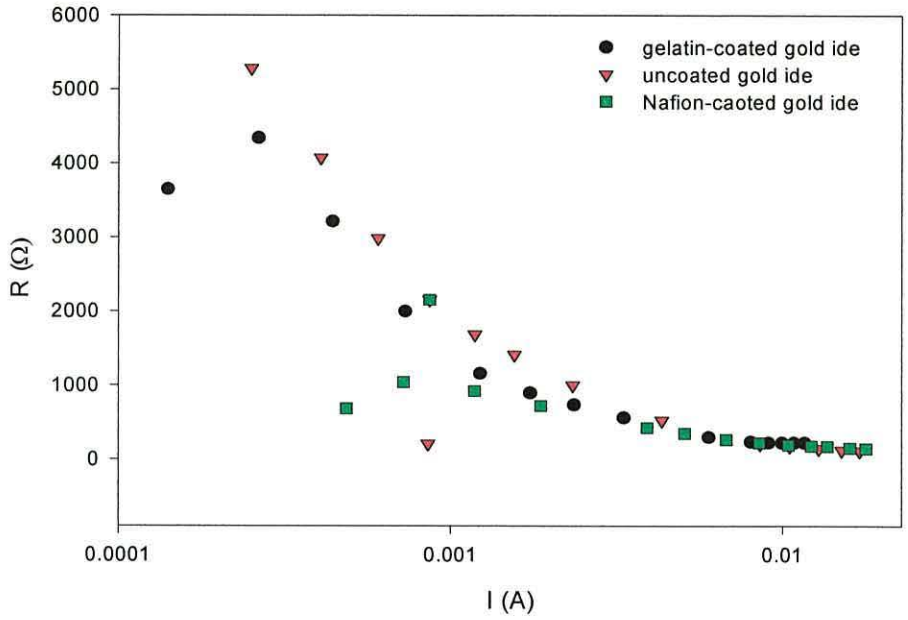


Fig. 6.20. Resistance of a typical gelatin-, Nafion- and uncoated interdigitated gold electrode in 150mM phosphate buffer for varying current at a frequency of 100 kHz

The role of Nafion in enhancing biosensors performance is not precisely understood, but it is clearly related to its complex structure, which acts as a selective membrane for ions (repelling anions and attracting cations). This has direct implications in urea and creatinine sensing in blood / plasma or urine, due both to the higher proportion of ammonium ions that will reach the electrode compared with other ionic species, and to the significant reduction of the effects of anionic interferants such as ascorbic acid in the use of the enzyme electrode in human samples.

Nafion's main drawback, on the other hand, lies with the additional diffusional barrier encountered by the substrate as it moves towards the enzyme phase. Moreover, it probably also affects the positioning of the enzyme molecules within its structure. Basically it will act as a trapping matrix, mechanically attaching the enzyme molecules in the spaces formed by the clusters and, maybe, also in the inter-spacing region; but the presence of ions and counter ions will give rise to electrostatic interactions too. As proteins themselves present relatively large polar entities, their orientation in the immobilisation matrix will depend on the exact structure of the Nafion region and

therefore, their catalytic ability could be dependant on the microstructure of the polymer.

Figure 6.21 shows the frequency response over the range 20 Hz to 300 kHz of the conductance of a typical Nafion immobilised urease electrodes for varying urea concentration in 150 mM phosphate buffer.

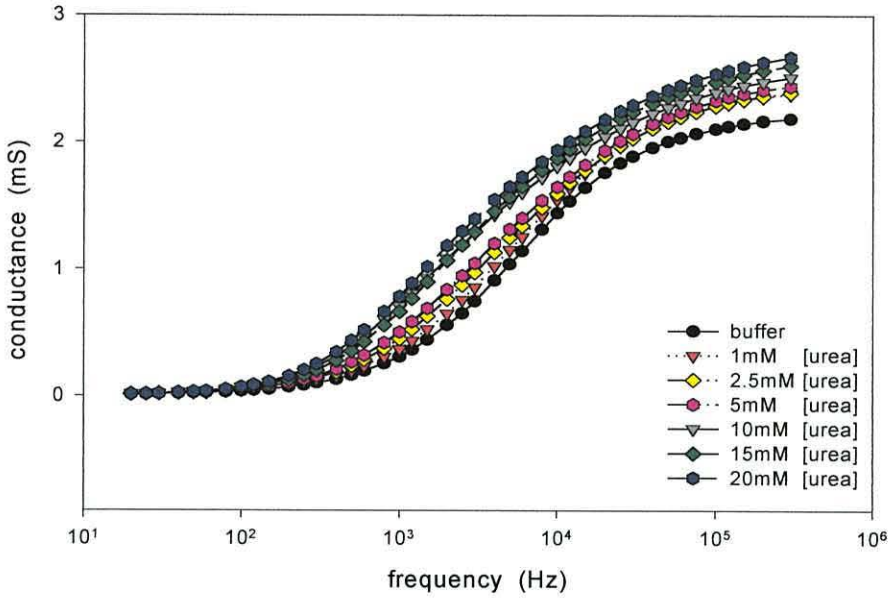


Fig 6.21. Frequency response of the conductance of a typical Nafion-immobilised urease electrode for varying urea concentration in 150 mM phosphate buffer at pH 7.4.

Consistently with all tests performed at different ionic strengths, 300 kHz was chosen to measure the conductance values in order to establish urea concentration because at this range of frequency measurements predominantly reflect the electrical properties of the enzyme layer and the electrolyte phase, and the results are consistent with a substrate concentration-dependent increase in the conductance of these phases. Figure 6.22 shows a calibration plot for a Nafion-immobilised urease electrodes derived from data in figure 6.21.

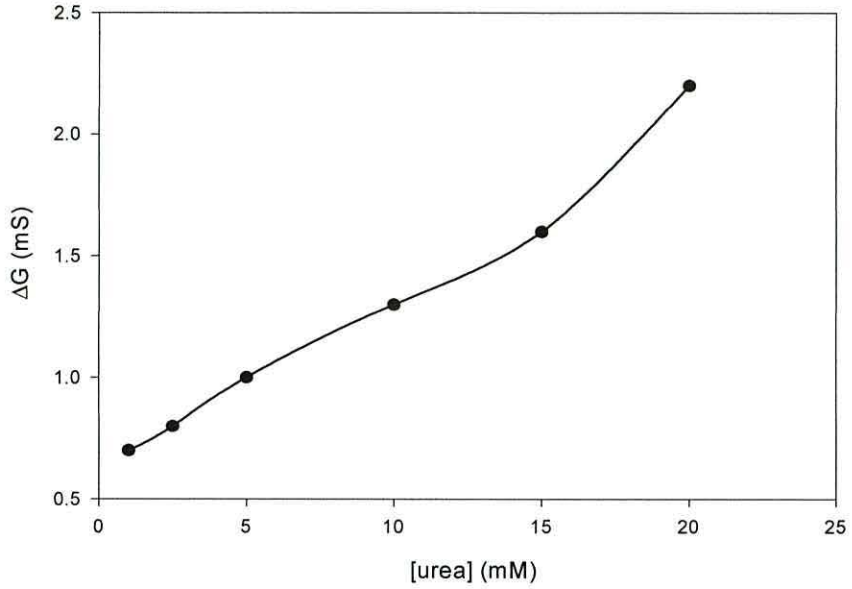


Fig 6.22. Calibration plot describing the change in conductance with urea concentration. Measurements are taken from data shown in figure 6.21 at a frequency of 300 kHz.

The incremental conductance which characterises the response of the sensor was calculated from the difference between the steady-state conductance for urea at each concentration and the conductance of the buffer (see equation [6.5]). The sensors showed a stable background conductance independent of the concentration of urea, which was attributed to the charge trapping abilities of the Nafion polymer.

Tests were carried out to establish the reproducibility of the response of Nafion-immobilised enzyme sensors both for electrodes within the same batch and between batches prepared on different days. An average CV of 3.9% was calculated for intra-batch reproducibility and of 20% for inter-batch reproducibility. The technique used to construct the sensors, which involves manual pipetting of the enzyme solution onto the surface of the electrode, makes it very difficult to obtain good reproducibility and a high variability factor must be expected due to experimental error.

Although platinum black gelatin-immobilised urease electrodes, gold polypyrrole-immobilised urease electrodes and gold Nafion-immobilised urease electrodes all show high sensitivities at high electrolyte concentrations, Nafion was chosen to pursue the study due to the simplicity of preparation and its lower price.

6.2.9. Urea measurement in serum

Figure 6.23 describes the response of Nafion-immobilised urease on gold electrodes to sheep serum superimposed on a calibration plot for the sensor against standard urea concentrations.

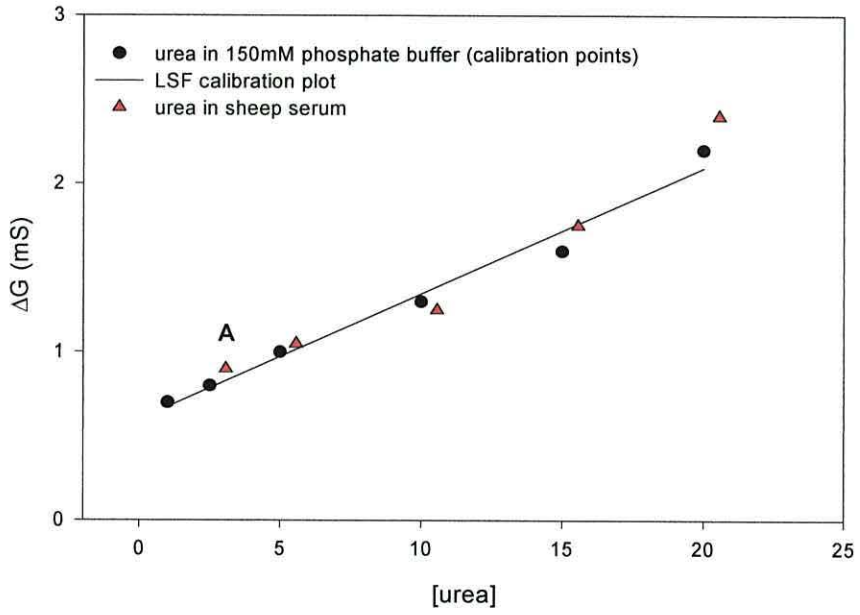


Fig 6.23. Response of a typical Nafion-immobilised urease electrode to samples of urea in serum. Calibration points and plot for standard urea concentration in 150mM phosphate buffer are shown for comparison.
Point A corresponds to the sensor response in untreated serum.

Point A corresponds to the measurement obtained in an untreated sample of serum. The urea concentration of this sample was independently obtained using the ammonia electrode to measure the ammonia gas concentration generated by addition of free

urease to the serum. The value obtained was 3.1 mM, which is within the normal range for urea in serum (1.6 mM to 10 mM). Additional urea was then added to the serum to produce a range of urea concentrations. The response of the sensor to these additions is plotted and can be seen to closely follow the calibration characteristic.

6.3. Conclusions

Model urease electrodes have been employed here to characterise and optimise ac impedance conductimetric sensors. The activity of urease both in solution and immobilised states has been investigated, as well as the effect of the method of immobilisation and enzyme loading on the sensors' performance. Furthermore, urease electrodes have been characterised as a function of temperature, pH and electrolyte concentration.

It has been shown that the type of immobilisation is critical for fabrication of devices with long shelf lives. In addition, for these ac conductance sensors, the electrode surface and immobilising matrix are crucial especially for measurements at higher electrolyte concentrations where electrode polarisation effects can significantly reduce the sensor response. This is of particular importance for conductimetric sensors designed for clinical use, where optimum sensitivity is required at physiological electrolyte concentrations.

Of the three immobilisation matrixes applied to urease electrodes, Nafion-based sensors offered the best performance, combining high sensitivity, long shelf life and freedom from electrode polarisation effects. Indeed, Nafion-immobilised urease electrodes were employed to detect and differentiate the concentration of urea in untreated serum samples and sera spiked with known concentrations of urea with positive results.

Chapter 7. Impedance Biosensors - Equivalent Circuit Model

Numerous publications on ac impedance-based biosensors have appeared over the last few years. Their broad applicability and relative simplicity of use have promoted extensive study in different fields, ranging from immunobiosensors (Billiard et al, 1991; Gebbert et al, 1994; McNeil et al, 1995) to microelectronic enzyme electrodes (Watson et al, 1988; Cullen et al, 1990). The sensor construction generally consists of metal electrodes covered by a thin immobilized enzyme layer entrapped behind a membrane. Changes in the characteristic impedance at a particular frequency, typically in the kHz range, which take place as a result of the enzyme catalysed reaction are recorded and correlated to the substrate concentration.

However, in spite of the extensive experimental effort dedicated to the development and optimisation of such sensors (Lawton et al, 1988; Yon Hin et al, 1990; Hintsche et al, 1991; Shul'ga et al, 1994) , very little theoretical analysis has appeared concerning their fundamental mechanisms of operation (Sheppard and Mears, 1996; Lee et al, 2000).

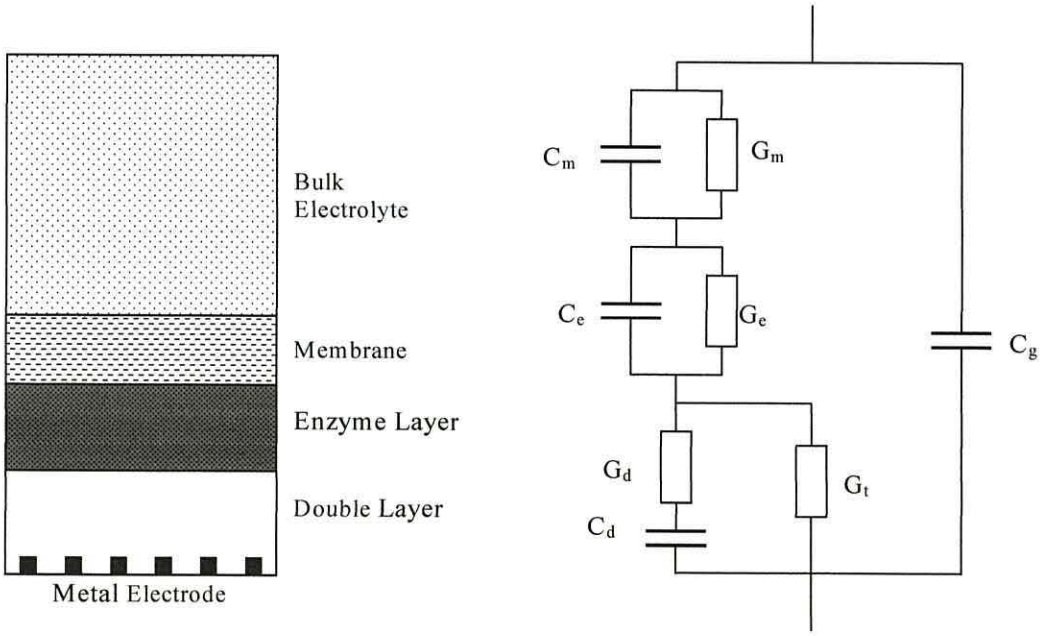
Following the work on impedance analysis carried out by Macdonald (1974, 1976) and by Taylor and Macdonald (1987), an equivalent circuit model representing the biosensor is presented here. This model attempts to identify and account for all electrical processes that contribute to the impedance of the system in an effort to obtain detailed information about the nature of the interactions that determine its response over a wide frequency range.

In order to verify the validity of the model, ac impedance measurements were made on urease electrodes prepared in our laboratory. Urease was chosen as a model enzyme due to its stability and activity characteristics and it was immobilised onto the surface of interdigitated gold or platinum black electrodes using gelatin or Nafion and an outer

cellulose acetate membrane. The sensors impedance characteristics were investigated over the frequency range 10 Hz to 300 kHz at electrolyte concentrations of 10 mM and 200 mM. In the presence of substrate, changes in conductance were measured and correlated with variations of urea concentration. Modelling of the data indicated that the dominant change resulting from the interaction between the enzyme and substrate was an increase in the electrolyte conductance rather than in the conductance of the enzyme layer. The processes occurring in each of the sensors constituent phases which are responsible for the sensors activity are discussed below.

7.1. Equivalent circuit model

The enzyme electrode and aqueous medium containing the substrate can be considered a multi-phase system described in figure 7.1a. Two equivalent circuits were considered; a simple series arrangement of parallel R-C combinations and a circuit proposed by Macdonald which was employed in ac admittance analysis of metal/insulator/electrolyte interfaces by Taylor and Macdonald. The equivalent circuit for this system is shown in figure 7.1b.



Figs. 7.1a and 7.1b. Multiphase system and equivalent circuit modelling biosensor performance.

When a potential is applied to an electrode in an aqueous environment, the electrode area will be surrounded by an electrical double layer associated with the electric field-induced accumulation of ions at the metal surface. The effect can be approximately modelled by an R-C circuit in which the double layer capacitor C_d is charged through an electrolyte conductance G_d.

The charge transfer conductance G_t allows for the existence of a current pathway although for inert electrodes and in the absence of electrochemically active species in the electrolyte the value of G_t will be very small relative to that of G_d.

The enzyme phase is usually composed of a hydrophilic polymeric material used to immobilize the enzyme. This system is likely to produce a dielectric relaxation process over the frequency range employed due to the electric field-induced translational motion of hydrated ions along the length of the polymer chain which produces large induced dipole moments. Therefore, a more exact description of the enzyme phase can be obtained by making the conductance and capacitance of this layer frequency-dependent according to the modified Debye equation:-

$$G_e + j\omega C_e = G_{es} + \frac{G_{e\infty} - G_{es}}{1 + (j\omega\tau)^{\beta}} \qquad [7.1]$$

where j is the complex operator(= $\sqrt{-1}$), ω is the angular frequency (= 2π x frequency) and G_s and G_∞ are the limiting low frequency and high frequency conductances which determine the range of C_e and G_e. β is the Cole-Cole parameter which determines the breadth of the distribution of relaxation times and τ is the characteristic relaxation time given by

$$\tau = \frac{1}{2\pi f_0} \qquad [7.2]$$

where f_0 is the relaxation frequency.

The membrane conductance, G_m, and capacitance, C_m, can be modelled simply as a parallel R-C combination in series. Finally the geometric capacitance of the inter-electrode space is described by C_g.

For simplicity and consistency, the analysis of the circuit (figure 7.1b) is performed in terms of the electrical impedance. The total impedance of the circuit can be shown to be

$$Z_T = \frac{1}{G_T + j\omega C_T} = \frac{Z_L}{(1 + j\omega C_g Z_L)} \qquad [7.3]$$

where G_T and C_T are the conductance and capacitance of the circuit as measured by the analyser. The impedance term Z_L is given by

$$Z_L = Z_a + Z_m + Z_e \qquad [7.4]$$

In the above equation Z_m and Z_e are the impedances of the membrane and enzyme layers which as parallel R-C combinations are simply

$$Z_{m,e} = \frac{1}{G_{m,e} + j\omega C_{m,e}} \qquad [7.5]$$

Z_a is the combined impedance of the double layer Z_d and charge transfer conductance G_t given by

$$Z_a = \frac{G_d + j\omega C_d}{j\omega C_d (G_d + G_t) + G_t G_d} \qquad [7.6]$$

7.2. Materials and Methods

7.2.1. Materials

Gold-coated interdigitated electrodes were manufactured in-house using glass as substrate and following standard photolithographic techniques. The interdigitated sensor square head had a length of 1 cm, with tracks of 0.1cm width and electrode spacings of 0.1 cm. A diagram of the interdigitated electrode array is shown in figure 6.1. Parallel plate gold electrodes were also assembled in-house, using gold discs of area 1.5 cm^2 and leaving an interelectrode separation of 0.9 cm.

All reagents employed are listed in section 6.1.1, as well as the procedures followed to prepare the different solutions required for the experiments.

7.2.2. Immobilisation methods

Urease electrodes were immobilised by entrapment in a gelatin matrix and by electrostatic bonding in the clusters of Nafion polymer. These immobilisation methods followed are described in sections 6.1.2.1. and 6.1.2.3.

7.2.3. Experimental Procedures

The experimental cell employed either interdigitated gold arrays mounted facing a hollow perspex housing or parallel plate gold electrodes.

A description of the electrical procedures followed is included in section 6.1.3.1, with diagrams of the experimental cell and the set-up employed.

7.3. Results and Discussion

The conductance characteristics of typical gel- and Nafion-immobilised urease on gold interdigitated electrodes in 10mM and 200mM phosphate buffer over the frequency range 20Hz to 300kHz are shown in figures 7.2a and 7.2b. Similar frequency response characteristics were exhibited by urease electrodes incorporated into parallel plate electrode configurations.

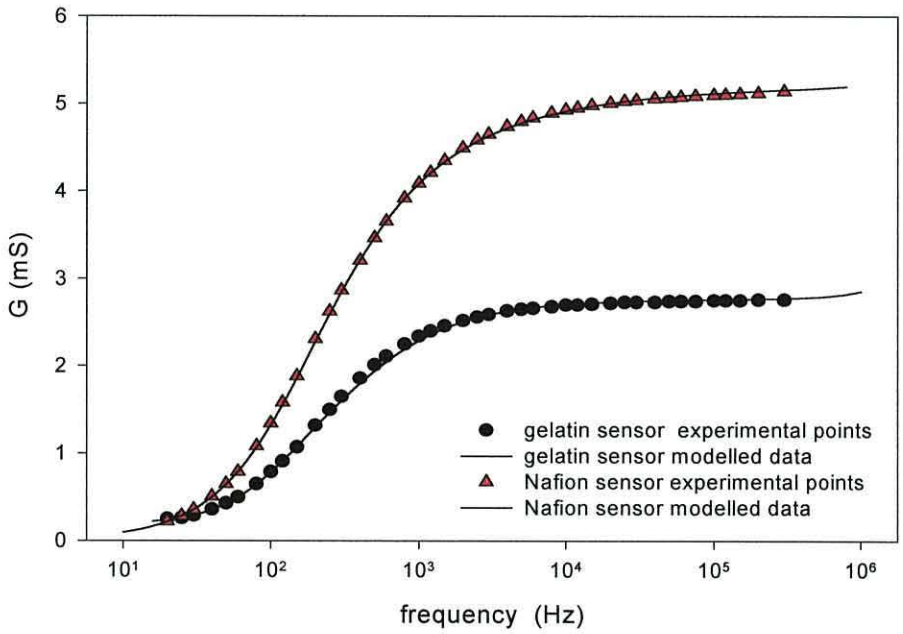


Fig 7.2a. Conductance characteristics of typical gel- and Nafion-immobilised urease on gold interdigitated electrodes in 10 mM phosphate buffer at pH 7.2 over the frequency range 20 Hz to 300 kHz.

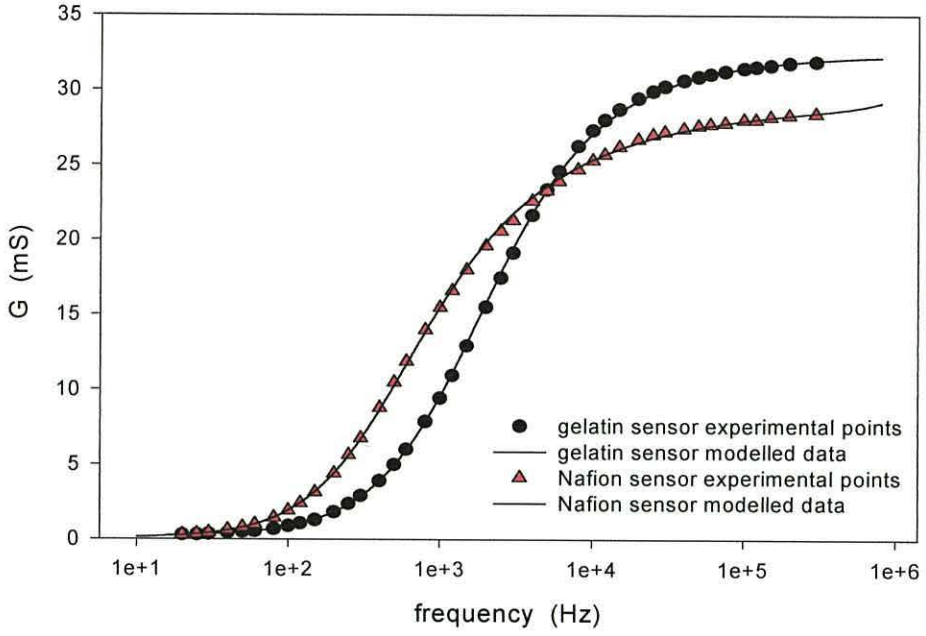


Fig 7.2b. Conductance characteristics of typical gel- and Nafion-immobilised urease on gold interdigitated electrodes in 200 mM phosphate buffer at pH 7.2 over the frequency range 20 Hz to 300 kHz.

The solid lines on these plots correspond to the conductance G_T modelled from equation 7.1 using values for conductances and capacitances of the constituent phases as given in Table 7.1.

Parameters	**Gelatin-immobilised on gold electrode**				**Nafion-immobilised on gold electrode**				**Gelatin-immobilised on Pt black electrode**	
	10mM buffer		***200mM buffer***		***10mM buffer***		***200mM buffer***		***170mM buffer***	
	Buffer	***Urea***	***Buffer***	***Urea***	*Buffer*	***Urea***	***Buffer***	***Urea***	***Buffer***	*Urea*
G_t ($\times 10^{-4}$ S)	2.1	2.1	3	7	0.05	0.05	1.1	1.1	400	475
G_d ($\times 10^{-2}$ S)	1	29.4	6	6	1.5	7.5	4.1	4.4	3	4
C_d ($\times 10^{-6}$ F)	2.6	4.1	6.5	16	5.8	4.82	11.2	10.2	124.5	144
G_{es} ($\times 10^{-3}$ S)	2.15	2.55	0.4	36	0.7	1	0.01	0.01	29	29.6
$G_{e\infty}$ ($\times 10^{-2}$ S)	0.46	0.51	16	16	1.22	1.22	95	100	5	5
$C_{e\infty}$ ($\times 10^{-10}$ F)	3	3	3	3	3	4.4	3	3	3	3
β_e	0.7	0.7	0.65	0.65	0.53	0.53	0.51	0.51	0.65	0.65
f_e (kHz)	0.6	0.6	13	13	0.45	0.45	300	300	1	1
C_m ($\times 10^{-10}$ F)	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.08
G_m ($\times 10^{-2}$ S)	2.144	2.144	12.5	12.5	2.144	2.144	11	11	12.5	12.5
C_g ($\times 10^{-11}$ F)	2.025	2.025	2.025	2.025	2.025	2.025	2.025	2.025	2.025	2.025

Table 7.1. Conductances and capacitances of the constituent phases employed to generate figures 7.2a and 7.2b

The model also fitted the data in the form G/f which has a preferential weighting towards the low frequency data and is proportional to the dielectric loss. Figures 7.3a and 7.3b are analogous to 7.2a and 7.2b.

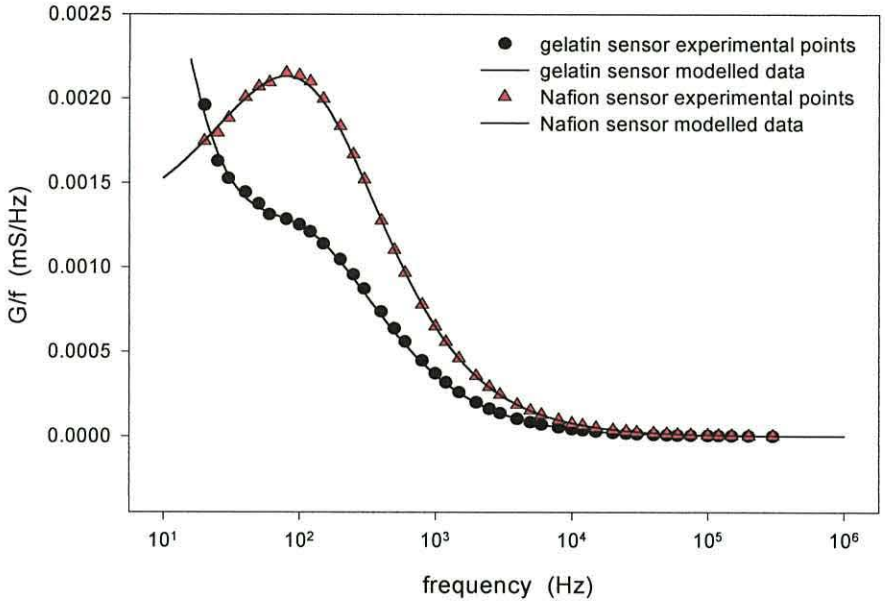


Fig 7.3a. G/f characteristics of typical gel- and Nafion-immobilised urease on gold interdigitated electrodes in 10 mM phosphate buffer at pH 7.2 over the frequency range 20 Hz to 300 kHz.

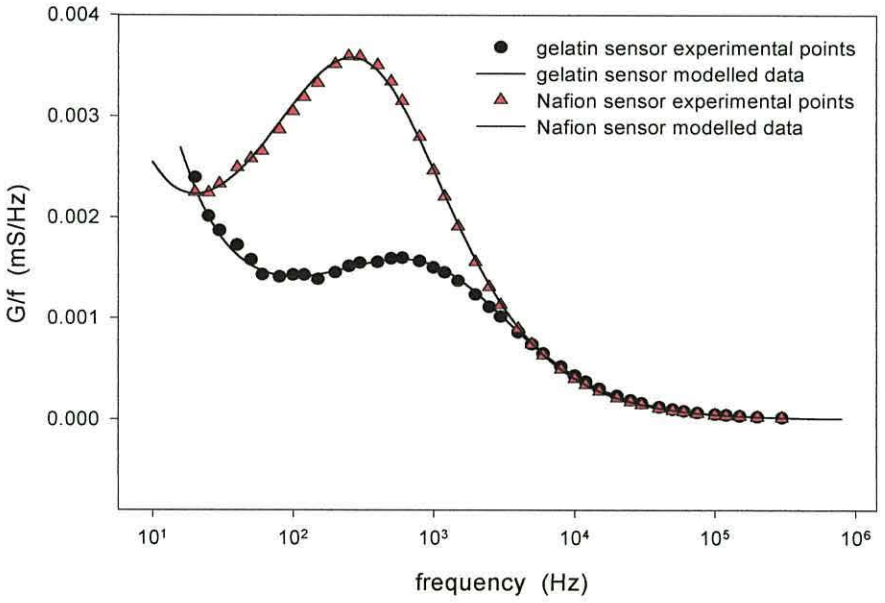


Fig 7.3b. G/f characteristics of typical gel- and Nafion-immobilised urease on gold interdigitated electrodes in 200 mM phosphate buffer at pH 7.2 over the frequency range 20 Hz to 300 kHz.

Modelling analysis was also carried out using the simple series sequence of R-C parallel combinations. It was found that identical trends were predicted to those of the equivalent circuit shown in figure 7.1b.

In the model employed here, the large number of variables made it difficult to ascribe unambiguous values to the component phases. To reduce this problem, the electrical values of the individual phases used in the model were independently measured where possible.

It can be seen that raising the electrolyte concentration from 10 mM to 200 mM produced increases in the double layer conductance and capacitance and large increases in the increment of the conductance and the characteristic relaxation frequency of the enzyme phase. The latter observations are consistent with the immobilising agents supporting a relaxation mechanism involving the formation of an induced dipole due to the translocation of ions along the polymer chain.

There are marked differences in the conductance characteristics exhibited by the two immobilizing polymers in the enzyme layer. The modelled values for the conductance increment and relaxation frequency are considerably higher for Nafion compared with gelatin, particularly at the lower electrolyte concentration. This is consistent with there being a larger number of ions associated with the Nafion polymer which exhibit a higher mobility along the polymer chain. It is likely that this reflects the chemical composition of Nafion, the clusters of negatively charged sulphonate ions being able to bind large numbers of positively charged metal ions which are able to transfer relatively easily between binding sites.

The family of curves in Figure 7.4 describes the conductance of the urease electrode over the frequency range 20 Hz to 300 kHz for urea concentrations of 1 mM to 20 mM.

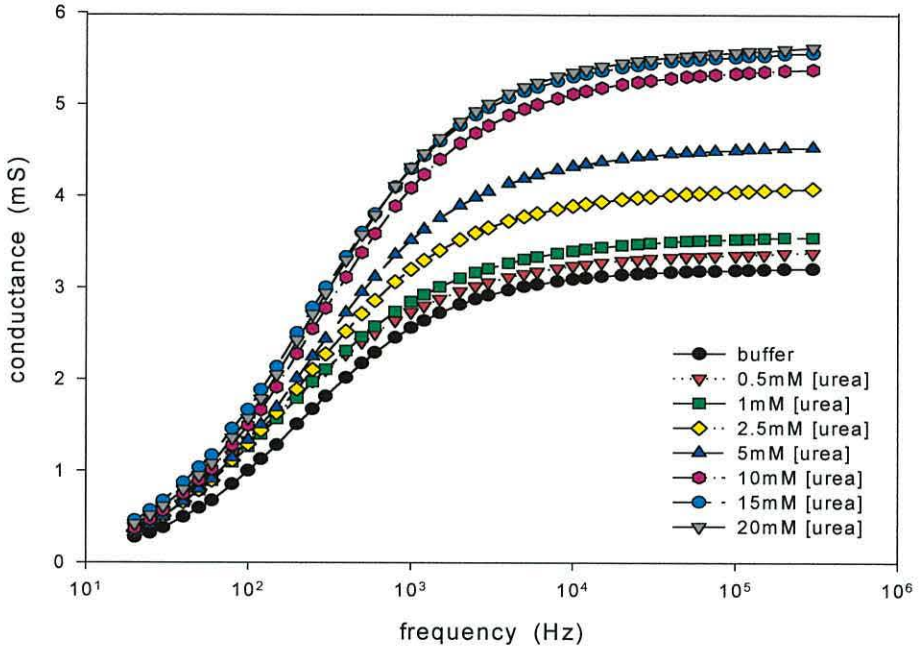


Fig 7.4. Conductance of a typical gel-immobilised urease electrode over the frequency range 20Hz to 300 kHz for urea concentrations of 1 mM to 20 mM in 10 mM phosphate buffer at pH 7.2.

By modelling the changes in the response characteristics of the enzyme electrode resulting from the addition of substrate, it was possible to determine the contributions from each of the composite phases. The sensor response (ΔG) was calculated from the change in the conductance of the sensor over the entire frequency range as follows:

$$\Delta G(\omega) = G_{urea+buffer}(\omega) - G_{buffer}(\omega) \qquad [7.7]$$

The difference plots represented in figures 7.5a and 7.5b were used since these showed in considerably more detail the effect over the whole frequency range of introducing substrate. It was found that for measurements in 10 mM electrolyte, a similar mechanism of operation was predicted by the equivalent circuit model for the gelatin and Nafion-immobilised urease electrodes. However, at 200 mM, there were significant differences between the measured frequency response characteristics and the model predicted a completely different mechanism of response for electrodes utilizing the two different immobilizing agents.

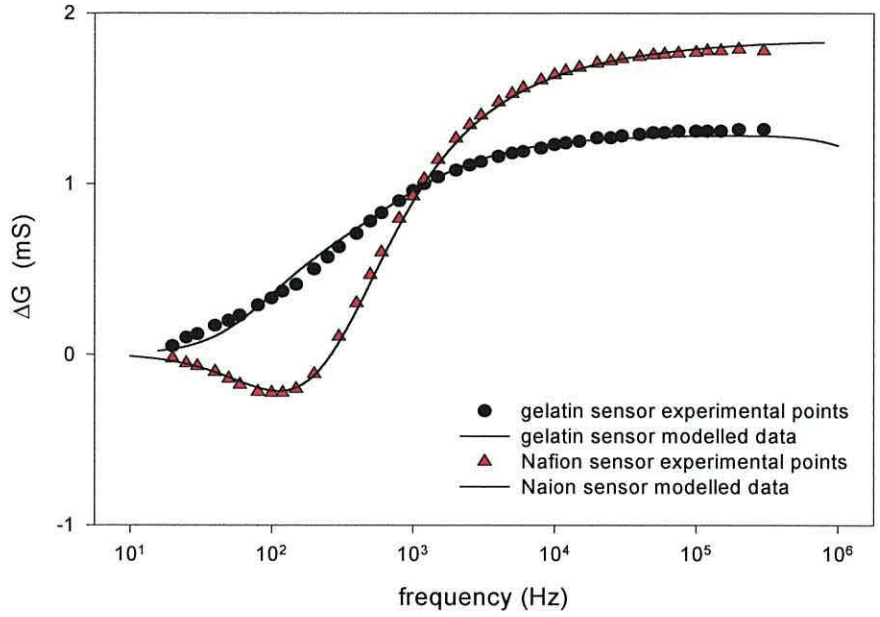


Fig 7.5a. ΔG of typical gel-immobilised and Nafion-immobilised urease electrodes in 10 mM phosphate buffer at pH 7.2. Data points correspond to 300 kHz and 5 mM concentration of urea

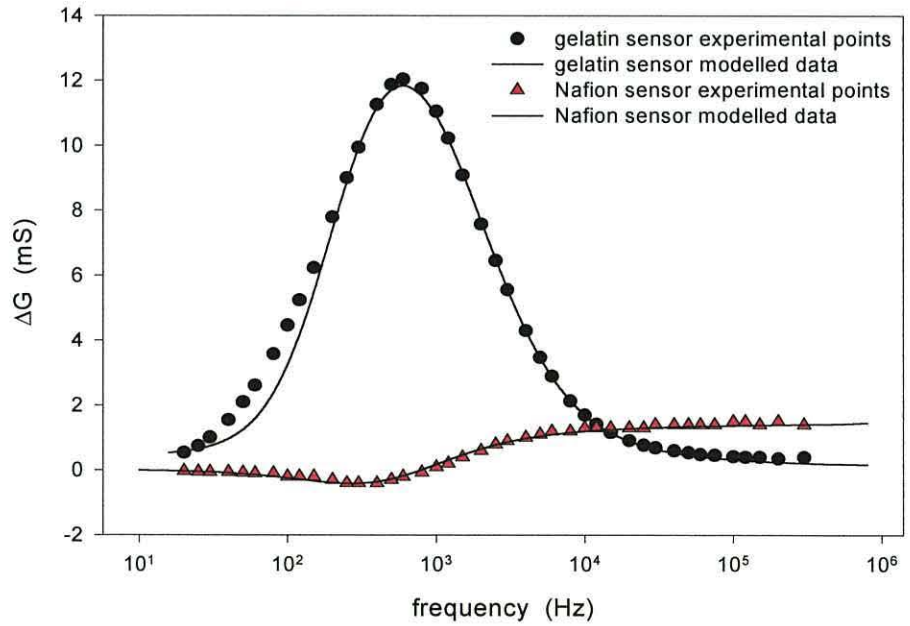


Fig 7.5b. ΔG of typical gel-immobilised and Nafion-immobilised urease electrodes in 200 mM phosphate buffer at pH 7.2. Data points correspond to 300 kHz and 5 mM concentration of urea

As can be seen in figure 7.5a, at 10mM electrolyte concentration, the values obtained by modelling the data indicate that the increases in the observed conductances for both gel- and Nafion-immobilised urease electrodes on introduction of substrate were the result of increases in the conductances of the enzyme and electrolyte phases. This is consistent with a scheme involving the generation of ionic product in the enzyme layer and diffusion of ions through the membrane into the electrolyte. The rise in electrolyte conductance was confirmed as being due to product diffusion by measurement of increased ammonium ion concentration in the buffer.

At 200 mM electrolyte concentration (figure 7.5b), for urease immobilized in Nafion, changes in the impedances of the component phases, on introduction of substrate, follow a similar trend to those seen at 10 mM; with significant increases in G_d and the high-frequency enzyme layer conductance, $G_{e\infty}$. However, for the gel-immobilised system the characteristic is very different and a different mechanism emerges from the modelling. In this case, the major changes elicited by product formation are large irreversible increases in the limiting low frequency conductance of the enzyme layer G_{es} and in the capacitance of the double layer, C_d. The values of G_d and $G_{e\infty}$ remain unchanged. These trends are consistent with a process involving the accumulation and trapping of ionic product in the enzyme layer giving rise to a significant increase in its conductance and in the magnitude of electrode polarization as described by the double layer capacitance, C_d.

Given that platinum black is known to dramatically reduce electrode polarisation effects, a gelatin-immobilised urease electrode fabricated on platinum black electrodes was tested in an effort to understand the polarisation mechanisms apparently responsible for the different behaviour of the sensors employing gelatin and Nafion as immobilising agents.

The results and modelled data from this enzyme electrode were quite different from those of the identical gel-enzyme system on gold electrodes at 200 mM electrolyte. In fact, the response of this Pt black-gel-urease electrode originated from an increase in G_d and $G_{e\infty}$, indicating a similar mechanism to that exhibited by Nafion-immobilised urease on gold. However, as discussed below, the frequency response characteristic and many of the modelled values were not the same, indicating different underlying processes. The

conductance and capacitance values for each of the different components is shown in Table 7.1.

The reduction of electrode polarisation by using platinum black is well documented (Schwan, 1966). The modelled electrical component values appear to confirm the accepted mechanism in which platinum black supports a very large increase in the microscopic electrode area. This is reflected by a 20 fold increase in the double layer capacitance and a 100 fold increase in the transfer conductance G_t compared with values derived for gold electrodes. Both equivalent circuit models also showed a large increase (60 fold) in the low frequency conductance of the enzyme phase as a result of using platinum black compared to gold electrodes, reflecting an increase in the long-range conductance of the ions in the enzyme layer. Since the number of ions is constant, the rise in conductance must be due to an increase in ion mobility.

The reason why varying the electrode material should alter this property is not immediately apparent but may be associated with the magnitude of the electric field applied to the gelatine / enzyme phase. The large transfer resistance predicted for bare gold electrodes implies that a large proportion of the voltage applied to the sensor will be dropped across the electrode / double layer interface. As a result, the electric field applied to the enzyme phase will be relatively small. When platinum black electrodes are employed the transfer resistance is low, implying a much smaller drop in voltage across this region and a relatively large electric field applied to the enzyme phase. Since the gelatin molecules possess a very large dipole moment, this is likely to result in some significant interaction with the applied field which may provide for relatively high mobility ion percolation pathways through the enzyme phase, as supported by the high values of G_{es}. This would then allow ions generated by the hydrolysis of urea to diffuse out of the enzyme layer into the electrolyte.

It is apparent from the values derived by modelling the Nafion / urease on gold electrode characteristics that, although the mechanism of operation involving increases in G_d and $G_{e\infty}$ is similar to that described above for the gel/urease on Pt black systems, the underlying processes are different. For the Nafion-based urease electrodes, the transfer conductance and the low frequency conductance of the enzyme phase are much smaller implying a low long-range ion mobility. However, the high frequency conductance of

the enzyme phase is greater than that of the Pt black / gelatine / urease sensor and the relaxation frequency is higher. This may reflect the existence of charge clusters in Nafion with ions able to exchange rapidly between adjacent binding sites. The fact that the ionic product is able to migrate through the enzyme phase implies that there must be connectivity between the charge clusters allowing ions generated by enzyme action to diffuse into the electrolyte by rapid short-range transitions between charge clusters.

7.4. Conclusions

Experimental data has shown that gel-immobilised urease in conjunction with platinum black electrodes and Nafion-immobilised urease on bare gold electrodes produce active impedance biosensors able to measure urea concentrations in electrolyte at physiological ionic strengths. The following chapters describe the characterisation of a model urease electrode capable of detecting urea in undiluted serum and the design of an applied creatinine biosensor to be used in undiluted urine.

Modelling has provided valuable information regarding the different underlying processes responsible for the action of these sensor systems involving ion migration through the enzyme layer into the electrolyte phase.

The accumulation of ions in the enzyme layer for gel-based sensors employing bare gold electrodes at 200 mM electrolyte concentration produces a very different response characteristic indicating increases in G_{es} and G_d rather than G_d and $G_{e\infty}$. Our studies have shown that, in this case, the rise in conductance of the enzyme electrode is time-dependent and to a large extent irreversible with the ions remaining trapped in the enzyme phase. As a result this system could not easily be employed to determine substrate concentration.

Chapter 8. Design of an Applied Sensor – Biosensor for Creatinine Detection

Following the characterisation study on impedance urease electrodes, further investigations were carried out to ascertain the applicability of this conductimetric biosensor to a medical situation: the measurement of creatinine in urine. In order to do this, two different enzyme electrodes were studied, one based on the single enzyme creatinine deiminase and another on the multienzyme system creatininase / creatinase / urease.

The detection concept, which is the basis of the model impedimetric urease electrode described in the previous chapter, applies to all conductimetric sensing devices. Changes in the conductance of the enzyme phase can be calculated over a particular frequency range (10 kHz – 300 kHz) by means of the circuit analysis of a three-phase enzyme electrode system comprising enzyme layer, membrane and bulk electrolyte. Such variation in the conductance is proportional to the concentration of product species generated in an enzyme catalysed reaction and therefore, a measurement of the conductance change will also determine the original concentration of substrate present in the sample.

The election of creatinine for the design of an applied biosensor came from a previous collaboration between the University of Wales, Bangor and British Nuclear Fuels Ltd. BNFL routinely measure creatinine concentrations in their employees because the level of creatinine is raised after exposure to radiation. They currently employ spectrophotometric / colorimetric methods, which involve hazardous chemicals (e.g. picric acid) and are time consuming.

As described in chapter 5, creatinine is the final product of creatine metabolism in mammals and has general clinical diagnosis importance for being not only an indicator of muscular, thyroid and renal disfunctions (Kim et al, 1999), but also of acute myocardial infarction (Khan and Wernet, 1997). The reference ranges of creatinine concentration in serum or plasma and 24 hour urine are 35 μM to 150 μM and 8.84 mM to 13 mM respectively but during kidney disfunction or muscle disorder the creatinine concentration in serum or plasma could rise to values higher than 1000 μM (Khan and Wernet, 1997; Jurkiewicz et al, 1998a).

The disadvantage of measuring urine levels compared to serum / blood levels is that a lag of 24 hours is needed, although, on the other hand, the collection of the samples is less intrusive. Moreover, the levels of creatinine in blood / serum can be employed only to evaluate kidney disfunction and urine creatinine levels can be used as screening test to evaluate kidney disfunction and as part of a creatinine clearance test. The low specificity and high cost of the commercially available analysers currently employed in general clinical practices have prompted the appearance of several enzymatic methods based on spectrometry.

The enzyme creatinine deiminase and the complex enzyme system creatininase / creatinase / urease were separately employed in the design of an impedimetric biosensor for the detection of creatinine. Such enzyme systems were immobilised onto interdigitated electrodes and creatinine sample solutions at different concentrations were detected by means of conductance changes.

Both single- and multi-enzyme systems were described in an attempt to characterise and optimise these two enzyme electrodes with the intention of assessing their practical application in clinical diagnosis.

8.1. Materials and Methods

8.1.1. Materials

Creatinase (creatine aminohydrolase from Actinobacillus, EC 3.5.3.3; 20 U/mg – 40 U/mg) and creatininase (creatinine amidohydrolase from Pseudomonas sp., EC 3.5.2.10; 100 U/mg – 300 U/mg) were also purchased from Sigma Chemical Company. Both creatininase and creatinase solutions were prepared at a concentration of 40 mg/ml in 10 mM potassium phosphate buffer at pH 7.2. Multi-enzyme creatininase / creatinase / urease solution was prepared mixing creatininase, creatinase and urease solution in no particular order, at a ratio of 50:50:1. Multi-enzyme creatinase / urease solution was prepared mixing creatinase and urease solution at a 1:1 ratio.

Creatinine deiminase (creatinine iminohydrolase, type III; 14 U/mg) was supplied by Toyobo. Creatinine deiminase solution was used at a concentration of 75 mg/ml in 10 mM potassium phosphate buffer at pH 7.2.

Creatinine was purchased from Sigma Chemical Company and different concentrations were prepared dissolving appropriate amounts in potassium phosphate buffer.

The rest of reagents and solutions employed can be found in section 6.1.1.

8.1.2. Immobilisation methods

8.1.2.1. Immobilisation with gelatin

Enzyme solution (creatinine deiminase at 75 mg/ml; creatininase at 40 mg/ml, creatinase at 40 mg/ml and urease at 40 mg/ml at 50:50:1 ratio; creatinase at 40 mg/ml and urease at 40 mg/ml at 1:1 ratio) was combined with 1% gelatin in 1:1 proportion. Both solutions were prepared in 10 mM potassium phosphate buffer (pH 7.2). 50µl of the enzyme / gel mixture was deposited over the electrode surface. The enzyme-coated electrodes were left at room temperature for around five hours after which a small section of cellulose acetate dialysis membrane was deposited on the surface. A hollow perspex housing was mounted on the electrode surface and covered with a gold coated

copper disc wrapped in Parafine to electrically insulate it from the solution. Such a test cell was then soaked in potassium phosphate buffer (pH 7.2) for approximately twelve hours.

8.1.2.2. Immobilisation with Nafion

Equal volumes of enzyme solution (urease 40 mg/ml; creatinine deiminase at 75 mg/ml; creatininase at 40 mg/ml, creatinase at 40 mg/ml and urease at 40 mg/ml at 50:50:1 ratio) and 1% Nafion were combined in 10 mM potassium phosphate buffer (pH 7.2) solution. 50µl of the enzyme / Nafion mixture was deposited on the electrode and left to dry. The enzyme-coated electrodes were left at room temperature for around five hours after which a small section of cellulose acetate dialysis membrane was deposited on the surface. A hollow perspex housing was mounted on the electrode surface and covered with a gold coated copper disc wrapped in Parafine to electrically insulate it from the solution. Such a test cell was then soaked in potassium phosphate buffer (pH 7.2) for approximately twelve hours.

8.1.3. Experimental methods: Dielectric Measurements

All experimental methods are described in section 6.1.3.1.

8.2. Results and discussion

8.2.1. Kinetics of the hydrolysis of creatinine catalysed by creatinine deiminase or creatininase / creatinase / urease multi-enzyme system

Following the thorough study on the kinetics of the hydrolysis of urea catalysed by urease (see chapter 6), general investigations were carried out for the kinetics of the hydrolysis of creatinine both by creatinine deiminase and by the multi-enzyme system creatininase / creatinase / urease.

Figure 8.1 shows the Lineweaver-Burk plot for a typical Nafion-immobilised creatinine deiminase electrode. Its characteristic parameters are found to be K_M =(19 ± 2) mM and v_{max}=(1.6 ± 0.1) mS/min.

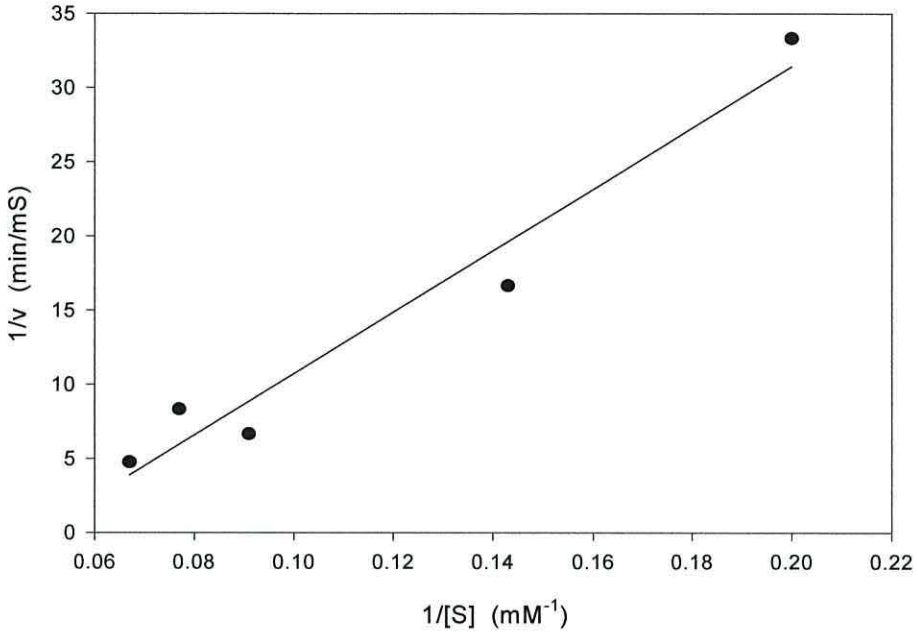


Fig 8.1. Lineweaver-Burk plot for a typical Nafion-immobilised creatinine deiminase electrode in 150 mM phosphate buffer at pH 7.2.

Figure 8.2 shows the Lineweaver-Burk plot for a typical creatininase / creatinase / urease electrode. The maximum velocity and K_M value for the catalysis of creatinine using a multi-enzyme system are found to be K_M =(31 ± 7) mM and v_{max} =(0.54 ± 0.06) mS/min

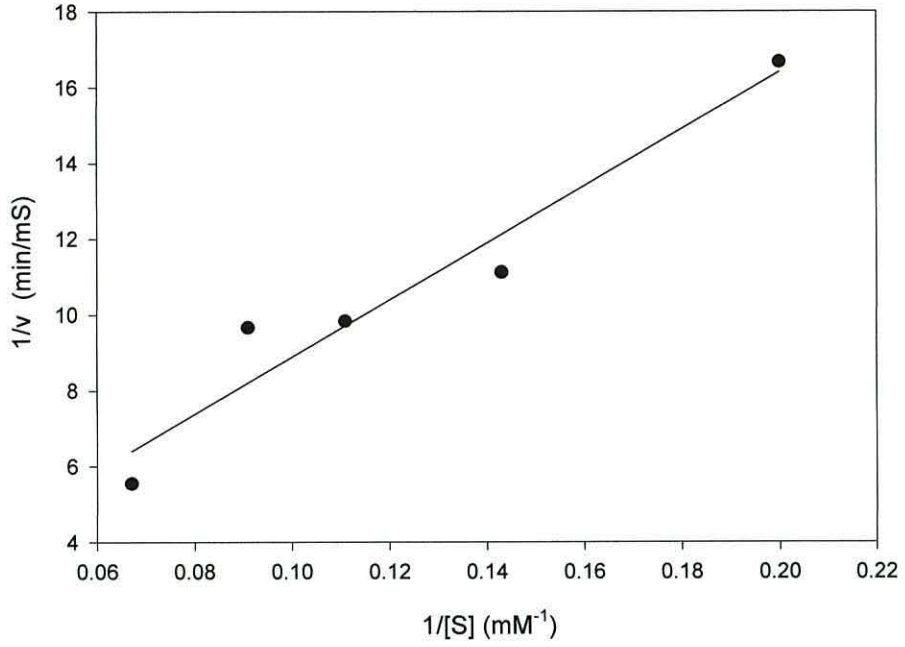


Fig 8.2. Lineweaver-Burk plot for a typical Nafion-immobilised creatininase / creatinase / urease electrode in 150 mM phosphate buffer at pH 7.2.

Some comparisons may be made between the kinetic parameters of the multi-enzyme and the single enzyme system. The Michaelis-Menten constant is higher for the multi-enzyme electrode, reflecting a lower enzyme-substrate affinity, probably due to the added structural difficulties involved in the co-immobilisation process that result in a reduction of the apparent binding efficiency of the enzyme / substrate complex.

For an enzyme in solution, the probability of interaction between a substrate molecule and an enzyme molecule depends on the kinetic energy of the reactant molecule, on the number of collisions and on the probability of a collision taking place with the right orientation. This steric factor is extremely important when dealing with immobilised enzymes, especially in multi-enzyme systems, where the co-existence of different enzymes in the immobilised layer might also have an effect on the probability of efficient collisions occurring.

On the other hand, the maximum velocity appears to be larger in the creatininase / creatinase / urease electrode, which seems to be unexpected at a first sight: the pace of

the reaction should not be enhanced but perturbed by the co-immobilising process. The necessity of three different catalysed reactions to generate a product capable of detection should lead to a more complex and less efficient sensor, which would need, under the same conditions, more substrate molecules to reach its maximum velocity than a single-enzyme based one. As explained in chapter 6 for immobilised urease electrodes, such an apparent higher maximum velocity is only the consequence of the displacement of the saturation point of the enzyme towards higher substrate concentrations.

The co-immobilisation of three enzymes in the Nafion polymer hinders the access of substrate molecules to the respective active sites, so the probability of successful bindings will be lower for multiple-enzyme systems than for single-enzyme ones. This means that, under the same conditions, a multi-enzyme system will need more substrate molecules to reach its maximum velocity than a single-enzyme one. Mathematically, this could be reflected by a lower slope on the linear part of the quadratic hyperbola that describes the dependence of the product formation velocity with the substrate concentration (see equation 6.2). This would describe a quadratic hyperbola with larger asymptotic values but this would only be the consequence of the displacement of the saturation point of the enzyme towards higher substrate concentrations.

8.2.2. Frequency response and sensitivity

8.2.2.1. Nafion-immobilised creatinine deiminase electrodes

Figures 8.3 and 8.4 include a previous study of the creatinine deiminase electrode at 10 mM phosphate buffer. They correspond to the frequency response and calibration plots for a typical gelatin-immobilised creatinine deiminase electrode. Frequency scans were carried out in the range 20 Hz – 300 kHz for the buffer solution and each particular creatinine concentration (5 mM - 15 mM).

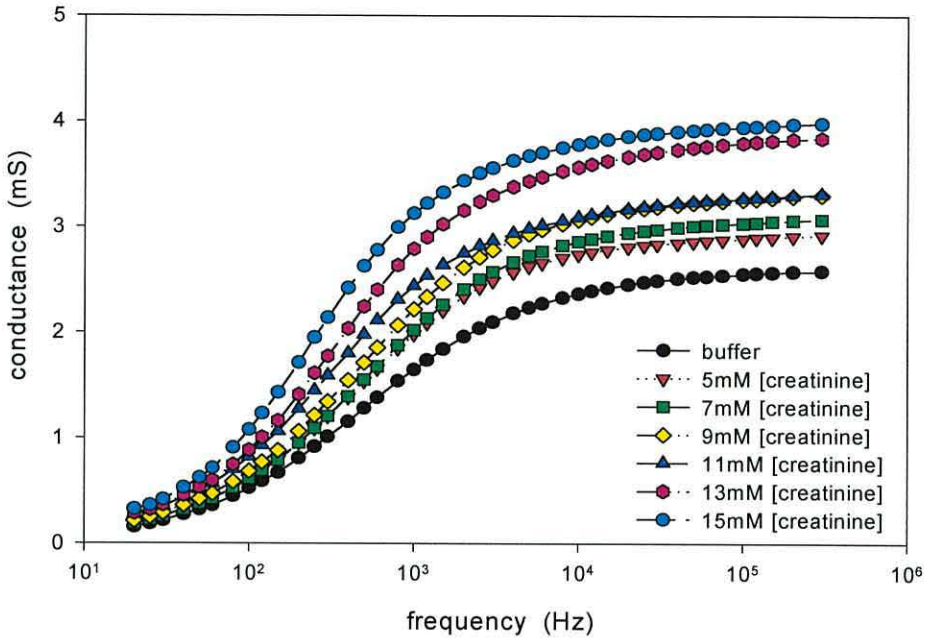


Fig 8.3. Frequency response of the conductance of a typical gelatin-immobilised creatinine deiminase electrode for varying creatinine concentration in 10 mM phosphate buffer at pH 7.2.

According to our previous work included in chapter 6, the region above 100 kHz shows the largest substrate concentration dependent change in the conductance of the enzyme layer. Therefore, figure 8.4 was constructed selecting the conductance of every sample at 300 kHz.

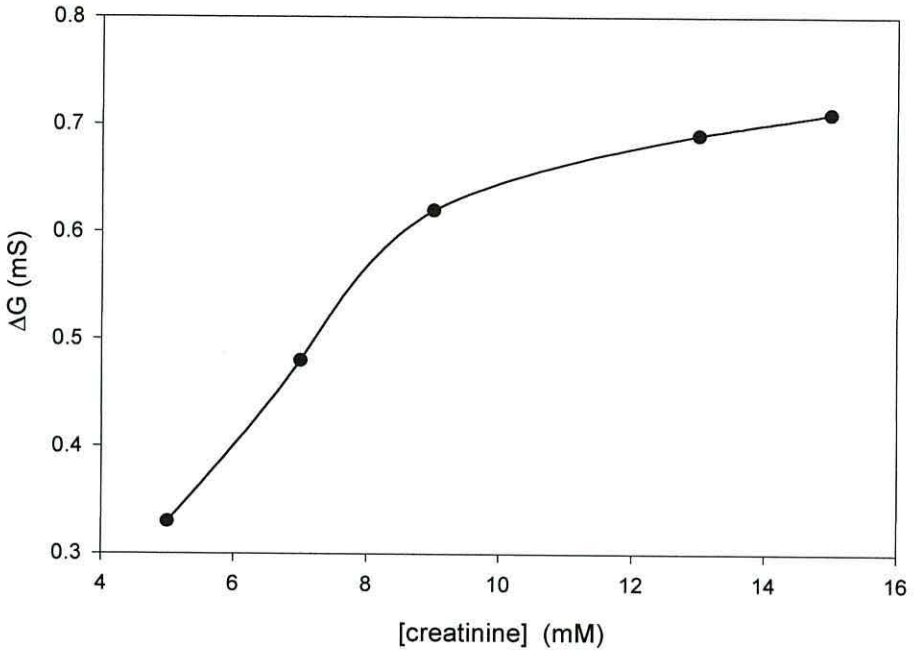


Fig 8.4. Calibration plot describing the change in conductance with creatinine concentration. Measurements are taken from data shown in figure 8.3 at a frequency of 300 kHz

The response of the sensor is the incremental conductance, ΔG, arising from the difference between the steady state conductance for each creatinine concentration and the conductance of the buffer.

$$\Delta G = G_{creatinine+buffer} - G_{buffer} \qquad [8.1]$$

The creatinine deiminase electrodes were found to exhibit sensitivities typically of the order of 65 μS/mM.

Figures 8.5 and 8.6 are analogous to 8.3 and 8.4 but measurements are made in 150 mM phosphate buffer at pH 7.2. As above, frequency scans are carried out from 20 Hz to 300 kHz for buffer solution and creatinine concentrations from 5 mM to 15 mM.

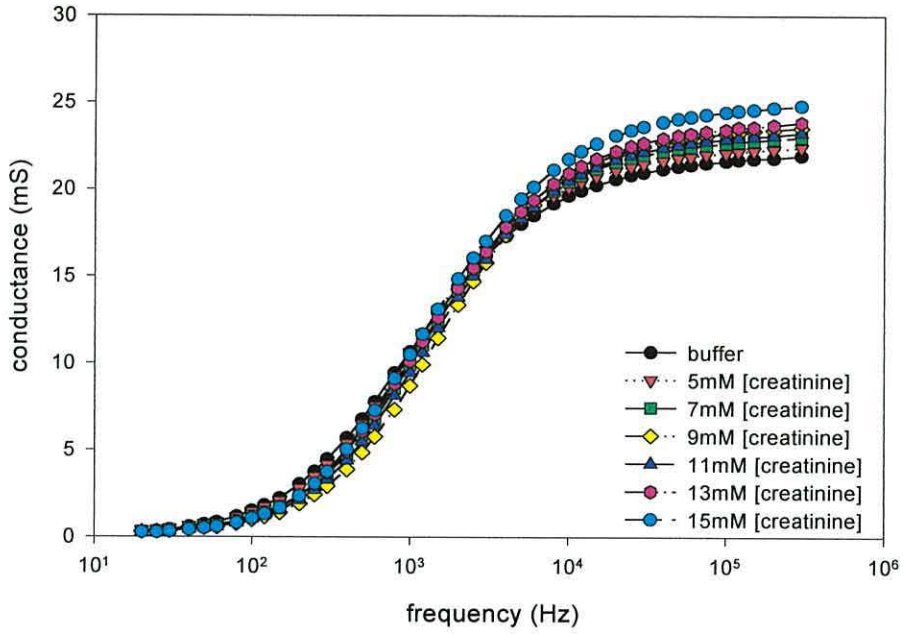


Fig 8.5. Frequency response of the conductance of a typical Nafion-immobilised creatinine deiminase electrode for varying creatinine concentration in 150 mM phosphate buffer at pH 7.2.

As for all previous calibration plots presented in this study, figure 8.6 was constructed from figure 8.5, using the conductance of every sample at 300 kHz.

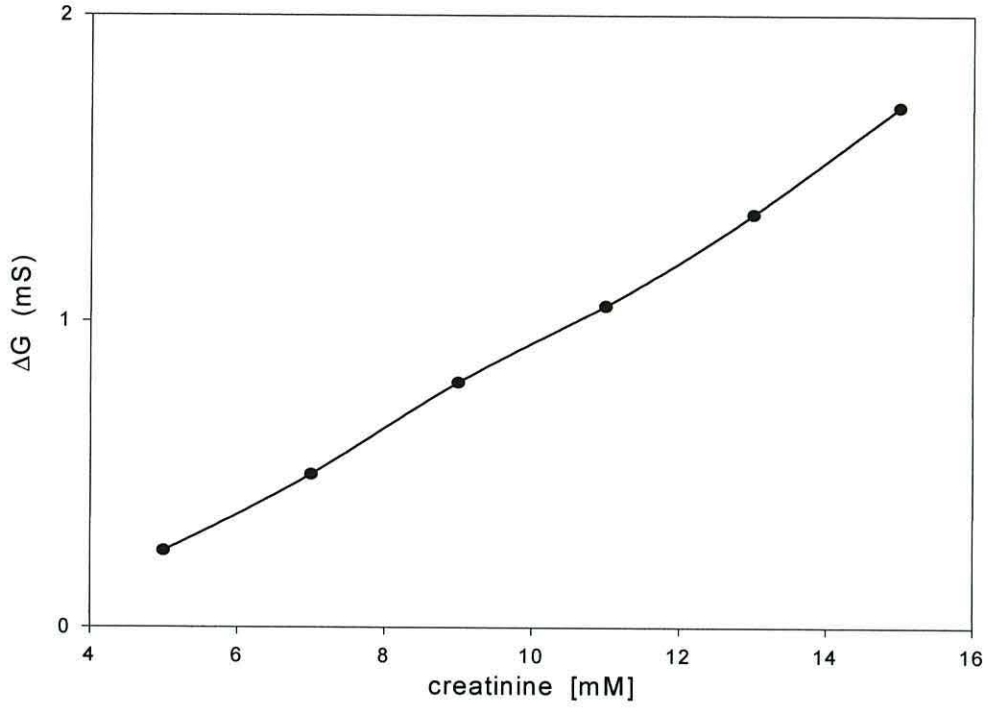


Fig 8.6. Calibration plot describing the change in conductance with creatinine concentration. Measurements are taken from data shown in figure 8.5 at a frequency of 300 kHz.

The characteristic shows a linear increase with increasing concentrations of creatinine and does not show any signs of saturation up to 15 mM creatinine, making it suitable for use in human urine. In this concentration range, the creatinine deiminase electrode showed a sensitivity of 94 μS/mM to creatinine.

8.2.2.2. Nafion-immobilised creatininase / creatinase / urease electrodes

8.2.2.2.1. Nafion-immobilised creatinase / urease electrodes

The multi-enzyme creatininase / creatinase / urease electrode was built from a bi-enzyme sensor system based on the co-immobilisation of creatinase and urease for the detection of creatine. The concept of both biosensors is described in chapter 2.

Creatinase catalyses the hydrolysis of creatine producing sacrosine and urea, the hydrolysis of the latter being catalysed by urease, generating ammonium ions. These

ions then generate an increase in the conductance of the enzyme layer which can be monitored and correlated to the concentration of creatinase present in the measured sample. The operation of the biosensor is therefore analogous to the two last steps of the creatininase / creatinase / urease electrode.

Figures 8.7 and 8.8 represent preliminary investigations carried out on gelatin immobilised creatinase / urease electrodes. Frequency scans and data analysis were carried out following the same procedure to that carried out for creatinine deiminase electrodes, described in the previous section.

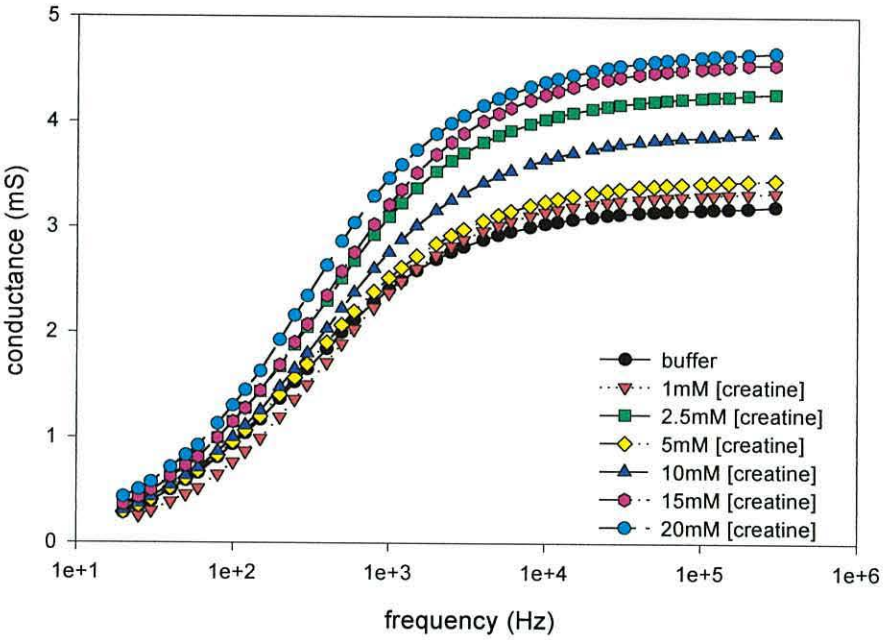


Fig 8.7. Frequency response of the conductance of a typical gelatin-immobilised creatinase / urease bi-enzyme electrode for varying creatine concentration in 10 mM phosphate buffer at pH 7.2.

As stated before, figure 8.8 was constructed by taking the conductance of every sample at 300 kHz.

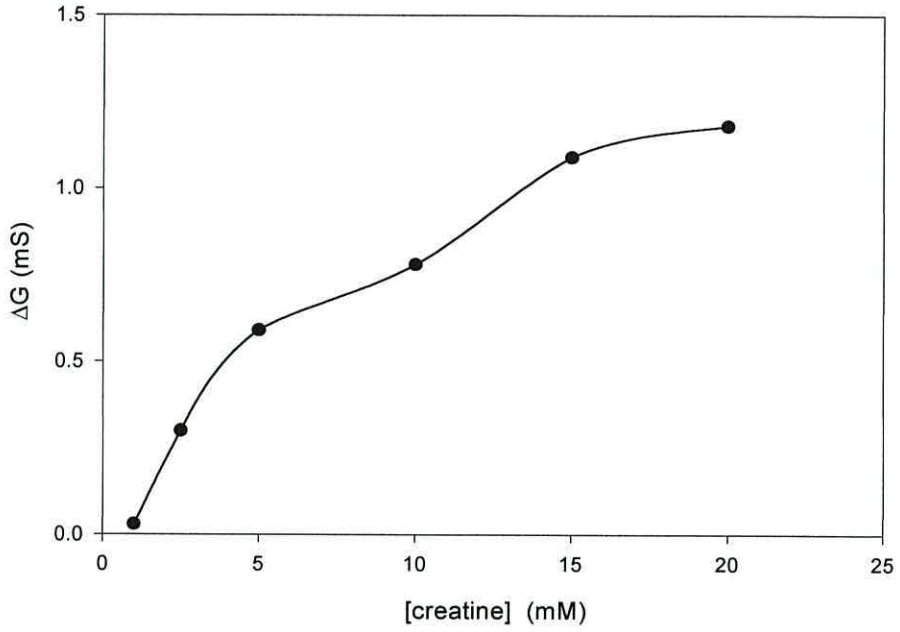


Fig 8.8. Calibration plot describing the change in conductance with creatine concentration in Measurements are taken from data shown in figure 8.7 at a frequency of 300 kHz.

The characteristic follows a linear trend below approximately 15 mM of creatine, showing clear signs of saturation at concentrations above that value. The creatinase / urease electrode showed a typical sensitivity of 74 μS/mM to creatine.

8.2.2.2.2 Nafion-immobilised creatininase / creatinase / urease electrodes

Figures 8.9 and 8.10 are analogous to 8.7 and 8.8, representing the frequency response and calibration plots for a typical gelatin-immobilised creatininase / creatinase / urease electrode in 10 mM phosphate buffer. Frequency scans were carried out in the range 20 Hz – 300 kHz for the buffer solution and creatinine concentrations from 5 mM to 20 mM.

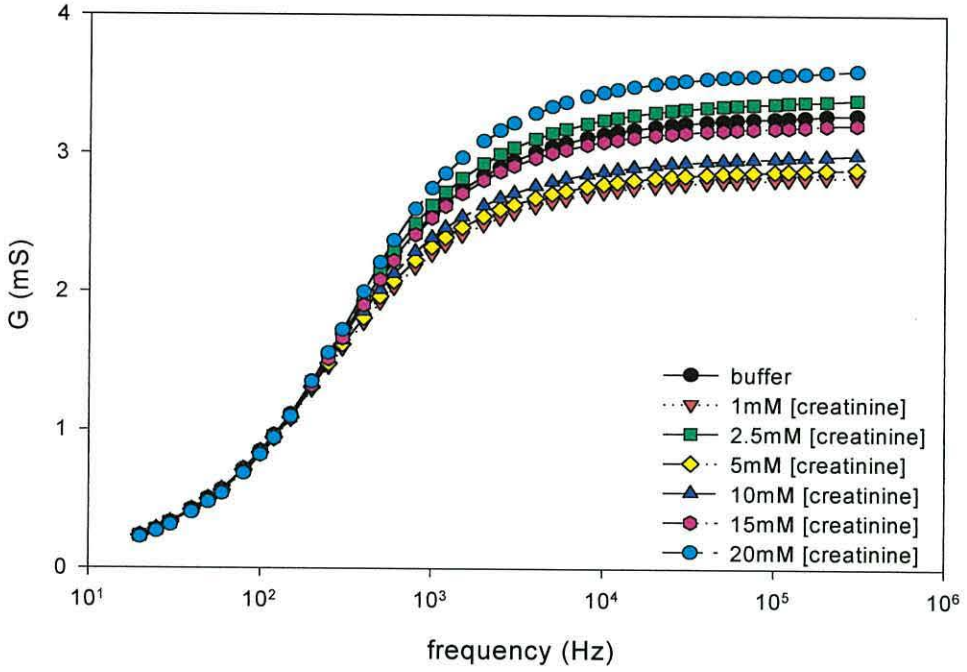


Fig 8.9. Frequency response of the conductance of a typical gelatin-immobilised creatininase / creatinase / urease tri-enzyme electrode for varying creatinine concentration in 10 mM phosphate buffer at pH 7.2.

As for calibration plots above, figure 8.10 was constructed with the conductance of every sample at 300 kHz.

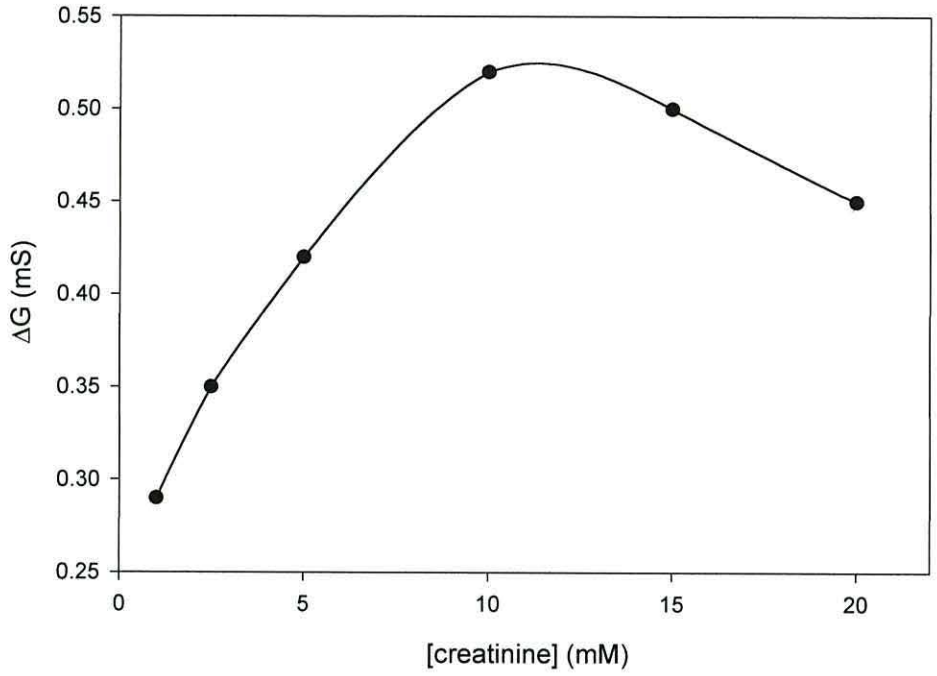


Fig 8.10. Calibration plot describing the change in conductance with creatinine concentration. Measurements are taken from data shown in figure 8.9 at a frequency of 300 kHz.

The characteristic follows a linear trend below approximately 10 mM of creatinine, showing a drop in the conductance response at concentrations above that value. The creatininase / creatinase / urease electrode showed a typical sensitivity of 52 μS/mM to creatinine.

Figures 8.11 and 8.12 are analogous to 8.6 And 8.7, representing the frequency response and calibration plots for a typical Nafion-immobilised creatininase /creatinase /urease electrode in 150mM phosphate buffer. Frequency scans and data analysis were carried out following the same procedure as that performed for creatinine deiminase electrodes. The conductance characteristics of Nafion-immobilised and gelatin-immobilised electrodes in 10 mM phosphate buffer are very similar. For 150 mM buffer, valid conductance responses, which enables us to differentiate varying creatininase concentrations, were only recorded for Nafion-immobilised sensors.

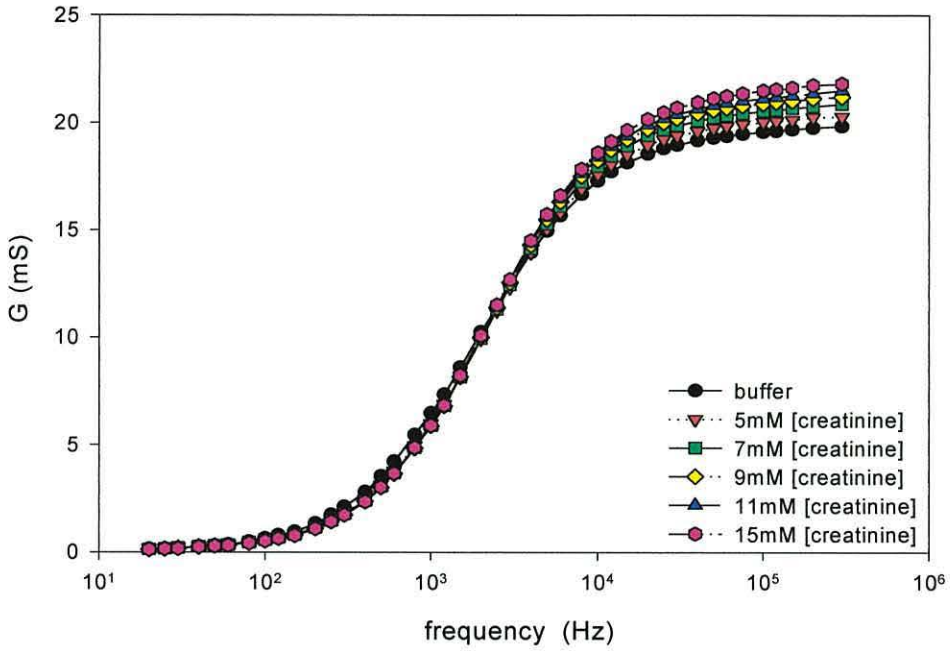


Fig 8.11. Frequency response of the conductance of a typical Nafion-immobilised creatininase / creatinase / urease electrode for varying creatinine concentration in 150 mM phosphate buffer at pH 7.2.

As for calibration plots previously reported, figure 8.12 was constructed with the conductance of every sample at 300 kHz.

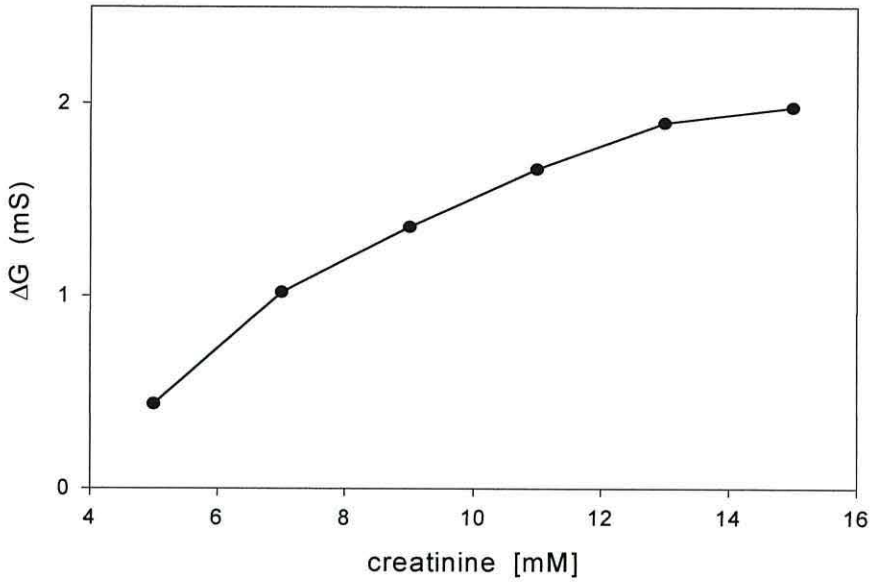


Fig 8.12. Calibration plot describing the change in conductance creatinine. Measurements are taken from data shown in figure 8.11 at a frequency of 300 kHz.

Creatininase / creatinase / urease electrodes showed higher sensitivities than the creatinine deiminase electrodes (120 μS/mM compared to 94 μS/mM) although signs of saturation appeared at lower concentrations of creatinine (around 15 mM). In spite of their lower efficiency at higher creatinine concentrations, the working range still encompasses the concentration range of creatinine in urine, so these electrodes could also be applied to measure creatinine in human samples.

8.2.3. Temperature dependence of creatinine biosensors

The knowledge of the sensitivity dependence on the temperature of a biosensor is important in a medical application in order to establish the optimum conditions for the test.

Frequency scans and time runs were carried out at different temperatures, from 0°C to 60°C, using 9 mM creatinine in 150 mM phosphate buffer at pH 7.2, for both creatinine deiminase and creatininase / creatinase / urease electrodes.

8.2.3.1. Nafion immobilised creatinine deiminase electrodes

Figure 8.13 shows the sensitivity at 9 mM creatinine of a typical creatinine deiminase electrode as a function of increasing temperature.

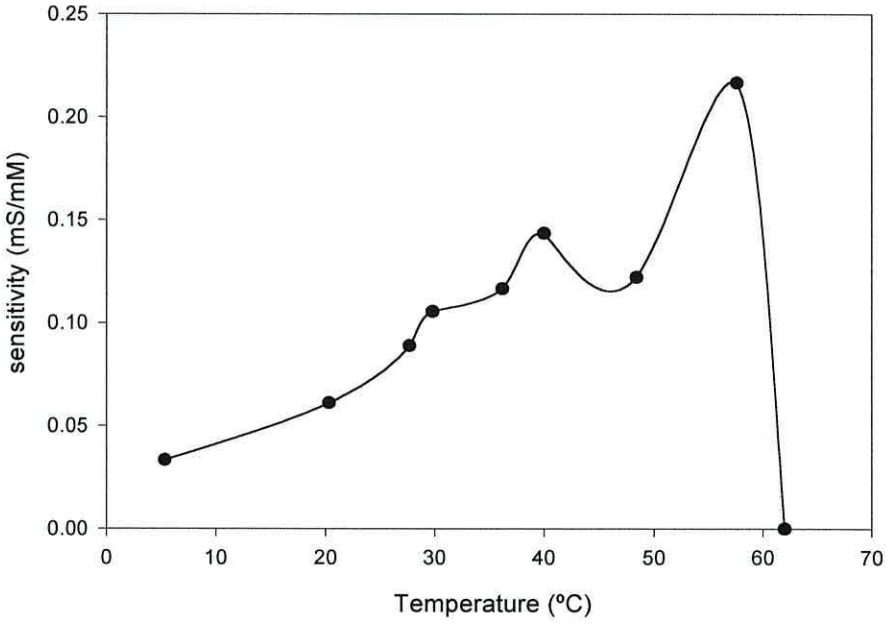


Fig 8.13. Temperature dependence of the sensitivity of a typical Nafion-immobilised creatinine deiminase electrode in 150mM phosphate buffer at pH 7.2

The dependence of the sensitivity on the temperature shows a sensitivity peak around 55 °C and a steep fall above that temperature which could be due to the denaturation of the enzyme. The temperature reported range of optimum activity of creatinine deiminase is 40 °C – 60 °C (Razumas et al, 1994). The rate of increase in sensitivity with temperature below 60 °C is approximately 3.5 μS/mM °C. This dependence would have to be taken into account if a creatinine deiminase electrode was designed for clinical sensing, possibly by monitoring the temperature of the samples and correcting the sensitivity accordingly.

8.2.3.2.Nafion-immobilised creatininase / creatinase / urease electrodes

Figure 8.14 shows the sensitivity at 9 mM creatinine of a typical creatininase / creatinase / urease electrode as a function of increasing temperature.

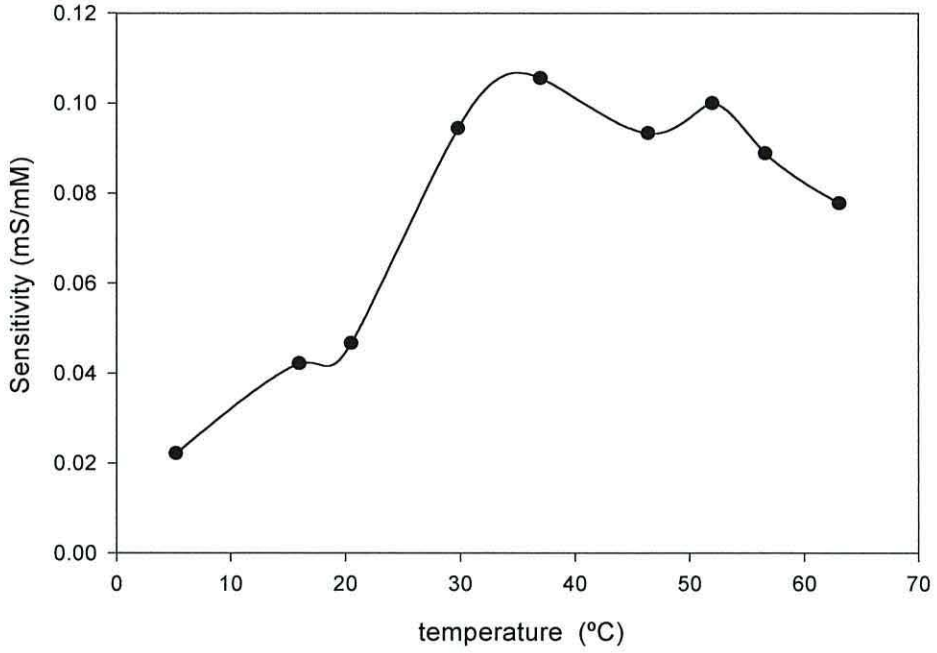


Fig 8.14. Temperature dependence of the sensitivity of a typical Nafion-immobilised creatininase / creatinase / urease in 150 mM phosphate buffer pH 7.2.

The multi-enzyme creatininase / creatinase / urease electrode shows a sensitivity maximum around 35 °C to 60 °C, followed by a smooth decrease in sensitivity with increasing temperature. The range of temperature of maximum activity for creatininase, creatinase and urease found in the literature are approximately 55 °C – 70 °C for creatininase (Sakslund and Hammerich, 1992), 35 °C – 45 °C for creatinase (Schumacher et al, 1993) and 60 °C for urease (Reithel, 1959; Scheller and Schubert, 1992). These ranges of temperature agree with our experimental data.

The rate of increase in sensitivity with temperature below 30 °C is approximately 2 μS/mM °C. As in the case of creatinine deiminase, this dependence would have to be taken into account if a multi-enzyme creatininase / creatinase / urease electrode was

designed for clinical sensing. The temperature of the samples should be monitored and the response of the biosensor corrected accordingly.

8.2.4. Shelf life of creatinine biosensors

The stability of single enzyme sensors fabricated by immobilising creatinine deiminase in Nafion and multi-enzyme sensors using a mixture of creatininase, creatinase and urease also in Nafion was also investigated. Sensors were immobilised following the procedure described previously and tested over a period of 30 days with 9 mM creatinine in 150 mM phosphate buffer solutions. Figure 8.15 describes the response of typical sensors recorded both for single- and multi-enzyme systems.

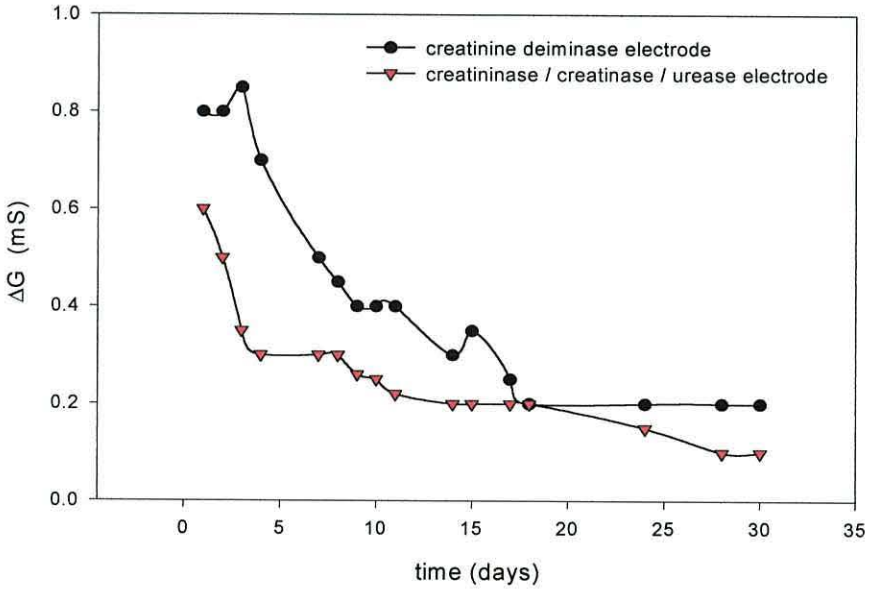


Fig 8.15. Shelf-life of a typical Nafion-immobilised creatinine deiminase electrode (black circle) and a Nafion-immobilised creatininase / creatinase / urease electrode (red triangle) in 150 mM phosphate buffer pH 7.2.

Creatinine deiminase and multi-enzyme creatininase / creatinase / urease electrodes both showed a steep decrease of the sensitivity with time, giving approximately half of the initial response after one week and one third after 20 days.

This short shelf-life would have negative implications in medical biosensors and could be due to the instability of the hydrated enzyme mixture. A way of increasing the stability of the devices could be to freeze dry or dehydrate the enzyme mixture, which is usual practice in medical sensing devices, and reconstitute it with water or appropriate buffers prior to its use.

No consistent results have been obtained on the dependence of these Nafion-immobilised creatinine deiminase electrodes and creatininase / creatinase / urease multi-enzyme electrodes on electrolyte concentration, an area which would be suitable for future work. Investigations on the effect of pH changes and possible interferants (vitamin C and paracetamol being the most common ones) to the catalaysed hydrolysis of creatininase would also have to be carried out in the design of a creatinine biosensor for medical use.

8.2.5. Creatinine detection in urine

As mentioned in the introduction, the concentration of creatinine in human urine falls between 9 mM and 13 mM for a healthy person, a range in which the two type of creatinine sensors studied give a linear response.

Prior to the immobilisation of the enzymes, it was necessary to determine the concentration of buffer that best matched the impedance characteristics of urine over the frequency range 20 Hz to 300 kHz. This was necessary to establish the correct baseline to use in order to calculate the sensors change in conductance ΔG, and compare such change between calibrated sensors and urine samples.

Performing frequency scans on interdigitated gold electrodes prepared by coating the electrodes only with Nafion only, and then testing them both with urine and changing concentrations of phosphate buffer, it was found that 90mM buffer concentration best matched the capacitance and conductance of urine for the frequency range investigated.

Electrodes immobilised with single enzyme creatinine deiminase and a multi-enzyme mixture of creatininase, creatinase and urease were employed. For each type of

electrode, the tests were carried out in two steps. The performance of the sensor was individually calibrated in terms of increments in conductance with changing concentration of creatinine solutions. Once characterised, the electrolyte solution was replaced with urine, and its conductance recorded until it reached steady state and fitted in the calibration lines in order to get the creatinine concentration present in it. The creatinine concentration of such a sample was also independently measured using an ammonia electrode to measure the ammonia gas concentration produced in the catalysed reaction taking place in the creatinine biosensor.

Following this, known concentrations of creatinine were added to the urine samples and further measurements taken to check the valid performance of the sensor. These concentrations were chosen to be 2.5 mM, 5 mM, 7.5 mM and 10 mM.

8.2.5.1. Nafion-immobilised creatinine deiminase electrodes

Figure 8.16 shows the response of Nafion-immobilised creatinine deiminase on gold electrodes to urine, superimposed on a calibration plot for the sensor against standard creatinine concentrations at 90 mM phosphate buffer. The black circles correspond to the calibration line obtained with creatinine in buffer and the red triangles to the creatinine in urine. Label *A* corresponds to the untreated urine sample, which gave a reading of 9.73 mM of creatinine.

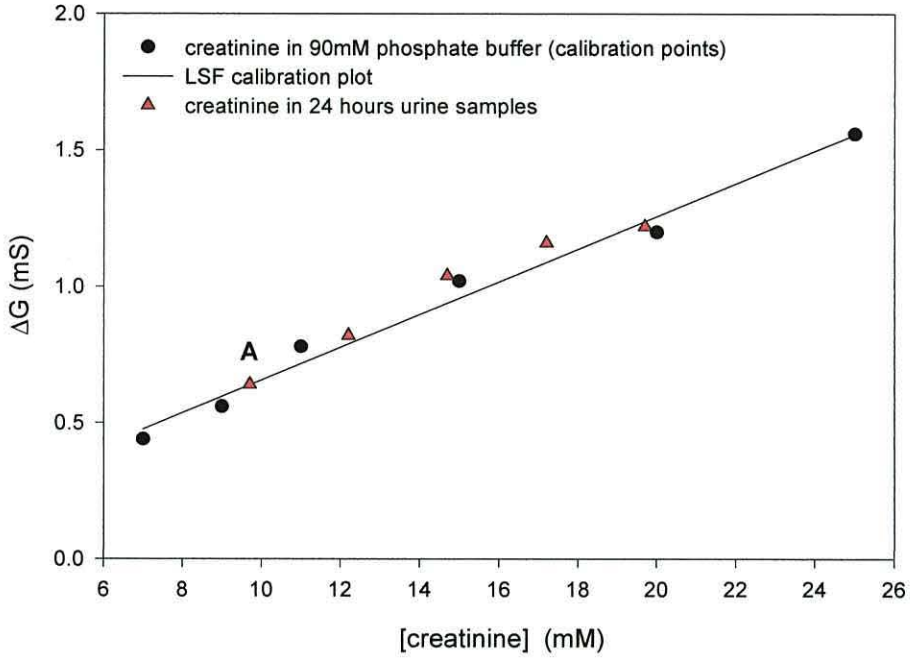


Fig 8.16. Response of a typical Nafion-immobilised creatinine deiminase electrode to samples of creatinine in urine. Calibration points and plot for standard creatinine concentration in 90mM phosphate buffer are shown for comparison. Point A corresponds to the sensor response in untreated urine.

8.2.5.2. Nafion-immobilised creatininase / creatinase / urease electrodes

Figure 8.17 is analogous to 8.16, describing the response of Nafion-immobilised creatininase / creatinase / urease multi-enzyme electrode to urine, superimposed on a calibration plot for the sensor against standard creatinine concentrations in 90 mM phosphate buffer.

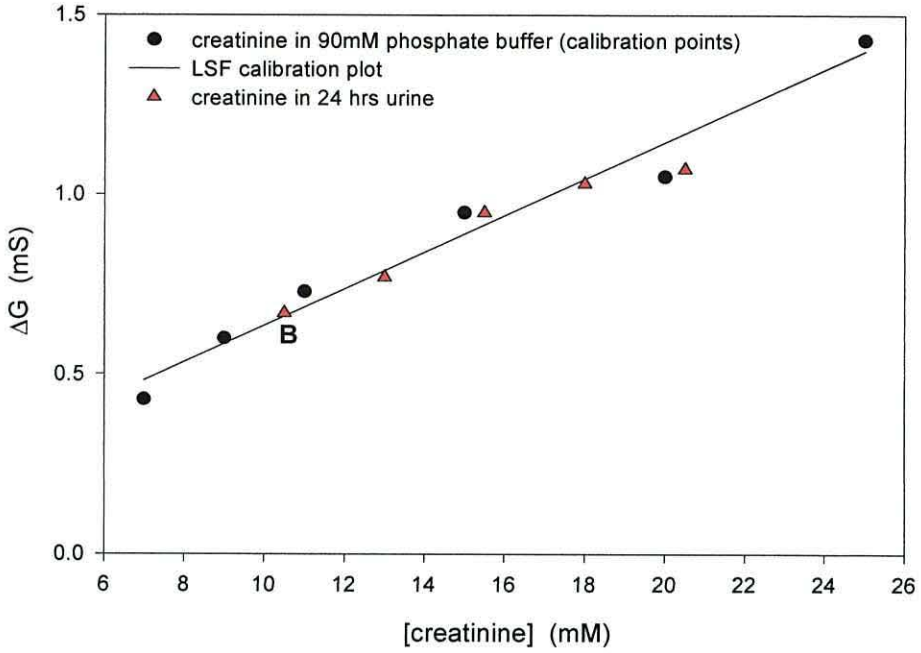


Fig 8.17. Response of a typical Nafion-immobilised creatininase / creatinase / urease electrode to samples of creatinine in urine. Calibration points and plot for standard creatinine concentration in 90mM phosphate buffer are shown for comparison. Point B corresponds to the sensor response in untreated urine.

As before, black circles correspond to the calibration line obtained with creatinine in buffer and the red triangles to the creatinine in urine. Point B corresponds to untreated urine, which resulted in a creatinine concentration of 10.5 mM.

The investigation presented here on the impedimetric detection of creatinine deiminase shows it is viable to apply such measurement techniques to practical diagnosis. Both single- and multi-enzyme electrodes detected consistently creatinine levels in untreated 24 hours urine and 24 hours urine spiked with known concentrations of creatinine.

8.3. Conclusions

Creatinine deiminase electrodes and creatininase / creatinase / urease electrodes have been investigated in order to assess the applicability of conductimetric biosensing to the medical detection of creatinine in urine. The activities of the single- and multi-enzyme array were investigated and their performance characterised.

Following findings on model urease conductimetric electrodes, Nafion was employed to immobilise the enzyme component at high ionic strengths. The sensors were able to differentiate the concentration of creatininase in a sample at physiological ionic strength. Furthermore, tests were carried out with both electrode arrays on 24 hour untreated urine and better sensitivities were reported for the creatinine deiminase electrodes.

Chapter 9. Conclusions and Future Work

The work included in this thesis is centred around ac impedance biosensors. It is widely accepted there is growing demand for hand-held sensors with disposable electrodes of the type studied here, especially for environmental applications and health and safety areas. In fact, previous collaboration existed between the University of Wales and British Nuclear Fuels Ltd. for the development of a creatinine sensor.

In medicine, biosensors have the potential to provide fast quantitative analysis on a reliable basis, as alternative to complicated, tedious and expensive laboratory procedures. The combination of high specificity and powerful recognition features with appropriate transducer systems in compact devices make them ideal for point-of-care-testing and near-patient technology. General practitioners, paramedics and certain patients (such as those belonging to risk groups or those suffering from chronic diseases who need continuous monitoring) can benefit from sensors designed for specific diseases. As an example, the overall market size for near-patient sensors for cardiac monitoring has been estimated to be approximately £20 to £30 million in the USA alone (1998) and it is considered to be still growing.

Despite the enormous market potential of biosensors, very few comprehensive studies have appeared to elucidate their characteristic operating features. The aim of the work described in this thesis is the investigation and development of ac impedance biosensors systems through the evaluation of the electrical, physical and chemical processes involved, in order to provide better understanding of the complex performance of these devices.

9.1. Conclusions

The investigations on impedance enzyme electrodes included here covered three main issues:

Analysis of the concepts behind the measurement techniques employed in ac impedance biosensors in terms of equivalent circuit analysis and proof of their applicability to sensing devices.

Description and characterisation of an impedance model enzyme electrode: a biosensor for urea detection.

Design and proof-of-feasibility for an impedance enzyme electrode for medical applications: a biosensor for creatinine detection in urine.

We have shown that changes in the conductance of a system comprised of an interdigitated electrode, enzyme layer and substrate solution are proportional to the concentration of product species generated in an enzyme catalysed reaction. A measurement of the conductance change will therefore also determine the original concentration of substrate present in the sample.

By means of the circuit analysis of this three-phase enzyme electrode system we have shown that it is possible to deduce separately the variations in the electrical parameters of each of the conforming phases originated by the chemical reaction. Depending on several critical factors (e.g. enzyme immobilisation method) different ionic profiles are expected in the different phases, each of them dominant at different frequency ranges.

Urease was chosen as a model enzyme to characterise and optimise a.c. impedance conductimetric enzyme electrodes because of its high stability and well-understood catalytic behaviour. The activity of urease was assessed in solution and immobilised form and the dependence of the biosensor performance on enzyme loading, immobilisation methods, temperature, pH and electrolyte concentration investigated. We found that the immobilising agent is not only critical in the determination of the

shelf-life of the sensor but, together with the electrode surface, it is also important in measurements at high ionic strengths where the sensor response can be significantly reduced. This is of particular importance in the design of conductimetric sensors for clinical use, as optimum sensitivity is required at physiological electrolyte concentrations. We showed that Nafion-immobilised urease sensors offer optimum performance in tests on serum samples, combining high sensitivity with long shelf-life and giving no signs of electrode polarisation effects.

Following the interest of BNFL in the detection and differentiation of creatinine levels in urine, we investigated potential creatinine biosensors constructed with two different enzymatic systems. Such a biosensor would be an attractive alternative to the currently used expensive benchtop instrumentation which employs hazardous chemicals and involves long testing times.

A single-enzyme creatinine deiminase electrode and a multi-enzyme creatininase / creatinase / urease electrode were characterised with similar results. Based on our previous work on Nafion and its outstanding performance at high ionic strengths, this polymer was employed to immobilise the enzymes on interdigitated gold electrodes. We showed it is feasible to design conductimetric biosensors to detect creatininase in undiluted 24-hour urine with good reproducibility and sensitivity.

9.2. Future work

Our low-frequency impedance work has demonstrated that, depending on the immobilisation process, the ionic product generated in the catalysed reaction taking place in enzyme electrodes either diffuses towards the electrolyte or remains trapped in the enzyme phase. Low sensitivity was recorded when the ions diffused towards the bulk phase, and high sensitivity, but low reproducibility was observed when the ions stayed trapped within the enzyme layer.

Further investigations should be carried out in order to produce enzyme electrodes which make use of both types of response (that from the bulk and the enzyme phase) in order to obtain high sensitivity detection and reusability.

Moreover, the circuit modelling of ac impedance enzyme electrodes presented here has predicted a relatively large response to substrate which would offer considerably higher sensitivities in the high frequency region above 100 MHz. Therefore, future work should include assessment, through experimentation and modelling, of the potential of biosensors operating on a high frequency impedance principle.

Although the performance of creatinine biosensors was positively assessed against urine samples, further investigations are required. Important features such as the dependence of the sensors sensitivity on electrolyte concentration and pH should also be investigated as a next step in the application of impedance sensing principles to the practical detection and differentiation of creatinine in clinical diagnosis. The potential interference effects due to some well-known substances commonly present in urine and / or blood or serum, such as vitamin C and paracetamol, should also be included as future work. It would also be interesting to investigate techniques to decrease the minimum creatinine concentration detectable with our impedance sensors (i.e. the differential conductance threshold) in order to develop a sensor capable of creatinine detection in blood and serum.

The use of capacitance measurements to measure changes in ion concentration should also be considered as future work. The change in capacitance resulting from accumulation of ions on the electrode surface for example (electrode polarisation) would be an appropriate indicator of the substrate concentration in a sample under test.

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