Novel Polypyrrole-Based Formate Biosensor

YONG J. YUAN  MRACI C. Chem
B.Sc. Ocean University of Qingdao
M.App.Sc. Royal Melbourne Institute of Technology

This thesis is submitted in fulfilment of the requirement for the
Degree of Doctor of Philosophy

Copyright©1998
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Formate Dehydrogenase in Motion

[2 interdomain linkages, 2 hinges, 7.5 degree rotation. Upon binding co-enzyme, one domain rotates relative to the other to close over the active site. Each of the two hinges occurs between α-helices.]

# CONTENTS

Declaration vi
Acknowledgements vii
Summary viii

Chapter One  
Fundamentals, Principles and Applications 1
1.1. Introduction 2
   1.1.1. Methods of Immobilisation 5
   1.1.2. Electrochemical Sensing 6
      1.1.2.1. Potentiometry 7
      1.1.2.2. Amperometry 7
      1.1.2.3. Conductometry 7
   1.2. Biosensors Designs 8
      1.2.1. Multilayer Design 8
      1.2.2. Chip-Based Electrode Devices 10
      1.2.3. Flow Based Biosensors 10
   1.3. Polypyrrole Films 11
      1.3.1. Applications of Polypyrrole Films 11
      1.3.2. Mechanisms of Enzymatic Reactions in Membrane 15
         1.3.2.1. Diffusion Limitations 15
         1.3.2.2. Kinetic Behaviour of Immobilised Enzymes 17
   1.4. Polypyrrole-Based Biosensors 19
      1.4.1. Introduction 19
      1.4.2. Mediated Enzyme Biosensors 19
   1.5. Aims of This Study 22
   1.6. References 23

Chapter Two  
Electrochemical Formation and Characterisation of Polypyrrole Films 31
2.1. Introduction 32
2.2. Experimental 35
  2.2.1. Reagents 35
  2.2.2. Polypyrrole Film Formation 35
2.3. Results and Discussion 36
  2.3.1. Positive Potential Limits and Pyrrole Concentration 38
  2.3.2. Stability of Polypyrrole 44
  2.3.3 Comparison of Dopants 45
2.4. Galvanostatic Polymerisation of Polypyrrole 50
  2.4.1. Comparison of Dopants 50
  2.4.2 Effect of $H^+$ on the Polymerisation of Polypyrrole 52
2.5. Conclusions 53
2.6. References 55

Chapter Three Ferrocyanide Mediated Formate Biosensor 58
3.1. Introduction 59
3.2. Experimental 60
  3.2.1. Reagents 60
  3.2.2. Preparation of Polypyrrole-Enzyme Electrodes 60
  3.2.3. Electrochemical Measurements 61
3.3. Results and Discussion 61
  3.3.1. Incorporation of Mediator 61
    3.3.1.1. Immobilisation of Ferrocyanide 61
    3.3.1.2. Effect of Galvanostatic conditions on Polypyrrole film 65
  3.3.2. Fabrication of Biosensing Membrane 68
  3.3.3. Microscopic Evidence of Enzyme Incorporation into Polypyrrole 70
  3.3.4. Cyclic Voltammetry on the Enzyme Film 72
  3.3.5. Amperometric Sensing of Formate 75
    3.3.5.1. Electron Mediation with Ferrocyanide 76
    3.3.5.2. Influence of Applied Potential on Amperometric Response 78
3.3.5.3. Amperometric Detection

3.4. Monolayer Biosensing Membrane

  3.4.1. Co-Electroimmobilisation of FDH/NAD/[Fe(CN)$_6$]$^{4-}$ into a Polypyrrole Film

  3.4.2. Effect of Pyrrole Concentration

  3.4.3. Effect of Ferrocyanide Concentration

  3.4.4. Effect of β-NAD Concentration

  3.4.5. Effect of Enzyme Loading

  3.4.6. Effect of O$_2$

  3.4.7. Effect of Current Density and Polymerisation Period

  3.4.8. Effect of Temperature

  3.4.9. Effect of pH and Electrolytes in Amperometric Measurements

3.5. Bilayer Biosensing Membrane

  3.5.1. Galvanostatic Film Formation

  3.5.2. Cyclic Voltammetric Behaviour

  3.5.3. Chronoamperometric Behaviour

3.6. Conclusions

3.7. References

Chapter Four

Ferrocyanide Mediated Flow Amperometric Formate Biosensor

4.1. Principle of Flow Injection Analysis

  4.1.1. Dynamic Behaviour in FIA

  4.1.2. Miniaturisation and Flow Cell

4.2. Experimental

  4.2.1. Reagents

  4.2.2. Preparation of Polypyrrole-Based Electrode

  4.2.3. Procedures of Electrochemical Measurements

4.3. Results and Discussion

  4.3.1. Fabrication of Monolayer Biosensing Membrane
4.3.2. Bilayer Formate Biosensing Membrane 117
4.3.3. SDS Modified Bilayer Membrane 123
4.3.4. Overoxidized Polypyrrole-Based Membrane 126
4.4. Triple Layers 129
  4.4.1. KCl Modified Triple-Layer Membrane 130
  4.4.2. Nafion Modified Triple-Layer Membrane 137
    4.5.2.1. Nafion Coated Film 137
    4.5.2.2. Nafion Electropolymerised Film 139
4.5. Conclusions 141
4.6. References 141

Chapter Five   Ferrocene and Prussian Blue Based Formate Biosensor Systems 144
5.1. Introduction 145
5.2. Experimental 146
  5.2.1. Reagents 146
  5.2.2. Preparation of Polypyrrole-Based Electrodes 146
  5.2.3. Procedures of Electrochemical Measurements 146
5.3. Results and Discussion 147
  5.3.1. Electrochemical Behaviours of Mediators 147
    5.3.1.1. Mediators in Aqueous Solutions 147
    5.3.1.2. Mediators in Polypyrrole Matrix 152
  5.3.2. Biosensing Membrane Fabrication 154
  5.3.3. Electrochemistry of Biosensing Membrane 155
    5.3.3.1. Cyclic Voltammetry 155
    5.3.3.2. Batch Amperometric Measurement 158
    5.3.3.3. Flow Amperometric Biosensing 160
5.4. Ferrocene Mediated Bilayer Biosensors 166
  5.4.1. Ferrocene Mediated Biosensor 166
  5.4.2. Ferrocene Carboxylic Acid Mediated Biosensor 169
5.5. Polypyrrole-Prussian Blue Bilayer  
5.5.1. Electrochemical Formation of Prussian Blue  
5.5.2. Prussian Blue Based Polypyrrole Bilayer  
5.5.3. Amperometric Response  
5.6. Conclusions  
5.7. References  

<table>
<thead>
<tr>
<th>Chapter Six</th>
<th>Improvement of the Performance of a Formate Biosensor by Use of Artificial Neural Networks</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1.</td>
<td>Introduction</td>
</tr>
<tr>
<td>6.1.1.</td>
<td>Artificial Neuron Model</td>
</tr>
<tr>
<td>6.1.2.</td>
<td>Multi-Layer Perceptron</td>
</tr>
<tr>
<td>6.1.3.</td>
<td>ANN in Analytical Chemistry</td>
</tr>
<tr>
<td>6.2.</td>
<td>Experimental</td>
</tr>
<tr>
<td>6.2.1.</td>
<td>Reagents</td>
</tr>
<tr>
<td>6.2.2.</td>
<td>Preparation of Polypyrrole-Based Electrodes</td>
</tr>
<tr>
<td>6.2.3.</td>
<td>Procedures of Electrochemical Measurements</td>
</tr>
<tr>
<td>6.3.</td>
<td>Results and Discussion</td>
</tr>
<tr>
<td>6.3.1.</td>
<td>Chemical stimuli Recognition</td>
</tr>
<tr>
<td>6.3.2.</td>
<td>ANN-Based Pattern Recognition and Detection</td>
</tr>
<tr>
<td>6.3.3.</td>
<td>Data Processing</td>
</tr>
<tr>
<td>6.3.4.</td>
<td>ANN Training</td>
</tr>
<tr>
<td>6.4.</td>
<td>References</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Seven</th>
<th>Conclusions and Further Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1.</td>
<td>Conclusions of the Project</td>
</tr>
<tr>
<td>7.2.</td>
<td>Further Recommendations</td>
</tr>
<tr>
<td>7.2.1.</td>
<td>Redox Polymers</td>
</tr>
<tr>
<td>7.2.2.</td>
<td>Co-Polymers</td>
</tr>
<tr>
<td>7.2.3.</td>
<td>Conductometric Measurements</td>
</tr>
</tbody>
</table>
DECLARATION

The material contained in this thesis has not been published elsewhere except as referenced and the work has been carried out by me. No other persons work has been used without due acknowledgement. This thesis has not been submitted in part or as a whole for the award of another degree or diploma in any other tertiary institution.

Yong J. Yuan

March 1998
ACKNOWLEDGEMENTS

I would like to thank my principal supervisor Associate Professor S. B. Adeloju for his valuable support, guidance and advice over the years. My appreciation also extends to my co-supervisor Professor G. G. Wallace for his contributions.

Thanks go to the members of the workshop of the Civil Engineering, without their prompt services this thesis would not have been possible.

I am grateful to my fellow sojourners and caffeine addicts at the Centre for Electrochemical Research and Analytical Technology, who made the Lab such an enjoyable place to work.

Finally, I would like to acknowledge the award of a 0.5 Resarch Assistantship under the ARC Small grants scheme. This award was valuable in providing for my living allowance over the three year period.
SUMMARY

The concepts of electroneutrality coupling and electron-hopping, which are useful for the incorporation of functional components and transportation of electrons, were applied in this project. Discrete layered structures were fabricated by sequential electropolymerization to modulate the performances of formate biosensors. Different types of layers, with or without enzyme, were successfully grown on the electrode surface. The presence of the enzyme (formate dehydrogenase), co-factor (β-nicotinamide adenine dinucleotide) and an electron mediator in the polypyrrole film was verified by scanning electron microscopy, chronopotentiometry, cyclic voltammetry and amperometric measurements. Monolayer, bilayer and trilayer formate biosensors were successfully fabricated for different analytical purposes.

The utilisation of the biosensing membrane for the reliable batch and FIA determination of formate based on an amperometric mode of detection are explored. Electron mediators such as ferrocyanide, Prussian Blue, ferrocene and ferrocene carboxylic acid were incorporated into the polypyrrole film to lower the required applied potential for amperometric sensing and to maintain the conductivity and stability of the polypyrrole backbone.

The application of artificial neural networks (ANN) to overcome the problem of reusability and reproducibility in a non-linear and complicated dynamic system is also considered. The resulting system was trained with a new neural network based software package, Turbo Neuron, for prediction of the concentration of formate, based on the entire collected data, which contain the history of the detection experiments. The proposed integrated ANN conducting polymer biosensor enables the determination of formate concentration, both online and in real time.
CHAPTER 1

Fundamentals, Principles and Applications
1.1. Introduction

Biosensors are in the forefront of current research in the area of bioanalytical chemistry. Unlike conventional chemical sensors, these new sensors use immobilised biomacromolecules, such as enzymes, and highly integrated systems to recognise analyte species. By IUPAC definition, a chemical sensor is a device which transforms chemical information (particularly the concentration of specific analytes) into an analytically useful electrical signal[1]. Biosensors are therefore a sub-set of chemical sensors in which biological reactions and recognition mechanisms are used for analyte detection and signal generation. These devices rely on the interaction of a biocatalyst, usually an isolated and purified enzyme, with the analyte.

![Diagram of biosensor operation]

Figure 1-1 Schematic of the operation of biosensors

As illustrated in Figure 1-1, biosensors are analytical devices which are composed of a biological recognition element (such as an enzyme, antibody, receptor, or microbe) coupled to chemical or physical transducer. There are four major types of transducers: electrochemical (electrodes), mass
(piezoelectric crystals or surface acoustic wave devices), optical (optodes) and thermal (thermistors or heat-sensitive sensors).

For many years biosensors have been hailed as the solution to many analytical problems[2a]. In addition to this, their application has been touted as being multi-industrial[2b]. There is general agreement that biosensors have the capacity to offer easy to use, cost-effective and rapid analyses in a wide variety of operating environments. However, biosensor technologies often do not lend themselves directly to manufacture, a factor underestimated by many scientists. In reality, only a few biosensor products have emerged, and even fewer have achieved mass-market commercial success. Owing to their high sensitivity, selectivity and simplicity, the analytical applications of biosensors have increased exponentially in recent years[2c]. The principles and applications of biosensors have been discussed in several monographs[2a-2i].

The potential for biosensor technology is enormous and is likely to revolutionise analysis and control of biological systems with a wide sphere of influence. In order to envisage areas where biosensors will find real commercial applications, it is firstly important to highlight the features and benefits of such devices as shown in Table 1-1 which provides a summary drawn up from several monographs[2a-2i].

**Table 1-1  Potential Benefits of Biosensors**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target specificity</td>
<td>Sensitivity and selectivity</td>
</tr>
<tr>
<td>Selective measurement in complex samples</td>
<td>Decreased user time</td>
</tr>
<tr>
<td></td>
<td>Ease of use (i.e., no sample preparation)</td>
</tr>
<tr>
<td>Fast measurement</td>
<td>Increased efficiency</td>
</tr>
<tr>
<td></td>
<td>Decreased user time</td>
</tr>
<tr>
<td>Continuous measurement</td>
<td>Increased accuracy and improved precision</td>
</tr>
<tr>
<td></td>
<td>Increased efficiency</td>
</tr>
<tr>
<td>Small size</td>
<td>Fit with current practice</td>
</tr>
<tr>
<td></td>
<td>Versatility</td>
</tr>
<tr>
<td></td>
<td>Economical</td>
</tr>
<tr>
<td>Electronic processing</td>
<td>Ease of use (i.e., easier data processing)</td>
</tr>
<tr>
<td></td>
<td>Versatility</td>
</tr>
<tr>
<td>Electrochemical engineering</td>
<td>Reproducibility</td>
</tr>
<tr>
<td>Associated disposables</td>
<td>Increased revenue</td>
</tr>
</tbody>
</table>
There are two broad areas of application for which biosensors would be most beneficial, such as:

- On-line, on-the-spot, indicators for use in food, environmental and other industries;
- Healthcare uses for near-patient monitoring.

In general biosensors enable simple, highly specific measurements to be carried out, both quickly and accurately, in complex matrices. Such devices can also be miniaturised, used continuously and integrated into other systems, facilitating the processing and storage of electronically generated data. These features are highly desirable in many applications. For simplicity, the following flow chart illustrates the applications and potential markets for biosensors in the 21st century. There is an opportunity for incredible returns for the R&D funds invested in such a fascinating area.

Biosensor research and development has been largely concentrated in the biomedical area because that area represents the largest market opportunity and that market is most receptive to short-lived or disposable devices.
1.1.1. Methods of Immobilisation

The first step in fabrication of biosensors usually involves the immobilisation of an enzyme or other bioactive substance onto/into a suitable polymer or other substrate. Figure 1-2 illustrates the different methods that have been used for immobilisation of enzymes and other bioactive substance onto/into polymer membrane[3].

![Typical Structures of Biosensors Membrane](image)

Figure 1-2  Typical Structures of Biosensors Membrane

Each of these methods has its own advantages and disadvantages. The main advantage of physical adsorption is that no reagents are required for the immobilisation[4-6]. Only weak interactions are involved between the support and the biomacromolecule due to Van der Waals forces, dipole-dipole interactions, hydrogen bonding, or the formation of electron transition complexes. Unfortunately, except in the latter, the reversible nature of the binding equilibrium is highlighted by its susceptibility to changes in pH, ionic strength, temperature, etc. The stability of adsorption can be influenced by hydrophobic, hydrophilic, ionic and polar interactions between the surface and the biomacromolecule. Also the biosensing function of the immobilised enzyme will depend on the orientation of the biomacromolecule on the surface of the transducer (or sensor).

Immobilisation by chemical binding is diverse and allows a reaction to be selected for specific biological system which can bind the biomacromolecule without loss of activity[7]. Covalent attachment or crosslinking of the biorecognition molecule to the sensor surface is generally the most irreversible of the immobilisation techniques[8]. Binding is usually effected through the use of the nucleophilic functional amino acid groups in the biomacromolecule, that will not affect the function and activity of the biorecognition site itself. The limitations of enzyme immobilisation by chemical binding include[7]:
• the relative irreversibility - this must be compared and contrasted critically with the merits and weaknesses of other methods;

• inhibition effects at high enzyme loadings.

Entrapment of the biomolecule in a three-dimensional polymer matrix may be accomplished by different polymerisation methods. The polymer may be an inert support, or may itself perform some function essential to the transduction of the analyte-dependent signal. In the former case polymers such as poly(vinyl chloride)[9], polyacrylamide[10] etc. are deposited on the sensor surface together with the recognition molecule, whereas in the latter case the support matrix may be modified to include a chemically active functionality. These can be classified as insulation and entrapment, respectively.

Conducting polymers and in particular electrochemically deposited polymers have gained considerable interest as support matrices, especially for electrochemical sensors, where they can be grown in situ under easily controlled electrochemical conditions[11]. The various applications of polypyrrole-based immobilisation method will be discussed in section 1.3 of this chapter. The electron transport properties of these polymers may in some cases be exploitable in the signal transduction process for the biosensor device.

1.1.2. Electrochemical Sensing

Electrochemical techniques such as amperometry, potentiometry and conductometry are among the more powerful detection methods commonly used for biosensing because of their selectivity, sensitivity and ability to detect low concentrations of analyte(s) without prior derivatization. The acceptability of these methods is further enhanced by the low cost of the required equipment.

An enzyme electrode consists of a thin layer of enzyme immobilised on the surface of a suitable substrate such as gold, platinum, silver, copper, or carbon. The enzyme is chosen to catalyse a reaction which generates a product or consumes a co-reactant which can be monitored electrochemically. Thus, the electrochemical signal (e.g., current, potential or resistance) provides a measure of the analyte concentration. An advantage of electrochemical biosensors is that the analytical signal is electrical in nature and this greatly reduces the complexity of the transducing system and controlling electronics[11].
1.1.2.1. Potentiometry

Potentiometry is defined as the measurement of the potential of non-polarised electrodes under conditions of zero current. Selectivity is derived from a membrane at which a charge separation is brought about that is dependent on the ion to be determined. The potential of an enzyme electrode can only be measured by comparison with a reference potential which has to be provided by a second electrode in contact with the sample. These electrodes are ideally suited to measurement in the FIA mode.

The potentiometric determination of urea$^{99,100,103-105}$ and glucose$^{49,67}$ with polypyrrole-based enzyme electrodes have been reported.

1.1.2.2. Amperometry

Amperometry is a voltammetric technique where the potential is kept constant as function of time. Current is measured as the compound undergoes an oxidation or reduction at the electrode held at a fixed operating potential. The measuring equipment is simple and the measured current is a linear function of the concentration of the substrate.

Some polypyrrole-based enzyme electrodes with amperometric detection have been developed for selective and sensitive determination of glucose$^{18,48,50,66,68,90}$, cholesterol$^{91}$, glutamate$^{92-94}$, dopamine$^{94,95}$, penicillin$^{96}$, fructose$^{97,98}$, urea$^{101,102}$, lactate$^{40,45,55,107}$, choline$^{401}$, nitrate$^{109}$, sulfite$^{110}$, creatinine$^{101}$, NADH$^{112-114}$, ascorbate$^{120}$, $\text{H}_2\text{O}_2$$^{115-117}$ and superoxide radical$^{115}$. Adeloju et al.$^{99}$ described an approach for amperometric detection of urea in a FIA system. An application of this technique for the detection of formate will be carried out in this research.

1.1.2.3. Conductometry

Conductometry (i.e., the measurement of the resistivity) is used frequently for analytical applications. There is a substantial reawakening of interest in the practical applications of conductometry. Recent electronic developments have resulted in automated precision conductometric instrumentation$^{26,27}$.

Up to now, only penicillin$^{96}$ was determined by a polypyrrole-based enzyme electrode with conductometric detection. It is found that the conductivity of
the polypyrrole film is sensitive to pH of the solution. A polypyrrole-coated electrode can serve as a pH-sensitive transducer for enzyme sensors.

1. 2. Biosensors Designs

The research and development of biosensors are at present in a rapidly growing phase due to the demands from a number of different areas. Many different types of biosensors have been developed, and the majority of the biosensor concepts are based on the combination of immobilised enzymes with classical sensors.

Electrochemical sensors make use of electrochemical reactions. As a parameter, the potential or conductance between two electrodes or the current through a polarised electrode is measured. A great variety of chemical substances can be measured in this way. Electrochemical measurement can be accomplished to a great extent by coupling portable flow system instruments and chip-based electrode devices.

1. 2. 1. Multilayer Design

Discrete layered structures have been fabricated by sequential electropolymerization to modulate the performances of the resulting biosensors\(^{56}\). Different types of layers, with or without enzyme, have been stacked on the electrode surface.

Polymer films that have electron donor (or acceptor) properties for reduced (or oxidised) enzyme would allow for mediated electron transfer from the enzyme to the electrode. Four typical structures of layered designs are illustrated in Figure 1-3.

Monolayer coverage of enzyme is commonly used for immobilisation onto the surface of a transducer. Bilayer designs were successful in biosensors. In this case, after immobilisation of the enzyme on the electrode surface, an anti-interference layer of polypyrrole film is grown on top to enable measurements in complex matrices\(^{50}\). A polypyrrole film coated with a lipid membrane\(^{19,20}\) or a poly(o-phenylenediamine) bilayer\(^{52}\) has been found to enhance the stability of glucose biosensors. Interferences from several species in physiological fluids were avoided by use of a polypyrrole/polyphenol bilayer lactate biosensor\(^{107}\). A polymetallophthalocyanine (PMePc) film
grown on a glassy carbon electrode, and followed by polymerisation of polypyrrole and glucose oxidase film showed good stability and reproducibility\cite{65}. A conducting polymer film could be used as a mediator in this case.

1) Monolayer

2) Bilayer

3) Trilayer

4) Sandwich

![Diagrams of electrode layer designs]

**Figure 1-3** Four Types of Electrode Layer Design

Tatsuma et. al.\cite{88} described a bilayer electrode carrying a glucose oxidase/polypyrrole (GOD/PPy) film as an inner layer and a horseradish peroxidase/polypyrrole (HRP/PPy) film as outer layer. It was found that this bilayer biosensor exhibited higher signal transfer efficiency than that of a monolayer GOD/HRP/PPy homogeneous film coated electrodes. Evidently, the multilayer film designs of enzyme-based electrode have potential benefits for biosensor design and fabrication.
1.2.2. Chip-Based Electrode Devices

Based on semiconductor technology, biosensors are characterised by their ruggedness, ability to be directly integrated with microelectronics and hence the possibility to be mass fabricated at low cost. A typical field effect device system for biosensing is shown in Figure 1-4.

![Diagram of typical field effect device](image)

**Figure 1-4** Schematics of Typical Field Effect Device

W: Enzyme-based working electrode, R: Reference electrode, A: Auxiliary electrode

1.2.3. Flow Based Biosensors

As most enzymatic reactions used in biosensors are irreversible, the depletion of enzyme on the electrode surface is a common problem. Polypyrrole-based film, such as a urea biosensor[99], was easy to poison by substrate and /or solution matrix which may bond strongly to the polymer and decrease the number of free active sites. One way of increasing the lifetime of immobilised enzyme is to use the biosensor in a flow system so that the sample is only in contact with the polypyrrole film for short periods.

So the commercial flow cells such as 'Wall-jet' cell, thin-layer flow through cell and tubular cell can be applied to biosensors. Several applications of polypyrrole-based biosensors[54-56,62,77,99,101,106,107] in flow systems have been reported. Due to the cell geometry, these biosensors seem to be very sensitive to changes in viscosity of the injected sample solutions. It is therefore very important to control solution flow. However, one of the advantages of enzyme-based electrodes is that they can be cleaned/flushed with buffer
solution after each measurement. This would extend the life of the biosensor and improve its reproducibility with further reuse.

The vitality of these methods is further enhanced by the low cost of the equipment and possibility for in-field measurement. The proposed polypyrrole-based formate biosensor in FIA mode will offer numerous possibilities for significantly increasing the flexibility and speed in comparison with existing methods.

1.3. Polypyrrrole Films

The three common processes in biosensing are analyte recognition, signal transduction, and readout. Intimate contact between the bioactive substrate and the transducer is achieved by its immobilisation on the surface of the transducer. This is accomplished generally by one of several methods as discussed in sections 1.1.1. and 1.2.1. Over a decade ago, Umana and Waller[12] described an interesting approach for trapping glucose oxidase in conducting polypyrrole film. As a consequence the products of the enzymatic reaction are generated in the immediate proximity of the electronically conducting material.

1.3.1. Applications of Polypyrrrole Films

Polypyrrole films provide a multi layered, dynamic polymeric coating which has a three dimensional reaction zone at the electrode surface on which various chemistries such as ion exchange, complexation, precipitation and enzyme reactions can be performed[13]. There has been significant interest in the preparation of polypyrrole and its various properties which have led to wide practical applications.

The physical entrapment of enzymes into conducting polymer based on electrochemical immobilisation offers the one of the best approaches for biosensor development and design. The common methods include galvanostatic, potentiostatic and potentiodynamic polymerisation. The mechanism of polymerisation of pyrrole is summarised as follows[14].

1) Monomers oxidation and resonance forms:
The neutral monomer is oxidised to a delocalised radical cation. The cation has possible resonance forms. Consequently, the monomers dimerise via radical-radical coupling at the α-position, and protons or hydrogen atoms are eliminated from the doubly-charged dihydridimer which results in a neutral species or dimer cations, respectively. In the solid state, a radical cation and dication that are partially delocalised over some polypyrrole segment are called a polaron and a bipolaron\(^{[15]}\), respectively. Polarons and bipolarons are produced in the doping process. Some possible species are revealed in the following steps.

2) Radical-radical coupling:

3) Chain propagation:

Chain propagation is accompanied by the addition of a delocalised radical cation to the already charged oligomers or dimers and the elimination of other protons. A study\(^{[14]}\) of the mechanism has proposed that the polymer film not only grows by the addition of monomers to a polymer chain end, but also that the precipitation of oligomer chains from solution influences the film growth processes.
When polypyrrole is oxidised by applying a positive potential, the pyrrole units have positive charges, which are balanced by a variety of so-called dopant anions. Thus, by reducing the film to the neutral state with the application of negative potential, anions are expelled from the polypyrrole film. This process is usually referred to as undoping.

Polypyrrole belongs to a group of semi-conducting polymers, an electron can be moved in their conjugated π-electron systems\textsuperscript{[16]}. Its advantages include ease of formation (chemically or electrochemically), flexibility of dopant selection, relatively good environmental stability, and good electrical and mechanical properties. The structure of polypyrrole differs from the ideal single infinite strand configuration in a number of ways, including a large degree of disorder which modifies the electron transport process. Chains are of short and random lengths and frequently cross-linked, thought to be every 5-10 monomer units\textsuperscript{[17]}. A variety of intrinsic structural defects exists such as
substitutions, nitrogen-nitrogen linkages, and the presence of dopant ions. Disorder is further enhanced by the fact that monomer units do not have complete rotational freedom with respect to one another, so polypyrrole chains are unable to seek a crystalline morphology.

In this case, the biorecognition site is incorporated by having the enzyme present during the electrodeposition of the polypyrrole film\(^{11}\):

![Structure of Polypyrrole](image)

**Figure 1-5** Structure of Polypyrrole

Some enzymes (see section 1.4.1) have been successfully incorporated into conductive polypyrrole film. The distinct advantages of incorporating enzymes into a polypyrrole backbone include:

1) better electrical properties due to the conductivity of polypyrrole;

2) ease of polymerisation and enzyme incorporation;

3) relatively fast and inexpensive polymerisation procedure;

4) immediate availability of a fresh polymer film.

The immobilisation of enzyme through electropolymerization has proven to be an interesting alternative to conventional methods of enzyme immobilisation for the preparation of electrochemical biosensors. Electropolymerization is usually performed in the galvanostatic mode. This approach often produces films with more uniform thickness than those prepared by the potentiostatic mode\(^{68,78,112,113,116,117}\).

In this study the electrochemical incorporation of formate dehydrogenase into a polypyrrole film will be explored for development of a biosensor for formate. A schematic diagram of immobilisation is predicted in Figure 1-6, with the use of ferrocyanide as a mediator.
Figure 1-6 Co-Electroimmobilisation of enzyme, co-factor and mediator into a single polypyrrole film.

1.3.2. Mechanisms of Enzymatic Reactions in Membrane

In the simplest case, molecular transport through a membrane occurs via a random walk mechanism which concludes with desorption of the analyte from the surface at the lower concentration. The total permeation process consists then of sorption, diffusion and desorption. A predominant process in membranes used for biosensor is penetrant (analyte) diffusion.

1.3.2.1. Diffusional Limitations

Biosensors which employ enzymes as recognition molecules often utilise the bioactive substance in an immobilised state, and so the enzyme kinetics should account for the diffusional limitations of the immobilised matrix. Molecular
diffusion across a polymer membrane can be described commonly by Fick's first law of diffusion:

\[ J = -D \frac{\partial c}{\partial x} \]

\( J \): Flux.
\( D \): Diffusion coefficient
\( \frac{\partial c}{\partial x} \): Concentration gradient.

The analyte diffuses from the bulk sample solution into the biocatalytic layer, where an enzyme reaction takes place (Figure 1-7). The time necessary to reach the steady-state signal (electrode response time) depends on the electrode type and assembly, on the thickness and permeability of the biocatalytic membrane, and on temperature, pH, stirring rate, and the type of substrate.

![Direction of Diffusion](image)

**Figure 1-7 Molecular Diffusion across a Polymer Membrane**

\( C_1 \): Concentration at the Bulk Solution.
\( C_2 \): Concentration at the Transducer Surface.

The kinetics of diffusion in polymer films is one of the significant influences in biosensors. Although conducting polymer materials are beneficial in the electron transfer, the diffusion dynamic behaviour of polymer films depends on the concentration gradient of species. The sensitivity of an enzyme
membrane under the diffusion-controlled conditions is proportional to the gradient of \( c_1 \) at \( x=0 \).

Unlike a soluble enzyme, a matrix-supported enzyme has to exercise catalytic action in a heterogeneous environment. As in conventional heterogeneous catalysis, at least five distinct steps can be identified in the overall enzymatic processes as follows:

1) diffusion of the analyte from the bulk phase to the polymer surface;

2) transport of the analyte from the polymer surface to the domain of the enzyme;

3) enzyme catalysed conversion of the analyte;

4) transport of the analyte and product from the domain of the enzyme to the polymer surface;

5) diffusion of the analyte and product from the polymer surface to the bulk phase.

1.3.2.2. Kinetic Behaviour of Immobilised Enzymes

One of the most important functions of proteins or enzymes is to act as catalysts for chemical reactions\(^{123}\). The immobilised sensing enzyme used in biosensor(s) reacts by the following mechanism:

\[
S + E_1 \xrightarrow{k_1} E_1S \xrightarrow{k_2} E_2P \xrightarrow{k_3} E_2 + P
\]

- \( S \): Substrate, \( E_1 \) and \( E_2 \): Sensing enzymes,
- \( P \): Product, \( k \): rate constants

The importance of transition state theory is that it relates the rate of a reaction to the difference in Gibbs free energy (\( \Delta G^\# \)) between the transition state and the ground state\(^{124}\). Substrate specificity by the enzyme is provided by the surface interactions. The non-covalent binding of the enzyme-substrate transition state lowers the activation energy for the reaction and thus catalyses
the reaction as illustrated in Figure 1-8. It shows two hypothetical free energy profiles, in which the catalyst offers a favourable alternative pathway involving an intermediate.

Covalent catalysis is particularly common among enzymes which catalyse group transfer reactions, or which depend on cofactors or coenzymes for activity. In the latter case the intermediate is formed from the substrate and the enzyme-bound cofactor.

![Diagram of Reaction Coordinate](image)

**Figure 1-8** Hypothetical Free-Energy Diagram of a Reaction

If an organic cofactor is brought into play during the catalytic mechanism, it is referred to as a coenzyme\(^{124}\). Dehydrogenases are enzymes that catalyse the transfer of a hydrogen from one substrate to another. This reversible system normally requires a coenzyme such as nicotinamide adenine dinucleotide (NAD). NAD is the coenzyme involved in dehydrogenase reactions and is reduced to NADH during the process. The reaction is stereospecific and only one isomer of NADH is produced. The kinetics of such reactions are obscured by several factors, such as\(^{124}\):

1) change in enzyme conformation;
2) steric effects;
3) microenvironmental effects;
4) bulk and internal diffusional effects.
1. 4. Polypyrrole-Based Biosensors

1. 4. 1. Introduction

An enzyme electrode is the first type of biosensor which combines a bioactive substance and an electrochemical sensor to detect an analyte involved in a biocatalytic reaction. The enzyme can be immobilised onto a membrane as discussed in section 1.1.1., and is tightly maintained in close contact with the electrochemical transducer. When the enzyme is in the presence of a target analyte an electrical signal is obtained and this can be correlated to the analyte concentration.

Potentiometric and amperometric modes of detection/sensing are commonly used with polypyrrole-based enzyme electrodes as summarised in Table 1-2. The resulting potential or current output gives a measure of the analyte concentration with a logarithmic or linear relationship, respectively. A strong interest arose for such analytical devices and numerous papers have appeared in the literature\(^\text{[18-120]}\). More recently a more efficient direct electrochemical method has been developed for pulsed electrochemical detection of urea\(^\text{[106]}\) in blood samples. In this case the potential is pulsed, respectively, between E\(_1\) and E\(_2\) with pulse widths of t\(_1\) and t\(_2\). The analytical signal is obtained by sampling the current flow at predetermined intervals.

As shown in Table 1-2, polypyrrole-based glucose biosensors\(^\text{[18-90]}\) have been investigated extensively due to their wide range of applications in clinical diagnosis, agriculture, process control, microbiological analysis and pharmaceutical analysis.

1.4.2. Mediated Enzyme Biosensors

Mediators are usually low molecular weight species which shuttle (Figure 1-9) electrons between the redox centre of the enzyme and the working electrode. Ideally, when used in an electrochemical device the mediator should react rapidly with the enzyme, exhibit reversible heterogeneous kinetics and possess a low overpotential for regeneration.
<table>
<thead>
<tr>
<th>Measured species</th>
<th>Enzyme</th>
<th>Mediator used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td></td>
<td>18-67</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose dehydrogenase</td>
<td></td>
<td>68,69</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Osmium</td>
<td>70</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Ferricyanide</td>
<td>71-74</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Ferrocene</td>
<td>75</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Ferrocenecarboxylate</td>
<td>76</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>1,1'-Dimethylferrocene</td>
<td>77</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Ferrocenecarboxaldehyde</td>
<td>78</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>1,4-Arenequinones</td>
<td>79</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>p-Benzoquinone</td>
<td>80-83</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>Benzoquinone sulfonate</td>
<td>84,85</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase</td>
<td>TTF-TCNQ</td>
<td>86,87</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose oxidase/ horseradish peroxidase</td>
<td>HRP/e^-</td>
<td>88-90</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Cholesterol oxidase</td>
<td>Ferrocenecarboxylate</td>
<td>91</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glutamate dehydrogenase</td>
<td>Phenazine methy sulfate</td>
<td>92</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glutamate oxidase</td>
<td></td>
<td>93,94</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Polyphenol oxidase</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Banana cells (polyphenol oxidase)</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Measured species</td>
<td>Enzyme</td>
<td>Mediator used</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------</td>
<td>------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Penicillinase</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Fructose</td>
<td>Fructose dehydrogenase</td>
<td>TTF-TCNQ</td>
<td>97</td>
</tr>
<tr>
<td>Fructose</td>
<td>Fructose dehydrogenase</td>
<td>Hexacyanoferrate(III) of ferrocene</td>
<td>98</td>
</tr>
<tr>
<td>Urea</td>
<td>Urease</td>
<td></td>
<td>99-106</td>
</tr>
<tr>
<td>Lactate</td>
<td>Lactate oxidase</td>
<td></td>
<td>40,45,55,107</td>
</tr>
<tr>
<td>Choline</td>
<td>Choline oxidase</td>
<td></td>
<td>40,45,56,108</td>
</tr>
<tr>
<td>Nitrate</td>
<td>Nitrate reductase</td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>Sulfite</td>
<td>Sulfite oxidase</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Creatinine iminohydrolase</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>Linamarin</td>
<td>Linamarase/glucose oxidase</td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>NADH</td>
<td>NADH dehydrogenase</td>
<td></td>
<td>112,113</td>
</tr>
<tr>
<td>NADH</td>
<td>NADH dehydrogenase/diaphorase</td>
<td></td>
<td>114</td>
</tr>
<tr>
<td>Superoxide radical</td>
<td>Superoxide dismutase</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Horseradish peroxidase</td>
<td></td>
<td>115-117</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Pyruvate oxidase</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>Alcohol dehydrogenase</td>
<td></td>
<td>119</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>Cucumber juice (ascorbate oxidase)</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>
Freshly prepared polypyrrole films are electrically conducting. This factor has gained considerable interest in the design of mediator based biosensors, where a direct electron transfer from mediator to the electrode is of great importance. However, the polypyrrole film can loose its conductivity when high potentials (e.g., 700mV vs Ag/AgCl) are applied\cite{21,121,122}. Therefore this film can only be used at low applied potentials to maintain its conductive state. The application of low potential to the working electrode will further improve the selectivity of the biosensors.

In recent years, several polypyrrole-based mediated electrochemical biosensors which utilised mediators such as quinones\cite{79-85} and ferrocene or its derivatives\cite{75,76-78,91,98} have been reported. The fabrication of a formate biosensor which incorporates a co-factor such as NAD and a mediator such ferrocene and ferrocyanide will be investigated in this study.

1.5. Aims of This Study

As discussed so far, the electrochemical immobilisation of β-NAD and formate dehydrogenase in polypyrrole films will be investigated in this study. The incorporation of a mediator in the polypyrrole film will be explored for lowering the required applied potential for amperometric sensing in order to maintain the conductivity and stability of the polypyrrole backbone. For this purpose, the use of ferrocyanide, Prussian Blue and ferrocene will be considered. The utilisation of the biosensing membrane for the reliable batch and FIA determination of formate based on an amperometric mode of
detection will be explored. The application of artificial neural networks (ANN) to overcome the problem of reusability and reproducibility in a non-linear and complicated dynamic system such as a conducting polypyrrole-based formate biosensor is also considered. The resulting system will be trained with a new neural network based software package, Turbo Neuron, for prediction of the concentration of formate, based on the entire collected data which contain the history of the detection experiments.

1.6. References


[77] W. Schuhmann, R. Lammert, B. Uhe and H. L. Schmidt, Sensors Actuators, (1990), B1, 537


CHAPTER 2

Electrochemical Formation and Characterisation of Polypyrrole Films
2.1. Introduction

The utilisation of electrochemical techniques for the synthesis and characterisation of conducting polymers has become very attractive for sensor development. Conjugated polymers, such as polypyrrole, can be oxidised and reduced in a way that is in principle similar to the redox polymers\cite{1}. During reduction anionic sites are formed that require cations for charge compensation (or anion expulsion). Upon oxidation cationic sites, such as polarons (PPy\textsuperscript{+}) and bipolarons (PPy\textsuperscript{2+}), are normally formed on the polypyrrole chains as follows\cite{1}:

\begin{equation}
\text{PPy} + X^-_{(s)} \rightleftharpoons \text{PPy}^+X^- + e^-
\end{equation}

\begin{equation}
\text{PPy}^+ + 2X^-_{(s)} \rightleftharpoons \text{PPy}^{2+}(X^-)_2 + e^-
\end{equation}

where $X^-$ are anions (the subscript (s) indicates that they are in the solution phase), and PPy symbolises a segment of the polypyrrole chain of a few monomer units length. The oxidation or reduction of polypyrrole can be readily accomplished electrochemically. The associated movement of anions ($X^-$) into the polymer matrix is illustrated in Figure 2-1.

![Figure 2-1](image.png)

**Figure 2-1** Schematic representation of electrochemical oxidation of a polypyrrole film. Reproduced from [2].

The diagram in Figure 2-1 demonstrates the principle of the *electroneutrality coupling*\cite{1,2} which requires a flux of ions into or out of the polymer film to provide internal charge compensation. During anodic oxidation of a polypyrrole film the anion $X^-$ compensates the positive charge of the resulting PPy\textsuperscript{+}. 

32
The versatility of this conductive polymer film enables the incorporation of a wide variety of counter-ions. Thus, the conductivity of polypyrrole may be increased by doping with anions. The range of dopants that have been incorporated into polypyrrole film is given in Table 2-1. In all such applications the coated metal is immersed in an appropriate electrolyte and subjected to an electrochemical treatment.

The most appropriate general model for describing electron transfer across the redox sites in conductive polymer is the electron-hopping concept for disordered systems\textsuperscript{[43,44]}. It is thus necessary to have occupied sites next to unoccupied ones for this process to take place. Hence, the ratio between the densities of acceptor and donor states is very important. The redox sites are oxidised or reduced by applying an appropriate electrode potential $E$, that is by raising or decreasing the free energy of electrons, $\mu_e$ depicted in Figure 2-2. The transport of electrons to or from the sites in the polymer phase is an essential part of this redox process.

![Figure 2-2 Schematic distribution of a possible electronic-energy-level in a conducting polymer (\(\cdots\)): occupied states; \(-\): unoccupied states). Reproduced from [2].](image)

The concepts of electroneutrality coupling and electron-hopping which are useful for the incorporation of functional components and transportation of electrons is important in this project. Therefore preliminary experiments and discussion of this chapter will focus on the fundamental aspects of electron and ion conduction that form the basis for understanding the electrochemical behaviour of a polypyrrole-based biosensor involving the use of a mediator for transferring electrons. A number of electrolytes, such as HNO$_3$, HCl, H$_2$SO$_4$, NaNO$_3$, NaCl and KCl, will be incorporated into polypyrrole films by electropolymerisation methods to explore the functions of cations and anions as a dopant in a conductive polymer, in order to verify the concepts of electroneutrality coupling and electron-hopping.
<table>
<thead>
<tr>
<th>Dopants</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl^-</td>
<td>3, 8, 9</td>
</tr>
<tr>
<td>NO_3^-</td>
<td>6, 10</td>
</tr>
<tr>
<td>SO_4^{2-}</td>
<td>4-8</td>
</tr>
<tr>
<td>H_2PO_4^-</td>
<td>8</td>
</tr>
<tr>
<td>ClO_4^-</td>
<td>3, 8, 11, 12, 13</td>
</tr>
<tr>
<td>I_2, or I^-</td>
<td>6, 37</td>
</tr>
<tr>
<td>[Fe(CN)_6]^{4-}</td>
<td>14</td>
</tr>
<tr>
<td>CH_3SO_3^-</td>
<td>8</td>
</tr>
<tr>
<td>CF_3SO_3^-</td>
<td>8</td>
</tr>
<tr>
<td>EDTA</td>
<td>15, 16</td>
</tr>
<tr>
<td>tetraethylammonium perchlorate</td>
<td>17, 18</td>
</tr>
<tr>
<td>tosylate</td>
<td>8</td>
</tr>
<tr>
<td>hectorite</td>
<td>19</td>
</tr>
<tr>
<td>tetrafluoroborate</td>
<td>4, 19-23</td>
</tr>
<tr>
<td>toluene sulfonate, or benzene sulfonate</td>
<td>3-7, 24-32</td>
</tr>
<tr>
<td>anthraquinone-2-sulfonate</td>
<td>33</td>
</tr>
<tr>
<td>polystyrene sulfonate</td>
<td>3, 8, 34</td>
</tr>
<tr>
<td>polyvinyl sulfonate</td>
<td>3, 8</td>
</tr>
<tr>
<td>naphthalene sulfonate, or disulfonate</td>
<td>4, 8</td>
</tr>
<tr>
<td>ethane sulfonate, or ethane disulfonate</td>
<td>3, 8</td>
</tr>
<tr>
<td>pentane sulfonate</td>
<td>3</td>
</tr>
<tr>
<td>poly(2-acrylamido-2-methyl-1-propane-sulfonate)</td>
<td>8</td>
</tr>
<tr>
<td>poly(4-vinyl-4'-methyl-2,2'-bipyridine)</td>
<td>35</td>
</tr>
<tr>
<td>dodecylsulfate</td>
<td>12</td>
</tr>
<tr>
<td>dodecylbenzenesulfate</td>
<td>8</td>
</tr>
<tr>
<td>polyethylene oxide</td>
<td>12</td>
</tr>
<tr>
<td>polyvinylalcohol</td>
<td>36</td>
</tr>
</tbody>
</table>
2. 2. **Experimental**

2. 2. 1. **Reagents**

All reagents used were of analytical-reagent grade, unless specified otherwise. All solutions were prepared with Milli-Q water (Millipore). Pyrrole was distilled prior to use. HNO₃, NaNO₃, HCl, NaCl, KCl, H₂SO₄, Na₂HPO₄, and NaH₂PO₄·2H₂O were supplied by Ajax Chemical Pty Ltd (NSW). Nitrogen (O₂-free) was used where necessary.

2. 2. 2. **Polypyrrole Film Formation**

Polypyrrole films were electropolymerised by potentiodynamic method (cyclic voltammetry) from deaerated solutions of pyrrole (0.1 or 0.5M) which contained 0.1M of these electrolytes: HNO₃, HCl, H₂SO₄, NaNO₃, NaCl and KCl. The cyclic voltammetric behaviour of the resulting polypyrrole films was examined in 0.1M phosphate buffer solution (pH 6.92). Polypyrrole films were also produced by galvanostatic polymerisation in a number of pyrrole-electrolyte solutions. Galvanostatic electropolymerisation was performed with an applied current density of 0.5mA/cm² for 180 seconds.

A BAS 100B Electrochemical Analyzer was used for the cyclic voltammetry. All experimental voltammograms were plotted on a FPG-315 Colour Plotter. A three-electrode, home-made cell was used, consisting of a gold working electrode (area=7mm²), a coiled platinum auxiliary electrode and a saturated KCl calomel reference electrode. The electrochemical cell arrangement used in this study is shown in Figure 2-3. A RS 232 Potentiostat/Galvanostat constructed in the Electronics Workshop of the Faculty of Science & Technology at UWS Nepean was used to produce polypyrrole films.
Figure 2-3  Cell arrangement for electropolymerisation and investigation of electrochemical behaviour of polypyrrole films.

R: reference electrode;  W: working electrode;  
A: auxiliary electrode.

2.3. Results and Discussion

In this section, polypyrrole films were synthesised by potentiodynamic (potential cycling) polymerisation from a range of aqueous pyrrole solutions which contained one of the following electrolytes: HNO₃, NaNO₃, HCl, NaCl, KCl and H₂SO₄. The electrochemical behaviour of a bare gold electrode in these electrolytes were initially examined by cyclic voltammetry to provide a background response in the absence of polypyrrole. Figure 2-4 shows the cyclic voltammograms obtained in the different electrolytes. The potential limit achieved in acid solutions is influenced by H₂ evolution and it is generally limited to -0.5V where the cathodic current increased considerably. In contrast, the limit is extented to -1.0V in basic solution and phosphate buffer (pH 6.92) solution.
Figure 2-4  Cyclic voltammograms of a gold electrode in some electrolytes. Concentration: 0.1M for each; Scan rate: 100mV/s.
2.3.1. Positive Potential Limits and Pyrrole Concentration

The positive limit for the potentiodynamic polymerisation of pyrrole was set in each case to accommodate only the first oxidation peak for the monomer. In most cases the evidence for the formation of polypyrrole is shown by the growth of the current-potential curves with repeated scans, as shown in Figure 2-5. A conducting film is usually formed on the electrode after the first cycle from -1.0V to +0.8V, and then back to -1.0V. The increase in the oxidation current of pyrrole in solution is indicative of effective growth of polypyrrole.

![Graph showing cyclic voltammetry with curves labeled a, b, c, and d.](image)

**Figure 2-5** Cyclic voltammetric polymerisation of pyrrole on gold Electrode. Composition: 0.1M NaCl and 0.1M pyrrole; Potential limits: +0.8V to -1.0V; Scan rate: 100mV/s; Sweep segments: 30.

a: scan 1, b: scan 5, c: scan 10, d: scan 15.
It is interesting to note that polypyrrole film can not be produced at low anodic potential, as demonstrated with a positive potential limit of +0.5V in Figure 2-6. In this case, even after 10 cycles, no black polypyrrole film was observed. Instead the cyclic voltammograms were similar to those obtained for the bare gold electrode in 0.1M NaCl (Figures 2-4e and 2-6).

![Graph](image)

**Figure 2-6** Cyclic voltammetric polymerisation of pyrrole on gold electrode. Composition: 0.1M NaCl and 0.1M pyrrole; Potential limits: +0.5V to -1.0V; Scan rate: 100mV/s; Sweep segments: 20. The comparison of two voltammograms in a dashed rectangle is for gold in 0.1M NaCl and 0.1M NaCl/0.1M pyrrole, respectively.

The choice of positive potential limits is therefore critical for potentiodynamic polymerisation of conducting polypyrrole on the gold surface. Figures 2-7a and 2-8a show that polypyrrole films did not grow sufficiently when low positive potential limits, +0.7V (in 0.1M pyrrole) and +0.6V (in 0.5M pyrrole), respectively, are employed. The use of more positive potential limits (≥+0.8V in 0.1M pyrrole, or ≥+0.7 in 0.5M pyrrole) is therefore necessary for rapid electropolymerisation of polypyrrole by cyclic voltammetry. This can be explained by the concept of electroneutrality coupling \(^{1,2}\). The anodic oxidation of polypyrrole, where the anions Cl\(^-\) compensate the positive charge of the produced PPy\(^+\), enhances the electropolymerisation of a polypyrrole film.
The rate of polymerisation is simply proportional to the applied potential. However, it is well known that polypyrrole films can be overoxidised at high positive potential\cite{38}. The evidence of overoxidation of polypyrrole films can be observed in the cyclic voltammograms in Figure 2-8. When the positive potential limits are $<-0.7\text{V}$, no cathodic peaks occur between $-0.5\text{V}$ and $-1.0\text{V}$. This could be due to rearrangement of the polypyrrole films when the positive potential limit exceeds $+0.7\text{V}$, and thus resulting in a corresponding cathodic reaction. Polypyrrole therefore loses its electrochemical activity and conductivity at an electrode potential of $+0.7\text{V}$ vs Ag/AgCl due to overoxidation\cite{38}. Care should therefore be exercised when using positive potential limits in biosensors applications where enzymes/mediators are immobilised in polypyrrole.

The concentration of pyrrole is one of the important factors in the electrosynthesis of conducting polypyrrole on a gold electrode. An increase in the monomer concentration to $0.5\text{M}$ in this study demonstrated that the required positive potential limit for potentiodynamic polymerisation of pyrrole can be lowered from $+0.8\text{V}$ (in $0.1\text{M}$ pyrrole) to $+0.7\text{V}$, as shown in Figures 2-7 and 2-8. On the other hand, the use of high concentration of pyrrole resulted in an increased conductivity of the polypyrrole film. Two PPy-Cl$^-$ films were grown by potential cycling between $+0.8\text{V}$ and $-1.0\text{V}$ for 15 cycles in $0.1\text{M}$ and $0.5\text{M}$ pyrrole with $0.1\text{M}$ NaCl as a dopant. As shown in Figure 2-9, an increase in the monomer concentration to $0.5\text{M}$ improved the conductivity of the polypyrrole film significantly. This observation can also be explained by the concept of electroneutrality coupling. The more PPy$^+$ sites produced for internal charge compensation in higher pyrrole concentration, the more efficiently the anions $X^-$ will compensate the positive charge of the resulting PPy$^+$. Furthermore, the higher pyrrole concentration may protect the polypyrrole film from overoxidation at $+0.7\text{V}$. The maximum anodic peak current at the positive potential limits of $+0.7\text{V}$ and $+0.8\text{V}$ are $85\mu\text{A}$ and $54\mu\text{A}$, respectively, as shown in Figure 2-8(b, c).
Figure 2-7  Comparison of positive potential limits for polymerisation of pyrrole(0.1M). Composition: 0.1M NaCl and 0.1M pyrrole; Scan rate: 100mV/s; Sweep segments: 20.

Positive potential limit: a, +0.7V; b, +0.8V; c, +1.0V.
**Figure 2-8** Comparison of positive potential limits for polymerisation of pyrrole (0.5M). Composition: 0.1M NaCl and 0.5M pyrrole; Scan rate: 100mV/s; Sweep segments: 40.

Positive potential limit: a, +0.6V; b, +0.7V; c, +0.8V.
**Figure 2-9** Effect of pyrrole concentration on polypyrrole films. Electrolyte: 0.1M phosphate buffer. Scan rate: 100mV/s.
2.3.2. Stability of Polypyrrole

As discussed early, polypyrrole film structure can be viewed as a three-layer structure, as shown in Figure 2-10. The inner layer is generated by initiation of polymerisation and followed by the development of polymer chain, producing the main layer. The main layer thickness depends on polymerisation time and factors such as current density and applied potential limit. The outer layer is produced by polymer termination, when the current or potential is turned off.

![Figure 2-10: Polypyrrole layers mode by electropolymerisation](image)

It has been observed that the outer layer of polypyrrole is fairly unstable, due to a short chain polypyrrole layer. The thickness of the main polypyrrole layer, in turn, influences film stability. As illustrated in Figure 2-11, the behaviour of PPy-Cl⁻ films were investigated in 0.1M phosphate buffer solution by cyclic voltammetry. Both films were formed by potentiodynamic polymerisation in 0.1M NaCl and 0.1M pyrrole monomer solution, within a potential range between +0.8V and -1.0V. Generally, the more the electrode is cycled, the thicker the polypyrrole film. The cyclic voltammograms indicated that the thin film was less stable due to the thinner main layer, as evident from the comparison of Figure 2-11a and 2-11b.
Figure 2-11 Voltammograms of PPy-Cl\textsuperscript{−} obtained by potential cycling polymerisation in 0.1M phosphate buffer solution. Scan rate: 100mV/s. Sweep segments: 20.

2.3.3. Comparison of Dopants

Dopant is one of the important co-factors in electropolymerisation, which results in increasing or decreasing conductivity of the polymers. Figures 2-12 and 2-13 illustrate the oxidative doping effect of some anions into polypyrrole films.
Figure 2-12  Growth of Polypyrrole Films by Potential Cycling. Composition: 0.5M pyrrole; Potential limits: +0.7V to -1.0V; Scan rate: 100mV/s; Sweep segments: 50.

Dopants: a, 0.1M KCl; b, 0.1M NaCl; c, 0.1M NaNO₃.
Figure 2.13  Growth of Polypyrrole Films by Potential Cycling. Composition: 0.5M pyrrole; Potential limits: +0.7V to -0.5V; Scan rate: 100mV/s; Sweep segments: 50.

Dopants: a, 0.1M HCl; b, 0.1M HNO₃; c, 0.1M H₂SO₄.
The anion ($X^-$) is one of the important factors of conducting polypyrrole films where the anions ($X^-$) compensate the positive charge of the produced PPy$^+$ for internal charge compensation. As shown in Figure 2-12, the cyclic voltammetric behaviour of polypyrrole in KCl and NaCl is very similar, it is slightly different between chloride and nitrate within the anodic potential window, scanning from +0.5V to +0.7. The effect of anionic charges is highlighted in Figure 2-13. The cyclic voltammogram for sulfate doped polypyrrole film is quite different compared to that of chloride and nitrate. This maybe due to neutralise polypyrrole film charges using the different numbers of anions.

Therefore the molecular structure and the anionic charges of an anion will affect the structures of conducting polypyrrole film due to the concepts of electroneutrality coupling and electron-hopping. It was further evidenced by a variety of polypyrrole films in 0.1M phosphate buffer as shown in Figure 2-14, cyclic voltammograms presented similar characteristics except sulfate-polypyrrole film.

It is very interesting to find that the polypyrrole cathodic peak depends on certain dopants, influenced both by the anionic and cationic components. Table 2-2 highlights a trend of the dopant's contribution to cathodic peak potential and current, respectively. Peak current indicated,

1) the polypyrrole conductivity in the presence of anion Cl$^-$ as:

\[ H^+ > K^+ > Na^+ \]

2) the polypyrrole conductivity in the presence of cation H$^+$ as:

\[ SO_4^{2-} > NO_3^- > Cl^- \]

**Table 2-2** A list of cathodic peaks of polypyrrole films

<table>
<thead>
<tr>
<th>Dopant</th>
<th>Potential(-mV)</th>
<th>Current(µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>666</td>
<td>26.2</td>
</tr>
<tr>
<td>KCl</td>
<td>712</td>
<td>37.5</td>
</tr>
<tr>
<td>HCl</td>
<td>720</td>
<td>48.0</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>746</td>
<td>41.0</td>
</tr>
<tr>
<td>HNO$_3$</td>
<td>752</td>
<td>83.8</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>596</td>
<td>98.6</td>
</tr>
</tbody>
</table>
Figure 2-14  Cyclic voltammetric behaviour of polypyrrole films in 0.1M phosphate buffer solution. Polypyrrole films were produced as shown in Figures 2-11 and 2-12. Scan rate: 100mV/s.
2. 4. Galvanostatic Polymerisation of Polypyrrole

2.4.1. Comparison of Dopants

It is interesting to note that the presence of phosphate in pyrrole monomer solution prevents formation of polypyrrole by galvanostatic polymerisation. The evidence of cyclic voltammogram was illustrated in Figure 2-15e. During film growth, anions (e.g., Cl\(^-\), NO\(_3\)\(^-\), HPO\(_4\)\(^2-\) and H\(_2\)PO\(_4\)\(^-\)) from the electrolyte are incorporated to maintain electrical neutrality. The electrical conductivity of polypyrrole films is quite sensitive to the counterion, as evident by comparison of behaviour in NaCl and NaNO\(_3\) (Figure 2-15b and Figure 2-15d), respectively.

It was observed in this study that cations (e.g., H\(^+\), K\(^+\) and Na\(^+\)) would affect electronic interaction between the polymer chain and the anions, resulting in differences in film morphology as a result of growth from different monomer solutions. This is also related to the voltammetric behaviour of the potential cycling polypyrrole films, addressed in section 2.3.3. Cations are indeed involved in the mechanisms of electropolymerisation. This provides remarkable three dimensional structures over the length of the polymer chain in the form of bands, analogous to those of a semiconductor.

The \(\pi\) electrons of the monomer units become delocalised after polymerisation due to the spatial extent of these electrons by a significant overlap integral of the units. The result is a full \(\pi\) band and an empty \(\pi^*\) band, a combination which offers no macroscopic conductivity unless modified in some way, e.g., by doping to create free electrons or holes.

Brédas and Pfluger et. al.\(^{[39,40]}\) calculated electronic structures for these materials, but the range of applicability of these calculations is limited by the assumption of crystallinity. Electrons or holes are the dominant charge carriers in most semiconductors, but in the case of polypyrrole extended composite excitations, such as polarons and bipolarons, have been suggested\(^{[41]}\). The presence of metal-like domains\(^{[42]}\), and their contribution to inhomogeneity in conductivity has also been suggested.
Figure 2-15  Voltammetric Behaviours of Galvanostatic Polymerised films in 0.1M phosphate buffer. 0.1M pyrrole, 0.1M dopants for each case and applied current density of 0.5mA/cm² for 180s on gold electrode. Scan range: -0.7V to +0.6V; Scan rate: 100mV/s.
2.4.2. Effect of H⁺ on the Polymerisation of Polypyrrole

Figure 2-16 shows the cyclic voltammograms obtained for PPy-Cl⁻ films formed by galvanostatic polymerisation in the presence of H⁺ and Na⁺ ions. The PPy-Cl⁻ cathodic peak details are given in Table 2-3. The stability and conductivity of the PPy-Cl⁻ film were improved in the presence of H⁺.

Figure 2-16 Voltammograms of PPy-Cl⁻ films in 0.1M phosphate buffer. The monomer solutions were (a) 0.1M NaCl/0.1M pyrrole and (b) 0.1M HCl/0.1M pyrrole. Two films were produced by galvanostatic polymerisation at 0.5mA/cm² for 180s. Scan rate: 100mV/s. Sweep Segments: 20.
Further investigation of the characteristics of films produced by galvanostatic polymerisation, such as anodic peaks and the plateau wave as shown in Figure 2-17, indicates the difference between NaCl and HCl incorporated films. The HCl incorporated film gave less anodic potential compared to an NaCl incorporated film. The experimental results are illustrated in Table 2-4. Therefore H⁺ and Na⁺ affect the polymerisation of PPy-Cl films by involving in the growth of a polypyrrole chain and electron transfer across the redox sites in the polymer.

2.5. Conclusions

The concepts of electroneutrality coupling and electron-hopping provide the fundamental aspects of electron and ion conduction. Various dopants (i.e., anions) were incorporated into polypyrrole for internal charge compensation, resulting in the different electrochemical behaviours due to their charges and structures. Cations, such as H⁺ and Na⁺, were evidently involved in the growth of polypyrrole, providing the different stability and conductivity of PPy-Cl films. This probably indicates one of the important factors, such a pH effect on a polypyrrole-based biosensor associated with a mediator for transferring electrons. Therefore the preliminary experiments and discussion of this chapter provide a basis for understanding the electrochemical behaviours.

Two electropolymerisation techniques have been used in a preliminary research on polypyrrole, namely potential cycling and galvanostatic modes. The cyclic voltammograms and chronopotentiograms can describe the process of the growth of polypyrrole. The cyclic voltammetry of a polypyrrole film in an electrolyte solution can further characterise the polypyrrole films electrochemically. These techniques, such as potentiometry and voltammetry, will be carried out throughout this research project in a conventional way.
Table 2-3  Effect of cation on polymerisation

<table>
<thead>
<tr>
<th>Dopants</th>
<th>Cathodic Peak</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential(-mV)</td>
<td>Current(μA)</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>532</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>508</td>
<td>29.3</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2-17  Schematic diagram of a chronopotentiogram obtained by galvanostatic polymerisation of a polypyrrole film

Table 2-4  Characteristics of chronopotentiometric films

<table>
<thead>
<tr>
<th>Dopants</th>
<th>Anodic Peak</th>
<th>Plateau wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential(mV)</td>
<td>Time(s)</td>
</tr>
<tr>
<td>NaCl</td>
<td>712</td>
<td>6</td>
</tr>
<tr>
<td>HCl</td>
<td>666</td>
<td>2</td>
</tr>
</tbody>
</table>
2. 6. References


CHAPTER 3

Ferrocyanide Mediated Formate Biosensor
3.1. Introduction

The role of formate in the early diagenesis of organic matter is of considerable importance in atmospheric and natural water chemistry\(^1,2\). The formation of formate occurs by photochemical oxidation of organic matter in the atmosphere or in natural waters\(^3\). It is also known to be an important substrate for bacteria in oxic and anoxic sediments and in pore waters\(^1,4\). Furthermore, dissolved organic matter in sea water often contains formate and its role in chemical and oceanographic processes has been the subject of numerous on-going research.

Formate is also involved in biochemical pathways\(^5\), where it is an important intermediate. It was found that the release of H\(^+\) ions in formaldehyde-induced acidification was coupled with the extrusion of formate anions into the extracellular medium\(^6\). The ability to utilise C\(_1\)-compounds like formate as a carbon source is also widespread among microbes\(^7\).

Despite its importance, the determination of trace concentrations of formate is relatively difficult due to either complicated, time-consuming sample preparation or inadequate sensitivity or selectivity of existing analytical methods. The methods commonly used for determination of formate include ion-exclusion chromatography\(^4\), liquid chromatography\(^8,9\), luminescence enzymatic assay\(^10\), and gas chromatography\(^11\). The enzymatic method is based on the oxidation of formate by formate dehydrogenase (FDH) with corresponding reduction of β-nicotinamide adenine dinucleotide (β-NAD) to reduced β-NAD form (β-NADH)\(^19\):

\[
\text{Formate} + \text{NAD} \xrightleftharpoons{\text{FDH}} \text{NADH} + \text{CO}_2
\]  

Subsequently, β-NADH can be determined by various detection techniques, such as fluorimetry\(^22,23\), spectrophotometry\(^24\), bioluminescence intensity\(^11\) and amperometry\(^25,26\) by the use of mediators such as phenazine methosulfate (PMS\(^+\)) and N-methylphenazinium tetracyanoquinodimethane (NMP\(^+\)TCNQ\(^-\)).

Whilst the enzymatic assay is more sensitive, more selective and more rapid than the other existing methods, the chemistry and biochemistry involved in the enzymatic reaction can possibly be more effectively and more robustly utilised if employed in electrochemical biosensing.
To date, no biosensor for formate has been reported in the literature. Yet, a biosensing device can be most beneficial for in-field monitoring or remote sensing in natural waters. The aims of the research in this chapter are:

- To develop a strategy for the electrochemical immobilisation of formate dehydrogenase into conductive polypyrrole films;

- To explore the use of the immobilised enzyme for the amperometric biosensing of formate;

- To study the effect of the co-immobilisation of the enzyme with a co-factor and an electron mediator on the performance of the polypyrrole-based formate biosensor.

The fabrication and optimisation of the biosensor performance involved careful consideration of important factors such as enzyme, co-factor, mediator and pyrrole concentration, magnitude of applied current density, temperature, pH, electrolyte and concentration.

3.2. Experimental

3.2.1. Reagents

All reagents were of analytical-reagent grade, unless specified otherwise. All solutions were prepared with Milli-Q water (Millipore). Formate dehydrogenase (EC 1.2.1.2., F-8649), β-nicotinamide adenine dinucleotide (N-7004) and pyrrole (P-4892) were obtained from Sigma Chemical Co. The pyrrole was distilled prior to use. Potassium ferrocyanide, NaCl, KCl, Na₂HPO₄, NaH₂PO₄·2H₂O and HCOONa were supplied by AJAX Chemical Pty Ltd. Nitrogen (O₂-free) was used where necessary.

3.2.2. Preparation of Polypyrrole-Enzyme Electrodes

Polypyrrole based enzyme electrodes were prepared by galvanostatic electropolymerisation in aqueous pyrrole monomer solution, after purging with N₂ for 10 minutes, onto a gold or platinum substrate with a surface area of 7mm². The electropolymerisation was performed on a computerised Potentiostat/Galvanostat used in conjunction with an IBM-PC. A three-electrode cell, consisting of a working electrode, a coiled platinum auxiliary electrode and a saturated KCl calomel reference electrode was used. The
working electrode was polished with 0.3μm alumina, and then ultrasonicated for 5 minutes to remove any residual. Galvanostatic polymerisation was accomplished in a monomer solution which contained pyrrole, FDH, NAD and K₄[Fe(CN)₆], with an applied current density for a given period.

3.2.3. Electrochemical Measurements

Prior to performing cyclic voltammetry the polymer based electrode was rinsed several times with Milli-Q water to remove any weakly bound enzyme molecules, after electropolymerisation. The electrode was then placed in an electrolyte solution, which contained either potassium chloride or phosphate buffer (pH7.0), or both. Cyclic voltammetry was performed on a BAS 100B Electrochemical Analyzer. All voltammograms were plotted on a FPG-315 colour plotter. A BAS LC-4C Amperometric Detector with PL-4 Recorder (J. J. Lloyd Instruments Ltd, UK) was used for amperometric measurements.

3.3. Results and Discussion

3.3.1. Incorporation of Mediator

In this section, the fabrication was accomplished by the incorporation of a mediator (ferrocyanide), co-factor (β-nicotinamide adenine dinucleotide) and enzyme (formate dehydrogenase) into a conducting polypyrrole membrane by galvanostatic electropolymerisation on gold and platinum electrodes. The presence of the electron mediator, cofactor and enzyme in the polypyrrole film was verified by chronopotentiometry, cyclic voltammetry (CV), amperometry and scanning electron microscopcy.

3.3.1.1. Immobilisation of Ferrocyanide

Amperometric biosensors undergo several steps to produce a measurable current which can be related to the analyte concentration. In order to lower the necessary applied potential, the use of redox mediators that can shuttle electrons from the active centre of the enzyme to the electrode surface was considered. The mediator of interest in this chapter is ferrocyanide.

Figure 3-1 shows the cyclic voltammogram of ferrocyanide in phosphate buffer on a bare Au electrode. It can be seen that well-defined cathodic and
anodic waves appeared at 0.134V and 0.240V, respectively. Evidently, these waves were due to the electrochemical redox reaction of ferrocyanide. The anodic peak was due to the oxidation of ferrocyanide while the cathodic peak was due to the reduction of ferricyanide. Owing to its redox behaviour, ferrocyanide is often found useful as an electron mediator.

![Graph](image)

**E(V) vs SCE**

**Figure 3-1** Electrochemical behaviour of ferrocyanide on gold substrate. Solution: 0.01M potassium ferrocyanide in 0.1M phosphate buffer. Scan rate: 25mV/s.

As indicated in Figure 3-2, similar redox peaks can be identified for the ferrocyanide incorporated into a polypyrrole matrix. Anodic and cathodic peak currents and potentials are summarised in Table 3-1. It can be seen that the redox potentials obtained for the immobilised [Fe(CN)$_6$]$^{3-/4-}$ are similar to those obtained for the compound on a bare gold electrode. The difference in the magnitude of current density on bare Au and PPy coated Au electrode is due to the difference in the ferrocyanide concentration in phosphate buffer solution and in the polypyrrole backbone. It indicates that the ferrocyanide concentration in the polypyrrole backbone is less than 0.01M. The additional cathodic wave at -0.468V illustrated in Table 3-1 corresponds to the reduction of PPy$^+$ (polarons) or PPy$^{2+}$ (bipolarons) by electroneutrality coupling as discussed in Chapter 2, due to the movement of anions in the polypyrrole backbone. As can be seen in Figure 3-2, a small anodic ripple which occurred
at -0.468V was not indicated in Table 3-1, as the current which is less than 1μA is limited by the sensitivity of a current gain.

**Table 3-1** Some Electrochemical Characteristics for Ferrocyanide on Bare Au and PPy Coated Au Electrodes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Bare Au</th>
<th>Polypyrrole coated Au</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cathodic</td>
<td>Anodic</td>
</tr>
<tr>
<td>Potential (mV)</td>
<td>134</td>
<td>240</td>
</tr>
<tr>
<td>Current (μA)</td>
<td>66.2</td>
<td>68.1</td>
</tr>
</tbody>
</table>

* An undetectable shape peak by a BAS 100B Electrochemical Analyzer.

**Figure 3-2** Electrochemical behaviour of ferrocyanide in polypyrrole matrix. Solution: 0.1M phosphate buffer. Scan rate: 25mV/s. Inset: comparing to a bare electrode in solution media. A ferrocyanide/polypyrrole coated Au electrode was fabricated by galvanostatic electropolymerisation at 1.0mA/cm² current density for 180 seconds, containing 10mM ferrocyanide in 0.1M pyrrole monomer solution.
Two pairs of waves were observed for the ferrocyanide/polypyrrole electrode in the first cycle. Similar observations were made for films cycled in KNO$_3$\textsuperscript{[12,13]} and KCl\textsuperscript{[14]}. The pair of waves which occurred at 0.13 and 0.23V is attributed to the [Fe(CN)$_6$]$^{3-/4-}$ redox couple. The pair of waves that appeared at -0.47 and -0.60V is assigned to the oxidation and reduction of the polymer backbone.

The [Fe(CN)$_6$]$^{3-/4-}$ redox waves are attenuated on the second cycle, as shown in Figure 3-3. It appears that the [Fe(CN)$_6$]$^{4-}$ ions are ejected from the PPy film in the first scan in order to maintain electroneutrality, reincorporation during the next cycle is inhibited by the much higher concentration of phosphate in the medium. Thus, the latter are assimilated into the film as charge-compensating dopant ions during the PPy oxidation. In this regard, the decreasing current magnitude of the [Fe(CN)$_6$]$^{3-/4-}$ feature in the voltammogram does not at all imply total ejection of the [Fe(CN)$_6$]$^{4-}$ species from the PPy/[Fe(CN)$_6$]$^{3-/4-}$ film. On the other hand, some regions of the polypyrrole film do not undergo reversible oxidation, and that once oxidised can not be re-reduced. It was also evident that the second and subsequent scans are consistently smaller than the first. Therefore, many reasons have been postulated for this phenomena\textsuperscript{[12-14]}, such as an irreversible phase transition within selected regions of the film and an ejection of [Fe(CN)$_6$]$^{4-}$ from the film.

![Graph showing cyclic voltammograms of PPy-[Fe(CN)$_6$]$^{3-/4-}$ film in 0.1M phosphate buffer solution. Galvanostatic electropolymerisation: same as Figure 3-2. Scan rate: 25mV/s. Sweep segments: 10.](image)

**Figure 3-3** Cyclic voltammograms of PPy-[Fe(CN)$_6$]$^{3-/4-}$ film in 0.1M phosphate buffer solution. Galvanostatic electropolymerisation: same as Figure 3-2. Scan rate: 25mV/s. Sweep segments: 10.
3.3.1.2. Effect of Galvanostatic Conditions on Polypyrrole Film

The thickness of polypyrrole film can be affected by the chosen electropolymerisation period and applied current density. This, in turn, will affect the redox behaviour of the polymer film. The cyclic voltammograms of PPy-[Fe(CN)$_6$]$^{3-/4-}$ films indicate that the redox behaviour of the electrode is influenced by the electropolymerisation period and current density, as shown in Figures 3-4 and 3-5. [Fe(CN)$_6$]$^{3-/4-}$ redox waves' current were enhanced by increasing polymerisation period at a fixed current density or increasing current density at a fixed polymerisation period. It appears that the thicker the film produced, the more [Fe(CN)$_6$]$^{3-/4-}$ is incorporated into the conductive polypyrrole film.

![Cyclic voltammograms](image)

**Figure 3-4** Effect of current density on characteristics of PPy-[Fe(CN)$_6$]$^{3-/4-}$ film. Monomer solution: 0.01M ferrocyanide in 0.1M pyrrole. Electrolyte: 0.1M phosphate buffer. Scan rate: 25mV/s.
Figure 3-5  Effect of the duration of electropolymerisation on characteristics of PPy-[Fe(CN)$_6$]$^{3-}/^{4-}$ film. Monomer solution: 0.01M ferrocyanide in 0.1M pyrrole. Scan rate: 25mV/s. Electrolyte: 0.1M phosphate buffer.
Evidently, the thicker the film, the higher the current produced. This is due to the increase in the total amount of \([\text{Fe(CN)}_6]^{3/-4}\) within the polypyrrole film resulting from the electron-hopping as discussed in Chapter 2. An interesting related observation is the much higher peak separation of \([\text{Fe(CN)}_6]^{3/-4}\) waves. As illustrated in Table 3-2, the minimum peak separation (\(\Delta mV\)) is 68 mV, which is close to 59 mV (potential difference between the redox pair due to one electron transfer, theoretically).

If the thickness of the film was estimated by assuming that 45 mC/cm\(^2\) of charge yields a film of 0.1 \(\mu\)m thickness\(^{[15]}\), it was observed that the application of a current density of 0.5 mA/cm\(^2\) for 360 second, as demonstrated in Table 3-2, gave the optimum film thickness of 0.4 \(\mu\)m and exhibits much more reversible waves for \([\text{Fe(CN)}_6]^{3/-4}\). It appears that the thicker film tends to prevent electron transfer due to the predominant formation and the resistance of polypyrrole. Therefore, it is evident that for a mediator to be effective there must be a balance between the amount of ferrocyanide incorporated and the thickness of the polypyrrole film.

**Table 3-2  Electrochemical parameters for PPy-Fe(CN)_6^{3/-4-} films obtained at different current densities**

| \(i_{app, \ (duration)}^{*}\) | \([\text{Fe(CN)}_6]^{3/-4-}\) | PPy/PPy\(^+\) |
|-----------------|----------------|----------------|----------------|
|                 | Anodic peak | Cathodic peak | Anodic peak | Cathodic peak |
|                 | Potential (mV) | Current (\(\mu\)A) | Potential (mV) | Current (\(\mu\)A) | Potential (mV) | Current (\(\mu\)A) |
| 0.5,(180)       | 208 5.2      | 140 3.1        | -468          | 0.57          |
| 0.5,(360)       | 252 7.3      | 116 16.9       | -468          | 11.1          |
| 0.5,(720)       | 220 9.6      | 128 5.4        | -394 35.6     | -540 16.7     |
| 2.0,(60)        | 284 38.0     | 76 21.6        | -386 50.0     | -532 23.8     |
| 2.0,(120)       | 92 20.7      | -508 13.9      |
| 1.5,(180)       | 254 24.6     | 108 16.0       |
| 1.0,(180)       | 238 16.3     | 126 12.7       |

* Applied current density (mA/cm\(^2\); duration (s).
It can also be observed from the data in Table 3-2 that PPy-[Fe(CN)$_6$]$^{3-/4-}$ films of similar thickness show different electrochemical characteristics. For example, variation of the applied current density and polymersation period, as indicated by use of 0.5mA/cm$^2$ for 720 second and 2.0mA/cm$^2$ for 180 second in Table 3-2, to give the same charge of 360mC/cm$^2$ and film thickness of 0.8µm gave films which demonstrate different electrochemical characteristics. In this regard the issue of film porosity or permeability becomes significant. The application of higher current density results in more porous polypyrrole films resulting in improved conductivity by the dopants.

3.3.2. Fabrication of Biosensing Membrane

Electrochemical polymerisation provides a simple means of incorporating a range of proteins into conducting polypyrrole film$^{[17]}$. The incorporation of enzyme into the film can be represented by the following reaction:

\[
\text{Py} \xrightarrow{E_{app}} [\text{Py}^+]_n \text{A}^- \quad \text{X}^{+} \text{A}^- \quad (3-2)
\]

where A$^-$ is a bulky immobile ion such as a mediator or a protein which is incorporated and n is normally between 2 and 4. Cations from solution can therefore move in and out of the polymer to provide charge compensation$^{[16]}$.

The stability and ease of polymerisation of polypyrrole film can be investigated by chronopotentiometry. The initial and highest potentials produced for polymerisation of a polypyrrole-based enzyme formate sensing film were 232 and 694mV, respectively, as shown in Figure 3-6.

It was observed that the potential was reduced, as the polymerisation proceeded within the first 10 seconds. The electrode potential then remained fairly constant as a function of time during the growth of the polymer film. This observation indicates that the film became more conductive as the polymerisation period is extended. Furthermore, the film grew thicker with the increasing polymerisation period.

Figure 3-7 shows a series of chronopotentiograms obtained during galvanostatic film formation in different monomer solutions. In all cases, the potential decreased to a stable value within 30 seconds and, thus, indicates that all films became more conductive with time. Based on the steady state potential
Figure 3-6  Chronopotentiogram for PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$. The pyrrole concentration was 0.5M mixed in 20 units FDH, 1mM β-NAD and 1mM ferrocyanide. A current density of 0.5mA/cm$^2$ for 8 min on a gold electrode was employed.

Figure 3-7  Chronopotentiograms for three compositions of polypyrrole film. The pyrrole concentration was 0.3M, 2.5mM potassium ferrocyanide, 5.0mM β-NAD and 10units FDH. A current density of 1.0mA/cm$^2$ for 5min on gold coated plastic film was employed.
it appears that PPy-[Fe(CN)$_6$]$^{4-}$ film is more conductive than the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ and PPy-NAD/[Fe(CN)$_6$]$^{4-}$ films. The incorporation of either NAD or both NAD and FDH did not seem to affect the conductivity of the film significantly. Normally, the enzyme (FDH and and co-factor β-NAD), as macromolecules, will not produce a conductive solution when dissolved in water without the addition of an electrolyte$^{[17]}$. Hence, the co-incorporation of ferrocyanide into the polymer film appears to serve a dual role in: (a) stabilising the polypyrrole network, and (b) acting as electron carriers.

3.3.3. Microscopic Evidence of Enzyme Incorporation into Polypyrrole

The examination of the galvanostatically polymerised films revealed that a vast morphological change occurred when the polypyrrole film was prepared in the presence or absence of FDH and NAD. In the absence of the enzyme and co-factor, the PPy-[Fe(CN)$_6$]$^{4-}$ film had, as shown in Figure 3-8a, three distinct layers under the scanning electron microscope: (i) a gold coated plastic film, (ii) a uniform thin polypyrrole-ferrocyanide film with nuclei present and (iii) propagated polypyrrole-ferrocyanide nuclei, which were crystallised nodules and sometimes clumped together.

Figure 3-8b illustrates the morphological change which occurred when β-NAD was added in pyrrole-ferrocyanide monomer solution. The most obvious difference was the size of the propagated polypyrrole nuclei, which were spherical nodules. The PPy-NAD/[Fe(CN)$_6$]$^{4-}$ film had a uniform thin layer of polypyrrole film, but no large crystallised and propagated polypyrrole-ferrocyanide nuclei were visible in the polypyrrole film, compared to the PPy-[Fe(CN)$_6$]$^{4-}$ film (Figure 3-8a). This observation clearly indicates that the morphology of the PPy-[Fe(CN)$_6$]$^{4-}$ film was altered due to the co-incorporation of NAD. However, the polymer had protrusions which were predominantly spherical, and similar to that of PPy-[Fe(CN)$_6$]$^{4-}$.

More interestingly, the polypyrrole film formed with the addition of FDH to the PPy-NAD/[Fe(CN)$_6$]$^{4-}$ monomer solution had a the 'sea-shell' nodules characteristic, as illustrated in Figure 3-8c. The distinct change, compared to Figure 3-8b, in the morphology of the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film appeared to be due to the presence of the enzyme.
Figure 3-8  SEM micrographs for polypyrrole films. Magnification 2600X.
(a) 2.5mM potassium ferrocyanide;
(b) 2.5mM potassium ferrocyanide/5.0mM β-NAD;
(c) 2.5mM potassium ferrocyanide/5.0mM β-NAD/10units FDH.
3.3.4. Cyclic Voltammetry on the Enzyme Film

The cyclic voltammograms obtained for the polypyrrole based enzyme electrodes in phosphate buffer solution are illustrated in Figures 3-9 and 3-10. It can be observed that two redox processes occurred on the surface of the film in this medium; one in the negative potential region and the other in the positive region. The two redox processes were observed both on platinum (Figure 3-9) and gold (Figure 3-10) electrodes. It was confirmed from the cyclic voltammogram obtained for the PPy-Cl film (Figure 3-9a) that the characteristic oxidation and reduction couple of polypyrrole appeared at the negative potential region. This therefore suggests that the second redox process observed in the positive potential region (Figure 3-9b) is associated with the oxidation/reduction couple for ferrocyanide.

Figure 3-9b indicates that the ferrocyanide redox peaks obtained with the PPy-[Fe(CN)$_6$]$^{4-}$ film were not well defined. The resolution of these peaks was even more affected with the co-incorporation of FDH in the PPy-FDH/[Fe(CN)$_6$]$^{4-}$ film (Figure 3-9d). However, the peak resolution was substantially improved with the co-incorporation of NAD (Figure 3-9c) or NAD and FDH (Figure 3-9e). These observations, and in particular comparison of Figure 3-9d and 3-9e, indicate that the improvement of redox behaviour of the mediator is due to reactions between the co-factor (β-NAD) and ferrocyanide. Similarly, comparison of Figure 3-9c and 3-9d indicates that the observed conductivity observation is consistent with those made from the chronopentiometric measurements.

The cyclic voltammograms obtained in phosphate buffer for the polypyrrole films on the gold substrate indicate that the oxidation and reduction couple of polypyrrole is a quasi-reversible process. Figure 3-10 shows that the anodic and cathodic current increased linearly as a function of scan rate. This observation is consistent with the expection for surface-confined redox systems$^{[18]}$. 
Figure 3-9  Cyclic voltammograms for polypyrrole films on a platinum electrode. Polymerisation conditions: 0.5mA/cm² for 5min; 0.1M pyrrole, 1mM K₄[Fe(CN)₆], 1mM β-NAD and 10 units FDH in 10mL water. Cyclic voltammograms were performed in 0.1M phosphate buffer, scan rate: 100mV/s.
Figure 3-10  Cyclic voltammograms of PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film on a gold electrode at different scan rates. (a) 10mV/s, (b) 30mV/s, (c) 50mV/s, (d) 75mV/s and (e) 100mV/s. Polymerisation conditions: same as for Figure 3-9, except 2mM K$_4$[Fe(CN)$_6$] was used. Cyclic voltammograms: 10mM formate + 0.1M phosphate buffer.

Figure 3-11a is also a cyclic voltammogram obtained for a PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film on the gold substrate in 0.1M phosphate buffer solution, within a narrower potential range. The second characteristic oxidation and reduction couple for the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film appeared at 436 mV and 38mV (vs SCE), respectively. It is interesting to note that the cyclic voltammetric behaviour of the film was altered (Figure 3-11b) when 20mM formate was added to the phosphate buffer solution, resulting in an enhanced oxidation peak at 294mV, and the cathodic peak shifted to 80mV. This is due to the catalytic action of FDH, which caused formate to be oxidised to CO$_2$ and to form β-NADH:

\[
\text{Formate } + \text{ NAD } \xrightarrow{\text{FDH}} \text{ NADH } + \text{ CO}_2
\]  

(3-1)

subsequently, this was oxidised to β-NAD$^+$ by K$_3$[Fe(CN)$_6$].
Figure 3-11  Influence of Formate on the Cyclic Voltammogram of PPy-FDH/NAD\([\text{Fe(CN)}_6]^{4-}\) Film on a Gold Electrode. 0.5M pyrrole, 1mM potassium ferrocyanide, 1mM β-NAD, 20units FDH and applied current density of 0.5mA/cm² for 8min on a gold electrode. Scan rate: 25mV/sec. (a) 0.1M phosphate buffer - no formate, (b) same as (a) + 20mM formate.

From the above results, it can be concluded that cyclic voltammetry is useful for obtaining indirect evidence of the incorporation of the enzyme and co-factor into a conducting polypyrrole film\[^{16,18}\]. When used in conjunction with other techniques, such as SEM, chronopotentiometry and amperometry, it provides a reliable confirmatory evidence of the presence of the enzyme in the film.

3.3.5. Amperometric Sensing of Formate

It is well-known that enzyme formate dehydrogenase (FDH), and co-factor, β-nicotinamide adenine dinucleotide (NAD), can catalyse the decomposition of formate by the following reaction\[^{19}\]:

\[
\text{Formate} + \text{NAD} \xrightarrow{\text{FDH}} \text{NADH} + \text{CO}_2
\]  (3-1)
Consequently, it is possible to determine the concentration of formate by measuring either NADH or carbon dioxide produced by this reaction. The reliable measurement of low levels of carbon dioxide in such systems is rather difficult. Also the measurement of NADH may be hindered by the resulting high overpotential. For example, on a carbon electrode, an overpotential of 1.1V at pH7.0[20] and 1.3V on platinum electrode have been reported[21]. In general, the high overpotential limits can possibly be the use of direct electrochemical detection approaches for measuring NADH formation in enzymatic reactions. As the required applied potential of such a device is quite high, it will be highly sensitive to many interfering electroactive species (ascorbic acid, uric acid, etc.) which are present in biological fluids. A viable solution to this problem is to use a modified FDH-NAD/mediator system, as shown in Figure 3-12, to enhance the electron transfer kinetics and, hence, improve the signal generation at the electrode.

![Figure 3-12 Electron Mediated Detection of Formate by Use of Ferrocyanide](image)

3.3.5.1. Electron Mediation with Ferrocyanide

The schematic diagram of the biological electron-transfer ‘chains’, shown in Figure 3-12, illustrates a number of processes which have come from a classical biochemical approach, viz. fractionation of a complex system into constituent parts, followed by analysis of those parts and finally reconstitution of the parts into a whole. This system involves the oxidation of formate, in the presence of FDH and NAD, to CO₂ and the formation of NADH which is then oxidised to NAD⁺ by K₃[Fe(CN)₆]. The chemically regenerated NAD⁺ continues the enzyme catalysed reaction. The resulting [Fe(CN)₆]⁴⁻ is oxidised at the electrode surface and the anodic current produced is dependent on
formate concentration. This approach is useful in overcoming the problem of poor selectivity and reproducibility caused by the high overpotential required for NADH.

The electrochemical response of the PPy-FDH/NAD electrode to formate was investigated in this study with ferrocyanide as an electron mediator. Figure 3-13 shows that the response of the PPy-FDH/NAD electrode to formate was substantially enhanced when ferrocyanide was present in the film. The reactions between the co-factor and the ferrocyanide are as follows:

$$\text{NADH} + \text{Fe(CN)}_6^{3-} \longrightarrow \text{NAD} + \text{Fe(CN)}_6^{4-} + \text{H}^+ \quad (3-3)$$

$$\text{Fe(CN)}_6^{4-} \xrightarrow{+0.35V} \text{Fe(CN)}_6^{3-} + e^- \quad (3-4)$$

**Figure 3-13** Schematic of the Electron Mediated Detection of Formate with Ferrocyanide. Applied potential was +0.4V. 5mM formate was measured in 0.1M KCl/0.01M phosphate buffer. The pyrrole concentration was 0.3M mixed in 10 units FDH, 5mM β-NAD and 2.5mM ferrocyanide. A current density of 1.0mA/cm² for 5 min on a gold electrode was employed.
3. 3. 5. 2. Influence of Applied Potential on Amperometric Response

The PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ electrode was immersed in a deoxygenated 0.1M phosphate buffer (pH 7.0). The response of the electrode to 5mM formate was investigated by applying various potentials to the electrode. A much higher amperometric response was obtained at higher electrode potentials, as shown in Figure 3-14. This result indicates that the oxidation of ferrocyanide generated by NADH in the conductive polypyrrole matrix is dependent on the applied potential. The steep increase in electrode output in Figure 3-14 is clearly observed at a potential higher than 0.2V where the incorporated ferrocyanide is oxidised in the polypyrrole membrane. At a potential below 0V, where reduced ferrocyanide is not oxidised, electron transfer from NADH to oxidise ferricyanide does not occur because all the [Fe(CN)$_6$]$^{3-/4-}$ in the membrane phase are in the reduced form. However, at a potential more positive than 0V, the electron flow from NADH to oxidise ferricyanide proceeds smoothly, since the reduced ferrocyanide is oxidised efficiently at the base of the electrode via the assistance of the conductive polymer. If the applied potential was beyond approximately 0.5V, the amperometric response started to decline as a function of time. This was caused by the instability of the conductive polypyrrole backbone which was overoxidised at the high potentials, and then inhibited the electron transfer in the polypyrrole backbone.

Therefore, the efficiency of the electron shuttling by a mediator improved as the applied potential became more positive. In the potential range from 0.2 to 0.4V (vs SCE), NADH is not oxidised on polypyrrole or Au electrode. The result in Figure 3-14 indicates that the enzyme activity can be controlled by changing the regeneration and recycling rates of co-factor and mediator in the conductive membrane.
Figure 3-14 Influence of applied potential on amperometric response of polypyrrole-based formate biosensor. 5mM formate was measured in 0.1M phosphate buffer(pH7.0). The pyrrole concentration was 0.5M mixed in 20 units FDH, 1mM β-NAD and 1mM ferrocyanide. A current density of 0.5mA/cm² for 8 min on a gold electrode was employed.

3. 3. 5. 3. Amperometric Detection

As previously shown in Figure 3-14, [Fe(CN)₆]⁴⁻ is oxidised efficiently at potentials greater than 0.30V vs SCE. Steady-state current measurements for formate were therefore recorded in the absence of oxygen at 0.35V vs SCE. Figure 3-15 shows a typical amperogram and a calibration curve obtained for increasing concentration of formate with the PPy-FDH/NAD/[Fe(CN)₆]⁴⁻ electrode. Evidently, the response increased with increasing formate concentration and this indicates that the catalytic action of the enzymes is quantitative. Under the present conditions, a linear concentration range of 0-2.5mM and a detection limit of 2.5mM were accomplished.
Figure 3-15  Amperometric response of polypyrrole-based formate biosensor. Applied potential was +0.35V. Other conditions same as Figure 3-14.
3. 4. Monolayer Biosensing Membrane

3. 4. 1. Co-Electroimmobilisation of FDH-NAD-[Fe(CN)$_6$]$^{4-}$ into a Polypyrrole Film

The amperometric response is based on a sequence of reactions as mentioned in section 3.3.5. Initially formate is oxidised to CO$_2$ to form β-NADH, which is then oxidised to β-NAD$^+$ by K$_3$[Fe(CN)$_6$]. Figure 3-16 illustrates the stereochemistry of the hydrogen transfer in the oxidation of formate by formate dehydrogenase. The immobilisation of multicomponents into a single polypyrrole film is intended to enhance and maintain the kinetic activity of each component involved.

![Chemical structure](image)

Figure 3-16 The stereochemistry of the hydrogen transfer
It should be remembered that ferrocyanide, β-NAD and FDH are entrapped into the polypyrrole film and that the mediator is predominantly in the reduced form [Fe(CN)$_6$]$^{4-}$. When formate is added to the stirred solution the enzymatic reaction shown in Eqn. 3-1 occurs to produce the reduced form of the co-factor (NADH). Following this, a chemical reaction proceeds to produce the co-factor- mediator complex, which dissociates to release the reduced form of [Fe(CN)$_6$]$^{4-}$. The electrochemical oxidation of [Fe(CN)$_6$]$^{4-}$ (Eqn. 3-4) yields an analytical signal which is proportional to the rate of formate oxidation, which is proportional to the formate concentration when the concentrations of the other reactants are held constant and the enzyme is unsaturated. A steady state current is achieved if the enzyme, co-factor and mediator are efficiently retained in the polymer membrane, allowing continuous recycling of NAD (Eqn. 3-3) and [Fe(CN)$_6$]$^{3-}$ (Eqn. 3-4). This also ensures that Eqn. 3-1 is driven towards the right hand side.

3.4.2. Effect of Pyrrole Concentration

Enzymes can be entrapped in polypyrrole as summarised in section 1.4.1. A minor loss of biological activity compared to chemical coupling methods is one of the main positive features of enzyme immobilisation by entrapment. Electrochemical polymerisation provides a simple means of incorporating a range of enzymes into conducting polypyrrole film$^{[17,27]}$

The effect of pyrrole concentration on the voltammetric behaviour of PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film is illustrated in Figure 3-17. It was observed that the reduction and oxidation currents of [Fe(CN)$_6$]$^{3-}\text{-}^{4-}$ peaks increased with increasing pyrrole concentration up to 0.3M, as demonstrated by the data in Table 3-3. This observation indicates that the film became more conductive and electroactive, as more [Fe(CN)$_6$]$^{4-}$ is entrapped into the polypyrrole film. Furthermore, amperometric measurement of formate confirmed that 0.3M pyrrole is the minimum monomer concentration for biosensing with polypyrrole enzyme-based film. The lowest pyrrole concentration for obtaining the amperometric response is 0.3M, as shown in Figure 3-18. As discussed in section 2.3.1., the concentration of pyrrole plays an important role as demonstrated previously by the concept of electroneutrality coupling. The more PPy$^+$ sites produced for internal charge compensation, the more efficiently anions X$^-$ can compensate the positive charge of the resulting PPy$^+$. In this case, the more co-factor (β-NAD, which is negatively charged as shown in Figure 3-16) is incorporated into the polypyrrole film.
Figure 3-17  Cyclic voltammograms of PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ films on a gold electrode at different pyrrole concentrations. (a) 0.1M, (b) 0.2M, (c) 0.3M, (d) 0.4M, (e) 0.5M and (f) 0.6M. Monomer solution contained 1mM [Fe(CN)$_6$]$^{4-}$, 1mM NAD and 10units FDH in 5mL water. Current density employed was 1mA/cm$^2$ for 300s. Cyclic voltammograms were performed in 0.1M KCl/ 0.01M phosphate buffer.
Figure 3-18 shows that the amperometric responses obtained increase with increasing pyrrole concentration. This suggests that the amount of formate dehydrogenase entrapped increases with increasing pyrrole concentration. This may be due to the more rapid polymerisation process at so higher pyrrole concentration that provide the more constructive three-dimensional structure which selectively accelerates a chemical reaction as shown in Eqn. 3-2.

<table>
<thead>
<tr>
<th>C_Py (M)</th>
<th>E_{Ferro} (mV)</th>
<th>I_{Ferro} (µA)</th>
<th>E_{Py} (mV)</th>
<th>I_{Py} (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_Ox</td>
<td>E_Re</td>
<td>I_Ox</td>
<td>I_Re</td>
</tr>
<tr>
<td>0.1</td>
<td>286</td>
<td>82</td>
<td>118</td>
<td>46</td>
</tr>
<tr>
<td>0.2</td>
<td>276</td>
<td>94</td>
<td>123</td>
<td>54</td>
</tr>
<tr>
<td>0.3</td>
<td>298</td>
<td>72</td>
<td>166</td>
<td>125</td>
</tr>
<tr>
<td>0.4</td>
<td>290</td>
<td>76</td>
<td>152</td>
<td>126</td>
</tr>
<tr>
<td>0.5</td>
<td>280</td>
<td>82</td>
<td>146</td>
<td>116</td>
</tr>
<tr>
<td>0.6</td>
<td>306</td>
<td>80</td>
<td>162</td>
<td>141</td>
</tr>
</tbody>
</table>

Polymerr film voltammograms were investigated in 0.1M KCI / 0.01M phosphate buffer. The monomer solution contains 1.0mM potassium ferrocyanide, 1.0mM β-NAD, 10units FDH and pyrrole in 5mL. A current density of 1.0mA/cm² for 5min on gold substrate was employed.
Figure 3-18  A plot of the amperometric response of 5mM formate in 0.1M KCl / 0.01M phosphate buffer when pyrrole concentration is varied in the monomer solution. Applied potential: 0.4V. Other conditions same as Table 3-3.
3.4.3 Effect of Ferrocyanide Concentration

Ferrocyanide can serve a dual role in the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film: (a) stabilise the polypyrrole network and (b) act as an electron carrier. Thus, it resulted in a rapid and easy co-immobilisation of formate dehydrogenase and $\beta$-NAD. This, in turn, resulted in improvement of selectivity and reproducibility of the amperometric response. The immobilised [Fe(CN)$_6$]$^{4-}$ is oxidised at the electrode surface and the anodic current produced is proportional to the formate concentration.

Figure 3-19 illustrates the effect of ferrocyanide concentration on the amperometric response of the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ electrode for formate. The increasing mediator concentration resulted in an increase in the amperometric response. It appears that the higher mediator concentration enhances the electron transfer kinetics and, hence, improves the signal generation at the electrode. The optimum concentration was found to be 2.5mM. No further increases in analytical signal for formate was obtained in the presence of [Fe(CN)$_6$]$^{4-}$ beyond the optimum value.

**Figure 3-19** A plot of the amperometric response of 5mM formate in 0.1M KCl / 0.01M phosphate buffer when ferrocyanide concentration was varied in the monomer solution. The monomer solution contained 0.3M pyrrole, 5.0mM $\beta$-NAD, 10units FDH and potassium ferrocyanide in 5mL. A current density of 1.0mA/cm$^2$ for 5min on gold substrate was employed. Applied potential: 0.4V.
3.4.4. Effect of β-NAD Concentration

Figure 3-20 shows the relationship between the amperometric response and β-NAD concentration. The optimum concentration range was found to be between 2.5mM and 5.0mM. Beyond 5.0mM, the signal dropped. This is thought to be due to β-NAD molecule occupying many sites within the polypyrrole film during the co-immobilisation and thus reducing the amount of FDH incorporated in the polypyrrole film. It was evident that the amperometric response was saturated after 2.5mM β-NAD and decreased at higher concentrations.

![Graph showing the relationship between NAD concentration and current](image)

**Figure 3-20** A plot of the amperometric response of 5mM formate in 0.1M KCl/0.01M phosphate buffer when β-NAD concentration was varied in the monomer solution. The monomer solution contained 0.3M pyrrole, 1.0mM potassium ferrocyanide, 10units FDH and β-NAD in 5mL. A current density of 1.0mA/cm² for 5min on gold substrate was employed. Applied potential: 0.4V.
3.4.5. Effect of Enzyme Loading

The catalytic function of formate dehydrogenase is limited by its solubility in the aqueous solution. Formate dehydrogenase (EC 1.2.1.2., F-8649) is a clear colourless solution when 6.4mg of its solid is dissolved in 1mL of water. It means that the maximum units in an aqueous solution are 6.4 units/mL for this particular batch of F-8649. Figure 3-21 shows the amperometric response for formate in the presence of varying enzyme concentration.

The signal declined between 2 and 5 units/mL. The formation of a precipitate was observed when $[\text{Fe(CN)}_6]^{4-}$ was added into the enzyme solution when more than 2 units/mL is present. This may be due to the alteration of the salt concentration in the enzyme solution. This, in turn, resulted in a decrease in the enzyme units in the monomer solution. The enzyme units in the monomer solution could be, however, saturated if up to 10 units/mL is used.

![Graph](image)

**Figure 3-21** A plot of the amperometric response of 2.5mM formate in 0.1M KCl/0.01M phosphate buffer when FDH units was varied in the monomer solution. The monomer solution contained 0.3M pyrrole, 2.5mM potassium ferrocyanide and 5.0mM β-NAD in 5mL. A current density of 1.5mA/cm² for 400s on gold substrate was employed. Applied potential: 0.4V.
3.4.6. Effect of O₂

The effect of O₂ on the polymerisation of polypyrrole film can be investigated by chronopotentiometry. The highest potentials produced for the polymerisation of a PPy-FDH/NAD/[Fe(CN)₆]⁴⁻ film with and without O₂ were 750 and 694mV (vs SCE), respectively, as shown in Figure 3-22. It can be seen that the O₂ might result in different formation of polymer films (Figure 3-22a versus 3-22b), due to O₂ involvement in the electropolymerisation of polypyrrole. These results suggest that a more conductive film is formed in the absence of oxygen, as much lower steady state potential is obtained in Figure 3-22b. Furthermore, Figure 3-23 illustrates the effect of O₂ on the amperometric response obtained for 5mM formate. Polypyrrole film formed in the absence of O₂ gave a more sensitive amperometric response than with those formed in the presence of O₂ (Figure 3-23b versus 3-23a). Further comparison of Figures 3-23b and 3-23c shows that the catalytic effect of FDH was more effective in the presence of O₂. This could be due to O₂ involvement in the hydrogen transfer reaction as shown in Figure 3-16. This observation will be particularly beneficial for biosensing of formate in environmental materials, without the need for N₂ purging.

\[\text{Figure 3-22} \quad \text{Effect of O₂ on chronopotentiograms of polymer films. The monomer solution contained 0.3M pyrrole, 1mM potassium ferrocyanide, 1.0mM β-NAD and 10units FDH in 5mL. A current density of 1.0mA/cm² for 5min on gold substrate was employed.}
\]
\[\text{a: With O₂ in PPy film growing, b: Without O₂ in PPy film growing}\]
Figure 3-23  Effect of O$_2$ on the amperometric response of 5mM formate in 0.1M KCl/0.01M phosphate buffer. Applied potential: 0.4V. Other conditions same as Figure 3-22.

a: Both with O$_2$ in PPy film making and amperometric detection,
b: Without O$_2$ in PPy film making and with O$_2$ in amperometric detection,
c: Both without O$_2$ in PPy film making and amperometric detection.
3.4.7. Effect of Current Density and Polymerisation Period

As indicated in section 3.3.1.2., the applied current density during the galvanostatic polymerisation affected the amount of ferrocyanide incorporated into the polypyrrole film due to charge compensation and physical entrapment. This principle can be applied to the physical entrapment of an enzyme, such as formate dehydrogenase. The applied current density during film formation is expected to influence the amount of formate dehydrogenase in the membrane, due to physical entrapment. According to the sequence of formate dehydrogenase\textsuperscript{[28]}, it contains 361 amino acids as represented below in three-letter code:

Lys Val Val Leu Val Leu Tyr Asp Ala Gly Lys His Ala Gln Asp Glu Glu Arg Leu Tyr Gly Cys Thr Glu Asn Ala Leu Gly Ile Arg Asp Trp Leu Glu Lys Gin Gly His Asp Val Val Val Thr Ser Asp Lys Glu Gly Gin Asn Ser Val Leu Glu Lys Asn Ile Ser Asp Ala Asp Val Ile Ile Ser Thr Pro Phe His Pro Ala Tyr Ile Thr Lys Glu Arg Ile Asp Lys Ala Lys Leu Leu Leu Val Val Ala Gly Val Gly Ser Asp His Ile Asp Leu Asp Tyr Ile Asn Gin Ser Gin Ser Gin Arg Asp Ile Ser Val Leu Glu Val Thr Gly Ser Asn Val Val Ser Val Ala Glu His Val Val Met Thr Met Leu Val Leu Val Arg Asn Phe Val Pro Ala His Glu Gin Ile Ile Ser Gly Gin Trp Asn Val Ala Glu Ile Ala Lys Asp Ser Phe Asp Ile Glu Gly Lys Val Ile Ala Thr Ile Gly Ala Gly Arg Ile Gly Tyr Arg Val Leu Glu Arg Leu Val Ala Phe Asn Pro Lys Glu Leu Tyr Tyr Asp Tyr Gin Ser Leu Ser Lys Glu Ala Glu Glu Lys Val Gly Ala Arg Arg Val His Asp Ile Lys Glu Leu Val Ala Gln Ala Asp Ile Val Thr Ile Asn Cys Pro Leu His Ala Gly Ser Lys Gly Leu Val Asn Ala Glu Leu Leu Lys His Phe Lys Lys Gly Ala Trp Leu Val Asn Thr Ala Arg Gly Ala Ile Cys Val Ala Glu Asp Val Ala Ala Ala Val Lys Ser Gin Gin Leu Arg Gly Tyr Gly Gin Asp Val Trp Phe Gin Pro Ala Pro Lys Asp His Pro Trp Arg Ser Met Ala Asn Lys Tyr Gly Ala Gly Asn Ala Met Thr Pro His Tyr Ser Gin Ser Val Ile Asp Ala Gln Val Arg Tyr Ala Gln Gly Thr Lys Asn Ile Leu Glu Ser Phe Phe Thr Gin Lys Phe Asp Tyr Arg Pro Gin Asp Ile Ile Leu Leu Asn Gly Lys Tyr Lys Thr Lys Ser Tyr Gly Ala Asp Lys

<table>
<thead>
<tr>
<th>Total number of negatively charged residues (Asp + Glu):</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of positively charged residues (Arg + His + Lys):</td>
<td>55</td>
</tr>
<tr>
<td>Net number of positively charged residues</td>
<td>10</td>
</tr>
</tbody>
</table>

According to the net number of positively charged residues, formate dehydrogenase may not be incorporated into polypyrrole backbone as $\mathbf{A^-}$ described in Eqn. 3-1 by the concept of electroneutrality coupling due to its overall positive charged. As shown in Figure 3-24, the amperometric response for formate on the biosensor increased significantly with increasing
applied current density from 0.25mA/cm² to 1.5mA/cm². This indicates that the applied current density during film formation plays a significant role in physical entrapment of formate dehydrogenase under this circumstance.

The thickness of PPy-FDH/NAD/[Fe(CN)_6]^{4-} film can be affected by both current density and polymerisation period. Thicker films may contain higher amount of the enzyme, co-factor and mediator, and may therefore exhibit higher sensitivity, as long as the response is not controlled by mass transfer, but by the enzymatic reaction. On the contrary, the background noise (mainly charging current and anion transportation) would be higher for thicker membranes because of the larger effective electrode area and the higher amount of redox species in the membrane, including PPy itself. Thus, an optimum thickness of the PPy-FDH/NAD/[Fe(CN)_6]^{4-} membrane was sought by controlling the current density and polymerisation period, as indicated in Figures 3-24 and 3-25, respectively. As indicated in these Figures, the optimum current density and polymerisation period are 1.5 mA/cm² and 400 seconds, respectively.

![Graph showing current density vs. amperometric response](image)

**Figure 3-24** A plot of the amperometric response of 20mM formate in 0.1M KCl/0.01M phosphate buffer when the current density of galvanostatic electropolymerization was varied. The monomer solution contained 0.3M pyrrole, 2.5mM potassium ferrocyanide, 5.0mM NAD and 10 units FDH in 5mL. Electropolymerization period for 5min on gold substrate was employed. Applied potential: 0.4V.
Figure 3-25 A plot of the amperometric response when the period of galvanostatic electropolymerization was varied. A current density of 1.0mA/cm² for electropolymerization on gold substrate was employed. Other conditions as Figure 3-24.

The effect of current density and polymerisation period on the thickness and porosity of a biosensor film is useful in controlling the permeability of an analyte or another interferent in a biosensing process. The permeability of conducting polymer modified electrodes to low molecular weight species will depend on morphology and thickness of the polymer layer. The permeability of the polymer is especially important for a biosensor employing formate dehydrogenase as the sensing element. Some biological interferents will be eliminated by a conducting polymer of suitable permeability. A similar approach has been used for a glucose biosensor based on entrapment of enzymes in a water-dispersed anionic polymer by Fortier et al[37].
3.4.8. Effect of Temperature

As electronic conduction is determined by the mobility (or diffusion) of dipoles in the material, it follows that the variation of resistivity with temperature can be represented by the Arrhenius equation as follows[29]:

\[ \rho = \rho_0 \exp \left( \frac{-\Delta H}{RT} \right) \]  \hspace{1cm} (3-5)

\( \rho \): resistivity of various polymers;
\( \rho_0 \): coefficient of resistivity of various polymers;
\( \Delta H \): activation energy;
\( R \): ideal gas constant;
\( T \): temperature.

Hence log \( \rho \) is inversely proportional to \( T \). Thus the mobility of dipoles will decrease with increasing temperature. Furthermore, conductivity will decrease very rapidly at high temperature.

In organic polymeric materials, conduction may occur through the movement of either electrons or ions, as described by the following equation[30]:

\[ \sigma = \mu q n \]  \hspace{1cm} (3-6)

In each case, the conductivity \( \sigma \) is equal to the product of the carrier mobility \( \mu \), its charge \( q \) and the number of carriers or the concentration \( n \).

As shown in Figure 3-26, two factors are involved in the mediated amperometric response of formate. Firstly, the electron transfer in the polypyrrole backbone is inversely proportional to temperature. Secondly, the optimum temperature for an enzymatic-catalytic reaction of formate dehydrogenase is approximately 35°C (stated by Sigma Chemical Co.). The experimental results indicated that the room temperature (23°C) was optimum for the measurement of formate under this condition. High temperature could decrease the extent rate of the electron transfer, inhibiting further conversion of intermediates to products. This fascinating observation has not been previously reported in the literature. The choice of room temperature is a compromise between the faster electron transfer and the more efficient enzymatic-catalytic reaction for the mediated amperometric response of formate.

As discussed in Chapter 2, the electron-hopping concept is the most appropriate general model for describing electron transfer across the redox sites in the disordered system. The electronic donor and acceptor states have
identical energy levels with respect to thermal fluctuations. The elementary step in electron transport is the transition from an occupied state (donor, reduction form) to an unoccupied state (acceptor, oxidation form).

The result obtained at 40°C, shown in Figure 3-26, shows that the background current suddenly declined after spiking 1mM formate into the solution. This appeared to be the consequence of a fast enzymatic-catalytic reaction and a slow electron transfer process. The intermediates, such as NADH, remained in the membrane for a while and did not get into the next reaction step immediately. It may result in a change in the conductivity of the film and interfere with the background current. These features will be further investigated in the next chapter.

![Graph showing electrochemical reaction at different temperatures](image)

**Figure 3-26** Effect of temperature on chronoamperograms of 1mM formate in 0.1M KCl/0.01M phosphate buffer. Applied Potential: 0.4V. Monomer solution contains 0.3M pyrrole, 2.5mM potassium ferrocyanide, 5mM NAD and 10 units FDH in 5mL. A current density of 1.5mA/cm² for 400s on gold substrate was employed.
3. 4. 9. Effect of pH and Electrolytes in Amperometric Measurements

It is expected that the amperometric response depends significantly on the buffering capacity of the sample. This dependence is due to the so called 'facilitated diffusion' generated from the biochemical reaction protons out of the membrane in the associated form with buffer species$^{[31]}$. Also, it is influenced by H$^+$ which affected the stability and structure of a polypyrrole film as discussed in section 2.4.2.

There are two reasons for pH in neutral or even high range. Proton deficiency can prevent the reaction from proceeding, if buffering is insufficient. The neutral pH is good for enzyme catalytic reaction and retention of the enzyme activity. The higher pH is useful in removing H$^+$ produced in Eqn. 3-3, where NADH is oxidised to NAD by K$_3$[Fe(CN)$_6$]. The H$^+$ produced will, therefore, rapidly approaches equilibrium at neutral pH values. Furthermore, assay at higher pH values may be useful in extending the linear range.

As shown in Figure 3-27, the amperometric response for formate declined dramatically at pH 6.0 and 6.4. It appears that the chemical regeneration of NAD$^+$ was inhibited and thus the enzyme catalytic reaction was not completed. In contrast, at higher pH regeneration of NAD$^+$ occurred readily and the enzyme catalytic reaction was completed.

As shown in Equation 3-6, the number of carriers or concentration n is proportional to the conductivity of polypyrrole-based membrane. The concentration of electrolytes, which are moving in and out of the polymer to provide charge compensation as described in Equation 3-1, can therefore impact on the amperometric current. This assumption is demonstrated in Figures 3-28 and 3-29. Figure 3-28 shows the different chronoamperograms obtained in various electrolytes. It can be observed that the background current, mainly contributed from polymer matrix, increased from 1085nA to 1982nA with increase in phosphate buffer concentration and decrease in KCl concentration. The concentration of phosphate buffer (pH 7.0) plays a significant role in removing the H$^+$ produced during the reactions, as shown in Figure 3-29. The amperometric current, for example, could reach as high as 162.5nA in 0.01M phosphate buffer/0.1M KCl compared to 80nA steady-state current in the same conditions. This suggests that basic physico-chemical processes inside the biomembrane determines the nature of the response of the biosensor.
Figure 3-27  Chronoamperometric response of 5mM formate in 0.1M phosphate buffer solution. A current density of 1.0mA/cm² for 5min on gold substrate was employed. Other conditions same as Figure 3-26.

Figure 3-28  Effect of various electrolytes on amperometric response of 5mM formate. Conditions same as Figure 3-27.
Amperometric steady-state current is shown in Figure 3-29, the maximum current is 80nA in 0.01M phosphate buffer/0.1M KCl. Phosphate buffer generates H⁺ out of the membrane in the associated form with phosphate species in the reaction system, KCl is present as a supporting electrolyte to decrease the contribution of migration to mass transfer of electroactive species and products. Moreover, both phosphate buffer and KCl serve the important function of decreasing the solution resistance. This function may also be useful in decreasing or eliminating matrix effects in analytical applications. Further investigation of KCl would be carried out in the next chapter.

**Figure 3-29** Effect of various electrolytes on steady-state amperometric current. All conditions are same as Figure 3-27.
3. 5. Bilayer Biosensing Membrane

So far, the use of ferrocyanide as a mediator in electron transfer between FDH and the Au electrode has proven to be successful. However, the limited long-term stability of the amperometric response appeared to be due to the leaking of the mediator from the sensor\[32\]. The use of bilayer membrane approach\[33-35\] appeared to be a useful way of overcoming this problem. In this case the enzyme layer will interact initially with the analyte and necessary oxygen to achieve the enzyme-formate reaction and to produce NADH. In this study, the ferrocyanide is used in the immobilised form in the inner membrane in order to prevent its leakage. The inner membrane with a permselective property may also be useful in preventing the interference by other electroactive species on the electrode\[36\].

3. 5. 1. Galvanostatic Film Formation

Fabrication of a bilayer of PPy-[Fe(CN)6]4- and PPy-FDH/NAD films was carried out in two monomer solutions as outlined in Table 3-4. A current density of 1.5mA/cm² was employed for the electropolymerisation for a period of 200s. Galvanostatic polymerisation of PPy-[Fe(CN)6]4- film was followed by the growth of a PPy-FDH/NAD film on top. The chronopotentiograms, shown in Figure 3-30, were recorded by in situ chronopotentiometry.

<table>
<thead>
<tr>
<th>Table 3-4 The composition of monomer solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>0.3M Pyrrole</td>
</tr>
<tr>
<td>2.5M Potassium Ferrocyanide</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5mL H₂O</td>
</tr>
</tbody>
</table>

Electrochemical deposition of such dynamic polymers provides a convenient method of enzyme immobilisation for biosensors and properties of the polymer layer, such as thickness or morphology, can be controlled according to the polymerisation conditions selected.
Figure 3.30  Choropotentiotograms of ferrocyanide mediated polypyrrole-based membranes formed in galvanostatic mode. A current density of 1.5mA/cm² for 200 second was employed. The composition of monomer solutions are illustrated as Table 3-4. The number indicated in bracket is represented as time and potential as (s, V).

(a) Ferrocyanide monolayer layer,
(b) Ferrocyanide and NAD/FDH bilayers.
3.5.2. Cyclic Voltammetric Behaviour

Figure 3-31 shows typical cyclic voltammetric behaviour of a bilayer of PPy-[Fe(CN)$_6$]$_{4^-}$ and PPy-FDH/NAD in phosphate buffer. The voltammogram displays the typical anodic peaks corresponding to the oxidation of the ferrocyanide immobilised in the inner membrane and polypyrrole, in addition to the cathodic peaks corresponding to the reduction of ferricyanide and polypyrrole. Two pairs of redox peaks are similar to the voltammetric behaviour of monomer film as discussed before, i.e., one for ferrocyanide at a positive potential, the other for polypyrrole in negative potential. An additional anodic peak occurred at approximately 0.13V. This may be associated with the immobilisation of more enzyme and co-factor into the outer layer of the bilayer membrane. This could result in a more sensitive amperometric response for formate than those obtained on a monolayer film of PPy-FDH/NAD/[Fe(CN)$_6$]$_{4^-}$.

![Cyclic voltammogram](image)

**Figure 3-31** Cyclic voltammogram of a bilayer membrane in 0.1M KCl/0.01M phosphate buffer. Scan rate: 100mV/s. Other conditions same as Figure 3-30.

3.5.3. Chronoamperometric Behaviour

Figure 3-32 shows a current-time curve obtained with a bilayer of PPy-[Fe(CN)$_6$]$_{4^-}$ and PPy-FDH/NAD on a gold electrode. The applied potential was
set at 0.4V vs SCE. The current on the electrode increased immediately after the addition of formate and reached a stead state within a few seconds.

As shown in Figure 3-32, the formate response on the bilayer membrane electrode was much larger than that obtained on the monolayer membrane electrode (Figure 3-15). The much higher activity on the electrode surface with a bilayer membrane is responsible for the larger formate response. It was 50 times higher compared to the monolayer membrane. This is due to the ability to prevent ferrocyanide from leaching out of the membrane, and the good permeability of formate in the outer enzymatic biomembrane as discussed in section 3.4.7. Figure 3-32 also shows that the response increased with increasing formate concentration.

**Figure 3-32** Amperometric response of a bilayer polypyrrole-based formate biosensor. Applied potential: 0.4V. Other conditions same as Figure 3-30 and 3-31.
3. 6. Conclusions

A polypyrrole-based formate biosensor which involved the use of [Fe(CN)$_6$]$^{4-}$ as a mediator has been successfully exploited, on the basis of the concepts of electroneutrality coupling and electron-hopping. A number of parameters were optimised for this biosensing system to understand the electrochemical behaviour and mechanisms involved. The fabrication of the biosensing membranes was extended to a bilayer film arrangement, which enabled detection of lower concentrations of formate, but the linear range was narrower compared to that obtained with the monolayer film.

Further research will be undertaken to improve the performance of the formate biosensor, particularly with regards to sensitivity and selectivity.

3. 7. References


CHAPTER 4

Ferrocyanide Mediated Flow Amperometric Formate Biosensor
4. 1. Principle of Flow Injection Analysis

FIA is a relatively new analytical technique which has shown great promise for rapid and precise analysis of discrete samples\(^{[1-3]}\). It is based on the injection of a liquid sample into a moving unsegmented continuous stream of a suitable liquid. The injected sample forms a narrow zone, mixed with reagents if necessary, which is then transported toward a detector which continuously records a physical parameter that changes as a result of the passage of the sample material through the flow cell\(^{[1-3]}\). Various processes such as chemical reaction, separation and detection can be made to occur at different parts of the manifold at different times in most of the FIA applications. Selectivity enhancement and increased sensitivity can be achieved through optimisation of parameters such as flow rate, reagent concentrations and other relevant conditions.

4. 1. 1 Dynamic Behaviour in FIA

Between the points of injection and detection, the sample will disperse physically to some degree in the flowing stream or, in those systems that involve the addition of a reagent, a chemical reaction may also occur. The resulting peak shape will be influenced by both processes. The physical dispersion consists of two processes, i.e., convection and diffusion\(^{[2]}\), as illustrated in Figure 4-1.

![Diagram of sample dispersion and signal profiles]

Figure 4-1 Sample Dispersion and Signal Profiles.

a: No dispersion;
b: Dispersion predominantly by convection;
c: Dispersion by convection and diffusion;
d: Dispersion predominantly by diffusion.
The dispersion \( D \) in FIA is defined as the ratio of concentrations before and after the dispersion process has taken place in the element of fluid that yields the analytical readout\(^1\),

\[
D = \frac{C^o}{C^{\text{max}}} = \frac{H^o}{H}
\]

\( C^o \): Original concentration of the injected sample solution;

\( C^{\text{max}} \): Corresponds to the maximum concentration of the recorded curve;

\( H^o \): No dispersion peak height; \( H \): Peak height (from recorder);

A typical recorder output, as shown in Figure 4-2, has the shape of a peak\(^1\), the peak height \( H \) being proportional to the concentration of the analyte. As it will take some time for the sample to travel from the injection point \( S \) to the peak maximum, this time is called the residence time \( T \).

![Figure 4-2 A Typical Recorder Output of FIA Comparison with no Dispersion](image)

\( C^o \): Original concentration of the injected sample solution;

\( C^{\text{max}} \): Corresponds to the maximum concentration of the recorded curve;

\( H^o \): No dispersion peak height;

\( H \): Peak height (from recorder);

\( S \): Sample injection;

\( T \): Residence time.
So the expression that takes both convective and diffusion transport into account and therefore best describes the overall physical dispersion phenomena is\(^2\):

\[
D \left( \frac{\partial^2 C}{\partial t^2} + \frac{\partial^2 C}{\partial r^2} + \frac{1}{r} \frac{\partial C}{\partial r} \right) = \frac{\partial C}{\partial t} + U_o \left( 1 - \frac{r^2}{R^2} \right) \frac{\partial C}{\partial t}
\]

D: Molecular diffusion coefficient; C: Concentration;
U_o: Maximum linear velocity; t_a: Travel time;
l: Partial tube length; r: Partial tube radius; R: Tube radius.

4.1.2. Miniaturisation and Flow Cell

One of the main goals of modern analytical chemistry is to reduce sample and reagent consumption and human participation in the analytical process. The simplest FIA system, as shown in Figure 4-3, consists of a pump, which is used to pump the carrier stream through a small bore tube; an injection port, through which a well-defined volume of a sample solution can be repeatedly injected into the carrier stream; and a reaction coil, in which the sample zone reacts with the components of the carrier stream to form a species which is responsive to the detector.

![Figure 4-3 Schematic Manifold for the Simplest FIA System](image)

C: Carrier; P: Pump; I: Injection valve;
M: Mixing coil; D: Detection; DP: Data processor; W: Waste.

Recently, a battery operated and computerised electrochemical instrumentation has been developed into a powerful technique for field based analysis of samples\(^4\). FIA is an ideal technique for implementing on-line separation, and the inclusion of an electrochemical detector endows the overall system with special features.

In flow-through biosensors, derivatization reactions are integrated with detection methods as the active microzone is located in a flow cell\(^5\). The flow
cell can be used to immobilise, on a suitable support, any of the participants of a chemical or biochemical reaction, e.g., the mediator, enzyme and co-enzyme. These continuous flow systems typically involve two major processes such as adsorption/desorption and chemical reaction, which are usually closely related to each other.

As discussed in Chapter 1, commercial flow cells such as 'wall-jet', thin-layer and tubular flow-through cells can be employed for biosensing. The schematic diagrams of the different cell designs are depicted in Figure 4-4. Owing to their geometry, the cells seemed to be very sensitive to changes in injected sample viscosity[1-3]. With proper control of solution flow, enzyme-based electrodes can be cleaned/flushed by buffer solution after each measurement. This may be beneficial in improving the reproducibility of biosensors.

![Schematic diagrams of the flow cells](image)

**Figure 4-4** Schematic diagrams of the flow cells

In Chapter 3, the incorporation of multiple components such as ferrocyanide, FDH and NAD, into a single polypyrrole film was successfully demonstrated for the fabrication of a biosensor for formate. In this chapter, a systematic study of the different ways of implementing this device as a flow-through biosensor with improved reproducibility was investigated. The biosensor was implemented with monolayer, bilayer and trilayer arrangements for the determination of various concentration ranges of formate. The amperometric responses of intermediates or products of the enzymatic reaction were observed by a flow through system, and eliminated by use of an overoxidised polypyrrole film.
4. 2.  Experimental

4. 2. 1.  Reagents

All reagents were of analytical-reagent grade, unless specified otherwise. All solutions were prepared with Milli-Q water (Millipore). Formate dehydrogenase (EC 1.2.1.2., F-8649), β-nicotinamide adenine dinucleotide (N-7004), and pyrrole (P-4892) were obtained from Sigma Chemical Co. 5%(wt) perfluoronated (Nafion® from Aldrich Chemical Co.) ion-exchanger powder was in a mixture of lower aliphatic alcohols and water. The pyrrole was distilled prior to use. Ferrocyanide, NaCl, KCl, Na₂HPO₄, NaH₂PO₄·2H₂O and HCOONa were supplied by AJAX Chemical Pty Ltd. Sodium dodecyl sulfate (SDS) was supplied by PROGEN Industries Ltd.

4. 2. 2.  Preparation of Polypyrrole-Based Electrodes

Polypyrrole based enzyme electrodes were prepared by galvanostatic electropolymerisation of pyrrole monomer from aqueous solution on to a flow through gold electrode with a surface area of 7mm² after purging with N₂ for 10 minutes. The working electrode was polished with 0.3μm alumina, and then ultrasonicated for 5 minutes to remove any residual. Galvanostatic polymerisation was accomplished in a monomer solution which contained pyrrole, FDH, NAD, K₄[Fe(CN)₆] or SDS with an applied current density for a given period. The compositon of a monomer solution is detailed in particular sections. 10μL of a 5% solution of Nafion was added to cover the PPy-FDH/NAD/[Fe(CN)₆]⁴⁺ membrane if necessary.

PPy-FDH/NAD/[Fe(CN)₆]⁴⁺ membranes were generated galvanostatically using a computerised Potentiostat/Galvanostat in conjunction with an IBM-PC.

4. 2. 3.  Procedures of Electrochemical Measurements

A BAS Unijet flow cell (MF-2061) was used. The cyclic voltammetry on the enzyme film and amperometric sensing of formate were investigated with a BAS CV-27 and a BAS LC-4C in conjunction with a MacLab®, respectively. After electropolymserisation of polypyrrole-based enzyme film, the electrode was then incorporated into an FIA system, which contains 0.1M potassium chloride and 0.01M phosphate buffer(pH7.0) as mobile phase.
4. 3. Results and Discussion

4. 3. 1. Fabrication of Monolayer Biosensing Membrane

As described in Chapter 3, electropolymerisation proved to be a feasible approach for fabricating a polypyrrole-based formate biosensor. A chronopotentiogram is shown in Figure 4-5 for the formation of PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film on the flow through electrode.

![Figure 4-5](image)

**Figure 4-5** Chronopotentiogram of PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ membrane formed in galvanostatic mode. A current density of 1.5mA/cm$^2$ for 400s on a gold electrode was employed in the monomer solution of 0.3M pyrrole, 2.5mM [Fe(CN)$_6$]$^{4-}$, 5mM NAD and 10 units FDH in 5mL.

The PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film coated onto the Au flow through electrode was incorporated into a FIA system in 0.1M KCl/0.01M phosphate buffer (pH7.0) as mobile phase. Figures 4-6 and 4-7 show the hydrodynamic behaviour of the electrode in this medium by use of cyclic voltammetry.

As shown in Figure 4-6, more distinct features of the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film were obtained at the higher flow rate due to the more rapid attainment of a steady state. Figure 4-7 suggests that the conformation of the PPy-FDH/NAD/[Fe(CN)$_6$]$^{4-}$ film may change after undergoing one or more biosensing reactions. It appears that the intermediates
produced, such as NADH; or the reaction products, such as CO₂ may affect the polypyrrole backbone and change the film permeability and porosity.

![Graph](image)

**Figure 4-6** Hydrodynamic cyclic voltammograms of PPy-FDH/NAD/\([\text{Fe(CN)}_6]^{4-}\) film in 0.1M KCl/0.01M phosphate buffer. Scan rate: 100mV/s. Other conditions same as Figure 4-5.

![Graph](image)

**Figure 4-7** Hydrodynamic cyclic voltammograms of PPy-FDH/NAD/\([\text{Fe(CN)}_6]^{4-}\) film in 0.1M KCl/0.01M phosphate buffer. Flow rate: 0.5mL/min; Scan rate: 100mV/s. Other conditions same as Figure 4-5.
Figure 4-8 illustrates the amperometric responses vs time curve obtained for 5mM formate in 0.1M KCl/0.01M phosphate buffer (pH 7.0) at an applied potential of 0.3V (vs Ag/AgCl). Upon application of the detection potential, the oxidation current of ferrocyanide quickly decreased to 255nA and 95nA after 10 and 20 minutes, respectively. The rapid decrease in the oxidation current of ferrocyanide which is reduced from ferricyanide by a catalytic reaction occurred in the initial stages. This is probably due to desorption of the enzyme bound loosely to the surface region of the PPy membrane. Then, the desorbed enzyme was flushed out of the mixing chamber by the mobile phase.

Other electrochemical properties could be considered to interpret the result shown in Figure 4-8. This, firstly, indicates the anodic oxidation of a polypyrrole film, where anions (such as Cl\textsuperscript{-}, H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-} and HPO\textsubscript{4}\textsuperscript{2-}) compound the positive charge of the produced PPy\textsuperscript{+}, is less favourite for formate dehydrogenase relevant to the functioning sites. Secondly, the double layer is made up in the aqueous phase. The inner Helmholtz plane is included among the anions from the solution. In the outer, those ions held by long-range electrostatic forces are within the diffusion layer. It prevents formate (HCOO\textsuperscript{-}) from penetrating effectively into the PPy-FDH/NAD/[Fe(CN)\textsubscript{6}]\textsuperscript{4-} membrane. It has to be taken into account that amperometric measurements should not be carried out until the constant current is obtained. Therefore, the amperometric measurements should be taken after establishing equilibrium for a while in order to obtain fairly stable responses.

![Graph](image)

**Figure 4-8** Variation of the amperometric response of formate in 0.1M KCl/0.01M phosphate buffer with time. Flow rate was 0.1mL/min; Injection volume was 500\textmu L; Applied potential was +0.3V; 5mM formate. Other conditions same as Figure 4-5.
According to the Marcus theory[7], a redox mediator with a low reorganisation energy after the electron transfer has to be able to penetrate into the active site of the enzyme to shorten the distance between the prosthetic group (e.g., NAD/NADH) and the mediator. Hence, the rate constant of the electron-transfer reaction can be increased. After this electron transfer the redox equivalents have to be transported to the electrode surface with a rate constant which is in the range of the turnover rate of the enzyme. In this kind of shuttle mechanism, the electron transport is connected to a mass transfer which is correlated to the diffusion of sufficiently soluble redox mediators.

The above discussion assumes that the rate of the electron transfer at the gold surface is not limiting, such that transfer of electrons to the ferricyanide limits the rate of reaction. This is not always the case, however, if the kinetics of the ferrocyanide/ferricyanide reaction control the overall reaction rate. As sample is dispersed by convection and diffusion at a high flow rate and in the thinner membrane, respectively, this causes a negative peak in the initial amperometric response, as shown in Figure 4-9. This observation suggests that the formation of ferrocyanide from ferricyanide is a rate-limiting step compared to the enzymatic reaction step. Furthermore, the conjugated polypyrrole backbone may be affected by reactive intermediates or products generated from the catalytic reactions.

As indicated in Figure 4-9, a negative peak corresponds to the sudden shift in the film conductivity due to a short-lived intermediate, such as NADH, or/and a product generated during the enzymatic reaction, such as CO₂. This is also achieved by subjecting the latter to a calibration curve while maintaining a constant potential applied to the enzyme membrane at a value characteristic of the limiting current of the species generated.

As shown in Figure 4-10, the magnitude of the negative response is proportional to the concentration of formate. The positive peak is treated as the enzymatic response due to the contribution of an anodic current of [Fe(CN)₆]⁴⁻. The magnitude of the difference between the positive and negative peaks for one sample injected is regarded as the total response for an enzymatic reaction. With hydrodynamic voltammetry it has been possible to identify short-lived intermediates which decompose within the time scale of the biosensors. The magnitude of both negative and positive peaks correlates with the concentration of formate in solution. Therefore, the response of each can be useful in determining formate under the different circumstances. However, further improvement of the biosensor to give a more selective response for formate is a more preferred option.
Figure 4-9  A chronoamperogram of formate biosensor recorded in 1mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 10μL. Applied potential was +0.4V. The pyrrole concentration was 0.3M mixed in 10 units FDH, 5mM NAD and 2.5mM ferrocyanide. A current density of 1.5mA/cm² for 100s on a gold electrode was employed.

Figure 4-10  Calibration curve of formate in 1mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Other conditions same as Figure 4-9.
4.3.2. Bilayer Formate Biosensing Membrane

The bilayer film used in this study was prepared by galvanostatic polymerisation as described in section 3.5.1. Figure 4-11 shows that with the use of a flow rate of 0.1mL/min and a sample injection volume of 500μL, a linear range between 0.1mM and 1mM formate was obtained with both peak height and peak area measurements. This linear range was narrower than that obtained with monolayer biosensor (Figure 4-9 and 4-10). However, the detection limits are identical. This could be caused by the less interaction between ferricyanide and NADH at the interface of PPy-[Fe(CN)$_6$]$^{4-}$ and PPy-FDH/NAD. The most appropriate general model for describing electron transfer across the redox sites in the polymer is the electron-hopping concept for disordered systems[8,9]. The elementary step in electron transport is the transition from an occupied state (donor) to an unoccupied state (acceptor). The electron hopping is a bimolecular process and it is thus necessary to have occupied sites next to unoccupied ones for this process to take place. Hence, the ratio between the densities of acceptor and donor states is very important.

To improve the reaction kinetics, a number of factors may be considered, such as:

(a) an increase in the amount of ferrocyanide and NAD in PPy-[Fe(CN)$_6$]$^{4-}$ and PPy-FDH/NAD layers, respectively;

(b) an increase in the porosity of PPy-[Fe(CN)$_6$]$^{4-}$ film to enable products/reactants from PPy-FDH/NAD layer to penetrate into the PPy-[Fe(CN)$_6$]$^{4-}$ layer.

The fabrication of a bilayer membrane was carried out galvanostatically in the monomer solutions, as outlined in Table 4-1, under the conditions listed in Table 4-2.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3M Pyrrole</td>
<td>0.3M pyrrole</td>
</tr>
<tr>
<td>10mM Potassium Ferrocyanide</td>
<td>5.0mM NAD</td>
</tr>
<tr>
<td>5mL H$_2$O</td>
<td>5mL H$_2$O</td>
</tr>
</tbody>
</table>

Table 4-1 The composition of monomer solutions
Figure 4-11 The mperometric response of a bilayer formate biosensor in 0.1mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 500μL. Applied potential was +0.3V. Other conditions are the same as Figure 3-30.

a: Calibration curve of peak area vs concentration;
b: Calibration curve of peak height vs concentration.
Table 4-2  The parameters of galvanostatic films formation

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-[Fe(CN)₆]⁴⁻</td>
<td>0.5mA/cm² for 100s</td>
<td>0.5mA/cm² for 100s</td>
<td>0.5mA/cm² for 100s</td>
</tr>
<tr>
<td>PPy-FDH/NAD</td>
<td>0.25mA/cm² for 800s</td>
<td>0.5mA/cm² for 400s</td>
<td>1.0mA/cm² for 200s</td>
</tr>
</tbody>
</table>

Figure 4-12 shows three types of PPy-[Fe(CN)₆]⁴⁻ + PPy-FDH/NAD bilayers where the PPy-FDH/NAD layer was electropolymerized on the top of the PPy-[Fe(CN)₆]⁴⁻ membrane which was dried under ambient conditions. The charge involved in growing the outer PPy-FDH/NAD layer was 200mC/cm², which yields a film of approximately 0.44μm, as discussed in section 3.3.1.2.

The different current density applied for the formation of the PPy-FDH/NAD layer altered/affected the porosity/permeability of each membrane. The maximum resulting potentials during the galvanostatic polymerisation were 1.01V, 0.78V and 0.71V at the current density of 1.0mA/cm², 0.5mA/cm² and 0.25mA/cm², respectively, as shown in Figure 4-12b.

Hydrodynamic voltammetry (Figure 4-13) shows the difference between the three types of bilayer due to the porosity/permeability of the outer PPy-FDH/NAD layer. The characteristics of PPy⁺ reduction peaks appeared are much correspondent to the porosity/permeability of the polypyrrole films due to the movement of cations in the polypyrrole backbone. The film polymerised at the current density of 0.25mA/cm² (curve C in Figure 4-13) gave a fairly small cathodic current of the PPy⁺ reduction compared to the current density of 0.5mA/cm² and 1.0mA/cm² in Figure 4-13B and 4-13A, respectively. Therefore, the porosity/permeability of the outer PPy-FDH/NAD layer does affect the redox behaviour of the films.

As described in section 3.4.7., current density and electropolymerisation period can affect the porosity/permeability of the polymer and the amount of enzyme, co-factor and mediator loaded in the film. It is evident that the application of higher current density results in more sensitivity. Figure 4-14 shows the amperometric responses for formate at various porosity/permeability films. The applied current density of 1.0mA/cm² for electropolymerisation of an enzymatic layer had a higher slope, indicating a more sensitive response.

As shown in Figure 4-15, as little as 10nM of formate can be detected with the bilayer membrane. The enhancement of sensitivity is due to the attainment of
much larger the rates of mass transfer at the electrode surface than the rates of diffusion.

**Figure 4.12** Chronopotentiograms of PPy-[Fe(CN)$_6$]$^{4-} +$ PPy-FDH/NAD films formed galvanostatically. (a) PPy-[Fe(CN)$_6$]$^{4-}$ monolayer, (b) PPy-[Fe(CN)$_6$]$^{4-} +$ PPy-FDH/NAD bilayers. Other conditions are the same as Table 4-1 and 4-2.
Figure 4-13  Cyclic voltammograms of a PPy-[Fe(CN)$_6$]$^{4-} +$ PPy-FDH/NAD bilayer membrane in 0.1M KCl/0.01M phosphate buffer(pH7.0) when a flow rate was 0.5mL/min. Scan rate: 100mV/s. The parameters of galvanostatic film formation are illustrated in Table 4-2.

Figure 4-14  Effect of current density and polymerisation period on amperometric responses in 0.1M KCl/0.01M phosphate buffer when flow rate was 1.0mL/min. Applied potential: 0.3V.
Figure 4-15  The amperometric response of a bilayer formate biosensor in 1.0mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 10µL. Applied potential was +0.3V. Other conditions are the same as Table 4-1 and 4-2B.
4. 3. 3. SDS Modified Bilayer Membrane

Sodium dodecyl sulfate has both hydrophilic and hydrophobic elements in its structure, forming a polar head and a non-polar tail. The result of this structural property is that in water and aqueous solutions of simple salts the hydrophobic hydrocarbon ‘tails’ come together, away from the solvent. If membrane surface regions with hydrophobic properties interact with the hydrophobic ‘core’ of the polypyrrole membrane, SDS could anchor the proteins within the membrane structure. The proteins within the insulating membrane can themselves be electronically or ionically conducting[10].

Based on the inner layer PPy-[Fe(CN)$_6$]$_{4^-}$ as shown in Figure 4-12a, a top layer of PPy-FDH/NAD/SDS was fabricated onto it. The outer enzyme’s layer was electopolymerised at a current density of 0.5mA/cm$^2$ for 400s in the monomer solution, which contained 0.3M pyrrole, 5mM NAD, 30 units FDH and 15mM SDS in 5mL. It was observed that the mechanical and hydrophobic properties of the polymer were improved, due to co-polymerisation. Membrane features can be converted from hydrophilic to hydrophobic by introducing some dopant and copolymerisation.

The highest potential measured during electropolymerisation as shown in a chronopotentiogram (see Figure 4-16) was 0.53V. This indicates that the conducting polypyrrole is more conductive than previous, resulting in efficient electron transfer. This may ultimately increase the sensitivity of the formate biosensor.

In addition, SDS is incorporated into a polypyrrole backbone as charge compensation anion as shown in Equation 3-1. The hydrophobic tails of SDS will easily attach some hydrophobic proteins in an enzyme via hydrophobic interactions[11]. Enzyme and co-enzyme were assembled in a polypyrrole-based outer membrane on a PPy-[Fe(CN)$_6$]$_{4^-}$ surface by a current-assisted self-assembly method as well as a physical entrapment method. These enzymes communicate electronically with the mediator through a molecular interface conducting copolymer. Self-assembly method[12-14] has been introduced into the biosensors recently. It can result in more stable and active immobilised enzyme layers[12].

The SDS modified bilayer membrane was further investigated by hydrodynamic cyclic voltammetry, as shown in Figure 4-17. It showed a significant difference of the cathodic currents of PPy$^+$ reduction compared to Figure 4-13. It indicated that the polymer backbone shown in Figure 4-17 was
not same as its shown in Figure 4-13, due to copolymerisation of pyrrole and SDS.

Figure 4-16  Chronopotentiogram of PPy-FDH/NAD/SDS membrane formed in galvanostatic mode. Monomer solution contained 0.3M pyrrole, 5mM NAD, 30 units FDH and 15mM SDS in 5mL. Current density was employed at 0.5mA/cm² for 400s.

Figure 4-17  Cyclic voltammogram of PPy-[Fe(CN)₆]⁴⁻ + PPy-FDH/NAD/SDS bilayer membrane in 0.1M KCl/0.01M phosphate buffer when a flow rate was 0.5mL/min. Scan rate: 100mV/s.
Hydrodynamic amperometric measurement was carried out to study the significance of electron transfer in this copolymer-based biosensor. Electron transportation is a very important characteristic of conducting polymer in amperometric measurements. The conductivity can be increased significantly by introducing into the polymer matrix various types of electronic states that can be reversibly occupied and emptied, thus constituting the basis for electronic conduction. As shown in Figure 4-18, as little as 2.5nM of formate can be detected with this bilayer.

Figure 4-18  The amperometric response of a PPy-[Fe(CN)$_6$]$^{4-}$ + PPy-FDH/NAD/SDS bilayer formate biosensor in 1.0mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 10μL. Applied potential was +0.3V.
4.3.4. Overoxidized Polypyrrole-Based Membrane

One of the advantages of conducting polymer is its ability to assist electron transfer during the process of signal generation. As observed in section 4.3.1., the intermediates or products from the enzymatic reactions can affect the charge transfer of the conducting polymer, resulting in negative amperometric responses. This is clearly of interest from the subject of electron conduction in polymers, in which electrochemistry plays an important role. It is possible to fabricate a biosensing membrane, in which an enzyme was immobilised, by changing the polymer conductivity alone with other parameters kept unchanged. An overoxidized polypyrrole-based membrane provides a way of sacrificing the conductance of the polypyrrole film, as a basis for eliminating the appearance of the negative peaks from the intermediates or products produced during the biosensing process.

Figure 4-19 shows the comparison of a conductive PPy-[Fe(CN)₆]⁴⁻ + PPy-FDH/NAD membrane and an overoxidized one. The hydrodynamic voltammogram obtained for the bilayer shrunk after being overoxidized at 0.8V for 20min. This indicates that an electroinactive and poorly conductive film was obtained.

![Graph](image)

**Figure 4-19** Comparison of hydrodynamic voltammograms of PPy-[Fe(CN)₆]⁴⁻ + PPy-FDH/NAD bilayer membranes. The monomer solutions were illustrated in Table 4-1. The electropolymerisation parameters were illustrated in Table 4-3 'Bilayer section'.
As discussed in section of 4.3.1., the negative current is caused by the influence of intermediates or products inside the biomembrane. The intermediates or products current decreased with increasing overoxidation of the conductive polymer membrane. As observed in Figure 4-20, 10mM of formate shows a significant negative current response on a normal conducting polymer (0 min), but decreased with increasing overoxidation of the polymer. Furthermore, the positive peaks obtained for the oxidation of ferrocyanide increased dramatically, compared with those obtained with normal conducting membrane. This is due to the stabilisation of the capacitance of the PPy-FDH/NAD/[Fe(CN)₆]⁴⁻ membrane in the overoxidized film.

![Graph showing amperometric responses of formate on the overoxidized polypyrrole-based formate biosensing membranes.](image)

**Figure 4-20** Amperometric responses of formate on the overoxidized polypyrrole-based formate biosensing membranes. Overoxidation time is indicated next to each response. Other conditions same as Figure 4-19.
Figure 4-21 was obtained as a function of the period of the overoxidation. It presented experimental analysis of enzyme electrodes with conducting polymer, in which the electrochemical reaction of a mediator in the polypyrrole film is sufficiently fast and thereby the enzyme-electrode charge transfer efficiency is necessarily unity. However, such an electrode reaction is usually not fast enough to prevent intermediates or products from changing the capacitance of the PPy-FDH/NAD/Fe(CN)$_6^{4-}$ membrane.

![Graph](image)

**Figure 4-21** Effect of overoxidation period on negative amperometric responses of a PPy-[Fe(CN)$_6^{4-}$]$_n$ + PPy-FDH/NAD bilayer membrane. Other conditions same as Figure 4-19.

As pointed out in Table 4-3, the rate of the heterogeneous electron-transfer reaction did affect the rate at which a Faradic reaction occurred, comparison of monolayer and bilayer membranes. The magnitude current of a negative response declined from 121nA to 30.5nA, if the polypyrrole film was overoxidized at 0.8V for 10min. It was further decreased to 3nA and 2nA at 20min and 30min overoxidation of polypyrrole films, respectively.
Table 4-3  Comparison of intermediates responses

<table>
<thead>
<tr>
<th>Polymerisation parameters</th>
<th>Monolayer</th>
<th>Bilayer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-FDH/NAD/[Fe(CN)_6]^{4-} (1.5mA/cm² for 100s)</td>
<td>-63</td>
<td>PPy-[Fe(CN)_6]^{4-} (0.5mA/cm² for 200s) &amp; PPy-FDH/NAD (0.5mA/cm² for 400s)</td>
</tr>
</tbody>
</table>

Although the overoxidized PPy films were reported to eliminate electroactive interferents in amperometric[15-17] and potentiometric[18] biosensors, the mechanism has not been discussed. From experimental results, it was evidenced that the intermediates exist in the polypyrrole conjugation system, resulting in a negative response.

4.4  Triple Layers

Another possible approach is to locate the enzyme layer between an inner and an outer membrane in a triple layer arrangement. Each of the two membranes can be tailored to a set of device characteristics. The new device design makes use of advances in membrane technology and can prevent leaching of the mediator from the membrane. It is also able to eliminate the negatively charged biological interferents, such as ascorbic acid, uric acid, etc., crossing the membrane and being oxidised at the underlying gold surface.

The outer membrane is tailored to control diffusion of the analyte from the sample to the enzyme layer such that the analytical range can be extended significantly to provide direct measurements on undiluted samples. To avoid leaching of the mediator from the enzymatic film as well as to block the entry of anionic biological interferents, coating of the diffusion-limiting membrane as shown Figure 4-22 was achieved by polymerising one thin PPy film or casting one thick coat from 5% Nafion. The denaturation or loss of enzyme
activity is very limited as shown by high responses obtained in amperometric measurements in following sections.

4. 4. 1. KCl Modified Triple-Layer Membrane

Based on a bilayer membrane, fabrication of a triple-layer membrane involves electropolymerisation in the following sequence: PPy-\([\text{Fe(CN)}_6]^{4-}\), PPy-NAD/FDH and PPy-Cl\(^{-}\) in the monomer solutions as shown in Table 4-4. The galvanostatic parameters were described in Table 4-5.

Figure 4-23 shows the electropolymerisation processes monitored by in-situ chronopotentiometry. The maximum potentials from the chronopotentiograms were 0.59V, 1.13V and 0.64V for PPy-\([\text{Fe(CN)}_6]^{4-}\), PPy-NAD/FDH and PPy-Cl\(^{-}\), respectively. The chronopotentiogram shape of the PPy-Cl\(^{-}\) membrane is different from the others, the highest potential is its steady-state potential. The PPy-Cl\(^{-}\) membrane may be useful as a diffusion-limiting membrane to avoid leaching of the mediator and block the entry of anionic biological interferents.
Table 4-4  The composition of monomer solutions

<table>
<thead>
<tr>
<th></th>
<th>PPy-[Fe(CN)₆]⁴⁻</th>
<th>PPy-NAD/FDH</th>
<th>PPy-Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3M Pyrrole</td>
<td>0.3M pyrrole</td>
<td>0.3M pyrrole</td>
<td></td>
</tr>
<tr>
<td>10mM K₄[Fe(CN)₆]</td>
<td>5.0mM NAD</td>
<td>10mM KCl</td>
<td></td>
</tr>
<tr>
<td>30units FDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5mL H₂O</td>
<td>5mL H₂O</td>
<td>5mL H₂O</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-5  The parameters of galvanostatic formation of the films

<table>
<thead>
<tr>
<th></th>
<th>Current density (mA/cm²)</th>
<th>Period (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-[Fe(CN)₆]⁴⁻</td>
<td>1.5</td>
<td>50</td>
</tr>
<tr>
<td>PPy-NAD/FDH</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>PPy-Cl⁻</td>
<td>0.5</td>
<td>100</td>
</tr>
</tbody>
</table>

A hydrodynamic voltammetric study was carried out to compare the properties of a triple layer membrane with bilayer and monolayer membranes. Some behaviours (Figure 4-24) were observed such as an anodic wave of the ferrocyanide oxidation and a cathodic wave of polypyrrole reduction, though some features were not very significant compared to those obtained for bilayer and monolayer. It must be emphasised that the cathodic current of PPy⁺ reduction is much less than the cathodic current obtained in Figure 4-17. This suggests that the movement of the cations is limited by the PPy-Cl⁻ diffusion-limiting membrane.
Figure 4-23  Chronopotentiograms of PPy-[Fe(CN)$_6$]$^{4-}$ + PPy-NAD/FDH + PPy-Cl$^-$ membranes formed in galvanostatic mode. Other conditions the same as Table 4-4 and 4-5.
Effect of flow rate at pH 7.0 over the range 0.1 - 2.5mL/min was studied, using the triple layer membrane prepared under the above conditions. The resulting biosensor response vs concentration of formate profile (Figure 4-25 and 4-26) indicates that the biosensing response depends on a compromise of such interaction and retention as follows:

1) The biosensing response decreased with increasing flow rate at the lower concentration range of formate. The reason for this phenomenon is that the flow rate was too high to conduct the enzyme catalysed formate reaction with NAD. A lower flow rate increases the biosensing response, due to the improvement of an intermolecular reaction.

2) The biosensing response increased with increasing flow rate at the higher concentration range of formate. The reason for this phenomenon is that CO₂ generated by the enzymatic reaction can be removed from the polymer backbone as quickly as possible. The reaction products or intermediates exist in a membrane so long that the conjugated polypyrrole backbone system would be easily affected. The interference of reaction products, therefore, can be eliminated by using the higher flow rate in an FIA system.
Figure 4-25  The biosensing responses vs concentration of formate at various flow rates. Amperometric measurements were carried out in 0.1M KCl/0.01M phosphate buffer at 0.3V vs Ag/AgCl. Injection volume was 100μL.

Figure 4-26  Effect of flow rate on the amperometric responses. Conditions same as Figure 4-25.
As shown in Figure 4-27, the analytical range was extended from 30nM (a bilayer membrane as shown in Figure 4-15) to 500nM, due to outer layer PPy-Cl\(^-\) as a diffusion-limiting membrane. The assumption was proven as described in Figure 4-22. Furthermore, the PPy-Cl\(^-\) membrane can prevent some negatively charged biological interferents from penetrating, and oxidised on the gold surface. This phenomenon can be interpreted as follows:

1) The negatively charged PPy-Cl\(^-\) outer layer repels negatively charged interferents, due to the repulsive forces.

2) The restrictedly permeable PPy-Cl\(^-\) outer layer keeps out large molecules, due to the electropolymerisation of PPy-Cl\(^-\) at the lower current density, such as 0.5mA/cm\(^2\).

Although a trilayer membrane which consists of a polypyrrole, a ferrocenemodified polypyrrole and a polypyrrole-glucose oxidase has been reported\(^{[19]}\), a more elaborate trilayer system which consists of mediating, biosensing and diffusion-limiting layers has been developed in this project. A functioned PPy-Cl\(^-\) outer layer shows the great contributions to avoid leaching of the mediator and enzyme from the enzymatic film as well as blocking the entry of anionic biological interferents.
Figure 4-27  The amperometric response of a PPy-[Fe(CN)$_6$]$^{4-}$ + PPy-FDH/NAD + PPy-Cl$^-$ triple layer formate biosensor in 1.5mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 100µL. Applied potential was +0.3V.
4. 4. 2. Nafion Modified Triple-Layer Membrane

As discussed in section 4.5.1., the negative charges of the polypyrrole-Cl\textsuperscript{−} film can act as a barrier for the negatively charged biological interferents found in samples. In this regard, extensive studies have been performed mainly using a perfluoronated ion-exchanger (Nafion\textsuperscript{20-27}). The use of Nafion films shows greater affinity to large, hydrophobic cations relative to small inorganic cations. Major interferents such as ascorbic acid and uric acid can therefore effectively be eliminated from the electrode surface, due to the characteristics of Nafion films. Two types of Nafion films will be used in a Nafion-modified membrane as an outer layer film to guard the biosensor electrode.

4. 4. 2. 1. Nafion Coated Film

The outer PPy-Cl\textsuperscript{−} layer of the membrane described in section 4.5.1. was replaced with a Nafion film. After polymerising PPy-FDH/NAD film, the enzymatic electrode was dried under ambient conditions and its surface was then covered by one coat of 5\% Nafion solution.

A Nafion-modified enzyme electrode was incorporated into an FIA system, Figure 4-28 shows the linear responses of hydrodynamic amperometry. The lower flow rate and the bigger sample loop were set as 0.1mL/min and 100μL, respectively. In contrast, a Nafion-modified outer layer plays a more efficient diffusion-limiting role to give a linear range between 100nM to 500nM, compared to a PPy-Cl\textsuperscript{−} modified enzyme electrode at flow rate of 0.1mL/min in Figure 4-25 and 4-26. For both approaches, as little as 100nM of formate can be detected, approximately 20nA. However, the PPy-Cl\textsuperscript{−} modified enzyme electrode required a higher flow rate of 1.5mL/min to improve the diffusion-limiting of PPy-Cl\textsuperscript{−} for extending the linear concentration range up to 500nM.
Figure 4-28  The amperometric response of a PPy-Fe[<(CN)₆]⁴⁻ + PPy-FDH/NAD + Nafion triple layer formate biosensor in 0.1mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 100μL. Applied potential was +0.3V.
4. 4. 2. 2. Nafion Electropolymerised Film

However, the practical utility of Nafion-based film is limited by their non-uniform thickness and poor reproducibility arising from the solvent evaporation method used in the film preparation. Undoubtedly, a new polymer composite is desirable for the further development of a Nafion modified triple-layer membrane.

The fabrication of this Nafion-modified enzyme membrane is the same as the procedures described in section 4.5.1.1., instead of using PPy-Cl\(^-\) as an outer layer. Sequential electropolymerisation was performed by the growth of PPy-Fe\(((CN)\_6\)\(^{4-}\), PPy-NAD/FDH and PPy-Nafion layers. The monomer solutions were shown in Table 4-6. The galvanostatic parameters were described in Table 4-7.

<table>
<thead>
<tr>
<th>PPy-[Fe(CN)(_6)](^{4-})</th>
<th>PPy-NAD/FDH</th>
<th>PPy-Nafion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3M Pyrrole</td>
<td>0.3M pyrrole</td>
<td>0.3M pyrrole</td>
</tr>
<tr>
<td>10mM K(_4)[Fe(CN)(_6)]</td>
<td>5.0mM NAD</td>
<td>200(\mu)L of a 5% solution of Nafion</td>
</tr>
<tr>
<td></td>
<td>30units FDH</td>
<td></td>
</tr>
<tr>
<td>5mL H(_2)O</td>
<td>5mL H(_2)O</td>
<td>5mL H(_2)O</td>
</tr>
</tbody>
</table>

**Table 4-7** The parameters for the galvanostatic formation of the films

<table>
<thead>
<tr>
<th></th>
<th>Current density (mA/cm(^2))</th>
<th>Period (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-Fe(CN)(_6)(^{4-})</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>PPy-NAD/FDH</td>
<td>1.0</td>
<td>200</td>
</tr>
<tr>
<td>PPy-Nafion</td>
<td>1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

A calibration curve is given in Figure 4-29, with a linear range between 25nM and 200nM. As little as 25nM of formate can be detected, compared to 100nM.
in Figure 4-28. The use of a PPy-Nafion outer layer proved to be more feasible and reproducible, compared to casting one thick coat from 5% Nafion.

Figure 4-29  The amperometric response of a PPy-[Fe(CN)$_6$]$_{4^-}$ + PPy-FDH/NAD + PPy-Nafion triple layer formate biosensor in 1.0mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer. Injection volume was 10μL. Applied potential was +0.3V.
4. 5. Conclusions

Ferrocyanide-mediated flow amperometric biosensors were explored in discrete layered structures fabricated by sequential electropolymerisation to modulate the performances of the formate biosensor. The linear ranges of various films for analytical purposes are summarised as follows:

<table>
<thead>
<tr>
<th>Film construction</th>
<th>Monolayer</th>
<th>Bilayer</th>
<th>Trilayer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer layer</td>
<td>-</td>
<td>Normal</td>
<td>PPy-Cl⁻</td>
</tr>
<tr>
<td>SDS Modified</td>
<td>Normal</td>
<td>SDS Modified</td>
<td>Nafion</td>
</tr>
<tr>
<td>Linear range</td>
<td>0.1-10mM</td>
<td>10-30nM</td>
<td>2.5-10nM</td>
</tr>
<tr>
<td></td>
<td>10-30nM</td>
<td></td>
<td>0.1-0.5μM</td>
</tr>
</tbody>
</table>

The linear range and the lowest concentration detected vary according to layered structures of the films. The various sensitivities obtained from different approaches do not compete against another. However, they are entirely complementary techniques for different analytical purposes.

The intermediates of the enzymatic reactions were observed in a flow amperometric system. This fact is a disadvantage with the respect to an amperometric response, due to the changing background current which overlaps with the response of the biosensing system. The solution to this problem is to use an overoxidized polypyrrole film for stabilising the background. On the other hand, it is also possible to establish a relationship between the response of intermediates and the analyte concentration by measuring the response of intermediates. Furthermore, the response of the intermediates may be useful for studying the mechanism of the biosensing system.

4. 6. References


CHAPTER 5

FERROCENE AND PRUSSIAN BLUE BASED FORMATE BIOSensor SYSTEMS
5. 1. Introduction

Much interest has been paid in recent years to the use of cofactors such as NAD for the improvement of the performance of biosensors\(^1\text{-}^{20}\). The aims of this chapter are to confirm the principle of mediators-based formate biosensors and explore new approaches for the mediator, ferrocyanide, leaking from the polypyrrole backbone. It has been found that in such systems the stability of the ferrocene-entrapped sensor is superior to that where ferrocyanide is employed as the mediator\(^{21,22}\). This is because the molecular size and structure of ferrocene derivatives are more favourable for stable immobilisation in the hydrophobic polypyrrole matrix than those of ferrocyanide. For example, ferrocene is bulky and has two hydrophobic heads, whereas ferrocyanide is hydrophilic and less bulky. Consequently, ferrocyanide can leak out of membranes more easily. The stability of the mediated biosensors seems to depend predominantly on the chemical structure of the mediator\(^{23}\). A macromolecular mediator such as ferrocene is therefore preferred to a hydrophilic mediator such as ferrocyanide in a thicker polypyrrole membrane. Various electron mediated biosensors have been developed with the ferrocene and its derivatives\(^{24-48}\). These mediators exhibit redox potentials in a region (100 to 400 mV vs SCE) where little interference from samples is noted\(^{33}\).

Another approach for preventing ferrocyanide from leaking is the intentional alteration of the solid-phase component of an electrode. This is able to promote electron transfer and stability during electrolytic applications. Yet, many studies\(^{49-56}\) have focused on the preparation and investigation of inorganic films, particularly for applications to electroanalysis. Inorganic films in this category have a variety of properties that are of interest to electroanalytical chemists. Prussian Blue films are electroactive\(^{49-54}\) and there are a few reports on their use for fabrication of glucose biosensor\(^{55,56}\).

Therefore, both approaches are useful in containing soluble or immobilised mediators which are able to oxidise the enzyme active site. The signal of the ferrocene-based biosensor is due to the current of ferrocene reoxidation via mediator recycling. Prussian Blue can be deposited electrochemically on the electrode surface, producing a dense redox active layer. It is demonstrated in this study that promising results were obtained when a polypyrrole-based enzyme electrode was used with ferrocene, ferrocene carboxylic acid or Prussian blue as a mediator in fabricating formate biosensors.
5. 2.  Experimental

5. 2. 1.  Reagents

All reagents were of analytical-reagent grade, unless specified otherwise. All solutions were prepared with Milli-Q water (Millipore). Formate dehydrogenase (EC 1.2.1.2., F-8649), β-nicotinamide adenine dinucleotide (N-7004), pyrrole (P-4892), ferrocene (F-3375) and ferrocene carboxylic acid (F-2641) were obtained from Sigma Chemical Co. The pyrrole was distilled prior to use. Ferricyanide, FeCl₃, NaCl, KCl, Na₂HPO₄, NaH₂PO₄·2H₂O and HCOONa were supplied by AJAX Chemical Pty Ltd.

5. 2. 2.  Preparation of Polypyrrole-Based Electrodes

Polypyrrole based enzyme electrodes were prepared by galvanostatic electropolymerisation of pyrrole monomer from aqueous solution on to a gold substrate with surface area of 7mm² after purging with N₂ for 10 minutes. A three-electrode cell, consisting of a working electrode, a coiled platinum auxiliary electrode and a saturated KCl calomel reference electrode was used. The working electrode was polished with 0.3mm alumina, and then ultrasonicated for 5 minutes to remove any residual. Monomer solution containing pyrrole, FDH, NAD and ferrocene or its derivatives was used with a certain current density to achieve electropolymerisation. Electrodeposition of Prussian Blue was achieved potentiostatically or galvanostatically by applying a constant potential of 0.4V or different current densities, respectively, in an equimolar mixture of 20mM K₃[Fe(CN)₆] and 20mM FeCl₃ solution.

5. 2. 3.  Procedures of Electrochemical Measurements

A three-electrode flow cell was used. The cyclic voltammetry on the enzyme film and amperometric sensing of formate were investigated with a BAS CV-27 and a BAS LC-4C in conjunction with a MacLab®, respectively. Electrochemical measurements were made in either batch or flow injection mode. In the former, measurements were made under stirred conditions, to provide convective transport, in a 10mL electrochemical cell (home-made). FIA was performed using a BAS Unijet flow cell (MF-2061). A HPLC pump (M-45, Waters Asso. Inc.) was used for the delivery of the mobile phase and sample. All the experiments were performed in 0.1M potassium chloride and 0.01M phosphate buffer (pH7.0), unless indicated otherwise.
5.3. Results and Discussion

5.3.1. Electrochemical Behaviours of Mediators

5.3.1.1. Mediators in Aqueous Solutions

A preliminary investigation into mediators, such as ferrocene and ferrocene carboxylic acid, was carried out in aqueous solutions. This aims to further development of a mediator as an electron shuttle in polypyrrole matrix.

Ferrocene carboxylic acid (Fcc), an acid, is easy to dissolve in a basic solution, such as KOH. For a system of redox reactions, the effect of moving the pH through six units away from neutral conditions is to increase the redox potential by 7.5x60mV, i.e. +450mV as indicated in Table 5-1 (First Cycle). A given gold-KOH solution interface shows a range of potentials (see Figure 5-1) where charge transfer reactions occur ineffectively because such reactions are thermodynamically or kinetically unfavourable in the alkaline media.

<table>
<thead>
<tr>
<th></th>
<th>First Cycle</th>
<th></th>
<th>Fifth cycle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential (mV)</td>
<td>Current (µA)</td>
<td>Potential (mV)</td>
<td>Current (µA)</td>
</tr>
<tr>
<td>Anodic</td>
<td>532</td>
<td>20.21</td>
<td>338</td>
<td>27.76</td>
</tr>
<tr>
<td>Cathodic</td>
<td>82</td>
<td>26.27</td>
<td>78</td>
<td>26.65</td>
</tr>
</tbody>
</table>

1mM ferrocene carboxylic acid in 0.1M KOH, scan rate: 100mV/s.

Further cyclic voltammetry was investigated in different media to improve the electrochemical processes. Ferrocene carboxylic acid was dissolved in KOH at the concentration ratio of 1:10, respectively, and then neutralised with addition of 0.1M phosphate buffer (pH7.0). Table 5-2 indicates that peak potential separation is 71mV which is much closer to 59mV compared to 450mV. Figure 5-2 shows the voltammetric behaviour of Fcc in phosphate buffer media. Electrolytes and pH play a significant role in all electron transfer so that the surface concentration of Fcc involved in the faradaic process can be described as quasi-reversible systems[57].
**Figure 5-1** Cyclic voltammograms of 1mM ferrocene carboxylic acid on gold electrode in 0.1M KOH. Scan rate: 100mV/s.

| Table 5-2 Characteristics of redox peaks of Fcc in phosphate buffer |
|------------------|-----------------|-----------------|
|                  | Anodic | Cathodic | Difference(|ΔE|) |
| Potential (mV)   | 319    | 248      | 71               |
| Current (μA)     | 3.21   | 3.22     | 0.01             |

1mM ferrocene carboxylic acid/10mM KOH in 0.1M phosphate buffer (pH 7.0), scan rate: 10mV/s.
Figure 5-2  Cyclic voltammograms of ferrocene carboxylic acid in 0.1M phosphate buffer (pH7.0). Scan rate: 10mV/s.

(a) 1mM Fcc/10mM KOH in 0.1M phosphate buffer;
(b) Comparison of 1mM Fcc, 0.1mM Fcc and 0.1M phosphate buffer.

The structure of a double layer probably affect the rates of electrode processes. The electrical double layer can be used to explain the electron transfer during the anodic process. The interaction of the solvated Fcc with positively charged gold was blocked by the inner Helmholtz plane of the high dense adsorbed OH⁻, preventing the electron from crossing the gold-solution interface.

The cyclic voltammograms shown in Figure 5-3 indicate that the anodic and cathodic current of Fcc increased linearly as a function of scan rate. This result is consistent with the expectations for a surface-confined redox system.
Figure 5-3  Cyclic voltammograms of 0.1mM ferrocene carboxylic acid/1mM KOH on gold electrode in 0.1M KCl/0.1M phosphate buffer.

Cyclic voltammetry of ferrocene (Fc) was investigated on gold electrode in 1M HNO₃ as shown in Figure 5-4. Since ferrocene is orange needles from methanol or ethanol, insoluble in water, it dissolved in diluted nitric and concentrated sulphuric acids to form a deep red solution with blue fluorescence[62]. The characteristics of redox peaks in 1M HNO₃ was illustrated in Table 5-3. Fc shows more electroactive than Fcc. The anodic current of Fc was near 3 times ones of Fcc, and the potential of Fc oxidation shifted in a negative direction compared to the ones of Fcc in Table 5-1.

Unfortunately, electrode processes are not always very facile or very sluggish. In this quasi-reversible case, the net current involves appreciable activated Fc from the forward and reverse charge transfers. Although the separation of redox peaks was around 150mV, it was observed that the increasing currents were as the function of time as shown in Figure 5-5 regarding to whole i-E.
E(V) vs SCE

Figure 5-4  Cyclic voltammograms of Ferrocene in 1M HNO₃. Scan rate: 100mV/s.

Table 5-3  Characteristics of redox peaks in 1M HNO₃

<table>
<thead>
<tr>
<th></th>
<th>First Cycle</th>
<th></th>
<th>Fifth cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential</td>
<td>Current</td>
<td>Potential</td>
</tr>
<tr>
<td></td>
<td>(mV)</td>
<td>(µA)</td>
<td>(mV)</td>
</tr>
<tr>
<td>Anodic</td>
<td>237</td>
<td>61.0</td>
<td>271</td>
</tr>
<tr>
<td>Cathodic</td>
<td>112</td>
<td>22.4</td>
<td>120</td>
</tr>
</tbody>
</table>

1mM ferrocene in 1M nitric acid, scan rate: 100mV/s.
Figure 5-5  Cyclic voltammograms of 1mM ferrocene in 1M HNO₃ on bare gold electrode.

5.3.1.2. Mediators in Polypyrrole Matrix

The electrochemical behaviour of Fcc in PPy matrix is of most interest for the fabrication of a Fcc-mediated biosensor. The dissolved Fcc in KOH was neutralised with the addition of an equivalent H₂SO₄. The monomer solution was made up of 1mM Fcc in 0.1M pyrrole. The PPy-Fcc film was electropolymerised at the current density of 0.5mA/cm² for 180s in the pyrrole monomer solution as mentioned above. Figure 5-6a shows the cyclic voltammogram of PPy-Fcc in 0.1M phosphate buffer (pH7.0). The result was similar to that shown in Figure 3-2 for PPy-[Fe(CN)₆]⁴⁻, but was more conductive compared to the films fabricated for the latter (Figure 3-4 and 3-5) at a current density of 0.5mA/cm² for 180s. This phenomenon may be due to the presence of H₂SO₄ in the polypyrrole backbone, and giving a more conductive film as proven in Chapter 2. Further experiments will be carried out in the absence of H₂SO₄.
A mixture of saturated Fcc concentration (3mM)$^{58}$ and 0.1M pyrrole was used to grow the PPy-Fcc film galvanostatically. The cyclic voltammogram of PPy-Fcc(Sat), shown in Figure 5-6b, indicates that the anodic currents were considerably suppressed, compared to those observed in Figure 5-6a. This may be due to low conductance of the resulting polypyrrole in the absence of H$_2$SO$_4$.

![Cyclic voltammograms](image)

**Figure 5-6** Cyclic voltammograms of galvanostatic polymerising PPy-Fcc films in 0.1M phosphate buffer. Scan rate: 100mV/s. The current density of 0.5mA/cm$^2$ was employed for 180s.

(a) 1mM Fcc in 1mM Na$_2$SO$_4$ and 0.1M pyrrole

(b) saturated Fcc and 0.1M pyrrole

To fabricate a ferrocene-mediated biosensor, the electrochemical behaviour of Fc in a polypyrrole matrix was further investigated. The cyclic voltammetric polymerisation was used to grow a PPy-Fc film onto a gold electrode. Figure 5-7a shows the growth process of the PPy-Fc film. All voltammograms gradually grow and tend to be more conductive with the increase of number scans, which demonstrates that the PPy-Fc film has been formed on the gold surface. After the PPy-Fc modified electrode was rinsed with Milli-Q water and immersed in the blank electrolyte, 0.1M phosphate buffer, the resulting cyclic voltammogram is shown in Figure 5-7b. Obviously, the redox reactions

153
of the PPy-Fc film undergo two reaction steps. A redox couple of PPy appeared at the negative potential region, and that of Fc appeared in the positive potential region. The redox characteristic of Fc was not obvious, it is probably due to little Fc in the polymer film. Or the mobility of phosphate ion in the polymer backbone was too low to enhance the conductivity of the polypyrrole backbone.

\[ \text{E(V) vs SCE} \]

**Figure 5.7** Cyclic voltammograms of PPy-Fc. Scan rate: 100mV/s.
(a) CV polymerisation of PPy-Fc film in 0.2mM Fc, 0.2M HNO\textsubscript{3} and 0.1M pyrrole. Sweep segments: 50. Every 5th cycle is shown.
(b) Cyclic voltammetry of film ‘a’ in 0.1M phosphate buffer.

### 5.3.2 Biosensing Membrane Fabrication

The fabrication of PPy-FDH/NAD/Fcc via a galvonostatic mode was monitored by in situ chronopotentiometry. As shown in Figure 5-8 the pretreatment of the enzyme solution by filtration through a 0.45μm filter membrane had an influence on the chronopotentiogram. This treatment reduced the cell potential dramatically during the galvanostatic growth. This is due to the
presence of other substances in the FDH and NAD solution which contributed to the high potential observed in the unfiltered medium. The lower cell potential would ensure that the destruction of the catalytical activity of the enzyme does not occur\[39].

![Graph showing potential vs time for filtered and unfiltered solutions.]

**Figure 5-8** Chronopotentiograms of PPy-FDH/NAD/Fcc films. 10 units FDH, 5.0mM NAD, saturated Fcc and 0.3M pyrrole in 5mL monomer solution. A current density of 1.5mA/cm\(^2\) for 400s was employed.

5. 3. 3. Electrochemistry of Biosensing Membrane

5. 3. 3. 1. Cyclic Voltammetry

Cyclic voltammograms in Figure 5-9 show different behaviours of PPy-FDH/NAD/Fcc films in various solutions. These results suggest that Cl\(^-\) has a significant influence on the behaviour of the film. It plays a main role in enhancing the conductivity of the polypyrrole backbone, and assisting electrons transfer in redox reactions. As shown in Figure 5-9, 0.1M KCl gave the more conductive film, compared to 0.01M KCl. 0.1M and 0.01M phosphate buffer solutions almost gave the same cyclic voltammograms in the presence of 0.1M KCl. This indicates that the conductivity of the PPy-FDH/NAD/Fcc film is most contributed by the movement of Cl\(^-\) in the polypyrrole backbone. Obviously, it gives a evidence to support a hypothesis in Figure 5-7b. The conductivity of a PPy backbone can be improved by introducing a electrolyte into its backbone via (1) electropolymerisation, and (2) diffusion.
Figure 5.9  Cyclic voltammograms of PPy-FDH/NAD/Fcc electrodes in various of media. Scan rate: 100mV/sec. Other conditions same as Figure 5-8.
Figure 5-10 shows that two redox processes occurred on the PPy-FDH/NAD/Fcc film in phosphate buffer/KCl solution (pH 7.0); one in the negative potential region and the other in the positive region. It was demonstrated in previous work (Chapter 4) that the characteristic oxidation and reduction couple of PPy appeared at the negative potential region. This therefore suggests that the second redox process observed in the positive potential region is associated with oxidation/reduction couple for Fcc.

It is well known that electrochemically reduced PPy is unstable. As shown in Figure 5-10, the waves obtained for the PPy redox reactions decreased with repeated cycles while those of Fcc redox reactions increased. The enhancement of the Fcc redox process may be due to the enhancement of the electron transfer in the positive potential region. As result of Cl⁻ penetration of the PPy backbone. In contrast, Cl⁻ tended to migrate out of the PPy backbone in the negative potential region. This further indicates that PPy is more conductive in the positive potential region rather than in the negative one.

![Cyclic voltammograms of PPy-FDH/NAD/Fcc electrode in 0.1M KCl/0.01M phosphate buffer (pH 7.0). Scan rate: 100mV/sec. Other conditions same as Figure 5-8.](image-url)
5.3.3.2. Batch Amperometric Measurement

The existence of a double-layer capacitance or the presence of a charging current in electrochemical experiments cannot be ignored. Indeed, during electrode reactions involving very low concentrations of electroactive species, the charging current can be much larger than the faradaic current for the reduction or oxidation reaction. For this reason, the total current output \( I_t \) is the sum of the faradaic current \( I_f \) and the charging current \( I_c \):

\[
I_t = I_f + I_c
\]  
(5-1)

A suddenly change in charging current during amperometric measurements would result in a non-linear calibration curve of an analytical application, and a negative peak as discussed in Chapter 4.

Figure 5-11 shows that charging current (background) was decreased by spiking KCl solution into a batch solution (0.01M phosphate buffer only). It suggests that ionic-strength could be a considerable factor in the interfacial reaction. It is therefore necessary that a certain amount of Cl\(^-\) should be added into the buffer solution for amperometric detection. As indicated in Figure 5-9, 0.1M Cl\(^-\) gives the significant electron transfer during the redox reactions, compared to 0.01M Cl\(^-\).

![Graph showing amperometric response](image)

**Figure 5-11** Amperometric response of Fcc mediated polypyrrole-based formate biosensor in 0.01M phosphate buffer. Applied potential was +0.40V. Other conditions same as Figure 5-8.
Figure 5-12 shows the time dependence of the steady-state catalytic current at a PPy-FDH/NAD/Fcc electrode. The electrode was stored in a 0.1M KCl/0.01M phosphate buffer solution at room temperature and occasionally measured the steady-state current upon addition of 1mM formate. The rapid decrease in the catalytic current in the initial hours is probably due to desorption of the loosely bound enzyme from the surface region of the PPy membrane, resulting in slow decline of the current with time. It suggests that the entrapped enzyme and mediator did not desorb from the interior of the PPy because of its large molecular size.

\[ y = 50.466x^{-0.456} \quad r = 0.997 \]

Figure 5-12 A plot of 1mM formate amperometric response as the function of the membrane storage in 0.1M KCl/0.01M phosphate buffer (pH 7.0) at room temperature. Applied potential: 0.4V. Other conditions same as Figure 5-8.

Figure 5-13 showed that the PPy-FDH/NAD/Fcc biosensor is more sensitive than a bilayer PPy-FDH/NAD/[Fe(CN)_6]^{4-} biosensor (Figure 3-32). It further confirmed that the structure of Fcc is more favourable for the stable immobilisation of the mediator in the hydrophobic polypyrrole matrix than its of ferrocyanide. Furthermore, the rate constant of the electron transfer between the mediator and the prosthetic group was improved.
Figure 5-13  The amperometric response of an Fcc mediated polypyrrole-based formate biosensor in 0.1M KCl/0.01M phosphate buffer (pH 7.0). Applied potential was +0.40V. Other conditions same as Figure 5-8.

5.3.3.3. Flow Amperometric Biosensing

As discussed in section 5.1., the structure of the immobilised molecule influenced the stable immobilisation of the mediator in the hydrophobic
polypyrrole matrix. Compared to mediators, the enzyme is hardly desorbed from the interior of the PPy because of its large molecular size and favourable peptide chains for the stable immobilisation in the hydrophobic polypyrrole matrix. The presence of a suitable mediator in the PPy membrane enhances the response as is observed with a mediator in solution. The data in Table 5-4 illustrate that the maintenance of the mediator in the PPy membrane plays a significant role in the amperometric biosensing of formate. In the absence of a mediator in the mobile phase, the amperometric currents decreased dramatically to zero in 2 hours as demonstrated in Table 5-4 and Figure 5-14.

<table>
<thead>
<tr>
<th>Current (nA)</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td></td>
</tr>
<tr>
<td>KCl/phosphate buffer</td>
<td>79</td>
</tr>
<tr>
<td>KCl/phosphate buffer/ferrocence</td>
<td>173</td>
</tr>
</tbody>
</table>

*Amperometric response of 5mM formate in an FIA system with a flow rate of 1mL/min. Injection volume was 10μL. Applied potential was +0.4V.

#The pyrrole concentration was 0.3M mixed in 5 units FDH, 5mM NAD and saturated Fcc. A current density of 0.5mA/cm² for 500s on a gold electrode was employed.

After 6 hours of amperometric measurements, the responses almost declined to nil as shown in Figure 5-15. It indicated that some enzyme and co-factor were lost from the PPy-FDH/NAD/Fcc membrane upon application of a positive potential (+0.4V), since the mediator concentration was maintained at 1mM in a mobile phase. This phenomenon is due to the positive charged FDH which was merely trapped in the interstices of the polymer network and not intimately bound to the polymer backbone. Such loss of enzyme will reduce sensitivity and, also affect the stability and linear range of the PPy-FDH/NAD/Fcc biosensor. A similar phenomenon, but at a negative potential, was found for a sulphite biosensor[61].
Figure 5-14  The stability of the amperometric response of 5mM formate in 0.1M KCl/0.01M phosphate buffer solution (pH 7.0). Other conditions same as Table 5-4.

Figure 5-15  The stability of the amperometric response of 5mM formate in 0.1M KCl/0.01M phosphate buffer and 1mM ferrocene carboxylic acid (pH 7.0). Other conditions same as Table 5-4.
A monolayer PPy-FDH/NAD/Fcc biosensor electrode which was used in a batch measurement can be also incorporated into a flow injection system. This approach dynamically combined two techniques into an integral form. 10μL of sample was injected into a flow stream, and then delivered to a mixing chamber for further enzymatic reactions. It results in a very broad analytical range for formate, as shown in Figure 5-16.

The potential of 0.4V was applied for 1 hour at a biosensing electrode to reach an equilibrium state, with respect to stabilisation of the charging current, i.e., the constant background current. It is due to a potential step input, current for charging the double-layer capacitance decays exponentially, as presented in Equation 5-2.

\[ i = \frac{E}{R_s} e^{-\frac{t}{R_sC_u}} \]  

Amperometric responses declined dramatically. Figure 5-16, for example, showed the signals of 10mM formate decreased by 30% after about half an hour. It unfortunately limited the biosensor application for long-time measurements due to the instability of a polypyrrole-based biosensor. However, there is a potential to develop a disposable biosensor regardless of the unstable background with the aid of patterns recognition which will be demonstrated in the following chapter.

A flow injection system was used to investigate the permeability of a PPy-FDH/NAD/Fcc membrane to electroactive species, such as ascorbic acid, oxalic acid and uric acid. This membrane has the ability to prevent direct oxidation of these species on a modified enzymatic electrode. This is due to a kinetic discrimination providing the possibility of selective enhancement of enzymatic chemistry in the stream. Biological interferants do not have enough time to penetrate the membrane, and can be flushed out of the mixing chamber, rather than oxidised on the gold surface. The PPy-based enzyme membrane did not oxidise these interferents at the applied potential of 0.4V. It is possible that the potential applied to the biosensor is less than 0.4V, due to the contribution from the capacitance and resistance of the film. At any time the sum of voltages across the resistor and capacitor must be equal to the applied voltage; hence:

\[ E = E_R + E_C = iR + \frac{q}{C} \]  

\( E \): total potential applied, \( E_R \): potential on a resistor, \( E_C \): potential on a capacitor, \( i \): current through a resistor, \( R \): resistance of a resistor, \( q \): electric charge of a capacitor, \( C \): capacitance of a capacitor.
Therefore the emergence of a PPY-based enzyme electrode and an FIA system provides a lot advantages for analytical applications and mechanistic studies.

**Figure 5-16** The amperometric response of a formate biosensor recorded in 1mL/min mobile phase of 0.1M KCl/0.01M phosphate buffer (pH 7.0). Other conditions same as Table 5-4.
Such an approach was applied to test a swamp water sample from the native wetlands at Kingswood (NSW, Australia) by a standard addition method. It provides a reliable method to determine the concentration of formate in an environmental sample. As illustrated in Figure 5-17, the concentration of formate was approximately 5mM.

Figure 5-17  Analysis of a swamp water sample by a standard addition method. Other conditions same as Figure 5-16
5. 4. Ferrocene Mediated Bilayer Biosensors

5. 4. 1. Ferrocene Mediated Biosensor

Enzymes cannot be dissolved in nitric acid solutions of ferrocene as this medium will cause changes in proteins conformation and lost of bioactivity of an enzyme\[63\]. The bilayer configuration developed in previous chapters can be used to polymerise a mediator and enzyme film separately in different monomer solutions, thus overcoming the problem of enzyme denaturation in acid media.

Two monomer solutions were used for galvanostatic polymerisation, as shown in Table 5-5. Gold substrate was immersed in monomer solution A for electropolymerisation of first layer, and then the polymer was rinsed several times with Milli-Q water to remove any weakly bound molecules. The electrode was then placed in monomer solution B for growing the enzyme-polymer layer.

Galvanostatic polymerisation was performed after deoxygenation of the monomer solution with N\(_2\) for 10 minutes, using a current density of 1.5mA/cm\(^2\) and a polymerisation period of 200 second. All polymers were produced without stirring.

Table 5-5 The composition of monomer solutions

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3M Pyrrole</td>
<td>0.3M pyrrole</td>
</tr>
<tr>
<td></td>
<td>2.5mM Ferrocene</td>
<td>5.0mM NAD</td>
</tr>
<tr>
<td></td>
<td>40mM HNO(_3)</td>
<td>10units FDH</td>
</tr>
<tr>
<td></td>
<td>5mL H(_2)O</td>
<td>5mL H(_2)O</td>
</tr>
</tbody>
</table>

Figure 5-18a shows a sharp increase in the electrode potential at the start of the galvanostatic polymerisation of PPy-Fc/NO\(_3\)^+ film. As the polymerisation proceeds the electrode potential decreased slightly and remained fairly constant as a function of time during the growth of the polymer film. This observation is similar to previous results. It indicates that the film became more conductive as the polymerisation period is extended. Figure 5-18b shows a
quite different chronopotentiogram to the one in Figure 5-18a, particularly in the first 10 seconds. The electrode potential increased sharply within 0.5 second, and then stabilised at 0.977V after 8.0 seconds. The higher steady state potential observed in this case is due to the fact that the enzyme and co-factor (FDH and NAD), as macromolecules, produce solution of low conductivity solutions when dissolved in water without the addition of an electrolyte.

Figure 5-18  Choronpotentiograms of Fe-mediated PPy-based membranes formed in galvanostatic mode. (a) PPy-Fe/HNO₃ monolayer, (b) PPy-Fe/HNO₃ + PPy-NAD/FDH bilayer. A current density of 1.5mA/cm² for 200 second was employed. The composition of monomer solutions are illustrated as Table 5-5.
The cyclic voltammograms obtained for the monolayer and bilayer polypyrrole films in 0.1M KCl/0.01M phosphate buffer (pH7.0) solution are illustrated in Figure 5-19. The ferrocene oxidation potential was dramatically shifted from -0.10V to +0.18V, after growing the enzyme layer on top of the ferrocene layer. This observation indicates that the second layer membrane may prevent KCl from penetrating into the ferrocene layer.

![Cyclic voltammograms](image)

**E(V) vs SCE**

**Figure 5-19** Cyclic voltammograms of ferrocene mediated polypyrrole-based membranes in 0.1M KCl/0.01M phosphate buffer. (a) PPy-Fc/HNO₃ monolayer, (b) PPy-Fc/HNO₃ + PPy-NAD/FDH bilayer. Scan rate: 100mV/s.

As shown in Figure 5-19b, ferrocene is oxidised at about 0.18V vs SCE. Steady-state current measurements for formate were therefore recorded at 0.4V vs SCE after 45min equilibration. Figure 5-20 shows a typical chronoamperogram for varying concentrations of formate with the PPy-FDH/NAD/Fc electrode.

As previously mentioned, Cl⁻ plays a significant role in a polypyrrole matrix. Figure 5-20 further indicates that the migration of Cl⁻ affected the current of electrochemical cell between the working and auxiliary electrodes, due to some interfaces in an electrolyte solution.
Figure 5-20 Chronoamperogram of ferrocene mediated polypyrrole-based membrane in 0.1M KCl/0.01M phosphate buffer at 0.4V. 1.0mM formate was spiked in batch solution for three times, and then followed by 1.0mM KCl spiking.

5.4.2. Ferrocene Carboxylic Acid Mediated Biosensor

As discussed in section 5.3., a monolayer Fcc-mediated biosensor gave very promising results. Further investigation of the Fcc-mediated biosensor in a bilayer arrangement was undertaken to improve its performance. Galvanostatic polymerisation procedures were the same as in section 5.4.1., two monomer solutions were replaced as shown in Table 5-6.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3M Pyrrole</td>
<td>0.3M pyrrole</td>
</tr>
</tbody>
</table>
| Ferrocene Carboxylic Acid (Saturated) | 5.0mM NAD
|                              | 10units FDH |
| 5mL H₂O                     | 5mL H₂O    |

Table 5-6 The composition of monomer solutions
The gold substrate was immersed in monomer solution A for electropolymerisation of the first layer. The electrode potential increased sharply up to 1.367V in 0.5 second, and then grew to 1.574V after 7.5 seconds. As the polymerisation of PPy-Fcc film proceeded the electrode potential decreased down to 1.40V after 36.5 seconds and then remained fairly constant. The shape of the chronopotentiogram, shown in Figure 5-21a, is similar that of PPy-Fc (Figure 5-18b), except for the different electrode potentials. This higher potential observed in the case of ferrocene carboxylic acid results in lower conductivity of the PPy-Fcc film, and affected the growth of the PPy-FDH/NAD film. This observation indicates saturated ferrocene carboxylic acid does not produce a conductive solution when dissolved in water, as previously discussed.

The PPy-Fcc film was rinsed several times with Milli-Q water to remove any weakly bound molecules. The electrode was then placed in monomer solution B for the growth of the enzyme layer. Figure 5-21b shows a sharp increase in the electrode potential at the start of the galvanostatic polymerisation of the PPy-FDH/NAD film onto PPy-Fcc film. As the polymerisation proceeded the electrode potential decreased slightly down to 1.02V and remained fairly constant as a function of the time during the growth of the polymer film. This observation is similar to that of the PPy-Fc/NO₃⁻ film in section 5.4.1., but the electrode potential is higher.

When the potential of the enzyme-modified electrode was measured against the SCE during the passage of current, a voltage drop equal to \( iR_s \) will always be included in the measured potential. Here \( R_s \) is the solution resistance between the electrodes which behaves as an ideal resistance over a wide range of conditions. It indicates low conductive solutions show high potentials in chronopotentiograms monitored by in situ potentiometry. The total potential monitored can be simply presented as follows:

\[
E_{total} = E + iR_{sol} = E_{PPy} + \eta + iR_{sol}
\]  \hspace{1cm} (5-4)

It does not mean the polypyrrole is overoxidised at very high overall potential shown in a chronopotentiogram, because most of potential contributed to the monomer solution is due to high resistance of a monomer solution.
Figure 5-21  Chronopotentiograms of ferrocene carboxylic acid mediated polypyrrole-based membranes formed in galvanostatic mode. (a) PPy-Fcc monolayer layer, (b) PPy-Fcc + PPy-NAD/FDH bilayer. A current density of 1.5mA/cm² for 200 second was employed. The composition of monomer solutions are illustrated as Table 5-6.
Cyclic voltammograms further proved the conductive property of the PPy-Fcc + PPy-FDH/NAD bilayer as shown in Figure 5-22. Two redox processes occurred, one in the negative potential region and the other in the positive region. The characteristic oxidation and reduction couple of PPy appeared at the negative potential region, while the second redox process observed in the positive potential region is associated with oxidation/reduction couple for Fcc.

Figure 5-22 shows a narrower cyclic voltammogram compared with Figure 5-10. This indicates that the conductivity of the PPy-Fcc + PPy-FDH/NAD bilayer is less than that of PPy-Fcc/FDH/NAD monolayer due to the interface between the PPy-Fcc and PPy-FDH/NAD layers.

![Cyclic voltammogram](image)

**Figure 5-22** Cyclic voltammogram of Fcc-mediated polypyrrole-based membrane in 0.1M KCl/0.01M phosphate buffer. Scan rate: 100mV/s. Other conditions same as Figure 5-21.

As found in sections 3.3.1.2., 3.4.7. and 4.4.2., the current density and polymerisation period control the permeability and thickness of the polypyrrole membrane during the galvanostatic polymerisation. It is interesting to find that the permeability and thickness of the inner PPy-Fcc layer affect the characteristics of Fcc redox reactions, though the outer PPy-FDH/NAD is kept same. It was interesting to investigate the effect of the parameters of galvanostatic films formation as shown in Table 5-7 and Figure 5-23. As discussed in previous sections, the use of the higher current density for polymerisation produces the more permeable film, and then enhancing charge transfer within a film. It was evident again that the same thickness of
PPy-Fcc films as shown in Figure 5-23a and 5-23b but the different permeability and porosity gave different electrochemical behaviour and may be indicative of the different electroactivity of the polypyrrole films. Furthermore, the same current density but a different polymerisation period will result in the different thickness of PPy-Fcc films, which affected the cyclic voltammograms as shown in Figure 5-23b and 5-23d. The cyclic voltammograms highlighted the higher conductivity of a thick polypyrrole film, due to the more polarons (PPy⁺) and bipolarons (PPy²⁺) sites produced and the more occupied sites next to unoccupied ones in a thick film.

Table 5-7 The parameters of galvanostatic films formation

<table>
<thead>
<tr>
<th></th>
<th>PPy-Fcc</th>
<th>PPy-FDH/NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.5mA/cm² for 200</td>
<td>1.5mA/cm² for 200s</td>
</tr>
<tr>
<td>b</td>
<td>0.5mA/cm² for 600</td>
<td>1.5mA/cm² for 200s</td>
</tr>
<tr>
<td>c</td>
<td>1.0mA/cm² for 200</td>
<td>1.5mA/cm² for 200s</td>
</tr>
<tr>
<td>d</td>
<td>0.5mA/cm² for 200</td>
<td>1.5mA/cm² for 200s</td>
</tr>
</tbody>
</table>

![Graph showing cyclic voltammograms](image)

**Figure 5-23** Comparison of cyclic voltammograms of ferrocene carboxylic acid mediated polypyrrole-based membranes in 0.1M KCl/0.01M phosphate buffer (pH 7.0). Scan rate: 100mV/s. The parameters of galvanostatic films formation is illustrated in Table 5-7. Monomer solution same as Table 5-6.
Therefore the concepts of electroneutrality coupling and electron-hopping as discussed earlier provide the basis for understanding the electrochemical behaviour of a polypyrrole-based biosensor associated with a mediator for transferring electrons.

Figure 5-24 shows the current-time curve formate response for PPy-Fcc and PPy-FDH/NAD bilayer membrane electrodes. The measured current increased immediately after the addition of formate and reached a steady state within a few seconds. The sensitivity of the formate response on the bilayer membrane electrode was close to that on the monolayer membrane electrode (Figure 5-15). It proves that Fcc does not leach easily from a hydrophobic polypyrrole backbone due to its structure. The major difference is the background current (charging current) between the two figures. The much higher resistance in a polypyrrole backbone with bilayer membrane is demonstrated in Figure 5-24, as described in the Ohm’s law as follows:

\[
I = \frac{U}{R}
\]  

(5-5)

**Figure 5-24** Chronoamperogram of ferrocene carboxylic acid mediated polypyrrole-based membrane in 0.1M KCl/0.01M phosphate buffer (pH 7.0) at 0.4V. 1.0mM formate was spiked in batch solution for three times. Other conditions same as Figure 5-21.
5. 5. Polypyrrole-Prussian Blue Bilayer

5. 5. 1. Electrochemical Formation of Prussian Blue

The electrodeposition of Prussian Blue (PB) is normally accomplished by applying a constant potential\(^{55}\), a constant current\(^{49, 54}\), or cyclic voltammetry\(^{56}\). The film of PB on the gold electrode was prepared in an aqueous ferric ferricyanide solution of an equimol mixture of 20mM FeCl\(_3\) and 20mM K\(_3\)[Fe(CN)\(_6\)]. The gold electrode was cathodically polarised in the above ferric ferricyanide solution by means of a galvanostatic condition. The analytical utility of PB is directly related to its structure and general redox characteristics\(^{64}\), such as the physico-chemical properties and fundamental electrochemistry of polynuclear transition metal hexacyanoferrates. The crystal structure of PB is a face-centred cubic lattice with a cell constant of 10.2Å, indicating a very roomy crystal structure\(^{65}\). PB has been known as a zeolite with channel diameters of about 3.2Å. It has been proven for only hydrated ions of K\(^+\), Rb\(^+\), Cs\(^+\) and NH\(_4\)^+ to transport through the crystal of PB during the reduction of the high-spin iron Fe\(^{3+}/Fe^{2+}\)^{51}. High-spin iron redox property is very useful for a mediated biosensor because of its low applied potential at 0.2 vs SCE. The electron transfer mechanism for the waves 0.2V have been formulated as follows, assuming the formula of Fe\(_4^{3+}\)[Fe\(_{II}\)(CN)\(_6\)]\(_3\) for PB\(^{49, 54}\):

\[
Fe_4^{3+}[Fe_{II}(CN)_6]_3 + 4e^- + 4K^+ \rightleftharpoons K_4^{+}Fe_4^{2+}[Fe_{II}(CN)_6]_3
\]  

(5-6)

Therefore the movement of the hydrated K\(^+\) in a crystal lattice results in increasing the conductivity of PB in an aqueous solution.

The galvanostatic transient behaviour for the growth of PB on gold is shown in the left column of Figure 5-25 as monitored by in situ chronopotentiometry vs SCE. A mechanistic study of the chemical reaction would be of special interest to PB. A very positive open-circuit potential in the solution of ferric ferricyanide was found to be about 0.82V vs SCE, and a similar value (0.85V) vs SCE was obtained on glassy carbon, Pt and SnO\(_2\) substrates\(^{51}\), suggesting that the mixture of the solutions is a very strong oxidant. The different chronopotentiograms as shown in Figure 5-25 indicated that different types of crystal lattice of PB were possibly formed. At the lower current density, less than 0.1mA/cm\(^2\), only a plateau was observed. The second plateau was clearly observed at about 0.4V with the current density of 0.2mA/cm\(^2\). At the higher current density, more than 0.3mA/cm\(^2\), only one plateau was observed due to overlapping of the two plateaus. The plateaus represent the reduction of FeCl\(_3\) and K\(_3\)[Fe(CN)\(_6\)] ions sequentially.
Figure 5.25  Galvanostatic transient and cyclic voltammetric behaviour of PB in 10mM ferric ferricyanide and 0.1M KCl/0.01M phosphate buffer (pH 7.0), respectively. Scan rate: 100mV/s.
And more, redox characteristics of PB were influenced by the different crystal lattice which were produced at the different current densities. The evidence is shown in the right column of Figure 5-25, cyclic voltammograms were responsible for the electron transfer during the redox reactions of high-spin iron ions in 0.1M KCl/0.01M phosphate buffer. This experimental result indicates that the current density of 0.2mA/cm² in 30s, which has a great impact on the occurrence of a 0.4V plateau in a chronopotentiogram, is a turning-point of the different PB lattice.

5.5.2. Prussian Blue Based Polypyrrole Bilayer

The bilayer configuration described previously was adopted in fabricating a Prussian Blue based biosensor. PB can be as a mediator to shuttle electrons between the enzymes and gold substrate. An enzyme and co-factor can be easily incorporated into a polypyrrole backbone via electropolymerisation. Two kinds of bilayer structure were used to investigate the stability of PB associated with PPy. The ferric ferricyanide and pyrrole monomer solutions used for this are given in Table 5-8. The conditions for potentiostatic deposition of PB and galvanostatic polymerisation of PPy parameters are given in Table 5-9.

<table>
<thead>
<tr>
<th>Table 5-8</th>
<th>The composition of monomer and PB solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td>PPy-FDH/NAD</td>
</tr>
<tr>
<td>10mL of 20mM FeCl₃</td>
<td>0.3M pyrrole</td>
</tr>
<tr>
<td>10mL of 20mM K₃[Fe(CN)₆]</td>
<td>5.0mM NAD</td>
</tr>
<tr>
<td></td>
<td>10units FDH</td>
</tr>
<tr>
<td>20mL H₂O</td>
<td>5mL H₂O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5-9</th>
<th>The parameters for the potentiostatic and galvanostatic formation of the films</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td>PPy-FDH/NAD</td>
</tr>
<tr>
<td>0.4V for 30s</td>
<td>0.2mA/cm² for 60s</td>
</tr>
</tbody>
</table>
The PB-mediated enzyme electrode was firstly fabricated as immobilised FDH and NAD onto a gold substrate, and followed by depositing a PB film onto a PPy-FDH/NAD film. The stability of PB was examined by cyclic voltammetry. Figure 5-26 illustrated the reproducibility of the cyclic voltammograms, and indirectly presenting the stability of a biosensing membrane. An anodic sharp spike at 0.2V was observed during the first cycle. A redox reaction seems to be strongly assisted by the structures of conductive polypyrrole. However, the sharp current decreased with repeated scanning. PB formed electrochemically on a PPy-FDH/NAD surface loses reactivity on repeated redox reaction. This may be due to the fact that the water-insoluble PB, $\text{Fe}_4^{3+}[\text{Fe}^{II}(\text{CN})_6]_3$, was reduced to $\text{K}_4\text{Fe}_4^{2+}[\text{Fe}^{II}(\text{CN})_6]_3$, Prussian White which is dissolved in 0.1M KCl/0.01M phosphate buffer (pH7.0) solution. It is also possible that some PB remained in PPy pores and, hence maintained the stable redox waves after 12 cycles.

A modification of the above electrode was fabricated by depositing a PB film first and then followed by a PPy-FDH/NAD film to protect water-soluble, $\text{K}_4\text{Fe}_4^{2+}[\text{Fe}^{II}(\text{CN})_6]_3$, leaching out of the membrane. Figure 5-27 indicates that PB becomes stabilised to show little change in cyclic voltammogram after 12 cycles by immobilising a PB film in the inner layer of the membrane.

A PB and PPy-FDH/NAD membrane coated glassy carbon electrode was further scanned up to 1.1V. Figure 5-28 shows a typical cyclic voltammogram in a 0.1M KCl/0.01M phosphate buffer (pH7.0). The electron transfer for the waves at 0.2V and 0.9V are as given by Equation 5-6 and 5-7, respectively.

$$\text{Fe}_4^{3+}[\text{Fe}^{II}(\text{CN})_6]_3 - 3e^- + 3\text{Cl}^- \Leftrightarrow \text{Fe}_4^{3+}[\text{Fe}^{III}(\text{CN})_6\text{Cl}^-]_3$$

(5-7)

It should be pointed out that the waves at 0.2V and 0.9V are due to the redox reactions for the high-spin iron ions, $\text{Fe}^{3+/2+}$, and the low-spin iron ions, $\text{Fe}^{III/II}(\text{CN})_6^{3+/4+}$, in the PB film[52].
Figure 5-26  Cyclic voltammograms of PPy-FDH/NAD + PB bilayer membrane in 0.1M KCl/ 0.01M phosphate buffer. Scan rate: 50mV/s. Cycles: 12. Other conditions same as Table 5-8 and 5-9.
Figure 5-27  Cyclic voltammograms of PB + PPy-FDH/NAD bilayer membrane in 0.1M KCl/ 0.01M phosphate buffer. Other conditions same as Figure 5-26.
Figure 5-28 Cyclic voltammogram of PB + PPy-FDH/NAD bilayer membrane coated onto a glassy carbon electrode in 0.1M KCl/0.01M phosphate buffer. Other conditions same as Figure 5-27.

5.5.3. Amperometric Responses

As discussed above, the high-spin iron ions in Prussian Blue are oxidised at about 0.2V vs SCE. Steady-state current measurements for formate were therefore recorded at 0.4V vs Ag/AgCl after 9 hour equilibrating. Figure 5-29 shows a typical chronoamperogram for varying concentrations of formate with the PB + PPy-FDH/NAD electrode.

Furthermore, the PB + PPy-FDH/NAD electrode was incorporated into a flow cell. Hydrodynamic current measurements for formate were carried out at 0.4V vs Ag/AgCl after equilibration for 3 hour to minimise the charging current of the amperometric response for formate as shown in Figure 5-30.

The Prussian Blue film formed electrochemically shows good inherent chemical and physical stability, and provides high electrocatalytic activity and sensitivity of electrochemical response to formate. This offers the advantage of producing amperometric biosensors, including a simple preparation procedure and easy control of the film thickness and the enzymes loading. There are, in principle, no limitation in the use of other enzymes for Prussian Blue based amperometric biosensors.
Figure 5-29  Amperometric response of a PB/PPy-based formate biosensor in 0.1 M KCl/0.01M phosphate buffer. Applied potential: 0.4V. Potentiostatic and galvanostatic parameters were 0.4V for 15s and 0.2mA/cm$^2$ for 300s, respectively. Other conditions same as Table 5-8.

Figure 5-30  Amperometric response of a PB/PPy-based formate biosensor in 0.1 M KCl/0.01M phosphate buffer when a flow rate was 1.0mL/min. Injection volume: 10µL. Applied potential: 0.4V. Potentiostatic and galvanostatic parameters were 0.4V for 20s and 0.5mA/cm$^2$ for 500s, respectively. Other conditions same as Table 5-8.
5.6. Conclusions

The concept of ferrocyanide-mediated formate biosensor is further extended to ferrocene derivatives and Prussian Blue mediators for transporting the electrons. A broad linear range was obtained from ferrocene carboxylic acid mediated formate biosensor, as shown in Figure 5-16. The major achievements in this chapter are not only of interest in analytical chemistry, but also fundamental aspects of electron and ion conduction that constitute the basis for understanding the electrochemical behaviour of a polypyrrole-based biosensor associated with a mediator for transporting electrons. The mediators, such as ferrocyanide, ferrocene derivatives and Prussian Blue, can be classified as an inorganic coordinator, an organic complex and a zeolite, respectively. This indicates that a biosensing research can be integrated into a number of academic disciplines.

5.7. References


184


CHAPTER 6

IMPROVEMENT OF THE
PERFORMANCE OF A FORMATE
BIOSensor BY USE OF
ARTIFICIAL NEURAL NETWORKS
6. 1. Introduction

With the new wave of research in the fields of artificial neural networks and learning systems there is a renewed hope that there will emerge artificial systems in the future with the capability of learning from experience in a similar manner as humans. But the human brain is used for a number of different tasks, such as logical thinking and advanced pattern recognition in addition to dynamic control, and there is no reason to believe that a learning system specialised for dynamic control need to have all the capabilities of biological neural systems. The study and modelling of biological neural networks may undoubtedly give us hints to how things could be done and lead to totally new computing paradigms, but these models should not limit our creativity when trying to design learning systems for specific areas.

The development of novel instruments for the rapid assessment of complex environmental and biochemical conditions has great potential, particularly in the determination of intermediates in biological systems with a wide sphere of influence. It offers the possibility of assessing real conditions with a rapid measurement in place of a lengthy or complex procedure. This effort is worthwhile in offering the possibility of acquiring information that was previously inaccessible, rather than just a more convenient way of acquiring and processing data that have been accessible to routine measurement for many decades.

6. 1. 1. Artificial Neuron Model

Artificial neural networks (ANN) have emerged from studies of how human and animal brains perform operations. The human brain is made up of many millions of individual processing elements, called neurons, that are highly interconnected. A schematic diagram of a single biological neuron is shown in Figure 6-1. Information from the outputs of other neurons, in the form of electrical pulses, are received by the cell at connections called synapses. The synapses connect to the cell inputs, or dendrites, and the single output of the neuron appears at the axon. An electrical pulse is sent down the axon (i.e., the neuron 'fires') when the total input stimuli from all of the dendrites exceeds a certain threshold[1].

188
Artificial neural networks are made up of individual models of the biological neuron (artificial neurons or nodes) that are connected together to form a network. The neuron models that are used are typically much simplified versions of the actions of a real neuron. Information is stored in the network often in the form of different connection strengths, or weights, associated with the synapses in the artificial neuron models.

The most commonly used neuron model is depicted in Figure 6-2 and is based on the model proposed by McCulloch and Pitts[2]. Each neuron input, \( x_1 - x_N \), is weighted by the values \( w_1 - w_N \). A bias, or offset, in the node is characterised by an additional constant input of 1 weighted by the value \( w_0 \). The output, \( y \), is obtained by summing the weighted inputs to the neuron and passing the result through a non-linear activation function, \( f(\cdot) \):

\[
y = f\left\{ \sum_{i=1}^{N} w_i x_i + w_0 \right\}
\]  

(6-1)
Various types of non-linearity are possible and some of these are shown in the diagram (e.g., hard limiter, threshold logic, sigmoidal and tanh functions).

6.1.2. Multi-Layer Perceptron

The most popular neural network architecture is the multi-layer perceptron. The network consists of an input layer, a number of hidden layers (typically only one or two hidden layers are used) and an output layer as shown in Figure 6-3. The output and hidden layers are made up of a number of nodes as described in section 6.1.1. However the input layer is essentially a direct link to the inputs of the first hidden layer and is included by convention. The outputs of each node in a layer are connected to the inputs of all of the nodes in the subsequent layer. Data flows through the network in one direction only, from input to output; hence, this type of network is called a feedforward network.
The network is trained in a supervised fashion. This means that during training both the network inputs and required, or target, outputs are used. A number of algorithms have been proposed for training the MLP and the most popular is the back-propagation algorithm\cite{3}. With this algorithm a set of input and corresponding output data is collected that the network is required to learn. An input pattern is applied to the network and an output is generated. This output is compared to the corresponding target output and an error is produced. The error is then propagated back through the network, from output to input, and the network weights are adjusted in such a way as to minimise a cost function, typically the sum of the errors squared. The procedure is repeated through all the data in the training set and numerous passes of the complete training data set are usually necessary before the cost function is reduced to a sufficiently low value.

An important feature of the MLP is that this network can accurately represent any continuous non-linear function relating the inputs and outputs\cite{4,5}. Hence, the MLP network exhibits potential for many applications, including modelling and control of real non-linear processes.
6.1.3. ANN in Analytical Chemistry

ANN techniques are able to be used in chemometrics to model the relationships in experimental data. The use of an ANN in identifying dynamic systems with highly complex nonlinear provides precise data extraction from signal measurement with very low signal-to-noise ratio\cite{6,7}. It has led to some recent advances in the process control field. The neural network adapts itself to the non-linear mechanism underlying the investigated system's input-output relationship using a hyperspace equation. During training, the coefficients of the equation are optimised such that the training data are satisfactorily fitted. Then for simulation or in situ applications, the optimised network can be used to predict system's output based on the inputs presented to it.

Its application has been demonstrated in a wide range of industrial and medical applications\cite{8,9}, spectral identification\cite{10-14}, multivariate calibration\cite{15,16}, sensors\cite{16-22} and prediction and control of chemical processes\cite{23,24}.

A new computerised approach to detection of trace concentrations of formate is considered. In this approach an integrated artificial neural network and conducting polymer biosensor is designed. The data collected (current signals) from amperometric detection of the polypyrrole formate biosensor were transferred into a MacLab® environment for data and signal processing. Later the data were transferred into an ANN trained computer for modelling and prediction of output. Such an integrated ANN/PPy-based biosensor system is capable of predicting formate concentration based on the created models and patterns. The method has advantages of being more selective and more accurate over conventional methods.
6.2. Experimental

6.2.1. Reagents

All reagents were of analytical-reagent grade, unless specified otherwise. All solutions were prepared with Milli-Q water (Millipore). Formate dehydrogenase (EC 1.2.1.2., F-8649), β-nicotinamide adenine dinucleotide (N-7004) and pyrrole (P-4892) were obtained from Sigma Chemical Co. The pyrrole was distilled prior to use. Ferrocyanide, NaCl, KCl, Na₂HPO₄, NaH₂PO₄·2H₂O and HCOONa were supplied by AJAX Chemical Pty Ltd.

6.2.2. Preparation of Polypyrrole-Based Electrodes

Polypyrrole based enzyme electrodes were prepared by galvanostatic electropolymerisation of pyrrole monomer from aqueous solution on to a gold substrate with surface area of 7mm² after purging with N₂ for 10 minutes. A three-electrode cell, consisting of a working electrode, a coiled platinum auxiliary electrode and a saturated KCl calomel reference electrode was used. The working electrode was polished with 0.3μm alumina, and then ultrasonicated for 5 minutes to remove any residual. Monomer solution contained 0.3M pyrrole, 10 units FDH, 5mM NAD and 2.5mM ferrocyanide. A current density of 1.5mA/cm² was applied to the gold electrode for 400s for this purpose.

6.2.3. Procedures of Electrochemical Measurements

The cyclic voltammetry on the enzyme film and amperometric sensing of formate were investigated with a BAS CV-27 and a BAS LC-4C in conjunction with a MacLab®, respectively. Amperometric sensing of formate with the biosensor was carried out by adding different concentrations (1, 2 and 5mM) of sodium formate at 2 min intervals. Measurements in a batch mode were made under stirred conditions, to provide convective transport.

The electrical current generated by the concentration changes was recorded with the application of a potential of 0.4V. The collected amperometric data were pre-processed for the ANN learning and predication process with the MacLab® software on an IBM computer. Turbo Neuron software was used to demonstrate the ability of ANN in model prediction of the concentration of formate on the biosensor.
6.3. Results and Discussion

6.3.1. Chemical Stimuli Recognition

As a molecule (the chemical stimuli) approaches a polymer surface, it is capable of undergoing several types of chemical interactions such as ionic, H bonding and hydrophobic interactions. The differences in the extent of these interactions is the basis of molecule (stimuli) recognition. Therefore, a polymer surface upon which selected molecular interactions are readily induced or discouraged is useful for the development of a generic recognition system. Materials such as polypyrrole can be prepared electrochemically, according to the electropolymerisation process as shown in Equation 3-2.

In this study the polymer membrane (PPy-FDH/NAD/[Fe(CN)₆]⁴⁺) was fabricated at constant current. The profile of the polymer growth is shown in Figure 6-4 where the change in the electrode potential is recorded versus time. A typical efficient growth is depicted by the sudden increase in potential to about 730mV, followed by a drop of 60mV while the polymer is growing. Since the polymer acts as a conductive material the resistance of the system decreases and the polymer electrode becomes more conductive as polymerisation proceeds. Consequently the drop in potential is an indication of the improvement in the conductivity of the polymer film with time.

![Graph showing potential vs time]

**Figure 6-4** Profile of PPy-base biosensor film grown at constant current with a current density of 1.5mA/cm² for 400s. Other conditions are explained in section 6.2.
To study the characteristics of the resulting polymer film the working electrode (gold coated with the polymer) was studied in 0.1M KCl/0.01M phosphate buffer by cyclic voltammetry. The quality of each polymer is verified by the presence of characteristic oxidation and reduction peaks, as illustrated in Figure 6-5 which showed the cyclic voltammograms of four PPy-FDH/NAD/[Fe(CN)₆]³⁺ monolayer films individually grown as shown in Figure 6-4.

Based on the established approach (PPy-FDH/NAD/[Fe(CN)₆]³⁺ monolayer biosensor) formate was detected by amperometric measurement with the application of a constant potential of 0.4V vs SCE to the biosensor. A typical profile of the amperometric signal versus time is shown in Figure 6-5 for varying concentration of formate.

From the amperograms it can be seen that baseline value of the electrical current is not stable, and it decays between addition of formate. It was found that the conventional steady-state measurement is not a reliable method for quantification of formate concentration as different amplitudes were observed for consecutive measurements of formate concentration. This was expected since the dynamic nature of the polypyrrole-based electrode was studied in a complex system consisting of enzyme, co-enzyme and mediator.

Although this trend was very disappointing for a biosensor in the stability point of view, an alternative solution, such as a computer modelling of the system using an artificial neural network for possible reliable detection, should be considered in the development of a formate biosensor.
Figure 6-5  Typical cyclic voltammetry results for four different PPy-FDH/NAD/[Fe(CN)$_6$]$^{3+}$ films in 0.1M KCl/0.01M phosphate buffer. Scan rate: 100mV/s.
Figure 6-6  Typical amperometric measurements. Applied potential: 0.4V. The current signals are for 4 or 5 consecutive addition of 1, 2 and 5mM concentration of formate on four different polymeric electrodes. The polymer was grown as shown in Figure 6-4.
6.3.2. ANN-Based Pattern Recognition and Detection

The operation of ANN involves implementation of processing nodes, that are linked to each other by variable strength connection weights. This is usually accomplished with software or special hardware on a computer. Although many neural architectures are possible, the most common one is the fully connected forward network with one input layer and two processing layers, one of which is called the 'hidden layer', the other one is the output layer. The neuron output, multiplied by the connection weights, is transferred to all neurons receiving inputs from this neuron as shown in Figure 6-3.

According to Equation 6-1 and Figure 6-2, the neuron input can be expressed as follows:

\[
\text{Neuron input} = WX + b = w_1x_1 + w_2x_2 + \ldots + w_mx_m + b
\]  \hspace{1cm} (6-2)

where \( W \) is the connection weight vector to the specific neuron, \( X \) is the input vector, and \( b \) is a bias (usually used as an extra weight to a constant input of unity).

The sum of all inputs to a neuron, if larger than a bias, activates the neuron according to an activation function. A sigmoid-shaped activation function illustrated in Figure 6-7 is usually used and this can be defined as follows:

\[
f(w, x, b) = \frac{1}{1 + e^{-\Sigma w_ix_i + b}}
\]  \hspace{1cm} (6-3)

![Figure 6-7 Feature of an artificial neuron](image-url)
ANN 'learns' from examples that are presented to the network. The ANN builds an internal model of the governing relationships embedded in the data base used for training. The training of an ANN is usually done by starting with random connection weights, presenting a known set of inputs and outputs of a system to the ANN, and adjusting the connection weights to decrease the error between the ANN outputs and the known system outputs (see Figure 6-8).

![Figure 6-8](image)

**Figure 6-8** Flow chart of an ANN modelling process

In the process shown in Figure 6-8, the general delta rule of error back propagation is

\[
W^{(new)} = W^{(old)} - \left( \frac{2\eta W^{(old)} X}{\|X\|^2} \right) X
\]

(6-4)

where \( W \) and \( X \) are the connection weights and data matrices, respectively, and \( \eta \) is a learning coefficient. This ANN modelling procedure was applied for the data collected from the formate detection experiments using Turbo Neuron software. To use this procedure the data need to be processed to match the requirement of the input data for the application of the software.
6.3.3. Data Processing

Six different experiments were carried out for the amperometric biosensing of formate. In each set of experiments, concentration of 1, 2 and 5mM were performed several times in the batch system. Consequently, 54 recorded current peaks were obtained and these were subsequently transferred (in a digital form on disk) to the data processing environment (MacLab®). To correct the baseline drift a simple ‘derivative’ of the electrical current signal was made by substrating the previous ‘nth’ value from each point. The delay ‘n’ value was chosen to be 4s, to allow for the relatively slow rise time of the signal. The highest derivative data time was taken as the centre of the response peak, and 11 data points before and after it (22 data) were chosen as the ANN training inputs. The normalisation technique employed was to zero-centre each column by substrating the mean of each data column, and then dividing it by the standard deviation of this column. This technique gives equal importance to each time measurement, and diminishes the influence of outliers caused by noise. The nominal formate concentration change, 1, 2 or 5mM, was given as the output, after a similar normalisation process with an additional reduction to the 0.1-0.9 range. The input ‘spectra’ (the selected 22 data) used with ANN are shown in Figure 6-9. The data in this figure are in fact the MacLab® processed data generated from Figure 6-6. The observed trend in Figure 6-9 supports the claim that the current peak height is not always a reliable parameter for the determination of formate concentration, as there are areas where the responses for formate concentration of 2 and 5mM are merged together.

6.3.4. ANN Training

The MacLab® development version of the algorithms incorporated in the TURBO-NEURON 1.1 software package was used to teach the ANN. Five data sets from different biosensing of the formate at different concentrations were used for the ANN training, and the data set from a sixth electrode was used as a test set to measure the generalisation capacity of the trained ANN to estimate correctly the response of a new electrode. This training was performed under the conditions as shown in Table 6-1.
Figure 6-9  Profile of the final data produced by MacLab for different concentrations to be transferred to ANN as inputs.

Figure 6-10  Profile of training and test during modelling vs error and percentage of improvements in error for prediction of the formate concentration. The training conditions are shown in Table 6-1.
Table 6-1  ANN training conditions

<table>
<thead>
<tr>
<th>Learning rule</th>
<th>Excitation</th>
<th>Training cycle (epoch)</th>
<th>Input</th>
<th>Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>conjugated gradient</td>
<td>sigmoid</td>
<td>40</td>
<td>22</td>
<td>1</td>
</tr>
</tbody>
</table>

It took only 16s on a Pentium 75MHz computer to converge to an error of 0.21 for training and 0.23 for test examples presented in Figure 6-10. Figure 6-11 also illustrates the percentage of error of 0.225 at epoch 40 (when the network adjust itself to minimise error) for the final test. Examination of the original data revealed that there were two suspicious measurements - one gave no response and the other one was due to a noise spike. By discarding both measurements and retraining (with 52 sets of data instead of 54) much better training and test results of predicted formate concentrations, as shown in Figures 6-12 and 6-13, were obtained.

![Figure 6-11](image.png)

**Figure 6-11**  Predicted output for formate concentration with the percentage of error at the last epoch (40). The prediction was carried out using the training presented in Figure 6-10.
Figure 6-12 Profile of training and test during modelling vs error and percentage of improvements in error for prediction of the formate concentration using conducting polypyrrole electrode while two suspicious data were discarded and therefore 52 current signals were considered. The training conditions are shown in Table 6-1.

According to Figure 6-12 for the same conditions applied for training, both test and training were much improved. The final error of 0.07 with 79% improvement (Figures 6-12 and 6-13) was achieved in comparison with the final error of 0.225 and 31% improvement in previous training (Figures 6-10 and 6-11).

The results for individual concentration (see Figure 6-14) also suggest a good prediction of different concentrations of formate. This demonstrates that the accuracy and flexibility of formate determination can be improved by using an integrated ANN trained computer/conducting polypyrrole-based biosensor system.

Nevertheless, the ANN was able to give quite a surprisingly good estimate in this investigation even when the differential concentration change signals were very close. This ability is carried out without any human intervention in the feature selection, with a minimum of computational and signal processing requirement.
Figure 6-13  Predicted output for formate concentration with the percentage of error at the last epoch (40) after discarding two suspicious data. The prediction was carried out using the training presented in Figure 6-10.

Figure 6-14  Comparison of target and predicted output for formate concentration using the system presented in Figures 6-12 and 6-13.
6. 4. References


205


CHAPTER 7

Conclusions and Further Recommendations
7.1. Conclusions of the Project

The multilayer membrane designs of enzyme-based electrode have been proven to be successful approach for biosensing of formate, with substantial improvement in sensitivity and selectivity. The biological interferents can be eliminated by multilayer designs and hydrodynamic analysis, prevented from oxidising on the gold substrate.

Broad linear ranges of ferrocyanide-mediated formate biosensors have been achieved by discrete layered fabrication, as summarised as follows:

![Concentration of formate (M)](image)

Conventional methods that used peak heights or plateaus as the main parameter for the determination of analyte concentration on conducting polymer sensors may in some cases be misleading. The use of an integrated ANN trained computer/conducting polymer biosensor, as demonstrated in this study, offers a unique solution to this problem. Unlike the conventional quantification method, the ANN not only considers the peak height or plateau, but it also takes into account the history of the polymer before and after the peak. Therefore, a much more reliable detection can be achieved, base on ANN modelling prediction.

7.2. Further Recommendations

7.2.1. Redox Polymers

The oxidised enzyme or co-factor can be reduced by highly flexible, insoluble redox polymers which can not diffuse out of the sensor. The use of redox polymers for mediated electron transfer can prevent the mediating species from diffusing away from the electrode surface, and the use of polymers may improve sensitivity and detection limits when the mediating reaction can take place in a volume, rather than at a surface. The electron transfer from the
active site of the polymer-entrapped enzyme to the electrode surface occurs to a first polymer-bound mediator which has sufficiently approached the prosthetic group to attain a fast rate constant for the electron-transfer reaction. In accordance with these possibilities, attempts can be made to make redox polymers which incorporate the necessary structures of a mediator.

7.3.2. Co-Polymers

Much research is being carried out on conducting polymers such as polypyrrole, polythiophene and polyacetylene. Attempts to overcome problems of instability and relatively poor mechanical properties of these polymers have been partly resolved by copolymerisation with compounds such as ionic polymers. The emphasis will be on polymers that have the ionic groups attached to the polymer matrix by chemical bonds (fixed-charge polymers). When the ionic polymers are in contact with polar solvents, the fixed ions and their counterions are solvated. An osmotic pressure develops that leads to swelling of the polymer. When the density of the fixed-charge sites is large, such polymer in contact with liquid electrolytes assume a solution like state; they constitute polyelectrolytes or polyelectrolyte gels.

7.3.3. Conductometry

Conductivity has been shown to be a good parameter to monitor. The technique has been proven to be adaptable to planar microelectrochemical sensors. Due to the high degree of miniaturisation, the signal of interest, being the resistive part of the measured admittance, tends to be masked by the overwhelming capacitive contribution. The sensor structure consists of two interdigitated thin film electrodes. There is no need in reference electrode in case of conductometric biosensors. Encapsulation requirements for conductometric biosensors are also much less stringent because driving voltage in this case is low-amplitude sine wave.