# The Fabrication of a Potentiometric Penicillin Biosensor for the Detection of β-Lactam Antibiotics in Pharmaceutical Preparations and Milk

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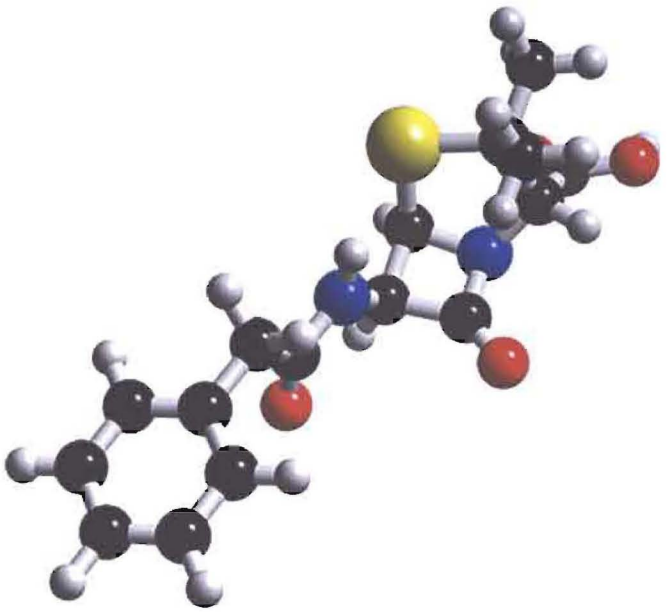

*3D structure of the Penicillin (Benzylpenicillin) molecule*

# The Fabrication of a Potentiometric Penicillin Biosensor for the Detection of β-Lactam Antibiotics in Pharmaceutical Preparations and Milk

**By**

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**B.Sc (Chem.), M. Chem. Tech. (UWS)**

*A Dissertation submitted in*
*fulfillment of the requirements for*
*the award of*
*Doctor of Philosophy*

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October 2010

# Abstract

A sensitive penicillin potentiometric biosensor was developed for the detection of β-lactam antibiotics in pharmaceutical formulations and in milk. This study outlines the approaches undertaken for the immobilisation of penicillinase (P'nase) in a number of polymer matrices. The first approach involved the immobilisation of P'nase in polypyrrole (PPy) by galvanostatic polymerisation of pyrrole (Py). The optimum conditions established for the formation of these films were 0.03 M Py, 50 U/mL P'nase, an applied current density of 0.9 $mA/cm^2$, polymerisation time of 40 s and 0.01 M penicillin (Pen) in the monomer solution. The incorporation of Pen in the monomer solution is important for the attainment of a steady state response. The biosensor was applied to raw milk samples spiked with penicillin G, as well as 3 different antibiotics, namely; abocillin 125 mg, flucoxacillin 500 mg and amoxycillin 875 mg.

The use of a PPy bilayer configuration was also investigated as a means of improving the sensitivity of the single layer biosensor. This was achieved through the immobilisation of P'nase in both the inner and outer layers which enhanced the response of the biosensor. The optimum conditions for the formation of the bilayer were: (a) outer layer: 0.1 M Py, 19 U/mL P'nase, 0.01 M Pen, current density of 0.9 $mA/cm^2$ and polymerisation time of 40 s; (b) inner layer: 0.1 M Py, 0.1 M $KNO_3$, current density of 0.9 $mA/cm^2$, polymerisation time of 40 s and 19 U/mL P'nase. The presence of the enzyme in the film was verified with the use of scanning electron microscopy (SEM) and x-ray photo electron spectroscopy (XPS).

The minimum detectable penicillin concentration with the bilayer potentiometric biosensor was 0.3 μM and the linear concentration range was 7.5 - 146 μM. The

bilayer biosensor was applied to the determination of penicillin in amoxycillin 500 mg and the average percentage recovery was 113 ± 24% indicating that the reproducibility was not very good, but similar observations were made for other bilayer configurations. Also the application of the biosensor to the determination of penicillin in milk was fraught with problems of non-specific binding of penicillin to the milk proteins resulting in poor reproducibility and low percentage recoveries.

The self-limiting growth of the non-conducting polymer, polytyramine (PTy), was exploited for the fabrication of a sensitive biosensor. The optimum conditions for the formation of the PTy-P'nase film were 0.03 M tyramine (Ty), 37 U/mL P'nase, 0.01 M $KNO_3$, 3 mM Pen, current density of 0.8 mA/cm$^2$ and an electropolymerisation time of 40 s. The linear concentration range obtained with this biosensor was 3 - 283 μM and the minimum detectable concentration was 0.3 μM. The average percentage recovery for amoxycillin of 102 ± 6%, was in close agreement with the average percentage recovery of 105 ± 5% obtained with the standard titrimetric method. Satisfactory percentage recoveries were also achieved for the detection of penicillin G in milk, particularly for concentrations ≤ 5 ppm penicillin G.

Other immobilisation methodologies, such as the cross-linking of penicillinase with glutaraldehyde (GLA) and bovine serum albumin (BSA), were also examined in this study. The optimum conditions established for the formation of the BSA-GLA-P'nase were 0.006% w/v BSA, 0.012% v/v GLA and 8 U/mL of P'nase. The linear concentration range obtained with the BSA-GLA-P'nase biosensor was 3 - 283 μM which is the same as that obtained with the PTy-P'nase electrode. The minimum detectable concentration obtained with the BSA-GLA-P'nase biosensor was 0.3 μM.

Relatively good percentage recoveries were obtained for the detection of penicillin in milk. The average percentage recovery of penicillin in amoxycillin of 103 ± 5% was also in close agreement with the results obtained with the standard titrimetric method.

P'nase was also cross-linked with poly (vinyl alcohol) (PVA) and BSA. The optimum conditions for the of BSA-PVA-P'nase film were: 2.5% w/v PVA, 0.006% w/v BSA, 2.4 mM Pen and 16 U/mL P'nase. The minimum detectable concentration was 1.7 μM. The linear concentration range obtained for the BSA-PVA film was 7.5 - 283 μM. The BSA-PVA-P'nase biosensor successfully detected penicillin in amoxycillin with an average percentage recovery of 97 ± 12 %. Higher penicillin concentrations (10 - 20 ppm) were detected more successfully than lower penicillin concentrations (≤ 5 ppm).

A comparison between the use of PTy/BSA-GLA and the PPy/BSA-PVA electrodes was undertaken. The minimum detectable concentration achieved with both electrodes was 3.3 μM. The linear concentration range obtained with the PPy/BSA-PVA and the PTy/BSA-GLA electrodes were 7.5 - 89 μM and 7.5 - 283 μM, respectively. The percentage recoveries obtained with these two electrodes was similar for the determination of penicillin in amoxycillin. The average percentage recovery obtained with the PPy/BSA-PVA electrode was 102 ± 15%, while that achieved with PTy/BSA-GLA was 100 ± 19%. These results indicated that the use of bilayer films with both conducting and non-conducting polymers, can be adequately used for the fabrication of penicillin potentiometric biosensors, but further improvement in the reproducibility is required when applied to real samples.

# List of Publications

1. Ismail, F., Adeloju, S.B., Moline, A.N., "Fabrication of a Single Layer and Bilayer Potentiometric Biosensors for Penicillin by Galvanostatic Entrapment of Penicillinase into Polypyrrole Films", Electroanalysis, submitted 2010.

2. Ismail, F., Adeloju, S.B., Moline, A.N., "Galvanostatic Entrapment of Penicillinase into Polytyramine Films and its Utilisation for the Potentiometric Determination of Penicillin", Sensors, 2010, 10, 2851-2868.

3. Ismail, F., and Adeloju, S.B., "Cross-linking Penicillinase with Glutaraldehyde and Bovine Serum Albumin for the Potentiometric Detection of Penicillin in Antibiotics and in Milk Samples", Talanta, submitted 2010.

4. Ismail, F., and Adeloju, S.B., "The Use of Poly (Vinyl Alcohol) to Cross-link Penicillinase for the Fabrication of a Penicillin Potentiometric Biosensor", Electroanalyis, submitted 2010.

5. Ismail, F., and Adeloju, S.B., "Comparison of a Polypyrrole-Based Bilayer Biosensor with a Polytyramine-Based Bilayer Biosensor for the Potentiometric Determination of Penicillin in Antibiotics and in Milk Samples" Talanta, submitted 2010.

# List of Conference Presentations

1. Ismail, F., Adeloju, S.B., "Bilayer Polypyrrole Biosensor for the Detection of Penicillin in Milk and in Pharmaceutical Preparations" *Postgraduate Research Expo,* 18th August 2004, Monash University, Melbourne, Australia

2. Ismail, F., Adeloju, S.B., "Bilayer Polypyrrole Biosensor for the Detection of Penicillin in Milk and in Pharmaceutical Preparations" *Interact-2004*, 4th-8th July 2004, Conrad Jupiters, Gold Coast, Queensland, Australia

3. Ismail, F., Adeloju, S.B., "The Development of a Potentiometric Penicillin Biosensor for the Detection of β-Lactam Antibiotics" *Interact 2002,* 21st-25th July 2002, University of Technology, Sydney, NSW, Australia

4. Ismail, F., Adeloju, S.B., Moline, A., "The Fabrication of a Bilayer Penicillin Biosensor" *CERAT 2001,* Sydney, Australia

5. Ismail, F., Adeloju, S.B., Moline, A., "Fabrication of a Polypyrrole-Based Penicillin Biosensor" *11$^{th}$ Australasian Electrochemistry Conference 2000,* 10th -14th December 2000, Sydney, Australia

6. Ismail, F., Adeloju, S.B., Moline, A., "Detection of Penicillin in Samples with a Polypyrrole-Based Penicillin Biosensor" *11$^{th}$ Royal Australian Chemical Institute Convention 2000,* 6th-11th February 2000, Canberra, Australia

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# LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| β | Beta |
| BIA | Biospecific interaction assay |
| BioFET | Bio-field-effect transistor |
| BSA | Bovine serum albumin |
| CA | Clavulanic acid |
| EIS | Electrolyte-insulator-semiconductor |
| ELISA | Enzyme-linked immunosorbent assays |
| Ev | Energy values |
| FET | Field-effect transistor |
| FPIA | Fluorescence polarisation immunoassays |
| G | Gram |
| GC | Gas chromatography |
| GCE | Glassy carbon electrode |
| GLA | Glutaraldehyde |
| HPLC | High pressure liquid chromatography |
| HPTLC | High pressure thin layer chromatography |
| LC | Liquid chromatography |
| LOQ | Limit of quantification |
| MRL | Maximum residue limit |
| μ | Micro ($10^{-6}$) |
| m | Milli |
| M | Molar |
| MWCNTs | Multi-walled carbon nanotubes |
| N | Nano |

| | |
|---|---|
| Ω | Ohm |
| oPPD | Poly (o-phenylenediamine) |
| PBPs | Penicillin–binding proteins |
| PEG | Poly (ethylene glycol) |
| Pen | Penicillin |
| PenFETs | Penicillin-sensitive field-effect transistor |
| PFA | Paraformaldehyde |
| P'nase | Penicillinase |
| ppb | Parts per billion |
| ppm | Parts per million |
| PPy | Polypyrrole |
| PTy | Polytyramine |
| PVA | Poly (vinyl alcohol) |
| Py | Pyrrole |
| RIA | Radio-immunoassays |
| secs | Second/s |
| SEM | Scanning electron microscopy |
| SPE | Screen printed electrodes |
| SPE | Solid–phase extraction |
| SPR | Surface plasmon resonance |
| TLC | Thin layer chromatography |
| U | Unit/s |
| V | Volt/s |
| XPS | X-ray photoelectron spectroscopy |

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# Chapter 1

## Introduction

## 1.1 General Introduction

The pharmaceutical and dairy industries need efficient and cost effective methods to assure the quality control of milk and antibiotics, particularly because the use of current analytical methods has proved to be time consuming and expensive. Some of these methods used to monitor penicillin include the use of bioassays for example, (Hassan et al. 1978), colorimetric (Van Opstal 1990) and titrimetric methods (Hoebus et al. 1996). However, these methods are fraught with several disadvantages, such as lengthy procedure times, high costs, extensive clean-up procedures and the inability to provide useful quantitative data. Hence, there is a need for the development of efficient devices such as penicillin biosensors that will enable rapid, cheap and reliable determination.

Penicillin G is a beta-lactam antibiotic that is used in veterinary medicine for the treatment of mastitis, urinary infections and septicaemia. The presence of excessive concentrations of penicillin in milk can have adverse effects, such as resistance to antibiotics, as well as hypersensitivity reactions in humans which can result if maximum residue levels (MRL) are exceeded. The MRL for benzylpenicillin in milk is $1.2 \times 10^{-8}$ M or 4 ppb (Thavarungkul et al. 2007). Penicillin is also used for the treatment of bacterial infections in humans. However the application of penicillin in humans is limited due to its ability to cause allergies and sensitivity in many individuals (Qin et al. 2005). These can include skin reactions and allergies that may be attributed to the presence of antigenic substances, such as penicillin polymers and the blue mould thiazole albumen that may be present as impurities (Qin et al. 2005). The determination of penicillin in milk is one way of controlling and minimising such adverse reactions.

## 1.2 The Structure and Chemistry of Penicillin

Penicillin belongs to one of the most important group of antibiotics, known as the beta-lactam antibiotics (Kadurina et al. 2003). The basic structure of penicillin consists of a thiazoldine ring, an attached beta-lactam ring and a side chain (R), as shown below in Figure 1.1.

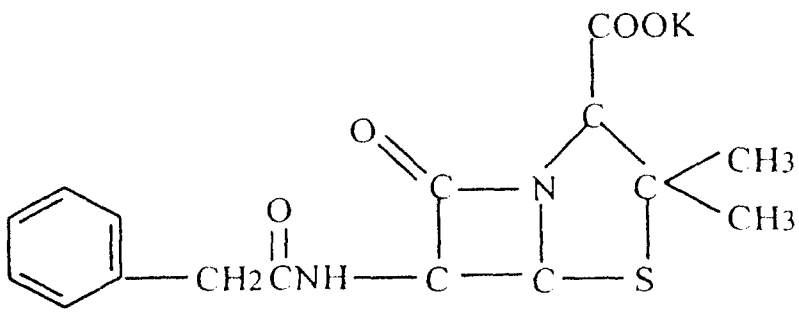



**Figure 1.1 Structure of Penicillin G (potassium salt). Reproduced from Guo et al. (2002).**

The side chain (R) controls the activity against bacteria, as well as determining the pharmacologic characteristics of penicillin G. R also determines the susceptibility of the molecule to the enzyme penicillinase (P'nase) which hydrolyses the β-lactam ring and inactivates penicillin G (Kadurina et al. 2003). Among the natural penicillins, benzylpenicillin or penicillin G has the greatest antimicrobial activity (Kadurina et al. 2003). Benzylpenicillin is produced through a fermentation process which contains strains of *Penicillium chrysogenum*. This process is followed by the separation and purification of the compound. The parent drug is available in four salt forms, namely

procaine, benzathine, potassium and sodium salts (Kadurina et al. 2003). The procaine and benzathine salts are used for slower absorption, and hence provide the opportunity for an extended period for action. The sodium and potassium salts are used to stabilise penicillin during storage (Miller 2002). The general mechanism for β-lactam antibiotics involves their attachment to penicillin–binding proteins (PBPs). This is followed by the inhibition of the peptidoglycan synthesis which occurs in the cell wall. The last step signifies the inactivation of the autolytic enzymes in the cell wall and the initiation of bacterial cell lysis and death (Kadurina et al. 2003).

Antibiotics such as cephalosporins are affected by the enzyme beta-lactamase otherwise referred to as P'nase. Members of the *Staphylococcus sp.* secrete beta-lactamase, which inactivates penicillin by cleaving the beta-lactam ring. Amoxycillin contains clavulanic acid (CA), which increases the effectiveness of the antibiotic against P'nase producing organisms (Miller 2002). Figure 1.2 shows the structure of amoxicillin which is an α-amino substituted β-lactam antibiotic that is used as an antibacterial formulation to combat β-lactamase producing bacteria.

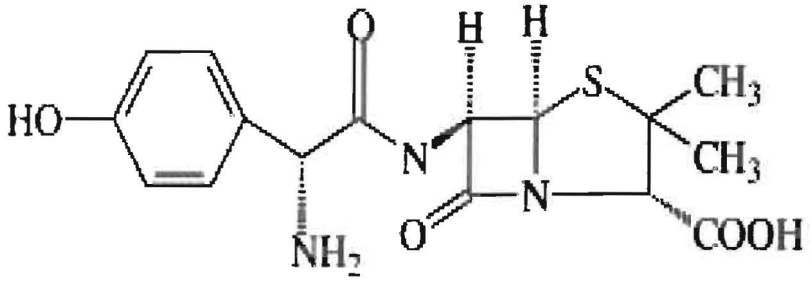



**Figure 1.2 Structure of amoxycillin. Reproduced from Cass et al. (2003).**

Amoxycillin is combined with CA (Tsou et al. 1997) and is usually prescribed as the potassium salt (Aghazadeh & Kazemifard 2001). CA is used to treat infections that are resistant to amoxycillin. CA is produced by the fermentation of *Streptomyces clavuliderus* (Aghazadeh & Kazemifard 2001) and is an irreversible binder of β-lactamases (Kadurina et al. 2003).

## 1.3 Therapeutic Uses and Detrimental Effects of Penicillin

Penicillin is administered to animals and humans for the treatment of a number of infectious diseases and for the promotion of growth (Cizman 2003). These include respiratory infections, soft tissue infections which result from the encroachment of sensitive bacteria, urinary tract infections and prostatitis (Liu et al. 2007). The treatment of mastitis is performed by intramammary infusion and this explains the presence of residual levels of penicillin in milk. The need to test milk samples a few days after treatment is crucial (Grunwald & Petz 2003) as penicillin residues in milk can lead to hypersensitivity and anaphylaxis. Other ramifications of excessive penicillin residues in milk include the inhibition of bacteria that are used in the production of milk products such as yoghurt and cheese which can incur financial losses to the farmer.

The presence of low levels of antibiotics in food for extended periods of time can also contribute to the spread of drug-resistant microorganisms (Grunwald & Petz 2003). Exposure to excessive levels of antibiotics can cause neuro- and nephrotoxicity, allergies and hematologic toxicity (Kadurina et al. 2003). The presence of the amide group in the penicillin molecule causes the initiation of the disease (Kadurina et al. 2003). Table 1.1

summarises the range of reactions that can occur due to exposure to excessive levels of penicillin. Allergy reactions are grouped according to the clinical syndrome, the time of onset, the type of reaction and the immune mechanism. The classification process also depends upon the underlying immune response. Hypersensitivity reactions occur less than half an hour after application. These reactions can occur in the form of angioedema and anaphylaxis and are a result of the cleavage of the β-lactam ring which renders the

**Table 1.1: Reactions to Penicillin (Adapted from Miller 2002)**

| Type of Reaction | Time of Onset | Characteristic Reaction |
|---|---|---|
| I | <1 h | Respiratory distress, hypotension, anaphylaxis and/or angioedema |
| II | >72 h | Splenomegaly, fever, arthralgia, lymphadenopathy. These symptoms disappear within weeks to days of stopping the use of penicillin. |
| III | >72 h | Autoimmune responses producing local ischemia and/or necrosis as a result of complement activation. |
| IV | >72 h | Contact dermatitis |
| Idiopathic | >72 h | Maculopapular rash or Stevens-Johnson syndrome |

tissue proteins immunogenic. This reaction leads to protein binding and allergy to the penicillin which occurs as a result of minor antigenic determinants such as enzymatic reaction products, penicilloic and penillic acids. The clinical symptoms of anaphylactic shock can include bronchospasm and abdominal pain, nausea, vomiting and severe hypotension (Kadurina et al. 2003). Angioedema and urticaria may occur simultaneously, and are marked by the swelling of face and periorbital tissues, lips and genitalia. Reactions that take more than 48 h, occur in the form of skin reactions, serum sickness and nephritis.

## 1.4 Milk and Quality Assurance

Milk is a vital dairy product which is rich in nutritional value (Splitt et al. 1996). Ninety

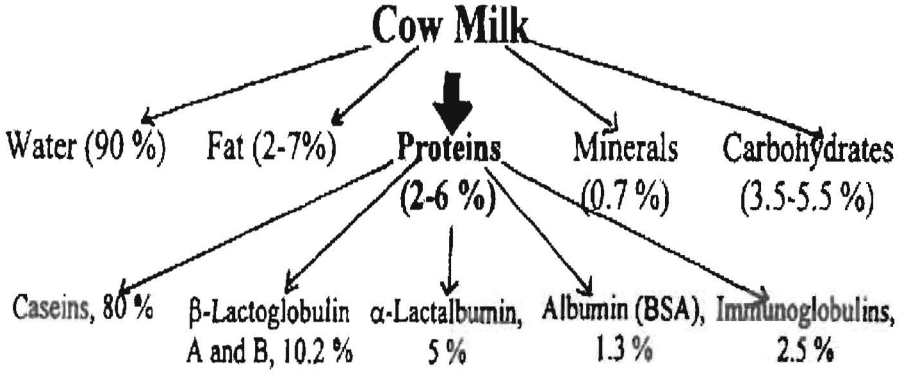



**Figure 1.3 Major constituents of milk. Reproduced from Splitt et al. (1996).**

per cent of milk is made up of water with the remaining 10% containing carbohydrates, fats and minerals as shown in Figure 1.3. Six per cent of milk is made up of peptides and proteins. The majority (80%) of milk proteins are composed of caseins which are removed through acid fractionation during the manufacture of cheese. This leaves behind the whey proteins which include immunoglobulins, bovine serum albumin (BSA), β-lactoglobulin and α-lactalbumin.

The presence of antibiotic residues in milk poses problems to consumers because of its ability to inhibit the growth of fermenting bacteria. This can be problematic for people who are allergic to antibiotics as it can have severe health consequences. Antibiotics are used for the treatment of mastitis or infection of the udder by bacteria, such as *E.coli, Streptococcus spp* and *Staphylococus aureus.* Exposure to low levels of antibiotics on a frequent basis can also lead to the emergence of resistant microorganisms. Such rapid analytical methods as biosensors are required for the control of these micro-organisms, providing a rationale for biosensor development (Rasooly 2004).

## 1.5 Current Methods of Penicillin Analysis

Several methods that have been used for the determination of penicillin have included bioassay, colorimetric, chromatographic, acidimetric, titrimetric and mercurimetric methods as well as spectrophotometric, fluorimetric and gravimetric analysis (Hassan et al. 1978). Brief features of some of these methods are described over the page:

### 1.5.1 Chromatographic Methods

Liquid chromatography, high pressure liquid chromatography (HPLC) and gas chromatography (GC) are some of the methods that are used for the analysis of antibiotics. Chromatographic methods, such as HPLC and GC, are more specific than microbiological methods (Joshi et al. 2002). Some of the challenges that are encountered with chromatographic methods include stability problems with β-lactam antibiotics and thus require specificity and rapidity (Joshi 2002). In addition to this, such methods are expensive and time-consuming (Gaudin et al. 2001), as well as requiring extraction and clean up steps (Joshi 2002).

Reversed-phase liquid chromatography combined with solid phase extraction and liquid chromatographic fractionation clean-up have proved to be inadequate. This is because penicillin-G has no useful ultra-violet maxima above 270 nm and does not fluoresce (Tarbin et al. 1995). The beta-lactam ring is also not stable towards acids and bases and presents problems for extraction and clean-up procedures.

Although chromatographic methods are sensitive, the limit of quantification (LOQ) achieved with this method is 0.3 - 0.5 μg/mL (Joshi 2002). In order to enhance the sensitivity, this method needs to be coupled with other methods such as electrochemical, fluorometric or mass spectrometric methods (Joshi 2002). Overall the scope of HPLC can be widened through the use of solvent systems and different types of columns (Joshi 2002). High pressure liquid chromatography/mass spectrometry (HPLC/MS) methods are reliable and are able to quantify antibiotics with sensitivities as low as 30-100 ppb for β-lactam antibiotics in milk and 4-6 $\mu g/kg^{-1}$ for penicillin (Setford et al. 1999; Gaudin et al.

2001; Su et al. 2010). However these methods require lengthy sample pre-treatment procedures as well as expensive instruments (Su et al. 2010).

### 1.5.2 Titrimetric Methods

The Karl Fisher method involves the titration of phenoxymethylpenicilloic acid with the Karl Fisher reagent which contains pyridine, iodine, sulfur dioxide and methanol. Penicillins contain 6 - 9% of penicilloic acid which may interfere with the Karl Fisher titration of water (Hoebus et al. 1996). However this method is fraught with disadvantages, such as the interference of the degradation product penicilloic acid, slow reaction time, as well as being pH-dependent. In addition, sodium amoxycillin contains a considerable amount of penicilloic acid (Hoebus et al. 1996) and may give unreliable results if analysed with this method.

EDTA titrations are another method that have been used for the determination of penicillin in pharmaceutical preparations. This method involves desulfurisation with potassium plumbite and the excess of lead ions is titrated with EDTA using a lead-ion selective electrode. The instability of some of the metal complexes or the solubility of some of the metal-penicillin complexes produces unsatisfactory results. The reported percentage recovery for amoxycillin with this method was 18% (Hoebus et al. 1996).

### 1.5.3 Colorimetric Methods

Colorimetric detection is an indirect method of detection which involves the on-line hydrolysis of penicillin with an immobilized enzyme reactor, followed by the iodometric detection of penicilloic acid (Van Opstal 1990). The consumption of iodine is detected colorimetrically by measuring the decrease in the absorbance of the iodine/starch complex. This is performed after the system has been optimised by using low back-pressure, high sensitivity as well as high sample throughput as the optimisation criteria. The achievable detection limit was 0.025 mM (Van Opstal 1990).

### 1.5.4 Atomic Absorption Spectrophotometric Methods

Atomic-absorption spectrometry (AAS) has been used for the determination of penicillin (Hassan et al. 1978). This has been done by extraction using nitrobenzene as an ion-pair complex with tris (1,10-phenanthroline)-cadmium. The cadmium in the nitrobenzene phase is then measured by AAS. The percentage recovery reported with this method ranges from 95-105%. However, this method is lengthy requiring 60 minutes per sample (Hassan et al. 1978).

### 1.5.5 Microbiological Methods

This method is based on the binding reaction between the functional groups of the drug and receptor sites on the added microbial cells. Cells from two different organisms provide the binding sites for the antibiotic concerned (Aerts et al. 1995). This approach is

regarded as a rather complex and sensitive screening method that is complimentary to microbiological control methods. It has also been used as a confirmatory test for samples which have been found to be positive with a microbiological screening method. This method has the tendency to produce false positive results, as well as detection limits which are above maximum residue levels (Aerts et al. 1995). Microbiological inhibition assays are inadequate for the determination of penicillin as they are not reproducible or selective, and their sensitivities differ with various antibiotics. These methods are also unable to detect inactive metabolites, are extremely slow requiring 3 - 16 h and not selective (Aerts et al. 1995). The limit of detection for microbiological multi-methods is 5 μg/L for penicillins and below 5 μg/L for β-lactams for microbial receptor-assay multi-method.

### 1.5.6 Physicochemical Methods

Physicochemical methods are advantageous as they are fast, economical and specific (Belal et al. 1998). However they require extensive clean-up procedures and they cannot be performed routinely in conventional laboratories (Aerts et al. 1995). These methods also have the tendency to produce false positive results.

The use of thin layer chromatography (TLC) for the detection of penicillin does not require automated on-line sample preparation techniques for biological samples (Aerts et al. 1995). Evaporation and centrifugation of inhomogeneous samples is difficult to automate without the use of expensive equipment. Extraction with an organic solvent requires the quantitative transfer of an analyte-containing polar phase, as well as the

complete removal of water to allow successful evaporation. In addition, emulsions may form as a result. Lipids and other apolar compounds that may be present in the biological sample may not be co-extracted and the denaturation of proteins will depend on the chosen pH. The addition of the extraction solvent dilutes the sample and, hence, decreases the drug-protein binding. This means that after centrifugation, the analytes are in the phase that can be directly applied to a suitable solid–phase extraction (SPE) system. In the case of milk, it can be injected onto a column-switching liquid chromatography (LC) system which allows automation. The drawbacks of aqueous extraction include the possibility of co-extracting interfering polar components. Depending on the chromatographic and/or spectroscopic properties of the penicillins, additional selectivity may need to be introduced before the final detection step. Other factors of importance include the extractability of the individual penicillin with solutions used, such as the buffer.

The selectivity of multi-residue screening methods can be defined as its potential to discriminate between the samples. That is the number of peaks that can be identified in a typical LC and capillary GC is 50 and 200, respectively, which is lower than the number of residues that can be found in veterinary drugs (Aerts et al. 1995). This is such that additional selectivity needs to be introduced into the assay. This is only if the analytes of interest can be selectively detected at long wavelengths UV-Vis absorption above 350 nm. However, this method is not able to detect all antimicrobials and thus raises questions about the feasibility of this method. The limit of detection for these methods are <10 μg/kg for ionophoric antibiotics (Aerts et al. 1995).

### 1.5.7 Polarographic Measurements

These methods have been applied to the determination of penicillins and its derivatives, including carbenicillin, ampicillin and benzylpenicillin (Belal et al. 1998). Polarographic measurements involve the treatment of these penicillins with nitrous acid to produce the abovementioned derivatives of penicillin, which is consequently measured. The percentage recovery for benzylpenicillin using the derivatisation method with differential pulse measurements (DPP) is 100.24 ± 0.84% (Belal et al. 1998). This method is less time-consuming, sensitive and more specific. However, penicillins cannot be analysed with this method without prior derivatisation.

### 1.5.8 Single-Residue Screening Methods

Single-residue screening methods are used to measure certain antibiotics, such as chloramphenicol and sulfonamides, which are unable to be detected by multi-residue methods (Aerts et al. 1995). Specific tests capable of detecting these compounds need to be used in addition to the microbiological methods that are suited to other microbials. These tests include immunoassays which are widely used in therapeutic drug monitoring for humans. This is because the antigen-antibody interaction is highly selective and enables analytical procedures to be carried out without sample pre-treatment. However, this method also suffers from non-specific binding of matrix components that are present in large amounts and can produce inaccurate results. The assay is performed by bringing the antibodies into contact with the analyte through the addition of radioenzymes or

fluorescent-labelled analyte. This competes with the non-labelled analyte for the available binding sites. The amount of labelled analyte bound is then determined directly or after the addition of a suitable substrate that is able to be transformed into a selectively detectable product. The majority of immunochemical residue methods are enzyme-linked immunosorbent assays (ELISA), radio-immunoassays (RIA) or fluorescence polarisation immunoassays (FPIA) (Aerts et al. 1995). A large number of ELISA-based methods are used for the detection of veterinary drug residues, such as the ELISA test for sulfadimidine in milk. Most of these methods show up to 10% cross reactivity between sulfamerazin which is a closely related to sulfonamide (Aerts et al. 1995). The detection limit for this method is 10 µg/L (Aerts et al. 1995). However, these tests are single-compound tests and require confirmatory tests in the case of positive findings.

### 1.5.9 Non-Spectrometric Methods

An alternative approach for the detection of antibiotics includes non-spectrometric methods (Aerts et al. 1995) such as microbiological screening tests. The identity of antibiotics determined through microbiological screening tests is confirmed by high-voltage electrophoresis. Although this method combines efficient separation with microbiological detection using different microorganisms, it does not have the ability to provide quantitative data.

The combination of liquid-liquid extraction, high pressure thin layer chromatography (HPTLC) and microbiological methods are alternative confirmation techniques for

antimicrobials (Aerts et al. 1995). False positive samples can be identified for penicillins by repeating the test in the presence of a beta-lactamase. The presence of the penicillin is confirmed if the inhibition zone disappears in the presence of the beta-lactamase. The detection limits for β-lactams are 20 mg/kg using micellar electrokinetic solution chromatography (Aerts et al. 1995). The repeatability and the detection process that are provided by the combined approach are poor and there is still a need for the development of sensitive and efficient devices, such as biosensors.

## 1.6 Penicillin Biosensors

Owing to the limitations of the methods discussed above in achieving the rapid and reliable determination of penicillin, considerable efforts have been devoted to the development of biosensors. These devices are capable of ensuring sensitive, cost-efficient and rapid measurements. Biosensors are self-contained devices that are made up of a transducer which is in contact with a biological selective membrane (Thevnot et al. 1999; Dzyadevych et al. 2008). It produces a signal that is detected and processed to generate a suitable analytical response (Purohit 2003), as shown in Figure 1.4. Biosensors are composed of a transducer and a sensing element which interacts with the target analyte producing a signal which is transmitted to the transducer and is then converted into an electrical signal. Biosensors can be applied to the determination of antibiotics in milk and for the quality control of antibiotics. Biologically selective membranes that are used for the development of biosensors can be prepared by various methods of enzyme

immobilisation. These include entrapment, cross-linking, adsorption, covalent binding and encapsulation.

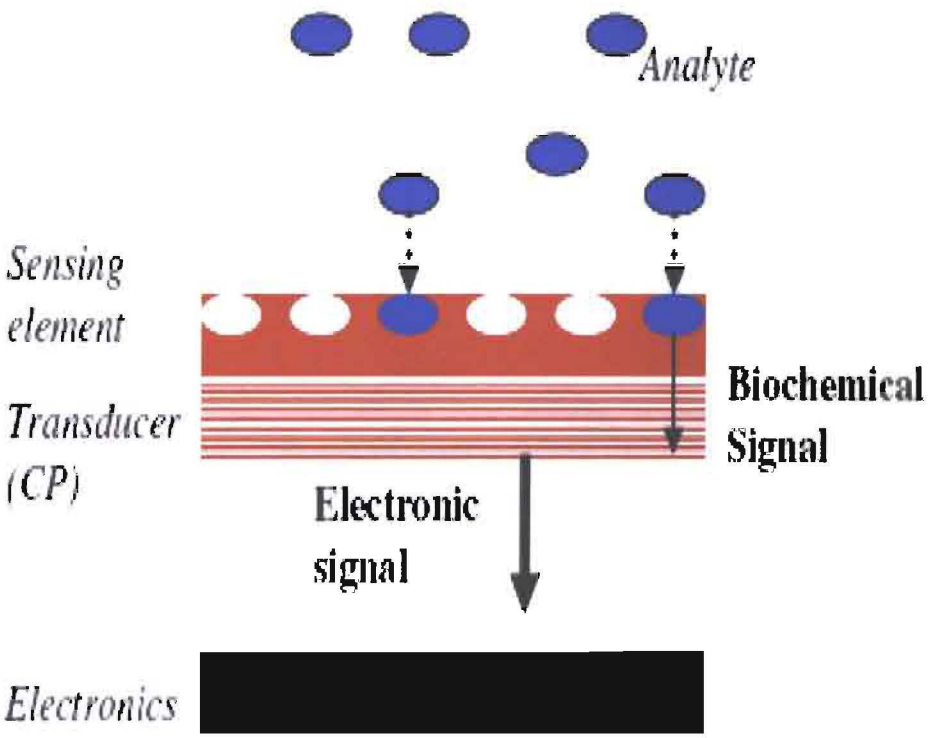



**Figure 1.4 Schematic layout of a biosensor. Reproduced from Guimard et al. (2007).**

Cross-linking of an enzyme can be performed by physical or chemical methods (Broun 1976; Bickerstaff 1997). This approach does not require support and involves joining enzymes or cells to form a complex structure as shown in Figure 1.5. Chemical cross-linking methods often involve the use of glutaraldehye (GLA) and bovine serum albumin (BSA) with the latter acting as a spacer whilst also increasing the number of protein molecules by using higher BSA concentrations. This helps to avoid the close-proximity problems that are caused by cross-linking an enzyme (Bickerstaff 1997).

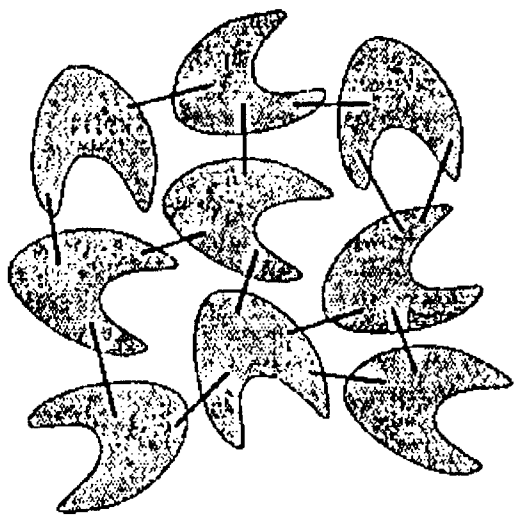


**Figure 1.5 Schematic diagram of cross-linking. Crescent like structures represent BSA and GLA. Adapted from Bickerstaff (1997).**

Entrapment is another method which has been used for the immobilisation of enzymes. This method involves the restriction of enzymatic molecules within a lattice structure of a gel, as shown in Figure 1.6. This can be achieved in two ways: (a) by mixing the enzyme with chemical monomers which are then polymerised. This traps the enzyme in the interstitial spaces of the lattice (Bickerstaff 1997), however the enzyme remains free in solution, or (b) by mixing an enzyme with a polyionic polymer and then cross-linking the polymer with multivalent cations in an ion-exchange reaction to form a lattice structure that traps the enzyme (Bickerstaff 1997).

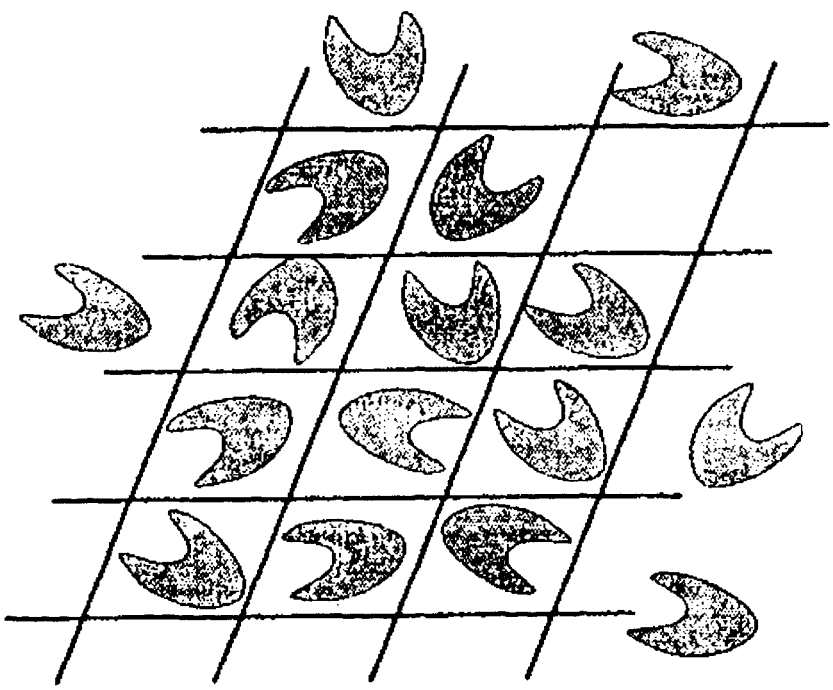


**Figure 1.6 Schematic diagram of physical entrapment. Reproduced from Bickerstaff (1997).**

Encapsulation is similar to entrapment where the enzymes are free in solution. However, this method involves enveloping the biological components in membranes that are semi-permeable, as shown in Figure 1.7. This enables substrates that are small in size to pass across the membrane, while large enzymes cannot pass through the membrane (Bickerstaff 1997).

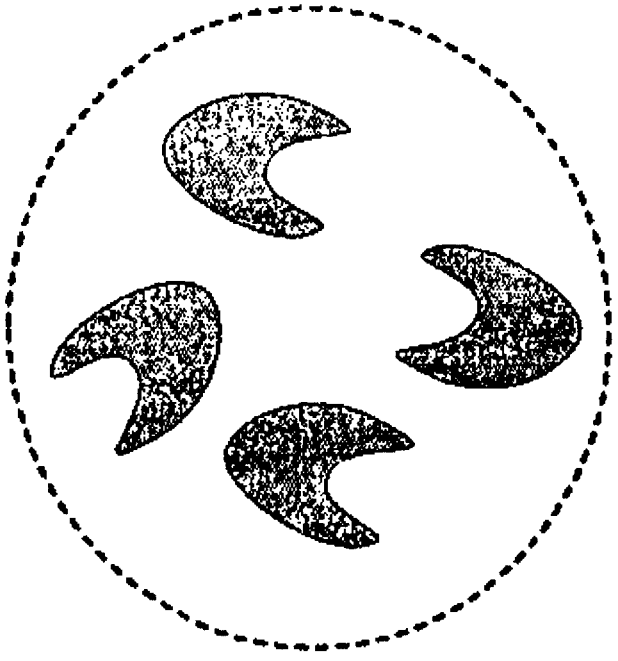


**Figure 1.7 Schematic diagram of encapsulation. Reproduced from Bickerstaff (1997).**

Covalent binding involves the formation of a covalent bond between the enzyme or the cell and the support material, as shown below in Figure 1.8. Despite the fact that a number of varied support materials are available, there is no ideal support as many are fraught with disadvantages (Bickerstaff 1997). An example of this is the use of starch and agarose, cellulose and dextran for enzyme immobilization where the sugar residues in the polymer chains contain hydroxyl groups which provide covalent bonds for chemical activation. However, the polysaccharide supports are susceptible to microbial and fungal disintegration. Porous glass and silica are also used as support but are less hydrophilic than sugar supports (Bickerstaff 1997).

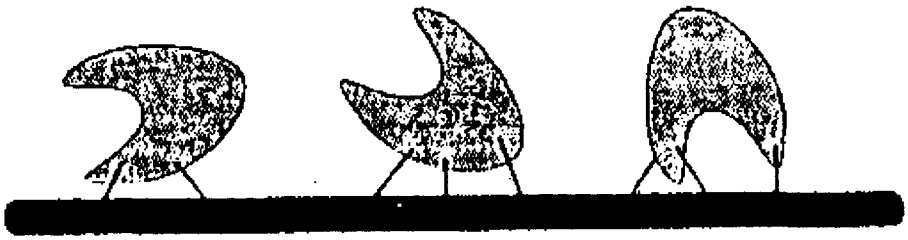


**Figure 1.8 Schematic diagram of covalent binding. Reproduced from Bickerstaff (1997).**

Adsorption is the simplest method that involves reversible interactions between the enzyme and the support. This method involves mixing the support and biological components for a period of time after which the biological component is removed and washed thoroughly. The pH and the ionic strength must be suitable while incubation takes place (Bickerstaff 1997). Desorption and leakage of the enzyme, are some of the disadvantages inherent with this method.

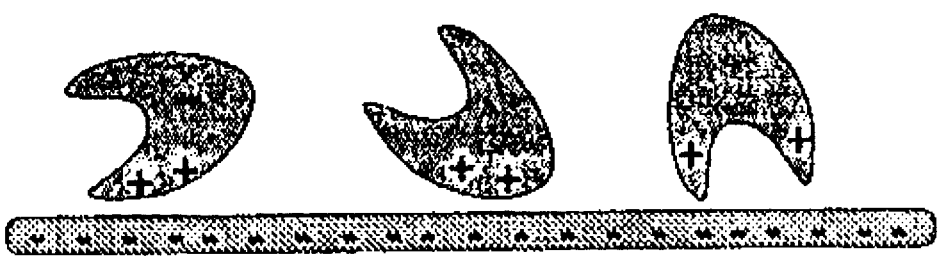


**Figure 1.9 Schematic diagram of adsorption. Reproduced from Bickerstaff (1997).**

The first penicillin biosensor was developed in 1973 and involved the immobilisation of P'nase in a polyacrylamide gel nylon net around the electrode (Papariello et al. 1973; Li et al. 1995). Since then several types of penicillin biosensors have been developed. These

include enzyme optrodes, amperometric and potentiometric biosensors, fiber-optic biosensors, microbial biosensors, bio-field transistors (BioFET), enzyme field-effect transistor (ENFET), electrolyte-insulator-semiconductor, capacitive sensors, silicon-based porous biosensors, surface plasmon resonance (SPR) biosensors, ion-sensitive field-effect transistor (ISFET) and whole cell enzyme biosensors. The different types of penicillin biosensors are briefly outlined and summarised in Table 1.2.

### 1.6.1 Enzyme Thermistor Biosensors (ET/FIA)

Enzyme thermistors measure the enthalpies of enzymatic reactions (Hundeck et al. 1990). This method involves the injection of a reproducible sample volume into an unsegmented flowing stream of solvent. This is performed within short intervals and the surroundings of this system are kept at a constant temperature. The heat that is produced from the enzymatic reaction is measured as a temperature change by the thermistor (Hundeck et al. 1990). Enzymatic reactions are carried out on small solid bed reactor columns.

The minimum detectable concentration of penicillin with the enzyme thermistor biosensor was found to be $10^{-6}$ g/L (Decristofor & Danielsson 1984). The linear concentration range achieved for penicillin and cephalosporin was 0.1 - 10 mg/mL. The analysis time for a sample was less than 80 s, however high enzyme cost is a main disadvantage. The selectivity achieved for analytes is dependent upon the specificity of the enzyme (Hundeck et al. 1990).

## Table 1.2 The Development of Penicillin Biosensors

| Electrode Type | Method of Immobilisation | Linear Concentration Range | References |
|---|---|---|---|
| Potentiometric pH electrode | Enzyme encaged in a PA gel | 0.1 - 0.5 mM | (Papariello et al. 1973) |
| Flow-through cell LED photoiodide | Bromothymol blue derivatised with glutathione and co-immobilised with enzyme to celloxaphane membrane | 5 - 20 mM | (Goldfinch & Lowe 1984) |
| Thermistor | GLA cross-linked to CPG and P'nase | 0.1 - 10 (mg/mL) | Decristofor & Danielsson 1984 |
| Potentiometric pH electrode | GLA cross-linked between porous glass and P'nase | 0.1 - 20 mM | (Olsson 1988) |
| Amperometric | Use of pH-sensitive redox active compounds (i.e. haematin) with pH affecting enzymes (i.e. P'nase) | 2 - 10 μM | Stred'ansky et al. 2000 |
| Potentiometric | Enzyme immobilisation was performed using dispersion polymers | $3x10^{-2}$ - $10^{-1}$ mol $dm^{-3}$ | Eppelsheim et al. 1995 |
| Fiber optic fluorescence | Polyacrylamide membrane with covalently bound fluorescein and P'nase | 0.25 - 10 mM | (Kulp et al. 1987) |
| Reflectance spectroscopy with fiber optics; FIA | P'nase cross-linked to cellulose with bovine serum albumin-glutaraldehyde (BSA-GLA) | 0.4 - 5 mM | (Yerian 1988) |
| Potentiometric pH electrode | GLA cross-linked between porous glass and P'nase | 0.05 - 0.5 mM | (Ghanasekaran & Monttola 1985) |

*Continued over the page...*

| | | | |
|---|---|---|---|
| Fiber optic fluorescence measurement | P'nase and FITC dextran immobilised in polyacrylamide gel | 0.5-50 mM | (Luo & Walt 1989) |
| Fiber optic fluorescence | P'nase and FITC dextran immobilized in biodyne B membrane | 0.1-50 mM | (Xie et al. 1992) |
| Potentiometric pH electrode | Recombinant *E. coli* adsorbed onto acetyl-cellulose membrane | 5-30 mM | (Galindo et al. 1990) |
| Potentiometric | n-type silicon substrates covered with $SiO_2$ and/or $Al_2O_3$ | 5-25 mM | Seki et al. 1998 |
| Potentiometry FET, FIA | P'nase-amidase cross-linked to BSA-GLA | 0.5-10 mM | (Brand et al. 1990) |
| Potentiometric pH electrode | P'nase cross-linked with BSA-GLA | 2.5-15 mM (in broth) | (Meier & Tran-Minh 1992) |
| CTTPS (charge-transfer technique) | Constructed by immobilising P'nase onto the ion-sensitive membrane ($Si_3N_4$) | 0-25 mM | Lee et al. 2009 |

Other types of reactors such as open tubular reactors have been reported, but these proved to be similar to packed bed enzyme reactors. This method was evaluated by comparison with HPLC and no statistical variations were found with these methods (Decristofor & Danielsson 1984). Packed bed enzyme reactors are more advantageous than the conventional methods such as chromatographic, acidimetric and mercurimetric methods to name a few. This is because of their ability to achieve total conversion of the substrate and shorten reaction times.

## 1.6.2 Immunosensors

A label-free impedimetric immunosensor which utilised anti-penicillin G based on self assembled thioctic acid monolayer (SAM) via covalent coupling on a gold electrode has been reported for the detection of penicillin G in raw milk samples. Quantitative determination involves the measurement of the increase in the impedance when penicillin binds to the immobilised anti-penicillin G on the electrode surface. This method reported an extremely low detection limit i.e. $3.0 \times 10^{-15}$ M and a wide linear range between $1.0 \times 10^{-13}$ and $1.0 \times 10^{-8}$M (Thavarungkul et al. 2007). However milk samples analysed with this method had to be diluted 10, 000 times due to the tendency of the fatty component of the milk to adhere to the electrode surface and has to be washed for a long time before it returns to the baseline. To minimise this problem, the matrix effect was reduced by substantial dilution of the sample. However, this increases the analysis time to 20-24 minutes per sample (Thavarungkul et al. 2007) which highlights another disadvantage of this method.

Another self-assembly method utilises the immobilisation of β-lactamase in thin films of poly (phenylenethylene). The polymer is sequenced in the conjugated chain with flexible groups containing sulfur atoms, synthesised by the Heck-Sonogashira coupling reaction (Vazquez et al. 2007). After examining the difference in the absorbance of the multilayer prior to and after the immobilization of the enzyme, it was found that 20% of the material was lost (Vazquez et al. 2007) which is a significant drawback of this immobilisation method.

### 1.6.3 Enzyme Optrodes

Fiber-optic chemical sensors have also been used for the determination of penicillin. These types of sensors are based on an integrated sensor consisting of a naturally occurring bioreceptor that is linked to a physical sensor. These sensors operate by detecting changes in the emissive or absorptive properties of the indicator that is fixed to the fiber tip (Luo & Walt 1989). The enzyme catalyses the conversion of the substrate into the product and this is accompanied by a change in the micro-environmental pH. The change in the pH is detected through the florescence change in the intensity of the pH-sensitive dye fluorescein isothiocyanate (FITC) that is attached to the enzyme (Luo & Walt 1989).

The first optical enzymatic biosensor was used for the determination of glucose (Lubbers & Opitz 1983). Arnold (1985) also demonstrated the use of an optrode for the determination of penicillin. Generally, penicillin optrodes operate by employing P'nase to catalyse the cleavage of the beta-lactam ring of penicillin to form penicilloic acid, which dissociates to form penicilloate and a proton. This produces a change in the pH of the sensor medium and the enzymatic reaction is monitored by a pH-dependent fluorescent dye. The surface of the sensor is coated with a dye and the enzyme is incorporated into the polymer which is attached to an optical fiber. In one approach, the enzyme is entrapped in polyacrylamide with the fluorescein covalently attached to it and bonded directly to the activated fiber tip (Kulp et al. 1987). In another approach, a derivatised form of hydroxypyrene trisulfonate (HPTS), a pH sensitive dye and BSA are intermolecularly cross-linked with GLA on top of an amino-silanised optical fiber (Kulp et al. 1987). No diffusional barrier exists between the membrane and the dye as the

enzyme is mixed with the dye. Both optrodes exhibited response times within 40 - 60 s and each electrode produced a linear concentration range 0.25 - 100 mM for penicillin G (Kulp et al. 1987). Each optrode reached a 95% steady state signal in 40 - 60 s. The recovery time of the optrode was 2 minutes and was measured without stirring. Although the use of penicillin optrodes has proven to be successful, this method has a number of disadvantages. This includes the need to use low buffer concentrations in order to obtain high sensitivities as the use of high buffer concentrations only results in poor sensitivities. Low buffer concentrations are necessary for the maintenance of strict control over the buffering strength of the solution (Kulp et al. 1987). These problems arise due to the fact that the measured parameters are pH and the $H^+$ concentration generated from the enzymatic reaction. Although several advantages have been reported with this enzyme immobilization method, the time required to reach a 95% steady state signal was less than 90 s (Luo & Walt 1989). This is despite the fact that this method has a low diffusion barrier, as well as enabling close proximity between the enzyme and fluorescent indicator.

### 1.6.4 Microbial Sensors

Microbial sensors use microorganisms that harbor plasmids which code for the synthesis of the enzyme (Galindo et al. 1990). *E. coli* was used to harbor a multicopy plasmid which codes for P'nase, and is immobilized in the vicinity of a combined flat pH electrode. Microbial sensors use large amounts of microbial cells which affect the response characteristics of the biosensor. This is one of the major disadvantages of

microbial sensors and may be overcome by increasing the specific activity of the enzyme that is used. This can be done by increasing the gene dosage of the coded enzyme that is contained in the bacteria by using multicopy recombinant plasmids (Galindo et al. 1990). However, the linear range (5 - 30 mM) that was obtained with the microbial sensor was narrow. The analysis of amoxycillin with the microbial sensor produced a lower sensitivity as well as a limited concentration range of 0 - 15 mM (Galindo et al. 1990). Microbial sensors are not capable of detecting penicillin below 5 mM, but can detect higher levels of antibiotics and can only be used for 13 days, whereas enzymatic sensors can be used for much longer (Galindo et al. 1990).

### 1.6.5 Field-Effect Transistors (FET) Based Biosensors

Different types of field effect transistors have been used in different forms, namely; BioFET, ENFET and ISFET. BioFET involves the measurement of the change in pH caused by the enzymatic reaction of penicillin hydrolysis which is catalysed by P'nase (Brand et al. 1990). This reaction occurs within the enzyme-loaded membrane which incorporates the pH-sensitive area of the FET (field-effect transistor). Very few applications have been reported for BioFETs and the only advantage that is associated with this method is that sterilisation can be performed without affecting or denaturing the enzyme (Brand et al. 1990).

ENFET is a type of FET that has been used to measure the variation of the $H^+$ ion concentration which is detected by the $H^+$ ISFET (Liu & Li 2000). The principle of ENFET operates on the principle of the pH change that is caused by the enzymatic reaction with the immobilised penicillin G acylase (Liu et al. 1998). The determination of the penicillin G concentration in fermentation broth was impossible due to the high specificity of the enzyme and the other constituents present in the broth which may have interfered with the signal.

ISFETs need to be used under thermostatic conditions due to their thermal instability. The reported detection limit was 5 μM and the linear concentration range was 0.05 - 1 mM (Poghossian et al. 2001). The need to use these sensors under thermostatic conditions is inconvenient for many applications. This is why the thermal behavior of penicillin-sensitive field-effect transistor (PenFETs) needs to be investigated in order to establish effective methods to improve their stabilities (Poghossian et al. 2001).

Lee et al. (2009) reported the use of ISFET and charge transfer technique (CTT) for the determination of penicillin. CTT involves a charged accumulation technique for the detection of penicilloic acid and a perception of a $H^+$ ions system, as shown in Figure 1.10. The principle of CTT is similar to that of an ISFET as it is based on the detection of $H^+$ ions by silicon nitride ($Si_3N_4$). Figure 1.10 shows that the [H+] ions are detected by a $Si_3Ni_4$ layer. The sensor signal is then measured with the change in the penicillin concentration.The potential varies with the production of hydrogen ions generated by the

enzymatic reaction (Lee et al. 2009). This method operates on the basis of the signal integration method where the changes in the penicillin concentration are transferred from

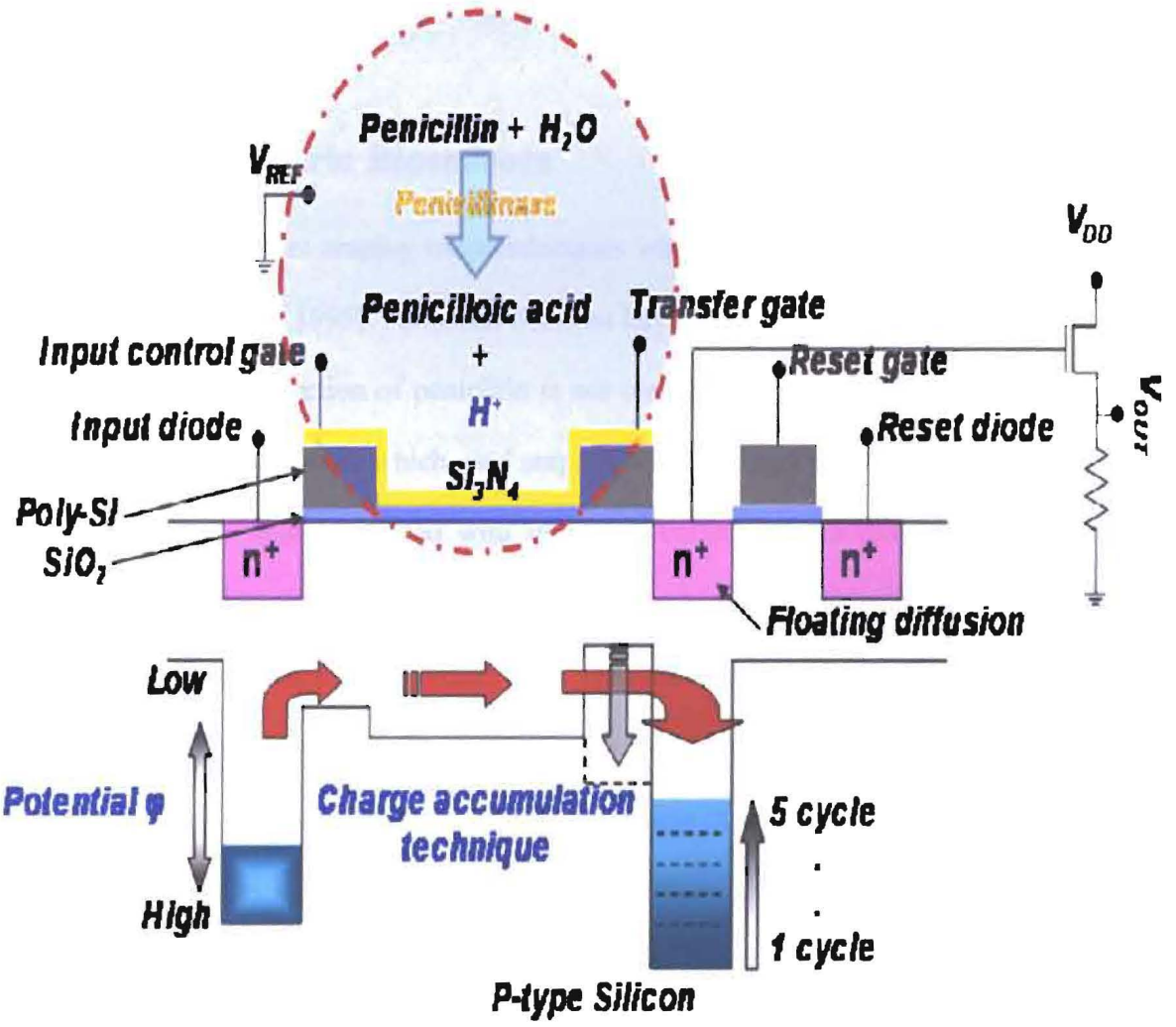



**Figure 1.10 Schematic representation of the CTTP method. Reproduced from Lee et al. (2009).**

the sensing part of the floating diffusion region several times, and the changes in the signal are accumulated. The CTTPS sensor is more sensitive than the ISFET. However,

the detection limit was 0.01 mM and the sensitivity of the sensor was 48 mV/mM (Lee et al. 2009). This method is more equipped to detect lower penicillin concentrations whereas potentiometric enzyme biosensors are able to detect high and low penicillin concentrations.

### 1.6.6 Amperometric Biosensors

These types of biosensors employ oxidoreductases which play a role in the catalysis of redox reactions (Li et al. 1995). Penicillin does not have an oxidoreductase and the use of amperometry for the detection of penicillin is not common. Li et al. (1995) reported on the iodometric penicillin assay which used amperometry to detect penicillin in broth. The resulting penicilloic acid is reacted with iodine in buffer and the excess iodine was detected amperometrically.

Other reported amperometric biosensors have required the use of pH-sensitive redox-active probe molecules such as haematin, which is a natural dye that is used in the selective straining of biological materials. It is also a water-soluble electroactive compound and used as a pH indicator (Stred'ansky 2000).

### 1.6.7 Potentiometric Biosensors

Potentiometry is another method that has been explored quite extensively in the literature. In one approach P'nase was cross-linked with GLA (Meier & Tran-Minh 1992). Four enzyme electrodes were constructed and each electrode was used in an FIA system for 4 h. The long-term stabilities of the electrodes were affected by the loss of enzyme activity (Meier & Tran-Minh 1992). The detection limit for the two cells is approximately 0.1

mM and the response for the new flow-through cell was non-linear above ca 15 mM and 20 mM for the wall-jet cell. This is attributed to the incomplete catalytic reaction of the enzyme. The low buffer capacity of the carrier resulted in the enzymatic reaction taking place under acidic conditions which was less than the optimum pH of the enzyme (Meier & Tran-Minh 1992).

Eppelsheim et al. (1995) reported the use of differential potentiometric thick-film sensors operating in differential mode. This method measures the relative potential change with reference to a non-selective electrode rather than the absolute potential change of a single selective electrode that is induced by the concentration of the analyte. A disadvantage of this method is that thick enzyme layers lead to decreased response times. Other potentiometric sensors for the detection of penicillin have used a pH-sensitive electrolyte (insulator) semi-conductor (EIS) layer structure that consist of Al/pSi/$SiO_2$ /$Si_3O_4$ by heterobifunctional cross-linking (Thust et al. 1996). The intermolecular cross-linking of the enzyme molecules was avoided by performing the immobilization process in two steps. The measurements were carried out in static mode which resulted in the production of a narrower linear range. Another disadvantage of this method is the observed decrease in the sensitivity at the beginning of the measurement period due to the loss of surplus enzyme. This may have been adsorbed on the surface of the sensor after the immobilisation procedure.

Other potentiometric biosensors include the construction of a pH electrode with tridodecylamine, as a hydrogen ion-selective ionophore, which has been used for the detection of penicillin (Leszczynska et al. 1998). In this study, the enzyme was covalently

bound to the membrane surface and pH-based biosensors can be cross-sensitive to pH which questions the feasibility of this method. All pH-based biosensor systems need to have their sample pH adjusted prior to measurement in order to ensure that a non-specific increase in the biosensor signal does not occur (Meier & Tran-Minh 1992; Carlsen et al. 1993; Leszczynska et al. 1998).

### 1.6.8 Capacitive Sensors

A capacitive penicillin biosensor is constructed by immobilising an enzyme in the pores of dielectric materials such as $SiO_2$ and $Si_3N_4$ porous pH penicillin sensors (Schoning et al. 2000). It works by performing measurements which are taken by using a fixed capacitance value with the use of a feedback circuit. A capacitive biosensor operates by registering a decrease in the pH value near the sensor surface which results from a change in the penicillin concentration and then translates into a shift in potential (Schoning et al. 2000). Some of the advantages and disadvantages of this method are capacitive electrolyte-insulator-semiconductor (EIS) sensors are cheaper in comparison to ENFETs and ISFETs and are easier to prepare as well as being stable in the long term. However ENFETs and ISFETs require corrosion-resistant encapsulation of electrical connections to shield from the surrounding liquid and can be unstable due to corrosion at the contact regions and since the sensitive area of an EIS is larger than the gate region of an ISFET, more analyte is required for analysis. This problem can be overcome by using a diffusion barrier (Poghossian et al. 2001). Also EIS has the ability to capitalise on such drawbacks by using a highly enlarged surface area, but they are less sensitive and not suitable for

miniaturisation (Luth et al. 2000). The linear range for the structured EIS sensor is (0.01 - 0.05 mM) and (0.01 - 1 mM) for the porous EIS sensor (Luth et al. 2000).

### 1.6.9 Electrolyte-Insulator-Semi-Conductor (EIS)

Macroporous type EIS structures are sometimes used for the detection of penicillin instead of planar silicon sensors due to the fact that the enlarged sensor surface area yields a higher capacitance (Simonis et al. 2003). This type of structure also provides a stable anchoring system, as well as the embedment of enzymes inside the pores. However, the deposition of the dielectric and sensitive materials requires the use of low temperatures inside the pores in order to produce a silicon-based sensor as the deposition is occurring inside the pores. These sensors are stable for a couple of weeks because of the non-ideal dielectric behavior, as well as the corrosive tendencies when placed in an electrolyte (Simonis et al. 2003). Such conditions have been overcome with the use of a different arrangement which utilises a porous layer during the anodic etching process. This is followed by the deposition of a double layer ($SiO_2/Si_3N_4$) insulator by high temperature processes, such as thermal oxidation, low-pressure chemical vapor deposition and lastly the adsorptive immobilisation of the enzyme. However, the inhomogenities in the thickness of the double layer insulator $SiO_2/Si_3N_4$, produced variances between the measured capacitance value and the theoretically estimated values. EIS structures employ simpler preparation processes and are stable in the long term. ISFETs on the other hand are electrochemically unstable due to corrosion at the contact regions. Poghossian et al. (2001) developed an EIS sensor that is based upon a pH-sensitive electrolyte-insulator-semiconductor which was not sensitive to cloxacillin. The

steady state potential was reached in more than 2.5 minutes. In addition to this, amounts less than 0.5 µg were not able to be detected (Poghossian et al. 2001), hence highlighting the disadvantages of this method.

### 1.6.10 SPR (Surface Plasmon Resonance Detection)

SPR affinity biosensors are optical sensors that exploit the excitation of special electromagnetic waves (Tobiska & Homola 2005). This technique is an optical phenomenon which occurs at the interface of a thin metal prism that is in contact with a solution. The resonance angle changes as the refractive index or mass changes at the solution surface as molecules bind or dissociate. SPR biosensors monitor the shift in the resonance angle as the analyte binds to a receptor that is immobilised at the solution/metal interface. This binding results in changes in the refractive index of the material at the solution/metal interface. This refractive index change is monitored in real time (Subramanian et al. 2006). This method involves the use of an antibody which has a higher affinity for an open beta-lactam ring. Two different pre-treatments were tested prior to the biosensor assay to open the ring in order to increase the assay sensitivity. This includes enzymatic methods, as well the chemical pre-treatment of the samples (Gaudin et al. 2001). Enzymatic pre-treatment proved to be non-feasible as the results obtained for penicillin in milk were 2.5 - 3.5 times higher than the results obtained for amoxicillin in milk (Gaudin et al. 2001). Other problems include the inability of antibodies to recognise the structure of the beta-lactam ring, as well as the surface of antibiotics, such as ampicillin. Another disadvantage of this method is that certain proteins in milk can bind to the electrode and interfere with measurements. Other studies have considered the use

of inhibition assays where a receptor protein, with carboxypeptidase activity, was used as the detection molecule (Gaudin et al. 2001). A receptor protein was used instead of an antibody which is not specific to the beta-lactam structure (Gustavsson et al. 2002). The mechanism of this reaction includes the formation of a stable complex between the receptor protein and the beta-lactam. This biosensor was specific for the detection of beta-lactam antibiotics in milk, in contrast to other sensors which are capable of detecting beta-lactams in antibiotic preparations as well as in milk. The percentage recovery of penicillin in milk with this biosensor was reported to be 108 - 118%. Percentage recoveries that were greater than 100% were attributed to the non-specific binding from the sample matrix (Gustavsson et al. 2002). The presence of whey proteins in milk was reported to be the most likely cause of these problems which also affected the detection limits.

Other methods for the detection of penicillin in milk, have included the use of assays based on the inhibition of the binding of digoxigenin-labelled ampicillin (DIG-AMPI) to a derivative of *Streptococcus pneumoniae* (Cacciatore et al. 2004). The resulting DIG-AMPI/PBP 2x complexes were detected in an SPR-based biospecific interaction assay (BIA) for digoxigenin with an antibody against digoxigenin that was immobilised on the sensor chip. Non-specific binding occurs as a result of the components of milk which is a common problem when analysing milk samples. This provides a rationale for the analysis of defatted samples. Non-specific binding could be reduced to a lower and more constant level by a heat-treatment step, a centrifugation step and the addition of carboxymethylated dextran to the samples (Cacciatore et al. 2004) which is quite a

lengthy procedure. The mechanism of this detection system involves the SPR-based detection system which monitors the change in mass density and occurs when a molecule binds to its specific binding partner on the sensor chip. This method could only be applied to processed milk due to the high level of non-specific binding to the sensor surface which occurs with raw milk samples. Cloxacillin could not be detected by the assay even in a modified test system with an improved sensitivity (Cacciatore et al. 2004).

### 1.6.11 Whole Cell Enzymes

The use of whole cell enzymes is one method which provides many advantages over the use of isolated enzymes. These advantages include entrapment of the whole cell enzyme, convenience in preparation and operational stability of the immobilized whole cell. However, the entrapment of whole cell enzymes also slows down the hydrolysis of penicillin, converting only 60% of the substrate to 6-aminopenicillanic acid (Nourouzian et al. 2002). Possible reasons for this are the accumulation of the reaction products such as phenylacetic acid which inhibits the activities of the immobilised penicillinase acylase (Prabhune et al. 1992).

## 1.7 Aims of Study

Owing to the limitations identified with current methods used for detection of β-lactam antibiotics, the general aim of this study is to develop a sensitive and efficient biosensor for the detection of β-lactam antibiotics in pharmaceutical preparations and milk. This will be achieved by investigating the effectiveness of the immobilisation of P'nase in

conducting and non-conducting polymers in different configurations of single layer and bilayer arrangements. The major focus of this study will be to monitor the potential change resulting from the enzymatic hydrolysis of penicillin rather than the change in the pH which has been more commonly used in other penicillin biosensors. The rationale behind this is that more sensitive measurements can be accomplished by measuring the potential instead of the pH change when protons are consumed or released.

More specifically this study will:

(a) investigate the immobilisation of P'nase in a number of conducting and non-conducting polymers, such as polypyrrole (PPy), polytyramine (PTy), bovine serum albumin-glutaraldehyde (BSA-GLA) and bovine serum albumin-poly (vinyl alcohol) (BSA-PVA), polypyrrole/bovine serum albumin-poly (vinyl alcohol) (PPy/BSA-PVA) and polytyramine/bovine serum albumin-glutaraldehyde (PTy/BSA-GLA);

(b) examine the influence of single and bilayer configurations on the sensitivity of the biosensor

(c) investigate the use of enzyme immobilisation methods, such as physical entrapment and cross-linking with BSA-GLA and BSA-PVA, on the potentiometric response for penicillin;

(d) compare the biosensor response with that of the standard titrimetric method; and

(e) apply the biosensor to the determination of penicillin in antibiotic preparations and in milk samples

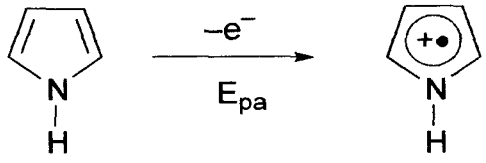
# Chapter 2

## The Development of a Polypyrrole-Based Penicillin Potentiometric Biosensor

## 2.1 Introduction

PPy has attracted a lot of interest for its use in the development of biosensors because of its low oxidation potential (Adeloju & Moline 2001). This facilitates the immobilisation of enzymes and allows pyrrole (Py) films to be electropolymerised from aqueous solutions that are compatible with many biomaterials (Scheller et al. 1979; Yon Hin et al. 1990; Nishizawa et al. 1992; Coche-Guerente et al. 1994; Brown & Luong 1995; Adeloju et al. 1996; Maines et al. 1996; Adeloju et al. 1997). Hence, this is one of the reasons for considering the use of PPy in this study.

Several mechanisms for the electropolymerisation of Py have been reported in the literature. However the mechanism reported by Diaz et al. (2000) and confirmed by Sadki et al. (2000), is the most cited in the literature. Figure 2.1 shows the mechanism for the electropolymerisation of Py. Scheme one of the mechanism shows the oxidation of the monomer R to form the radical cation $\mathbf{R}^{+\cdot}$. This is followed by the coupling of the two radical cations which results in the formation of the dihydromer dication **5**. Scheme two shows the several resonance forms of the cation which then dimerises via resonance form **3** as shown in scheme three. Scheme four depicts the stabilisation step where the loss of two protons forms the aromatic dimer **6.** The dimer is then oxidised to the radical cation, followed by the polymerisation reaction as shown in scheme 5. The stabilisation reaction allows the dimer to become less reactive than the monomer. Scheme 6 shows the formation of the trimer dication that deprotonates to produce the neutral trimer. This is followed by the electro-oxidation of **12** to produce the radical cation **13** as shown in scheme 7. The propagation step, oxidation, coupling and deprotonation continue until the final product is obtained as shown in scheme 8. The electropolymerisation reaction produces the doped form of the polymer i.e. the oxidised conducting form as shown in

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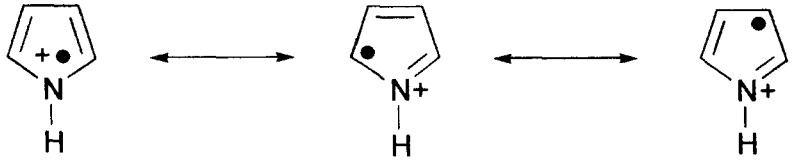
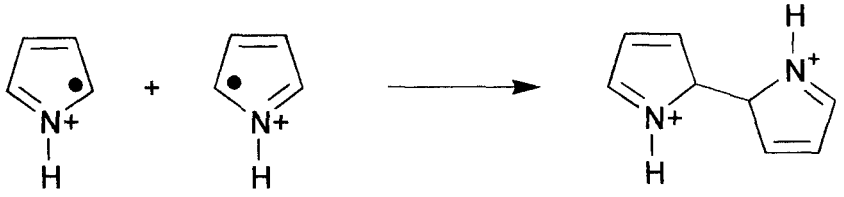
Scheme 1



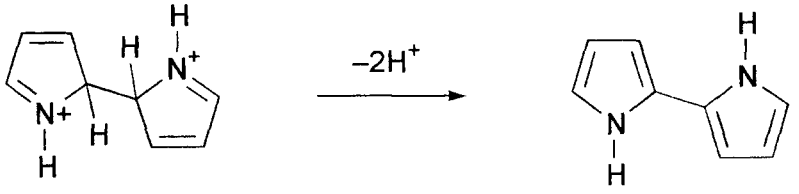

2 3 4

Scheme 2




3 3 5

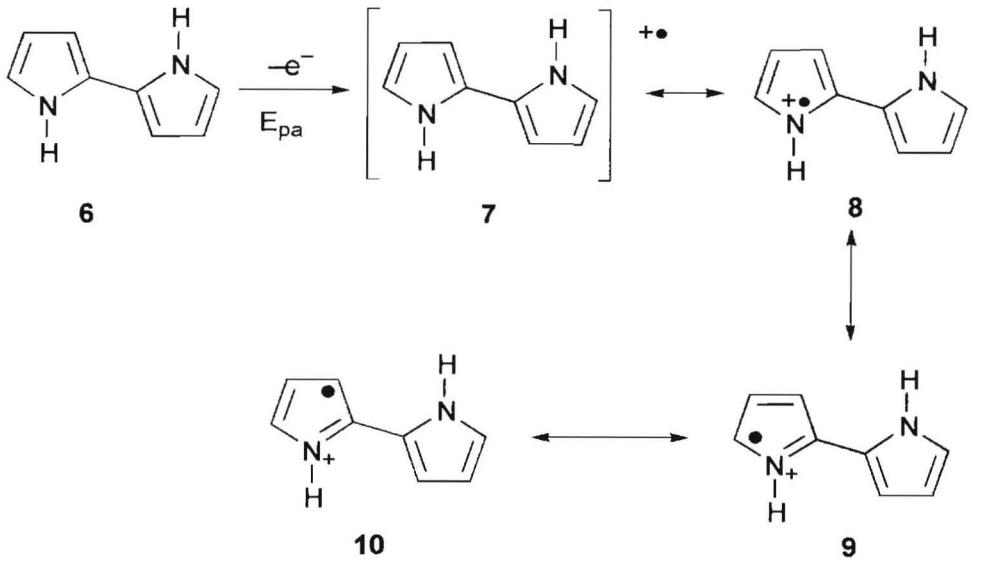
Scheme 3



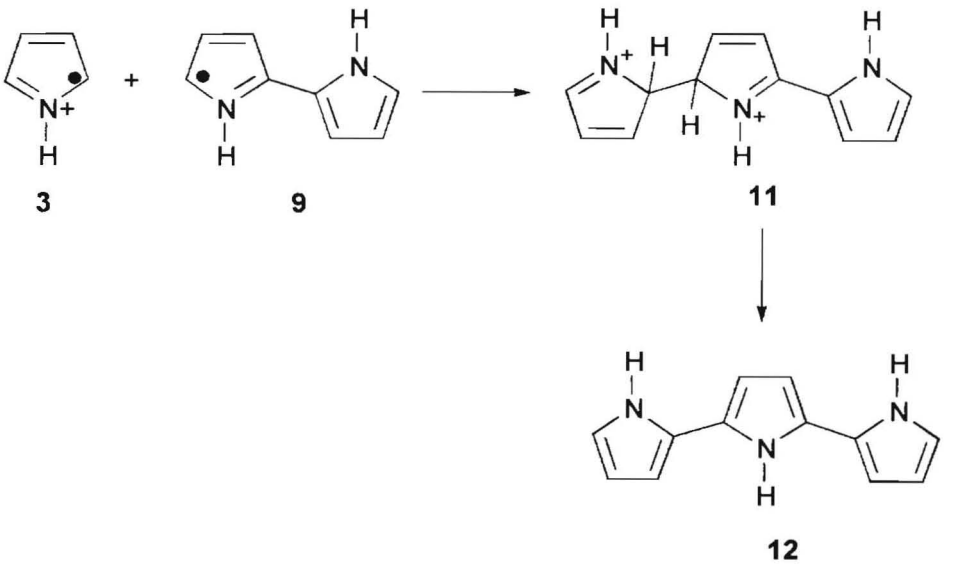

5 6

Scheme 4

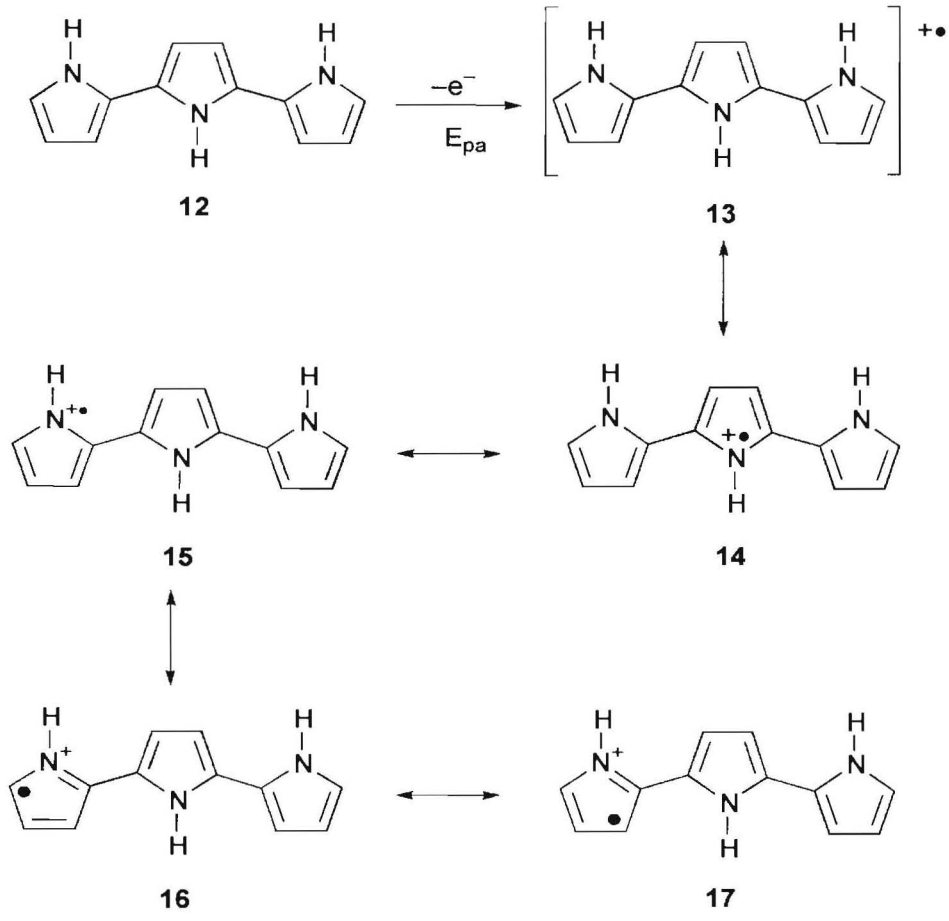
**Figure 2.1 The electropolymerisation of pyrrole. Reproduced from Sadki et al. (2000).**



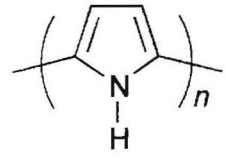

**Scheme 5**



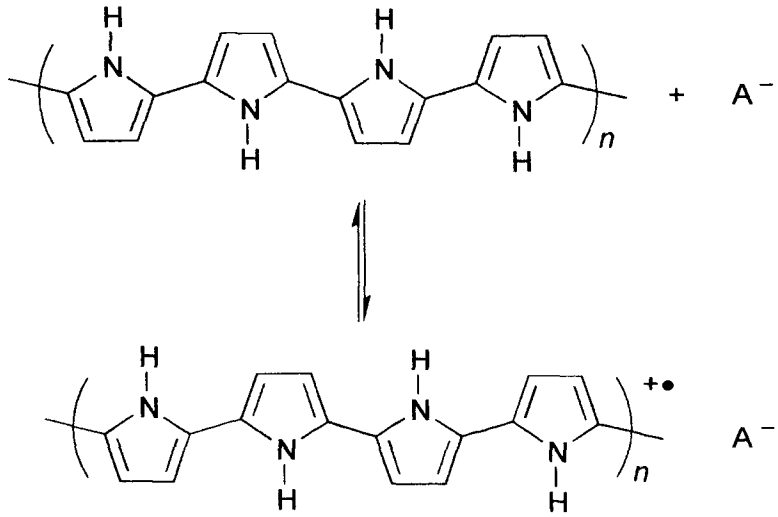

**Scheme 6**



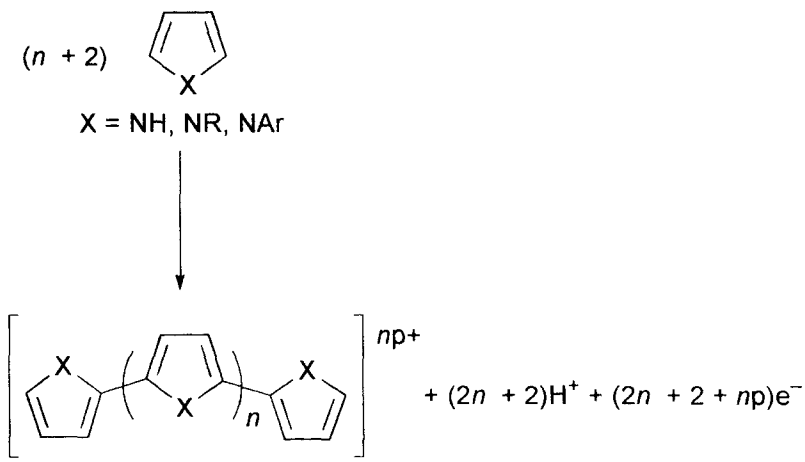

**Scheme 7**



**Scheme 8**



**Scheme 9**



**Scheme 10**

scheme 9, where A represents the electrolyte anion. The polymerisation reaction is shown in scheme 10. This study will investigate the entrapment of P'nase during the electropolymerisation of Py. Different methods have been used to immobilise P'nase for

the fabrication of penicillin biosensors. These have included covalent binding (Decristofor & Danielsson 1984; Leszczynska et al. 1998), self-assembled immobilisation (Thavarungkul et al. 2007; Vazquez et al. 2007), physical adsorption (Schoning et al. 2000; Luth et al. 2000) and entrapment (Kulp et al. 1987).

Nishizawa et al. (1992) used a BSA-GLA cross-linked membrane coated with a PPy film which monitored the change in the pH. To date, PPy has not been used to fabricate potentiometric penicillin biosensors that monitor the potential change. The primary objective of this chapter is to explore the use of P'nase entrapped in a PPy film for the potentiometric detection of penicillin. More specifically, the aim of this study is to investigate the polymerisation of Py as a means of immobilising P'nase in a PPy film. Factors such as the influence of the P'nase concentration, current density, inclusion of penicillin (Pen) in the monomer solution, polymerisation time and buffer concentration will be examined to obtain an optimum potentiometric response for penicillin. The effectiveness of the resulting biosensor will be investigated by applying it to the determination of penicillin in milk samples and to pharmaceutical preparations.

## 2.2 Experimental

### 2.2.1 Reagents and Chemicals

All chemicals were of analytical grade unless otherwise specified. All solutions were prepared with Milli-Q water (18 $\Omega$ $cm^{-1}$). Py was supplied by Sigma Aldrich (USA) and was distilled at 130°C before use due to its sensitivity to heat and light. The distilled Py was refrigerated and covered with aluminium foil to prevent ultra-violet degradation.

P'nase (3.5.2.6) from *Bacillus cereus* was purchased from Sigma Aldrich (USA). A stock P'nase solution (200 U/mL) was prepared by dissolving 0.00639 g (which contains 11.3 mg protein) in 10 mL Milli-Q water. Penicillin was purchased from Sigma Aldrich (USA) and a 0.01 M stock solution was prepared and stored in the refrigerator.

A 0.05 M (pH 8.0) phosphate buffer solution was prepared by diluting 3.4 mL phosphoric acid (14.75 M) to 1 L. This was then used to prepare a 0.01 M stock buffer solution by diluting 200 mL to 1 L. 0.1 M potassium nitrate ($KNO_3$) was added to the buffer as the supporting electrolyte and the pH of the buffer was adjusted with 5 M NaOH. The buffer solution was stored in the refrigerator and diluted further as required.

## 2.2.2 Instrumentation/Electrodes

A potentiostat/galvanostat built at UWS, later transferred to Monash University, was employed for electropolymerisation and potentiometric measurements. Platinum electrodes were polished prior to use with 0.3 μm alumina on a micropolishing cloth and then rinsed with Milli-Q water. Electrodes were also electrochemically cleaned periodically with 1.0 M $H_2SO_4$ by cycling the electrode potential between -200 and +1450 mV versus Ag/AgCl at a sweep rate of 75 mV/s for 10 minutes or until a constant current-voltage was observed.

The electropolymerisation of monomer solutions was performed galvanostatically in a three-electrode cell which consisted of a platinum working electrode (0.08 $cm^2$), pseudo platinum auxiliary and pseudo platinum reference electrodes. Monomer solutions were purged with nitrogen for 5 minutes to remove dissolved oxygen. Aluminium coated

electrodes were used for scanning electron microscopy (SEM) and x-ray photoelectron spectroscopy (XPS) analysis.

Potentiometric measurements were carried out with a two-electrode cell using a PPy-P'nase working electrode and a silver/silver chloride reference electrode. Solutions were stirred during potentiometric measurements with a Sybron Thermolyne stirrer (model S-17410).

### 2.2.3 Preparation of Antibiotic Samples

Abbocillin 125 mg tablets were weighed and ground into a fine powder and dissolved in 20 mL of 0.01 mM phosphate buffer solution. The same procedure was applied to amoxycillin 875 mg tablets and to the contents of the flucoxacillin 500 mg capsules.

### 2.2.4 Preparation of Milk Samples

Milk samples were obtained from the University of Western Sydney, research farm (Faculty of Science, Hawkesbury campus). Three 10 mL raw milk samples were spiked with 250 µL of 0.01 M penicillin G. Each sample was then diluted with 10 mL of 0.01 mM buffer solution.

### 2.2.5 Potentiometric Biosensing

A two-electrode electrochemical cell was used for all potentiometric measurements. The cell contained 30 mL of 0.1 mM phosphate buffer solution (pH 8.0). Solutions were

stirred during potentiometric measurements with a magnetic stirrer. Each potentiometric experiment was repeated 3 times.

### 2.2.6 Preparation of Samples for SEM Analysis

PPy films were electropolymerised on aluminium coated electrodes (4 cm x 1 cm). The monomer solution consisted of 0.1 M Py, 0.01 M Pen and 50 U/mL P'nase. The electropolymerisation process was undertaken for 5 minutes at 0.9 $mA/cm^2$.

SEM was carried out using a JEOL, JSM-6300F scanning electron microscope at Monash University, Clayton campus, Melbourne. The morphologies of the films were characterised at an acceleration voltage of 15 kV and magnifications of 5, 000X, 10, 000X and 20, 000X.

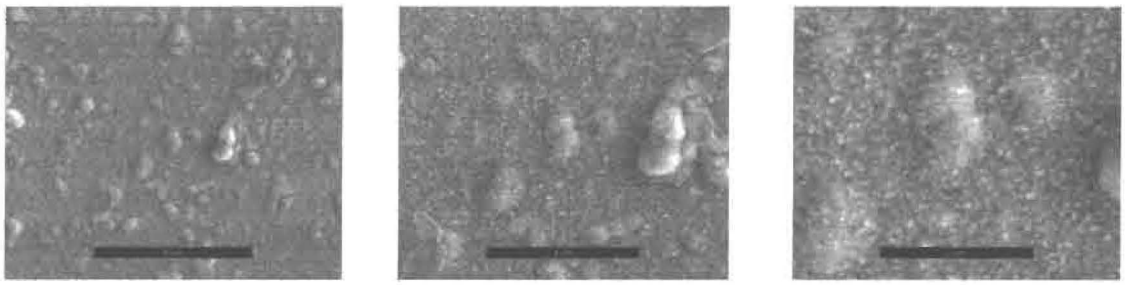
### 2.2.7 Preparation of Samples for XPS Analysis

The PPy films were prepared as indicated above in section 2.2.6. XPS analysis was performed using a KRATOS Analytical AXIS-HSi at CSIRO, Molecular Science, Clayton, Melbourne. Survey spectra were performed to determine the elements present in the films. The analysis was conducted with a monochromatic rotating anode source which provided A1Kα radiation (12 kV x 15 mA). The pressure in the analysis chamber was between 2 x $10^{-8}$ and 5 x $10^{-8}$ mbar. The angle between the normal to the sample surface was $0^{o}$. The area of analysis of the film was ca. 1 $mm^2$ and probed at a sampling depth of ca.10 nanometres.

## 2.3 Results and Discussion

### 2.3.1 Surface Analysis using Scanning Electron Microscopy

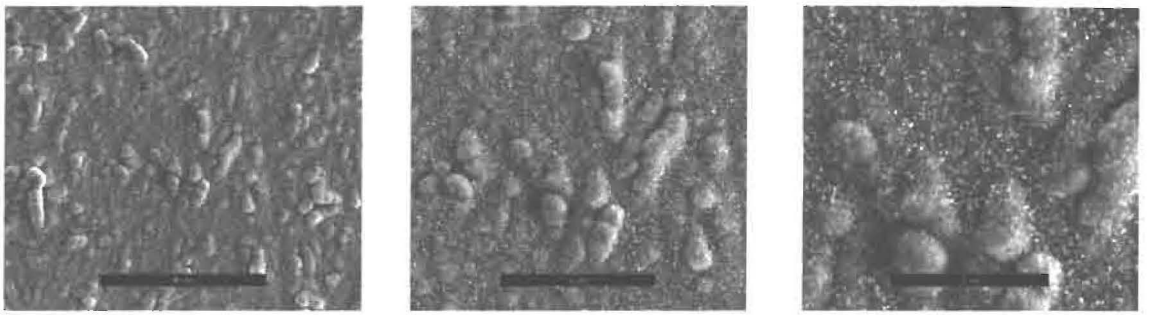
Figure 2.2 shows the scanning electron (SE) micrographs obtained for the polypyrrole-penicillin (PPy-Pen) films grown in the absence of P'nase. The absence of the enzyme shows a more uniform surface morphology with the appearance of a few nodules in comparison to the PPy films shown in Figure 2.3. The very few nodules are attributed to the presence of Pen in the PPy film whereas Figure 2.3 shows a rougher film surface



(a) (b) (c)

**Figure 2.2 SE micrographs showing the surface morphology of PPy films grown in the absence of the enzyme. Monomer solution contained 0.1 M PPy and 0.01 M Pen where (a) 5, 000X (b) 10, 000X (c) 20, 000X. The films were electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

with the appearance of more nodules than in Figure 2.2. The non-uniform surface of the film is attributed to the incorporation of the enzyme in the film. This change in the appearance of the film and hence the porosity of the film further indicates the incorporation of the enzyme in the film. The porosity of the film also affects the



(d) (e) (f)

**Figure 2.3 SE micrographs showing the variation in the surface morphology of PPy films in the presence of the enzyme. Monomer solution contained 0.1 M PPy, 50 U/mL P'nase and 0.01 M Pen where (d) 5, 000X (e) 10, 000X (f) 20, 000. The films were electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

permeability of the film which is influenced by the concentration of the enzyme (Shin & Kim 1996). These factors affect the sensitivity of the electrode, as will be demonstrated in other parts of this chapter. It also highlights the need to optimise enzyme concentrations to obtain optimum performance of the electrode.

## 2.3.2 XPS Analysis of Film

XPS is a useful analytical tool for the surface analysis of a film which involves the elemental analysis of the film. This is useful for verifying the presence of the enzyme within the film and identifying the PPy backbone. Figure 2.4 shows a wide scan XPS spectrum for the PPy-P'nase film. The presence of the photoelectron peak detected for the N 1s peak at 398.3 eV is indicative of the presence of the C-N bond of Py.

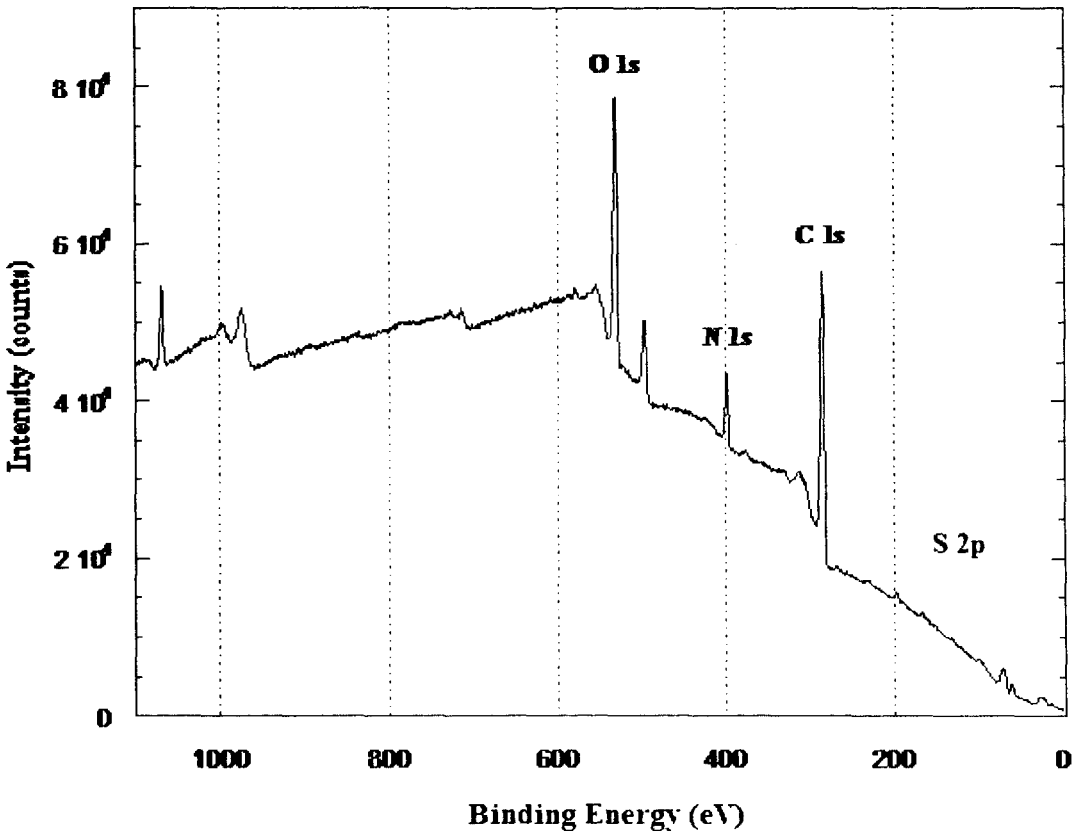



**Figure 2.4 XPS wide scan of PPy-P'nase film. Monomer solution contained 0.1 M PPy, 50 U/mL P'nase and 0.01 M Pen. The films were electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

The C 1s (3) peak indicated at 286.5 eV also corresponds to the C-N bond, thus confirming the structure of the PPy backbone which contains C-N and C-C bonds. The incorporation of the enzyme in the film was verified by the appearance of the S 2p peak at 168 eV in the XPS widescan shown in Figure 2.4. This confirms the incorporation of the enzyme within the film as all enzymes are made up of amino acids that contain

sulfur bonds and the identification of the sulfur peak enables the verification of the presence of the enzyme in the film (Adeloju et al. 1993).

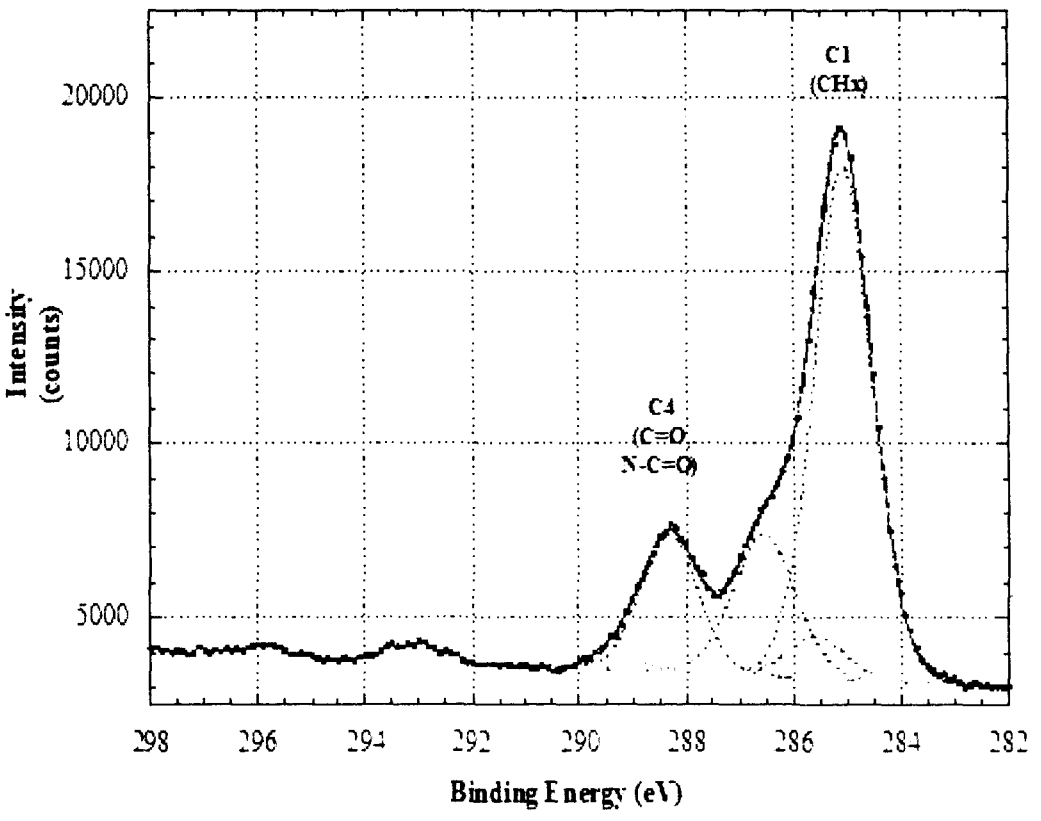



**Figure 2.5 C 1s spectrum for the PPy-P'nase film. Monomer solution contained 0.1 M PPy, 50 U/mL P'nase and 0.01 M Pen. The films were electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

The change in the morphology of the film, shown in Figure 2.3, also confirms the presence of the enzyme in the film. The peak which appeared at approximately 200 eV in Figure 2.4 is attributable to impurities or background noise.

Figure 2.5 shows the presence of C 1s (4) peak which is characteristic of the N-C=O or C=O groups which appeared at 288.3 eV. Also, the O 1s peak at 531.5 eV in Figure 2.4 corresponds to the presence of C=O bond. The identification of these groups confirms the presence of Pen in the film. Evidently, this was due to the addition of Pen to the monomer solution. Table 2.1 lists all the relevant photoelectron signals detected and the corresponding binding energy values. The mean

**Table 2.1 Mean Binding Energy Values and Assignments for Photoelectron Peaks Detected for the PPy Sample**

| Element | Mean Binding Energy (eV) | Standard Deviation (eV) | Peak Assignment |
|---|---|---|---|
| C 1s (1,2) | 285.0* | 0.0 | Hydrocarbons $CH_x$ reference carbon |
| C 1s (3) | 286.5* | 0.1 | C-N |
| C 1s (4) | 288.3* | 0.1 | C=O |
| S 2p | 168.0* | 0.8 | Sulfur |
| O 1s | 531.5* | 0.1 | C=O, $COO^-$ |
| N 1s | 398.3* | 0.1 | C-N |

* Mean of 5 values

values of 5 measurements plus the standard deviations are shown with peak assignments. The assignments shown in Table 2.1 were based on the binding energy of the photoelectron peak corresponding to a particular element or species. These values

were obtained by using the C 1s peak at 285.0 eV as a reference (Contour & Mouvier 1975; Dubois et al. 1981; Beamson & Briggs 1992; NIST XPS 2000).

### 2.3.3 Optimisation of the Pyrrole Concentration

The concentration of Py in the monomer solution is important for the attainment of an optimum response as it influences the sensitivity of the biosensor. The sensitivity was

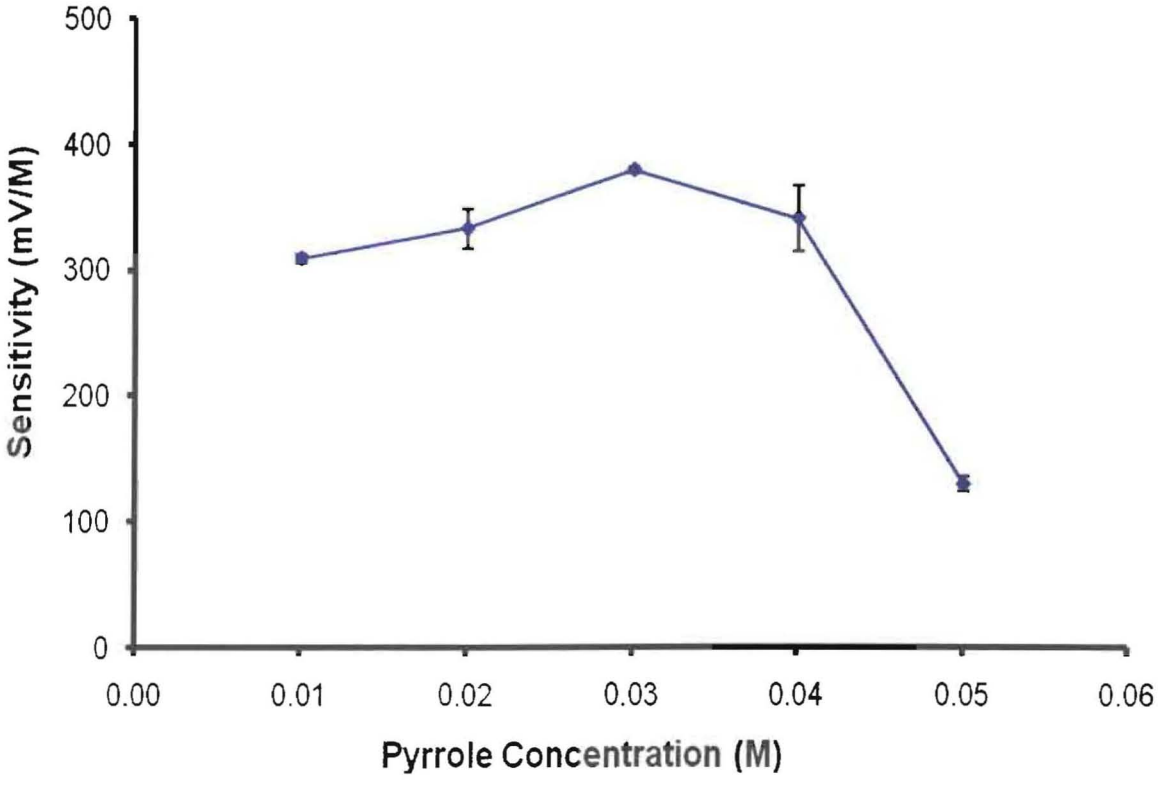



**Figure 2.6 Influence of the pyrrole concentration used for film formation on the penicillin response obtained with PPy-P'nase biosensor. The following conditions were kept constant during electropolymerisation: 19 U/mL P'nase, current density 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

determined using the slope of the linear calibration plot. Figure 2.6 illustrates the effect of the increasing Py concentration on the sensitivity of the PPy-P'nase electrode to penicillin. The use of Py concentrations >0.03 M resulted in a significant reduction in the sensitivity of the biosensor. This is attributed to an increase in the film thickness which reduces the ability of the catalytic products to reach the electrode surface (Adeloju & Sohail 2008; Adeloju et al. 2008). The conditions that do not increase the diffusion barrier significantly are more suited for the sensitive potentiometric detection of penicillin. The optimum sensitivity for the penicillin response was obtained by using 0.03 M Py and for this reason this monomer concentration was used for the formation of PPy-P'nase electrode in other investigations.

### 2.3.4 Influence of the Inclusion of Penicillin in the Monomer Solution

The inclusion of Pen in the monomer solution was an important aspect of this study. This is because the potentiometric response obtained with the PPy-P'nase electrode was unstable. Each response decreased rapidly after reaching a maximum potential with each penicillin addition. According to previous reports, this reduction in the potentiometric response is due to a drop in the pH of the membrane caused by the presence of negative charge on the membrane (Gorchkov et al. 1996). Although it was clearly obvious that the potentiometric response increased with increasing penicillin concentration, a steady state response was not achieved and the quality of the response was inadequate to ensure reliable quantification of penicillin. Figure 2.7 shows that with the addition of Pen to the monomer solution, a significant improvement in the

attainment of a steady state response was achieved. Also, the sensitivity of the response was improved dramatically. Possible reasons for the improvement in the potentiometric response include the increased conductivity of the PPy film in the presence of Pen

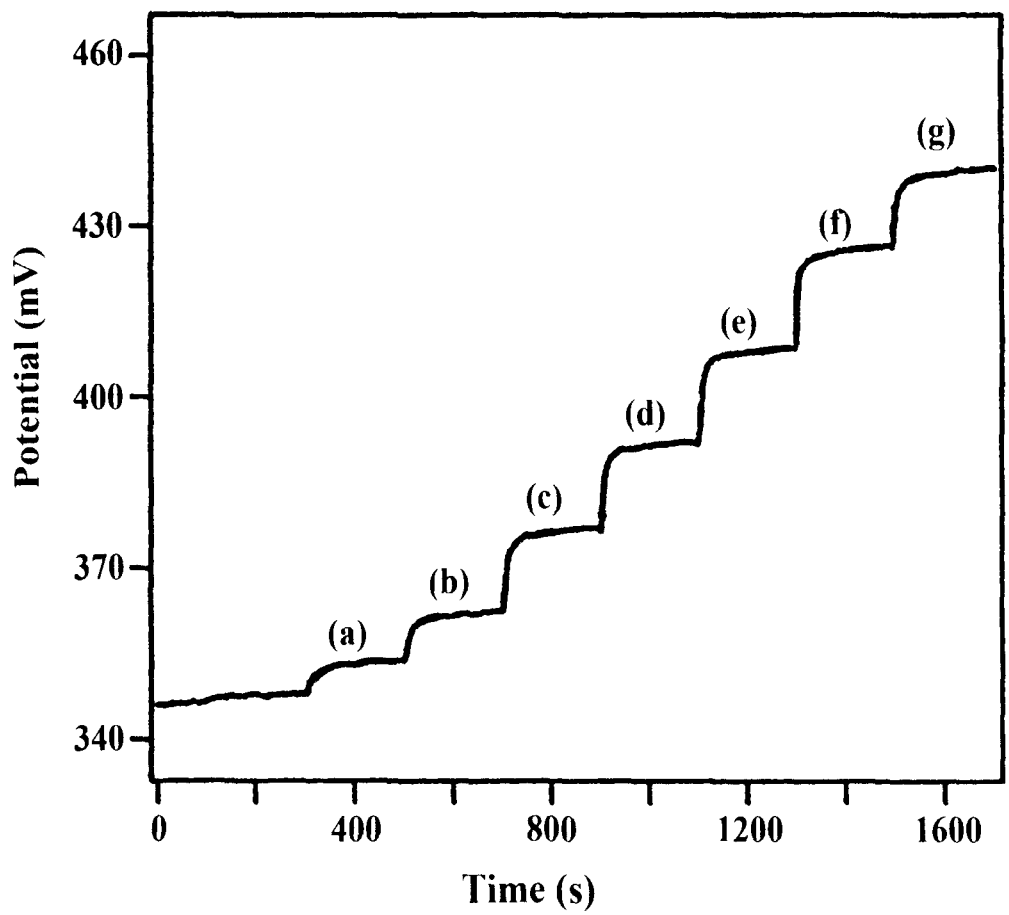



**Figure 2.7 Typical potentiometric response of the PPy-P'nase-Pen electrode. Film conditions: 0.03 M Py, 19 U/mL P'nase, 0.01 M Pen, current density 0.9 mA/cm$^2$, electropolymerisation time 40 s. Potentiometric biosensing was carried out in 0.01 mM buffer solution, (a) 0.002 (b) 0.003 (c) 0.008 (d) 0.02 (e) 0.05 (f) 0.1 and (g) 0.2 mM penicillin.**

(Nishizawa et al. 1992), as will be demonstrated in chapter 3. The presence of a positive charge on the polymeric membrane will also attract more penicillin, which is a criterion for an increase in the potentiometric signal (Gorchkov et al. 1996). It appears that the presence of Pen in the film influenced the charge on the membrane and increased the conductivity of the PPy membrane. The charge on the membrane is difficult to predict (Gorchkov et al. 1996) due to the presence of the polyanionic enzyme and the partial positive charge on the PPy backbone (Shin & Kim 1994). However, it is important to note that the charge of the membrane also has an electrostatic influence on the charged enzymatic products and the charged substrate (Gorchkov et al. 1996). This provides a reasonable rationale for the achievement of a steady state response when the production and the loss of hydrogen ions are balanced (Kulp et al. 1987). The loss and production rates of hydrogen ions can also be influenced by the retention of penicilloate in the membrane which accounts for the negative charge on the membrane and the resultant decrease in the potentiometric response. Penicilloate is negatively charged (2 charges per molecule) and is generated during the enzymatic reaction through the dissociation of penicilloic acid. In order to influence the charge on the membrane and avoid the decline in the potentiometric response, Pen was added to the monomer solution.

Figure 2.8 provides a diagrammatic illustration to explain the process of the retention of the enzymatic product, penicilloate, in the film and the resultant decline in the potentiometric response. Mechanistically, P'nase catalyses the cleavage of the β-lactam ring of penicillin to form penicilloic acid which dissociates into penicilloate and a proton and produces a change in the pH of the membrane (Kulp et al. 1987), as shown in reaction 1 (Stred'ansky 2000).

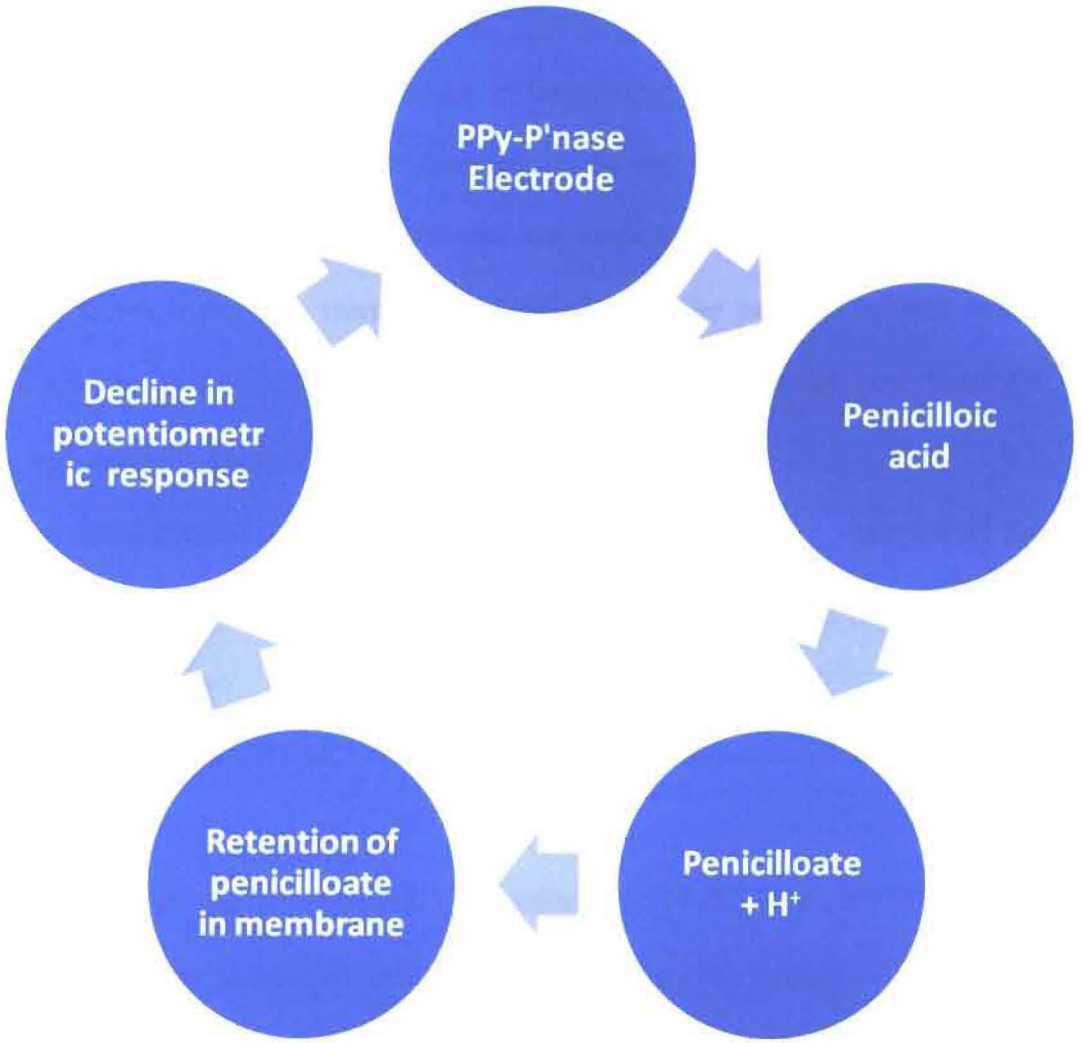



**Figure 2.8 Model illustrating the process of the retention of penicilloate in the film.**

$$\text{Penicillin} + H_2O \longrightarrow \text{Penicilloate}^- + H^+ \qquad \text{(Reaction 1)}$$

This produces an analytical signal through an increase in the potential caused by the generation of $H^+$ ions. However the decrease in the pH of the membrane and the resultant negative charge on the membrane are responsible for the decline in the response. It seems reasonable therefore to conclude that the presence of Pen in the monomer solution was necessary to ensure the attainment of a stable potentiometric response and was used in other studies.

The relationship between the concentration of $[H^+]$ and the relative potential of the electrode is demonstrated by the Nernst equation:

$$E_{cell} = RT/nF \ln [H^+] \qquad \text{(Reaction 2)}$$

(where $E_{cell}$ = emf of cell, R= universal gas constant (8.314 $JK^{-1}$ mol); T= temperature (in degrees kelvin); n= number of electrons transferred for each reaction; F= Faraday's constant (96485 C $mol^{-1}$) (Ewing 1985).

## 2.3.5 Effect of the Penicillinase Concentration

The effect of the P'nase concentration on the response of the film was studied by varying the concentrations of P'nase in the monomer solution. Figure 2.9 illustrates the behaviour of the electrode produced with varying enzyme concentrations. A dramatic decrease in the electrode sensitivity was observed when enzyme concentrations greater than 50 U/mL were added to the monomer solution. The incorporation of higher

enzyme concentrations to the monomer solution resulted in a less sensitive film due to the fact that the polymerisation of Py is hindered at higher enzyme concentrations because of the high molecular weight of P'nase which contributes to steric hinderance

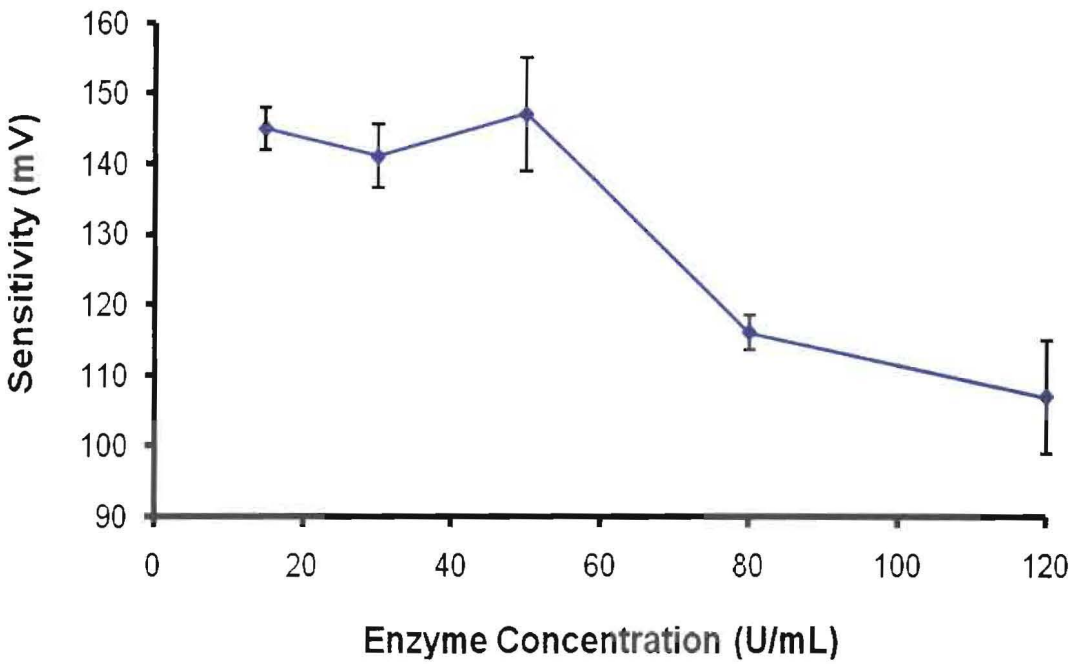



**Figure 2.9 The influence of the penicillinase concentration on the sensitivity of the electrode. The following conditions were kept constant during electropolymerisation: 0.03 M Py, 0.01 M Pen, current density 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

(Almeida et al. 1993). These results indicate that the use of 50 U/mL of P'nase for the formation of the PPy-P'nase film was optimal. Alternatively, the use of enzyme concentrations greater than 50 U/mL appears to have had a negative impact on the

sensitivity of the film. This can be attributed to the fact that high enzyme concentrations can affect the nucleation process during the formation of the PPy film (Schneider & Schwitzgebel 1993). This is due to the fact that at such high enzyme concentrations a more compact film was obtained. In this case, the saturation of the PPy layer with the enzyme renders the film less permeable to the substrate (Griffith et al. 1996; Shin & Kim 1996; Njagi & Andreescu 2007).

## 2.3.6 Effect of Applied Current Density and Polymerisation Time

The applied current density and the polymerisation time can affect the formation of the

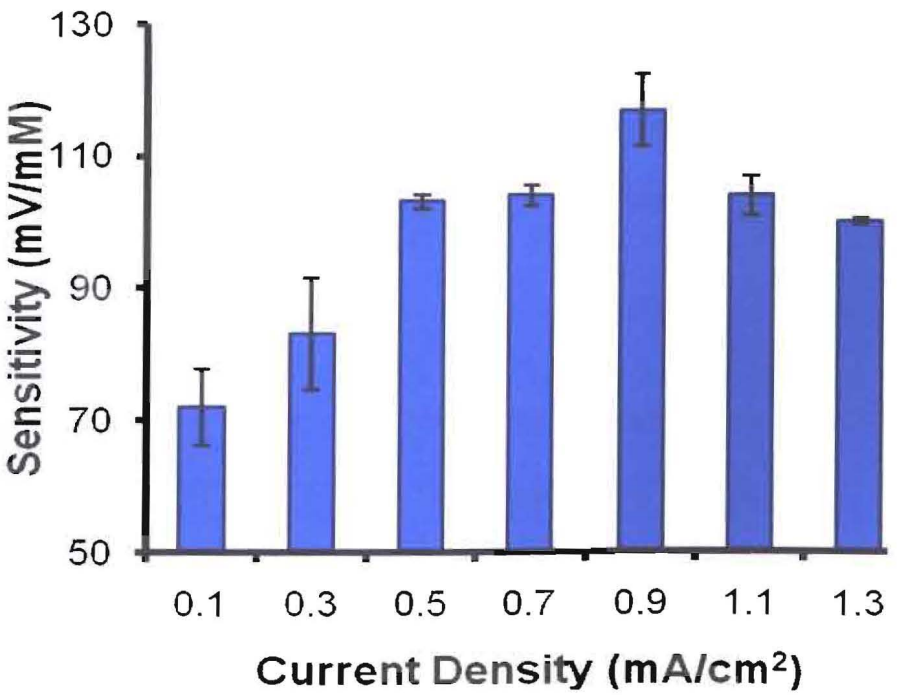



**Figure 2.10 The dependance of the penicillin response on the applied current density used for film formation. The following conditions were kept constant: 0.03 M Py, 19 U/mL P'nase, 0.01 M Pen, electropolymerisation time 40 s.**

PPy-P'nase film and influence the sensitivity of the response. Figure 2.10 illustrates the effect of the applied current density on the formation of the PPy-P'nase film and the associated sensitivity to penicillin. The highest sensitivity was achieved when a current density of 0.9 $mA/cm^2$ was applied for film formation. Beyond this applied current density, the sensitivity of the electrode decreased due to the increased film thickness and the corresponding increase in the diffusion barrier which limits the ability of the reaction products to reach the electrode surface (Adeloju & Moline 2001; Adeloju & Sohail 2008).

The use of lower current densities for the formation of the PPy-P'nase film affected the sensitivity of the film because the slower growth rate of the film results in less enzyme being entrapped (Diaz & Hall 1983; Shin & Kim 1994). This suggests that the formation of the film was slower with lower enzyme retention between the current densities of 0.01 - 0.7 $mA/cm^2$ and, hence, resulted in a lower sensitivity. This is caused by the rapidity of the formation of the PPy film which is also accompanied by a concomitant decrease in the amount of enzyme incorporated (Adeloju & Moline 2001). The highest sensitivity was achieved with an applied current density of 0.9 $mA/cm^2$ and was used in other studies.

Figure 2.11 shows the effect of increasing the polymerisation time used for the formation of the PPy-P'nase film on the sensitivity of the electrode. The highest sensitivity was achieved with a polymerisation time of 40 s. Beyond this polymerisation time, the sensitivity of the electrode to penicillin was substantially lower. This is due to the increased film thickness and the associated increase in the diffusion barrier. The lowest sensitivities were obtained for films formed with polymerisation times of 10 - 20

minutes. At these polymerisation times the response of the electrode was influenced by the relatively thick film and the increased diffusion barrier which affects the porosity of the membrane and the response of the film. The optimum sensitivity for the potentiometric detection of penicillin was achieved with a PPy-P'nase film formed with a polymerisation time of 40 s. Hence, a polymerisation time of 40 s was used in other studies.

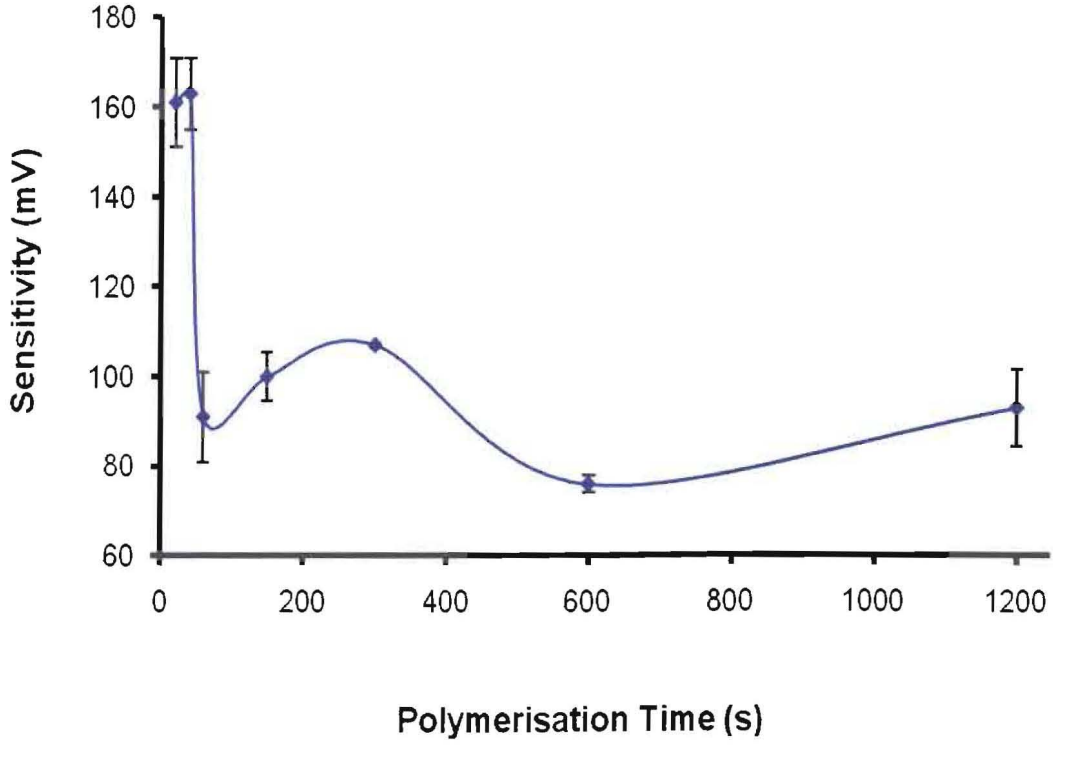



**Figure 2.11 Dependence of the penicillin response on the polymerisation time used for film formation. The following conditions were kept constant: 0.03 M Py, 19 U/mL P'nase, 0.01 M Pen, current density 0.9mA/cm$^2$.**

## 2.3.7 Influence of Buffer Concentration

The penicillin potentiometric response of the PPy-P'nase electrode can also be influenced by the buffer concentration. Figure 2.12 shows a sharp decrease in the response of the electrode when the buffer concentration was increased from 0.05 to 1 mM. This is due to the fact that the buffer concentration must be low enough to ensure detection of the relatively low $H^+$ ion concentration produced by the enzyme catalysed hydrolysis of penicillin (Situmorang et al. 2001). In addition, the reduction of the sensitivity and the measurement range of the biosensor were also decreased due to an increase in the concentration of the buffer molecules in the enzymatic membrane thereby affecting the protons produced by the enzymatic reaction (Brand et al. 1990) and decreasing the electrode sensitivity.

On the other hand, a decrease in the buffer capacity also decreases the effective diffusion coefficient of hydrogen ions and also affects the sensitivity. This is evident with the use of 0.01 mM buffer where the potentiometric response of the electrode to the hydrogen ions produced from the dissociation of penicilloic acid is decreased. At this buffer concentration, the response to higher penicillin concentrations is compromised by the low buffer capacity. Consequently, the acid produced from the enzymatic reaction at higher penicillin concentrations far exceeds the low buffer capacity. As a result, a non-linear calibration curve is obtained when 0.01 mM buffer was used. At these lower concentrations, the buffer capacity is inadequate (Chao & Lee 2000) and the response of the electrode is affected.

The highest potentiometric responses were obtained when 0.1 and 0.05 mM buffer solutions were used. Plausible reasons for this include the fact that higher buffer

concentrations shorten the response time due to the enhancement of proton diffusion (Chao & Lee 2000) which is evident with both buffer concentrations.

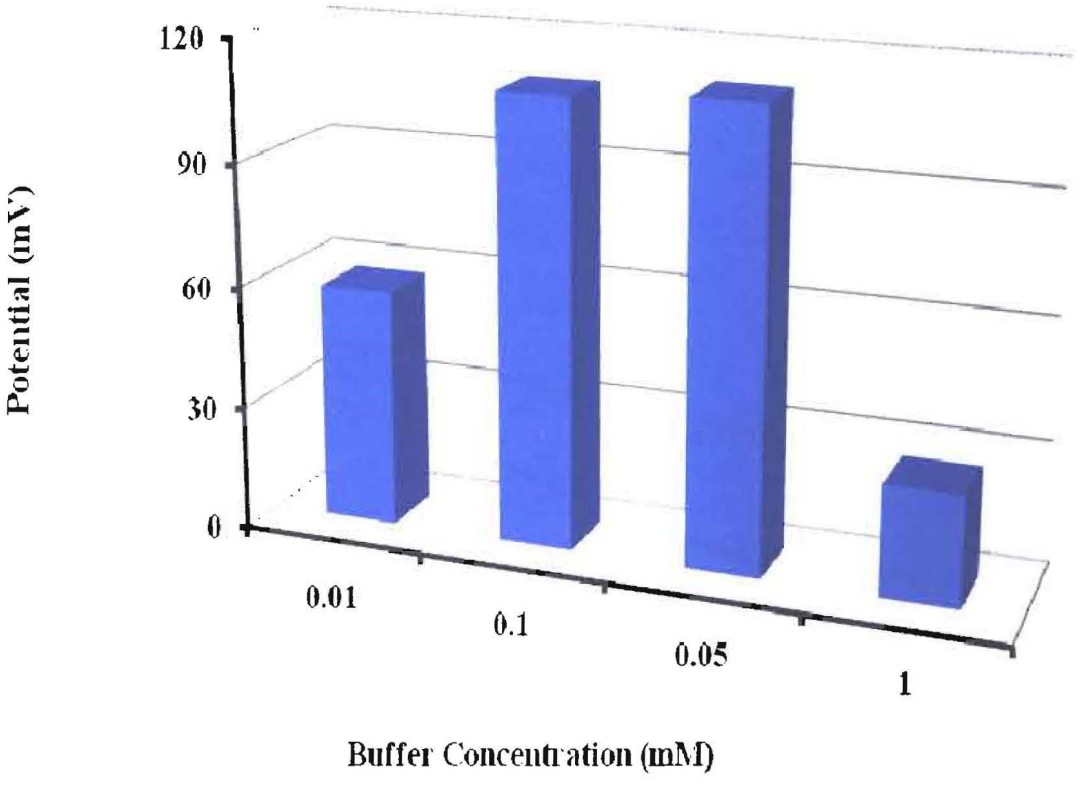



**Figure 2.12 Influence of the buffer concentration on the potentiometric response of penicillin. The following conditions were kept constant: 0.03 M Py, 19 U/mL P'nase, 0.01 M Pen, current density 0.9mA/cm$^2$, electropolymerisation time 40 s.**

The linear concentration range of the biosensor is also influenced by the buffer concentration. A plot of potential versus the logarithmic concentration of penicillin showed that the slope for 0.05 mM buffer was more sensitive than that obtained for 0.1 mM buffer. Both coefficients of determination shown in Figure 2.13 appear to be low. The statistical significance of the correlation coefficient at the 95% confidence level is (0.896 ± 0.02), the slope (35.754 ± 1.37) and the y-intercept is (607.28 ± 25.65).

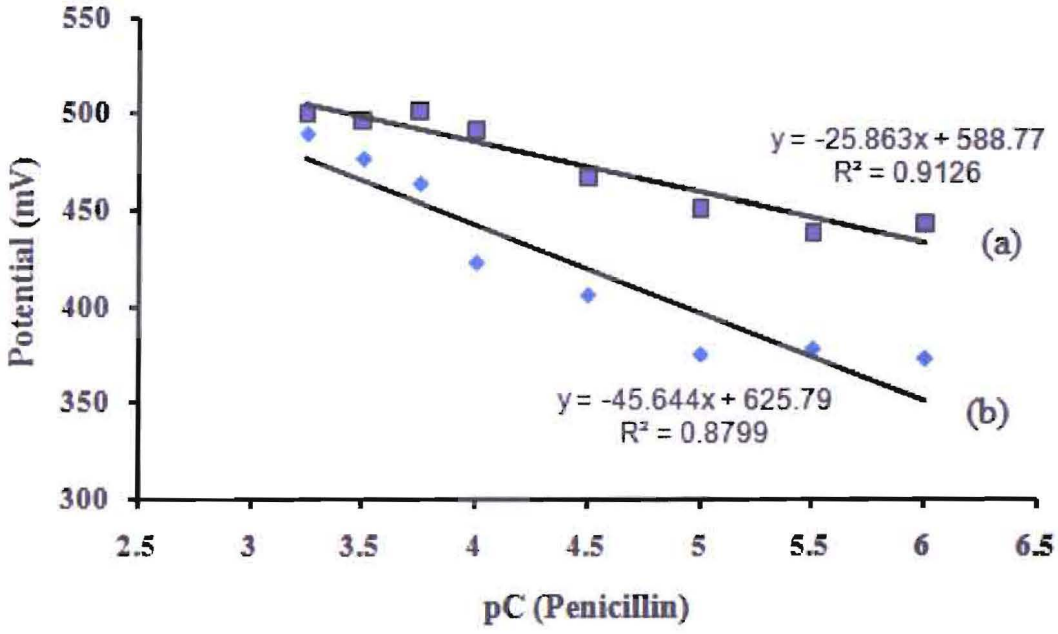



**Figure 2.13 Logarithmic concentration of penicillin response obtained with PPy-P'nase film. The following film conditions were applied: 0.03 M Py, 19 U/mL P'nase, 0.01 M Pen, current density 0.9 mA/cm$^2$, electropolymerisation time 40 s where (a) 0.1 mM phosphate buffer and (b) 0.05 mM phosphate buffer.**

However, 0.05 mM buffer was not sensitive for penicillin concentrations >156 μM. Figure 2.13 shows that the penicillin response in 0.1 mM buffer is more linear ($R^2$=0.9126) than that obtained in 0.05 mM buffer ($R^2$=0.8799). The linear concentration range for 0.1 mM buffer is 45 - 283 μM and is wider than the response obtained in 0.05 mM buffer (146 – 283 μM). The decrease in the buffer concentration decreases the number of buffer molecules in the enzymatic membrane required to buffer the protons generated by the dissociation of penicilloic acid (Brand et al. 1990). This compromises the linear range of the biosensor. Suitably low buffer concentrations are required in order to maintain control over the buffering capacity of the solution (Kulp et al. 1987). For this reason a buffer concentration of 0.1 mM was employed in other studies.

### 2.3.8 Analytical Applications

The biosensor was applied to the potentiometric detection of penicillin in raw milk samples. Figure 2.14 shows that the injection of a 300 μL aliquot of the spiked raw milk sample, produced a potential change of 12 mV. Subsequent additions of 300 and 500 μL of 0.01 M penicillin G resulted in increases in the potentiometric response. These observations confirmed that the penicillin present in the raw milk sample can be detected with the PPy-P'nase biosensor. However, reliable quantification of penicillin in milk could not be achieved as the observed increase in the potentiometric response for the added penicillin standard was lower than expected. This is due to a high level of non-specific binding of milk proteins to the sensor surface (Gustavsson et al. 2002;

Cacciatore et al. 2004) which interfered with the potentiometric response. Hence, further optimisation of sample preparation is still necessary.

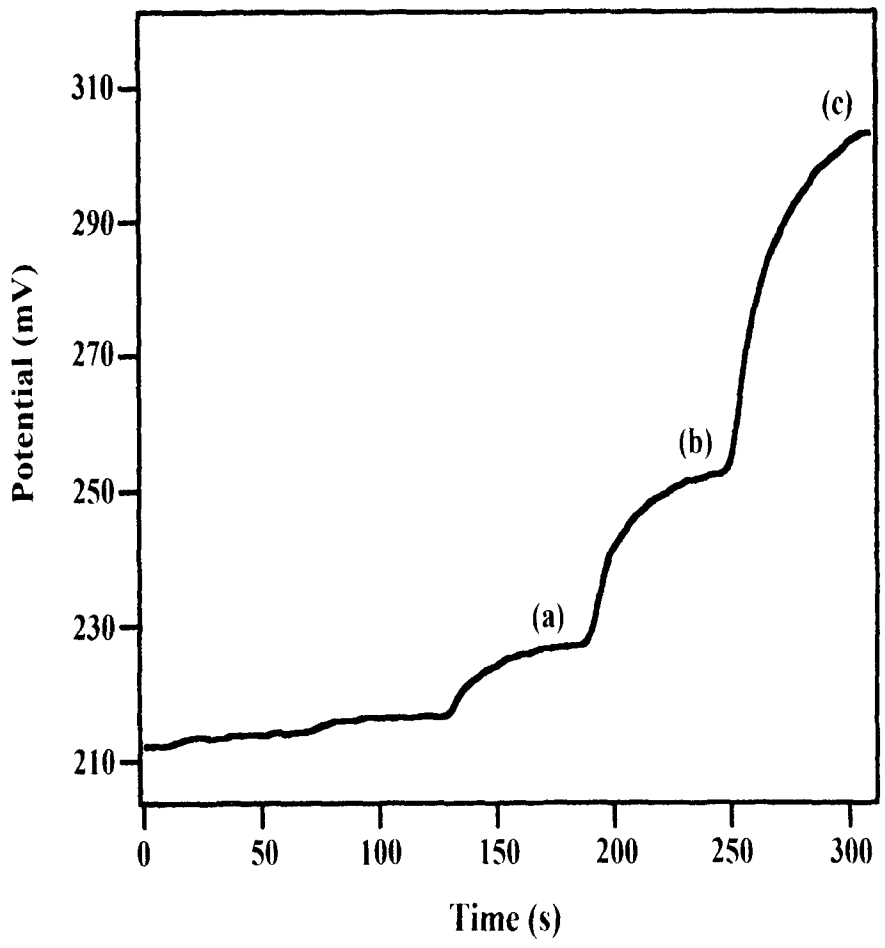



**Figure 2.14 Potentiometric response obtained for penicillin in a raw milk sample obtained with PPy-P'nase-Pen electrode where (a) 300 µL aliquot of spiked milk sample (b) 0.1 mM penicillin and (c) 0.2 mM penicillin.**

The biosensor was also applied to the determination of penicillin in pharmaceutical preparations, such as abbocillin 125 mg, flucoxacillin 500 mg and amoxycillin 875 mg.

Figure 2.15 shows the potentiometric response of the biosensor to a 300 μL addition of abbocillin 125 mg tablet which produced a potential change of 13 mV. Again, subsequent additions of 300 and 500 μL of 0.01 M penicillin G resulted in increases in the potentiometric response and, thus, confirmed the presence of penicillin in the tablet. However, quantification was again not possible at this stage, and further optimisation of sample preparation is necessary.

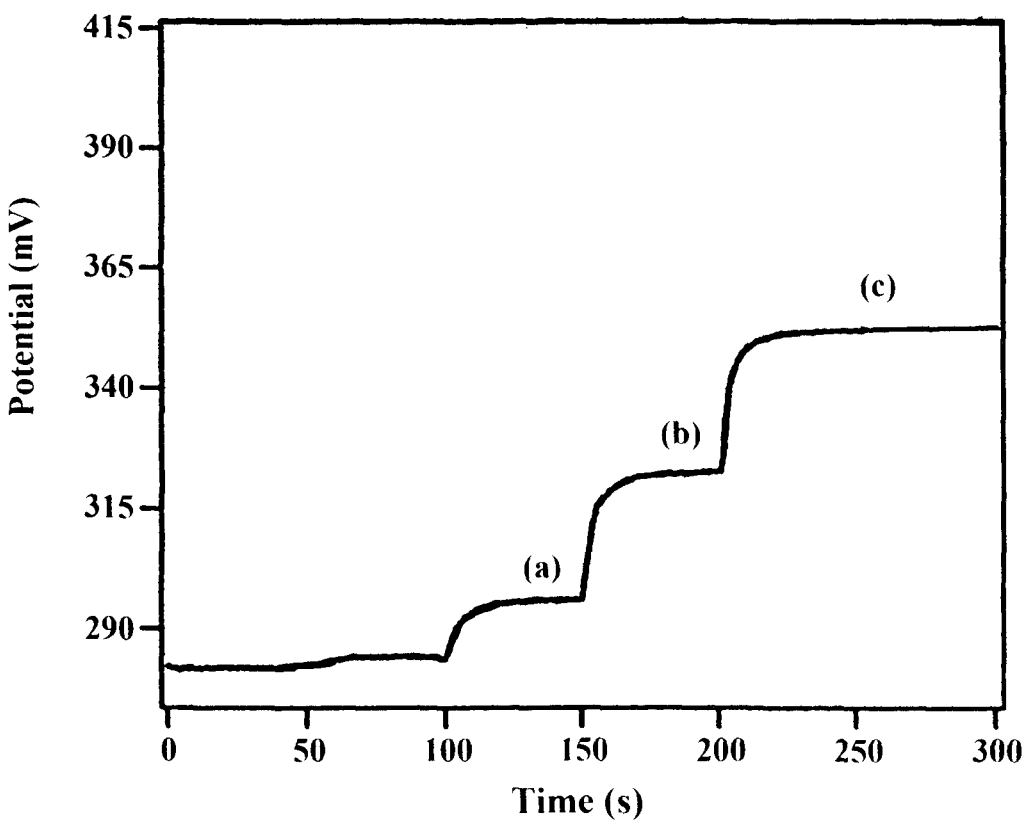



**Figure 2.15 Potentiometric response obtained for penicillin in abbocillin 125 mg sample obtained with PPy-P'nase-Pen electrode where (a) 300 μL of abbocillin sample (b) 0.1 mM penicillin standard (c) 0.2 mM penicillin standard.**

Figure 2.16 shows the penicillin response obtained with the addition of a 300 µL aliquot of flucoxacillin 500 mg tablet which produced a potential change of 34 mV. This potential change is higher than that shown for abbocillin 125 mg which corresponds to the different penicillin concentrations present in both antibiotic tablets. The consecutive addition of 300 and 500 µL of 0.01 M penicillin G increased the penicillin response and confirmed the presence of penicillin. However, sample preparation requires further optimisation in order to achieve reliable quantification of penicillin.

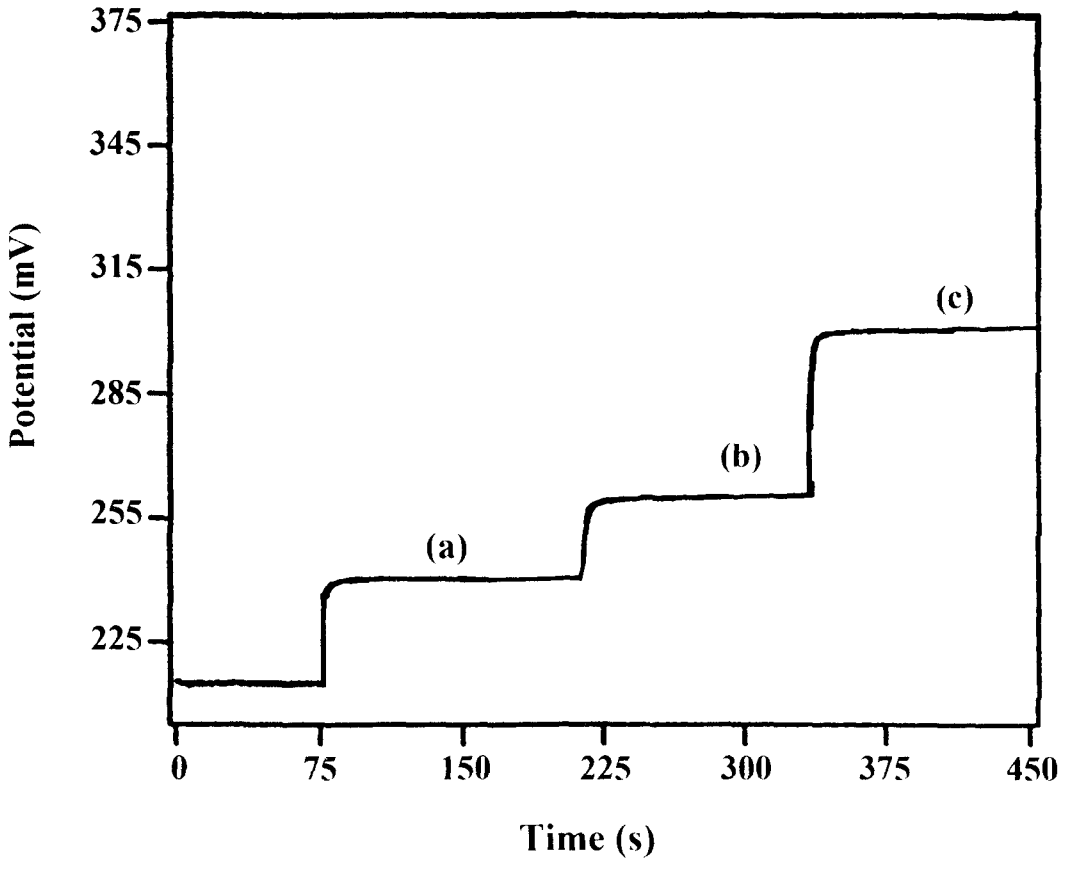



**Figure 2.16 Potentiometric response of flucloxacillin 500 mg obtained with PPy-P'nase-Pen electrode (a) 300 µL of flucloxacillin sample where (b) 0.1 mM penicillin standard (c) 0.2 mM penicillin standard.**

Figure 2.17 shows that the addition of a 300 μL aliquot of amoxycillin 875 mg tablet also produced a potential change of 65 mV which is higher than the potential changes achieved for both abbocillin 125 mg (13 mV) and flucoxacillin 500 mg (34 mV). The differences between the potential changes obtained for flucoxacillin 500 mg, abbocillin

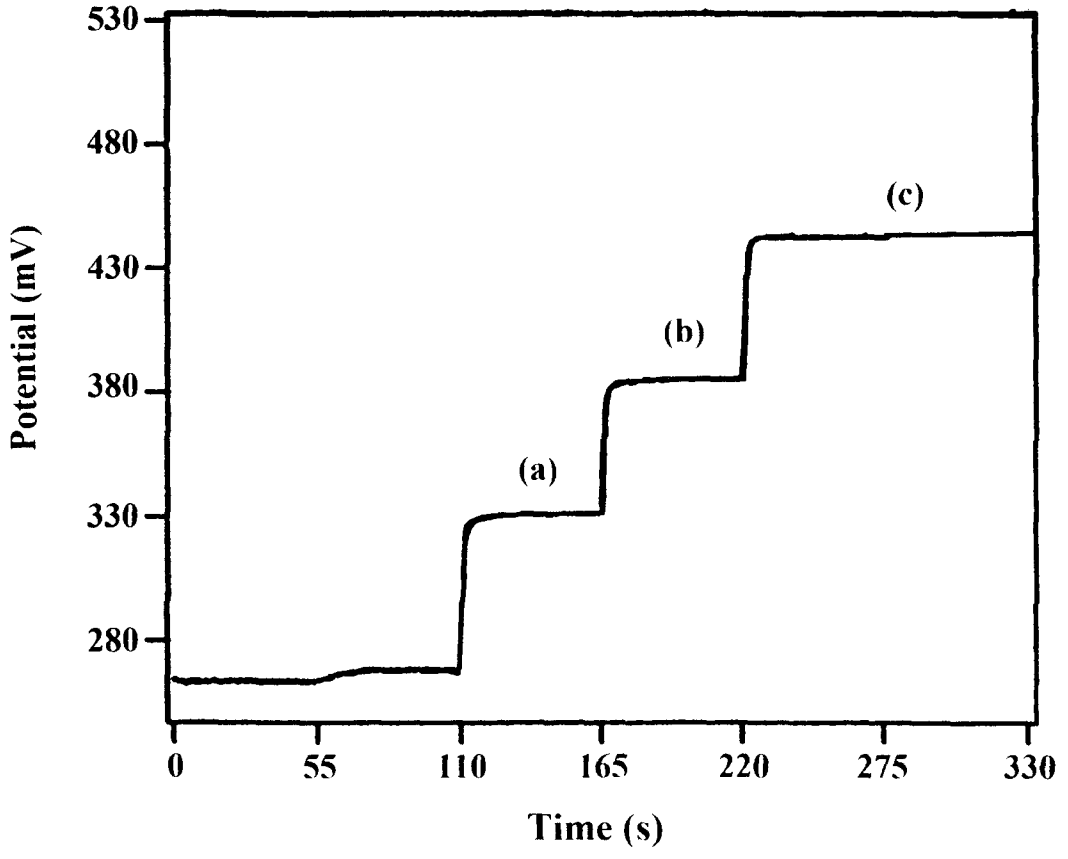



**Figure 2.17 Potentiometric response obtained for amoxycillin 875 mg obtained with PPy-P'nase-Pen electrode where (a) 300 μL of amoxycillin sample (b) 0.1 mM penicillin (c) 0.2 mM penicillin.**

125 mg and amoxycillin 875 mg clearly indicates that the biosensor is able to distinguish between the varying penicillin concentrations present in the pharmaceutical preparations. However, further optimisation of sample preparation is required and will be examined in chapter 3.

## 2.4 Conclusions

The fabrication of a penicillin biosensor by physical entrapment of P'nase in a PPy film has been successfully accomplished. The optimum conditions established for the film formation of the PPy-P'nase biosensor were 0.03 M Py, 50 U/mL P'nase, 0.01 M Pen, applied current density of 0.9 $mA/cm^2$ and a polymerisation time of 40 s. The inclusion of Pen in the monomer solution was necessary to achieve a steady state response. The potentiometric response produced for films without the incorporation of Pen in the monomer solution, was unstable due to the negative charge on the membrane as a result of the retention of penicilloate in the membrane. The presence of Pen in the membrane influenced the charge on the membrane and facilitated the attainment of a steady state response. The buffer concentration which produced the most sensitive response for penicillin was 0.1 mM buffer. The linear concentration range obtained with the PPy-P'nase biosensor was 45 - 283 μM.

The PPy-P'nase electrode was applied to the determination of penicillin in raw milk samples. However, it was evident that the sample preparation method required further optimisation in order to reduce interferences resulting from specific and non-specific binding of milk proteins to the surface of the sensor. The biosensor was also applied to the determination of the active constituent in 3 pharmaceutical formulations, namely abbocillin 125 mg, flucoxacillin 500 mg and amoxycillin 875 mg. The ability of the

electrode to distinguish between the different concentrations of the active constituents was established. However, further sample preparation was again required to achieve reliable quantification of the active constituent in the antibiotics. Further work will be undertaken in chapter 3 to improve the ability of the biosensor to quantify penicillin in milk and in pharmaceutical preparations.

# Chapter 3

## The use of a Bi-Layer Configuration for the Development of a Penicillin Biosensor

## 3.1 Introduction

The entrapment of P'nase in a single layer PPy film was demonstrated in chapter 2, however the sensitivity of the biosensor required further improvement. One way to achieve this is to use a bilayer configuration whereby the enzyme is immobilised in both layers. The reason for this is that bilayer biosensors are more sensitive than single layer biosensors (Vidal et al. 1999) and have the ability to expand the scope of the single layer biosensor as they provide more alternatives for the improvement of the overall properties of the biosensor. Specifically, the benefits of using a bilayer configuration include considerable sensitivity enhancement through the efficiency of electron transfer between the enzyme and the electrode. For example, the use of peroxidase and oxidase enzymes in different layers provides significant benefits than with the use of a single layer bi-enzyme biosensor (Tatsuma et al. 1993; Vidal et al. 1999).

According to several reports, bilayer configurations exhibit unique properties (Abruna et al. 1981; Leidner et al. 1985; Hillman et al. 1990; Torres et al. 1990; Bobacka et al. 1993; Gao et al. 1993; Maksymiuk 1994; Maksymiuk 1996; Wojda et al. 1997; Wojda et al. 1998). These include the choice of the polymers used in the bilayer system, such as the use of polymers that exhibit lower conductivities as one of the components of the bilayers. This limits the response of the system, whereas the use of two highly conductive polymers, in a bilayer configuration, is more advantageous as it maximises the response of the biosensor. It is also important to note that the electrochemical modification of the layers during the preparation of the bilayers should not affect the overall stability of the biosensor. The conductivity of the inner layer should be preserved to enable new layers to be grown over the inner layer (Vidal et al. 1999).

Overall, the nature of the bilayer arrangement has a significant influence on the diffusion of the substrate and, hence, on the sensitivity and selectivity of the electrode. To this end, serious consideration must be given to ensure careful control of the porosity and the thickness of these layers in order to maximise sensitivity.

Bilayer biosensors can also be prepared with different polymers where the choice of polymers allows the separation of the energy levels of the polymers used and enables the maintenance of a stable charge in the bilayer (Hillman & Mallen 1990). However the use of same conducting polymer in both layers provides more advantages. Cosnier et al. (1998) reported on the use of PPy in both layers of a bilayer system. Several studies have also reported that bilayer systems are made of polymers with well separated redox properties, such as PPy and poly (N-methylpyrrole), as well as PPy and polythiophene (Hillman & Mallen 1990; Wojda & Maksymiuk 1998; Alumaa et al. 2004). However, this study will focus on the spatial separation of consecutively prepared PPy bilayers.

Nikolelis & Tzanelis (1994) reported on a penicillin biosensor which used a lipid bilayer membrane that monitored hydrolytic enzyme reactions at the membrane surface. No other work has been reported on the use of a PPy bilayer to fabricate a potentiometric penicillin biosensor which employs the same polymer in both layers. This study will investigate the immobilisation of P'nase in a PPy bilayer configuration for the fabrication of a sensitive potentiometric biosensor for penicillin. The adequacy of the bilayer configuration for the potentiometric detection of penicillin will be determined through the optimisation of the inner layer, Py concentration, enzyme concentration, Pen concentration in the monomer solution, applied current density,

polymerisation time, and the influence of buffer concentration. The resulting biosensor will be applied to the determination of penicillin in amoxycillin and in milk samples.

## 3.2 Experimental

### 3.2.1 Reagents and Chemicals

All experimental conditions were the same as those described in section 2.2 in chapter 2, unless otherwise indicated.

### 3.2.2 Preparation of Milk Samples

The determination of penicillin concentration in milk was conducted with homogenised and pasteurised milk (total fat content 3.4 %, saturated fat content 2.2 %) which was purchased from a local supermarket (Sydney, NSW). Four milk samples (30 mL each) were spiked with 0, 250, 500 and 1000 μL of 0.01 M penicillin G, consecutively. Acid fractionation was then applied to each sample by adding 0.1 M hydrochloric acid until the pH reached 4.6. This separated the proteins into 2 components, namely: caseins in the precipitate, and the whey proteins in the supernatant which was extracted using a pasteur pipette leaving behind an insoluble precipitate, the caseins. The supernatant was then centrifuged at 6000 rpm for 25 minutes and filtered through Whatman filter paper no. 541. The resulting clear solution was diluted with 15 mL of 0.01 mM phosphate buffer. This procedure was repeated for all 4 samples. The milk samples were refrigerated at 4 $^{o}$C.

### 3.2.3 Preparation of Amoxycillin Samples

Twelve amoxycillin 500 mg tablets were ground into a fine powder. The powdered samples were then dissolved in 100 mL of 0.01 mM phosphate buffer solution. The solution was filtered through Whatman filter paper no. 541. The filtrate was then collected and centrifuged at 2000 rpm for 10 minutes.

The concentration of amoxycillin was determined by injecting a 30 μL aliquot of the dissolved sample into the buffer solution. This was followed by sequential additions of 100, 200 and 800 μL of the 0.01 M penicillin G standard solution.

### 3.2.4 Preparation of Samples for XPS Analysis

Bilayer PPy films were electropolymerised on aluminium coated electrodes (4 cm x 1 cm). The monomer solution for the inner layer of the film contained 0.1 M Py, 19 U/mL P'nase and 0.1 M $KNO_3$. The inner layer of the film was electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes.

The monomer solution for the outer layer of the film contained 0.1 M Py, 19 U/mL P'nase and 0.01 M Pen. The outer layer of the film was electropolymerised at a current density of 0.9 mA/cm$^2$ for 5 minutes. The conditions for the XPS analysis were the same as those described in chapter 2 (section 2.2.7).

### 3.2.5 Preparation of Samples for SEM Analysis

Bilayer PPy films were prepared as described in section 3.2.4. The SEM conditions were the same as those specified in chapter 2 (section 2.2.6).

### 3.2.6 Titration Procedure

Amoxycillin 500 mg tablets were crushed and 1.08 g was transferred to a 100 mL volumetric flask and diluted to the mark with 0.01 mM phosphate buffer. Following this, the mixture was centrifuged for 10 minutes at 6, 000 rpm. Next 10 mL of this solution was transferred to a 250 mL volumetric flask and 40 mL of 11.77 M HCl and 10 mL chloroform were added. The mixture was then titrated with 0.05 M potassium iodate with vigorous shaking. The colour of the chloroform layer changed from colourless to brown/deep red to colourless again. The end point was taken as the first permanent decolourisation of the chloroform layer. This procedure was repeated for three tablets.

### 3.2.7 Film Formation

#### 3.2.7.1 Formation of the Inner Layer

The galvanostatic electropolymerisation of Py was performed in two steps. The first step involved the formation of the PPy film from a solution containing 0.1 M Py, 19 U/mL P'nase and 0.1 M $KNO_3$. A current density of 0.9 $mA/cm^2$ was applied for 40 s.

#### 3.2.7.2 Formation of the Outer Layer

The second step involved the formation of the second layer of the PPy film from a solution containing 0.1 M Py, 19 U/mL P'nase and 0.01 M Pen. A current density of 0.9 $mA/cm^2$ was applied for 40 s.

### 3.2.7.3 Optimisation of the Bilayer

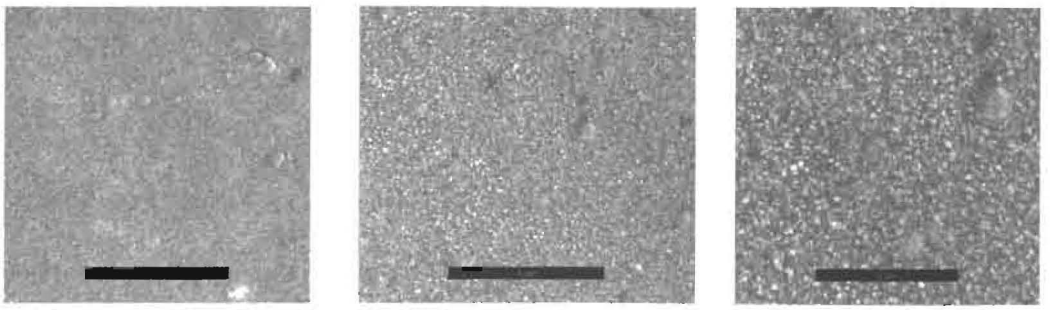
The optimum conditions for the potentiometric detection of penicillin with the bilayer film were determined by varying the components in the monomer solution used for layer 2. The varied components include the enzyme concentration, effect of Py concentration, influence of Pen concentration in monomer solution, influence of buffer concentration, effect of current density, and polymerisation time. The mean deviation was calculated for each film to determine the reproducibility of each film. The sensitivities of the films, $R^2$ values and linear concentrations were compared.

## 3.3 Results and Discussion

### 3.3.1 SEM Analysis

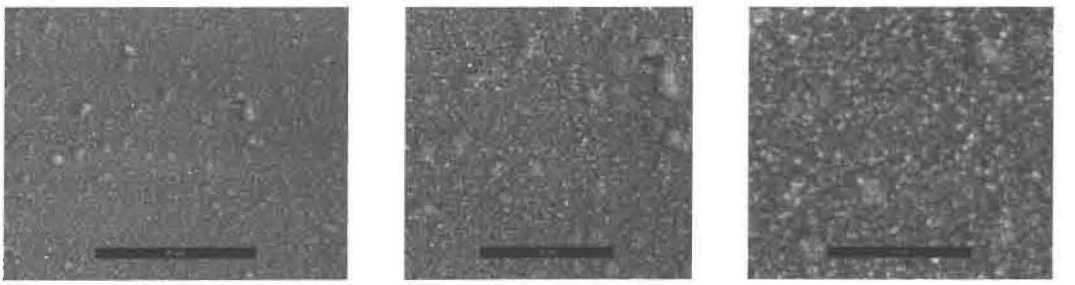
The presence of the enzyme in the outer layer was verified by SEM. Figures 3.1 and 3.2 show the changes in the morphology of the films obtained for the PPy and PPy-P'nase films at different magnifications.

The absence of the enzyme in the monomer solution for the outer layer, exhibited a more uniform surface, as shown in Figure 3.1. The appearance of the increased number of nodules shown in Figure 3.2 is attributable to the incorporation of the enzyme in the outer layer of the PPy bilayer film. However, it is important to note that the appearance of the nodules is not distinct due to the lower enzyme concentration incorporated in the outer layer of the bilayer film (19 U/mL) compared with the 50 U/mL enzyme that was used for the formation of the single layer PPy-P'nase film (shown in chapter 2) where the changes in the morphology of the film were more apparent. This is because the optimised enzyme concentration for the single layer (chapter 2) is lower than the optimised enzyme concentration used for the bilayer (chapter 3).



(a) (b) (c)

**Figure 3.1 SE micrographs showing the surface morphology of PPy bilayer films in the absence of the enzyme in the outer layer. Monomer solution of outer layer contained 0.1 M Py, 0.01 M Pen where (a) 5, 000X (b) 10, 000X (c) 20, 000X. The films were grown at a current density of 0.9 mA/cm$^2$ for 5 minutes.**



(a) (b) (c)

**Figure 3.2 SE micrographs showing the variation in surface morphology of PPy bilayer films in the presence of the enzyme in the outer layer. Monomer solution of outer layer contained 0.1 M Py, 19 U/mL P'nase, 0.01 M Pen where (a) 5, 000X (b) 10, 000X (c) 20, 000. The films were grown at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

### 3.3.2 Surface Analysis using XPS

The PPy-P'nase bilayer film was analysed with XPS to characterise the PPy backbone and verify the presence of P'nase within the film. All spectra were referenced by setting the hydrocarbon C 1s peak to 285.0 eV (Beamson & Briggs 1992) to compensate for the effects of residual charging (Sharma et al. 2004).

Figure 3.3 shows a wide scan XPS spectrum of the PPy-P'nase film. The photoelectron peak detected for C 1s at 286.5 eV is attributable to the C-N of PPy. The N 1s peak which appeared at 401.8 eV, confirms the structure of the PPy backbone which contains C-N and C-C bonds. The peak detected for O 1s at 532.9 eV corresponds to the presence of $KNO_3$ which has N-O bonds. $KNO_3$ was incorporated in the inner layer of the film.

The presence of P'nase was again confirmed with the S 2p peak which appears at 168 eV (Adeloju et al. 1993). The verification of sulfur in the XPS analysis along with the morphological changes observed in the SEM analysis provides conclusive evidence that P'nase was incorporated into the film.

Figure 3.4 shows the presence of the C 1s (4) peak shown at 288.3 eV which is characteristic of the N-C=O or the C=O groups. The N 1s peak at 400.2 eV is also attributable to the N-C=O groups pertaining to the presence of Pen in the monomer solution. This is similar to the results shown in Chapter 2 for the formation of the PPy single layer where the C 1s (4) appeared at 288.3 eV also pertaining to the C=O or the

N-C=O groups. Hence, this confirms the incorporation of Pen in the film as it was added to the monomer solution.

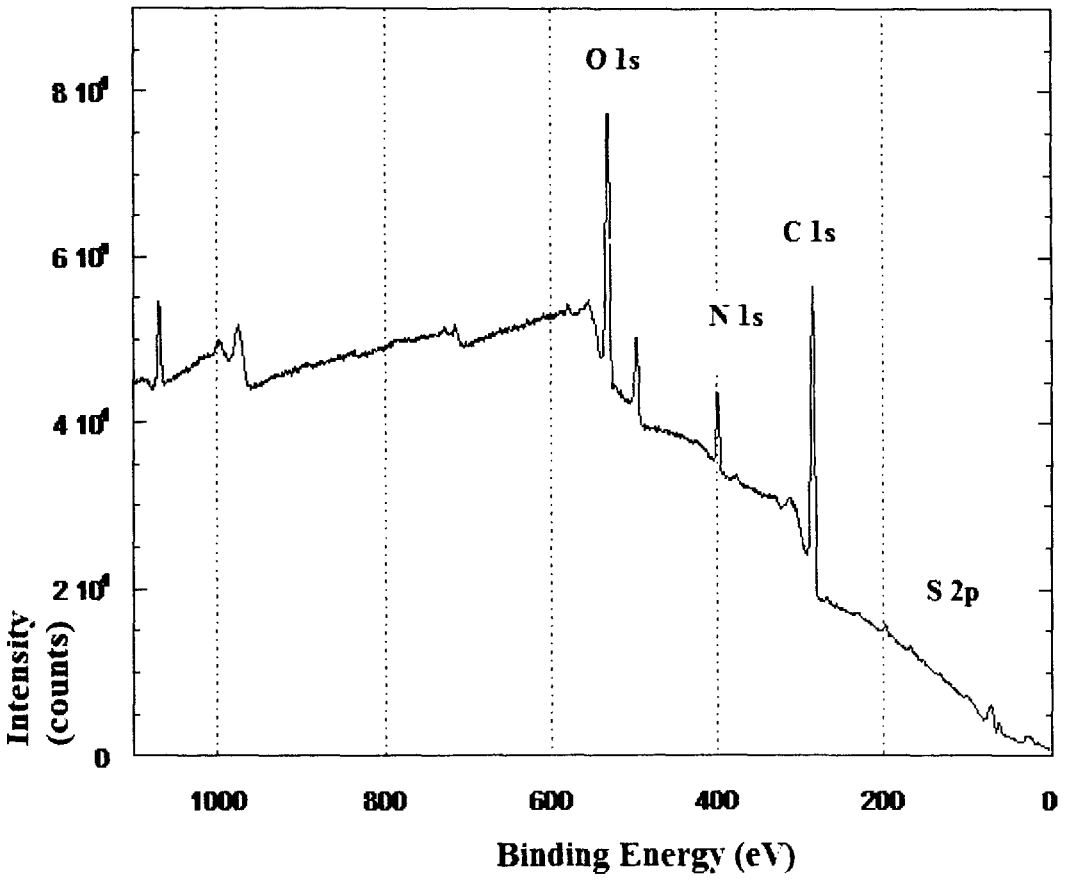



**Figure 3.3 Representative XPS wide scan of PPy-P'nase film. Monomer solution contained 0.1 M PPy, 19 U/mL P'nase and 0.01 M Pen. The films were grown at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

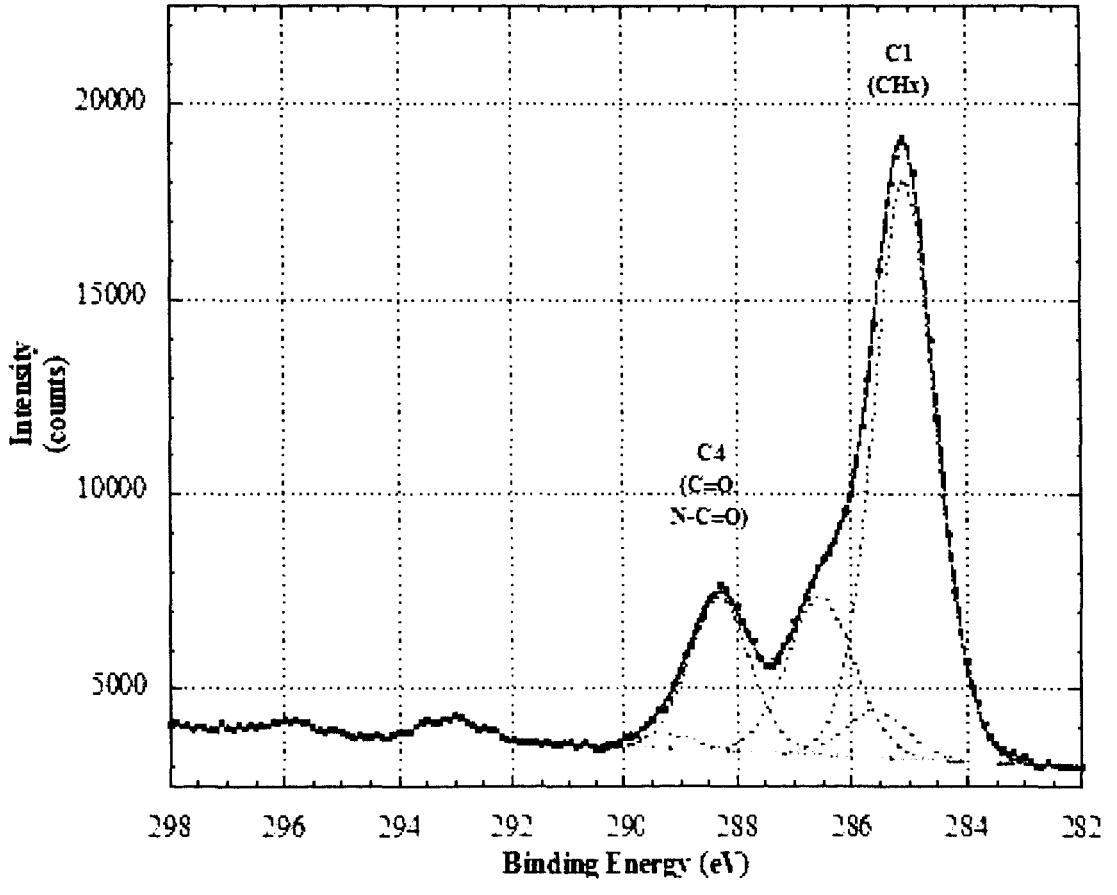



**Figure 3.4 Representative C 1s spectrum for the PPy- P'nase film. Monomer solution contained 0.1 M PPy, 19 U/mL P'nase and 0.01 M Pen. The films were grown at a current density of 0.9 mA/cm$^2$ for 5 minutes.**

### 3.3.3 Optimisation of the Inner Layer

The optimisation of the conditions used for the formation of the inner layer of the bilayer biosensor, is important for achieving optimum sensitivity of the penicillin response. The optimum conditions for the formation of the inner layer of the film were determined by varying the components of layer 1 as shown in Table 3.1. The components of layer 2 were held constant i.e. PPy-P'nase-Pen. Optimum film conditions were determined using the film sensitivity, correlation coefficient and the linear concentration range. The data in Table 3.1 indicate that the highest sensitivities for penicillin were obtained with the use of PPy-P'nase-Pen-KCl or PPy-P'nase-KCl film as the inner layer.

**Table 3.1- Influence of the Inner Layer on the Potentiometric Response obtained for Penicillin**

| Composition of Inner Layer of Film | Sensitivity (mV/mM) | $R^2$ | Linear Concentration Range (μM) |
|---|---|---|---|
| PPy-P'nase-Pen* | 120 | 0.89 | 20 - 300 |
| PPy-P'nase-Pen-KCl* | 464 | 0.93 | 14 - 250 |
| PPy-P'nase* | 450 | 0.97 | 8 - 300 |
| PPy-P'nase-KCl* | 461 | 0.97 | 8 - 300 |
| PPy-P'nase-$KNO_3$* | 448 | 0.99 | 3 - 300 |
| PPy-P'nase-Pen-$KNO_3$* | 393 | 0.94 | 8 - 300 |

*outer layer comprised of PPy-P'nase-Pen film

Evidently the incorporation of KCl in the monomer solution slightly enhanced the sensitivity of the penicillin response, caused by an increase in the conductivity of the film. The PPy-P'nase-Pen-KCl film was the most sensitive film but with a narrower linear concentration range whereas the PPy-P'nase-KCl film had a wider concentration range. It seems that the incorporation of Pen in the PPy-P'nase-Pen-KCl film had a negative effect on the achievement of a wider linear concentration range.

The PPy-P'nase-Pen film gave the lowest sensitivity and $R^2$ values due to the absence of KCl in the film. The incorporation of $KNO_3$ in the PPy-P'nase film produced a similar sensitivity to the film that did not contain $KNO_3$ (450 mV/mM for the PPy-P'nase film and 448 mV/mM for the PPy-P'nase-$KNO_3$ film). Hence the incorporation of $KNO_3$ into the PPy-P'nase-Pen film produced a less sensitive electrode but with a wider linear concentration range. Evidently, the differences in the sensitivities obtained with the incorporation of KCl and $KNO_3$ into the respective films is indicative of the difference in the conductivity of the films. It is also important to note that the linear concentration range (8 - 300 μM) and the $R^2$ value of 0.97 obtained with the PPy-P'nase film is identical to that obtained with the PPy-P'nase-KCl film. This suggests that the incorporation of KCl in the film increased the sensitivity of the film however the concentration range obtained with this film was narrower (8 - 300 μM) than that obtained for PPy-P'nase-$KNO_3$ (3 - 300 μM). The PPy-P'nase-$NO_3$ film produced the highest $R^2$ value of 0.99 and a wider linear concentration range of 3 - 300 μM. On the basis of the above observations, this configuration was chosen as the inner layer for the bilayer biosensor. However, the linear concentration range obtained with the PPy-P'nase-Pen-KCl film was narrower than that obtained with the PPy-P'nase-KCl film. Consequently, the PPy-P'nase-KCl film was used as the inner layer.

The optimum conditions established for the polymerisation of the inner layer are 0.1 M Py, 19 U/mL P'nase and 0.1 M $KNO_3$. A current density of 0.9 $mA/cm^2$ was applied for 40 s. These conditions were used in further investigations.

### 3.3.4 Optimisation of the Outer Layer

### 3.3.5 Influence of the Pyrrole Concentration

The conductive nature of PPy enables control of the film thickness by the amount of charge passed during electropolymerisation (Pantano & Kuhr 1995). This highlights the distinct benefit of using a conductive polymer, such as PPy in both layers. The use of a conducting polymer in the outer layer is advantageous in enabling the diffusion of the substrate across the two layers. Without this conducting outer layer, the enhancement of the properties of the bilayer system (Bobacka et al. 1993; Tamm et al. 2001), seem less possible.

Figure 3.5 shows that the sensitivity of the penicillin response increased with increasing Py concentration used for the formation of the outer layer. The optimum penicillin response was obtained when 0.04 M Py was used in the monomer solution. Beyond this Py concentration, a sharp decrease in the sensitivity was observed. This is due to the increased resistance to the diffusion of penicillin through the outer layer (Vidal et al. 1999). Another relevant factor is the consecutive electropolymerisation of the outer PPy layer which may have caused some changes in the properties of the inner layer (Tamm et al. 2001) and resulted in a decrease in the sensitivity of the biosensor. Specifically, the consecutive layers in the bilayer system are not distinctly separated and synthesis

can occur in the pores located in the surface of the outer layer whilst the outer layer is being formed (Tamm et al. 2001) which may have contributed to the sharp decrease in the sensitivity of the biosensor. The most obvious cause is the net increase in the thickness of the film which resulted when 0.05 M Py was used for the formation of the outer layer.

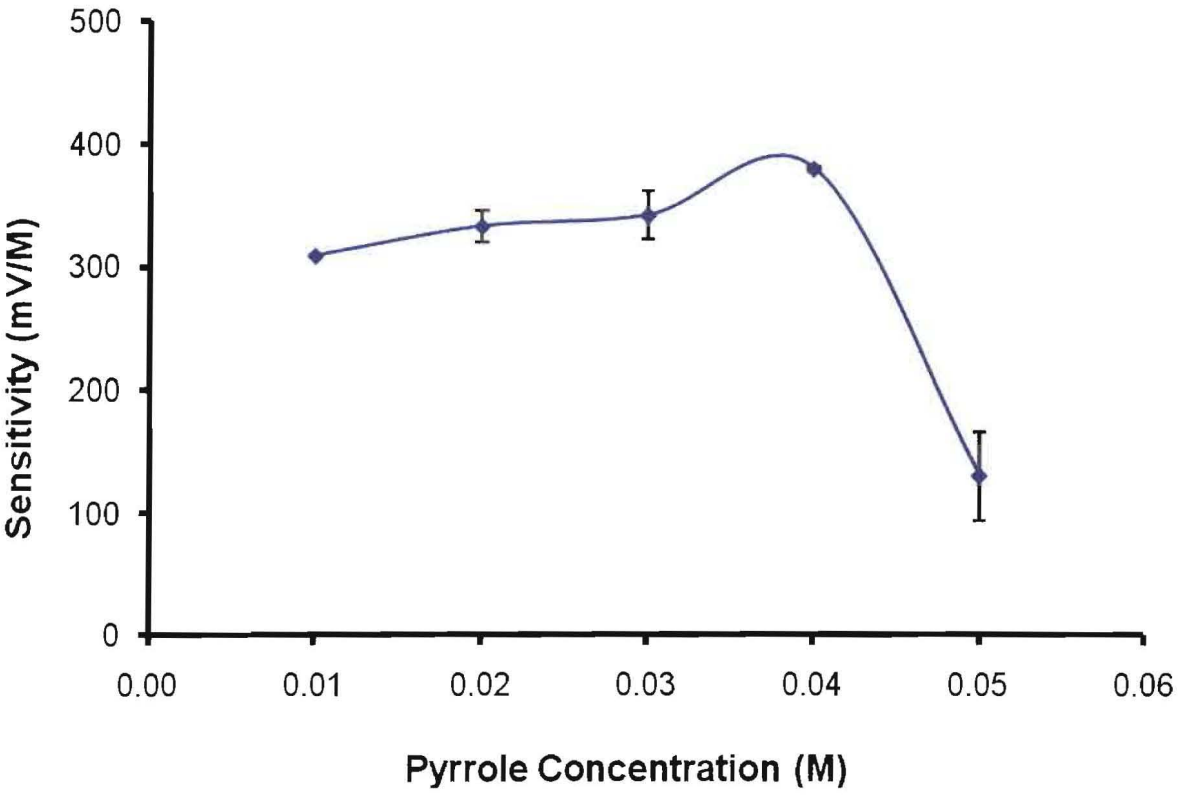



**Figure 3.5 Influence of the pyrrole concentration used for the formation of the outer layer of the bilayer biosensor. The following conditions were kept constant in the outer layer: 19 U/mL P'nase, 0.01 M Pen, current density applied 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

The reproducibility of the bilayer electrode was also examined. Table 3.2 provides the individual measurements and the mean with mean deviations at different penicillin concentrations.

**Table 3.2 Reproducibility of the Response**

| Penicillin Addition (μM) | Response 1 (mV) | Response 2 (mV) | Response 3 (mV) | Mean (mV) |
|---|---|---|---|---|
| 0.1 | 62 | 67 | 54 | 61 ± 5 |
| 0.2 | 106 | 110 | 100 | 105 ± 4 |
| 0.3 | 136 | 130 | 129 | 132 ± 3 |

The mean deviation for the penicillin measurement of 0.1 μM was slightly higher than those obtained for 0.2 and 0.3 μM, indicating that the reproducibility improved with increasing penicillin concentration. The F-test showed no significant difference between the standard deviations at the 95% confidence level.

## 3.3.6 Influence of Enzyme Concentration

Vidal et al. (1999) reported that the immobilisation of enzyme in both layers is tantamount with that of a single layer biosensor with a thickness higher than the optimal

value. However, Figure 3.6 shows that this is only the case when P'nase concentrations higher than 19 U/mL were used for film formation. In fact Figure 3.6 shows that the incorporation of P'nase in the outer layer improved the sensitivity of the penicillin response. This view is further supported by the fact that the response of the biosensor increases with an increase in the number of enzyme layers (Kobayashi & Anzai 2001) which has been demonstrated with the bilayer system.

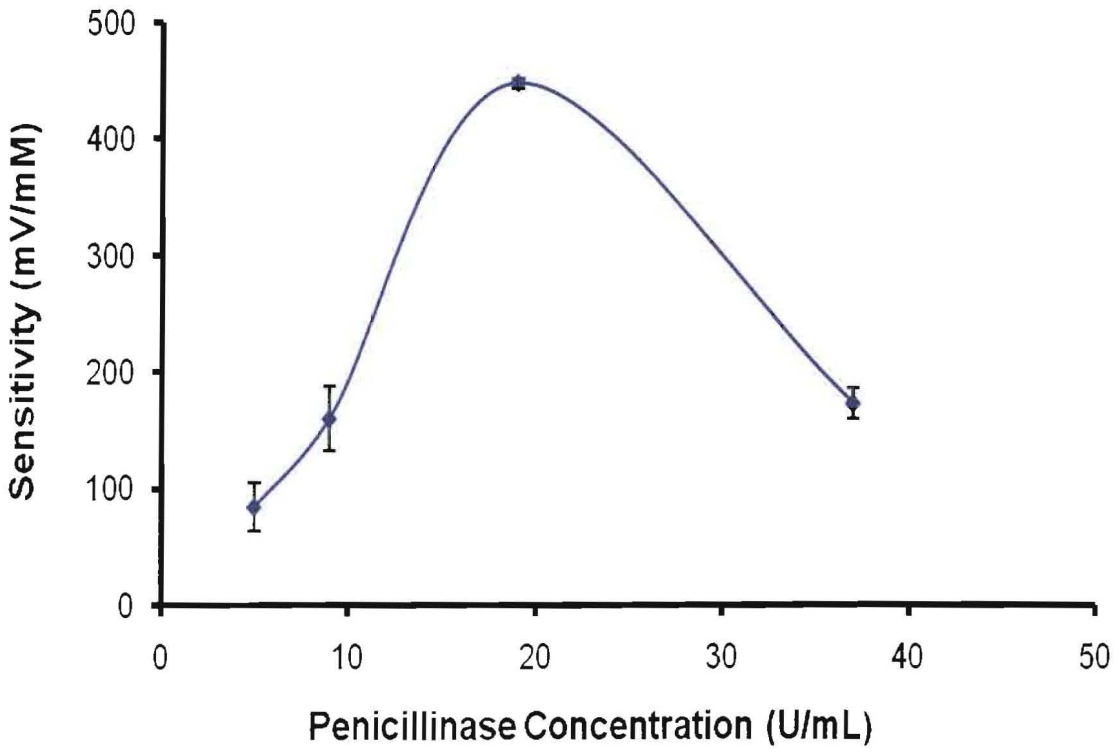



**Figure 3.6 Influence of the enzyme concentration used for the formation of the outer layer on the potentiometric response of the bilayer biosensor. The following conditions were kept constant: 0.04 M Py, 0.01 M Pen, current density applied 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

The optimum response for penicillin was obtained in the presence of 19 U/mL of P'nase in the monomer solution. At optimum enzyme concentrations, the polymer swells thereby facilitating a more active conformation of the enzyme. This occurs after the potential distortion of the protein during the entrapment process (Hammerle et al. 1992; Vidal et al. 1999). Beyond this concentration the sensitivity of the biosensor decreased rapidly. Another possible reason for this behaviour is the formation of a thicker film with the use of higher enzyme concentrations which rendered the film less permeable because of its more compact PPy structure. The immobilisation of the enzyme in both layers means that the penicillin does not have to diffuse to the inner layer to reach the enzyme, as this would also restrict the substrate concentration that is supplied for the reaction (Vidal et al.1999). Hence, the use of 19 U/mL of P'nase is recommended for the preparation of the outer layer.

### 3.3.7 Effect of Penicillin Concentration

The influence of the Pen concentration added to the monomer solution used for the formation of the outer layer was also investigated. As mentioned in Chapter 2, the presence of Pen in the monomer solution is useful for the stabilisation of the potentiometric response, as well as achieving further enhancement of the sensitivity of the electrode. This is evident with the sharp increase in the sensitivity of the film when 0.7 mM Pen was present in the monomer solution, as shown in Figure 3.7. In fact there was a 95 mV/mM increase in the sensitivity of the film when Pen was added to the monomer solution. Possible reasons for this include the fact that when Pen is added to the monomer solution, the pH of the membrane becomes more positive as it counteracts

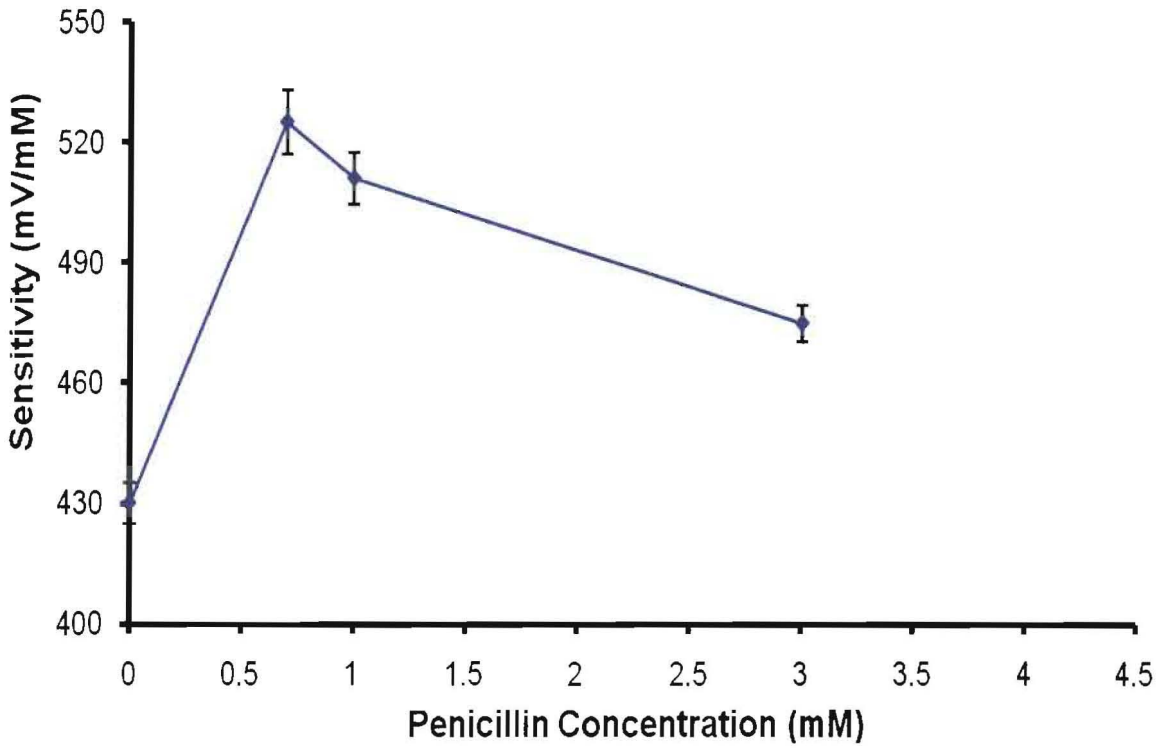



**Figure 3.7 Influence of the penicillin concentration in the monomer solution used for the formation of the outer layer on the penicillin response. The following conditions were kept constant: 0.04 M Py, 19 U/mL P'nase, current density applied 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

the effects of the retention of penicilloate in the film. These effects include a decrease in the pH of the membrane which is accompanied by a negative charge on the membrane and is a criterion for the decline in the potentiometric response of the film (Gorchkov et al. 1996). A change in the pH of the membrane is produced through the catalysis of P'nase which cleaves the β-lactam ring of penicillin to form penicilloic acid and dissociates into penicilloate and a proton (Kulp et al. 1987), as shown in reaction 1 in chapter 2.

The charge on the membrane has an electrostatic influence on the substrate and the enzyme which provides a possible rationale for the attainment of a higher electrode sensitivity when 0.7 mM Pen was added to the monomer solution. Other reasons for this behaviour include the fact that penicillin increases the conductivity of PPy (Nishizawa et al. 1992) which improves the electrode response. It is interesting to note that the absence of Pen in the monomer solution produced the least sensitive film. It seems that the incorporation of Pen in the film improved the response of the electrode as well as the sensitivity. Possible reasons for this include the negative charge on the membrane due to the retention of the negatively charged penicilloate in the membrane. The negative charge on the membrane does not promote an increase in the potentometric response and also decreases the sensitivity of the film. The retention of penicilloate in the membrane influences the charge on the membrane and could explain why 0.7 mM Pen enhanced the sensitivity of the film. Thus the more positive the membrane, the greater its ability to attract the substrate, Pen, which in turn promotes the increase in the potentiometric response as well as the sensitivity of the biosensor. Figure 3.7 shows that Pen concentrations greater than 0.7 mM decreased the sensitivity of the electrode. This can be attributed to the inability of the film to incorporate higher Pen concentrations due

to the saturation of the membrane with Pen. In particular, a notable decrease in the sensitivity of the electrode results when the Pen concentration is increased to 3 mM. For this reason, 0.7 mM of Pen was incorporated into the monomer solution for the formation of the outer layer.

### 3.3.8 Influence of Applied Current Density and Polymerisation Time

The current density applied during the electropolymerisation process provides a useful means for controlling the film thickness, particularly in the case of conducting polymers, such as PPy (Vidal et al. 1999). Figure 3.8 shows that an increase in the applied current density from 0.2 - 0.5 $mA/cm^2$ was accompanied by a significant increase in the sensitivity of the biosensor. The sensitivity of the film formed with an applied current density from 0.2 - 0.5 $mA/cm^2$ increased more rapidly than those formed at higher current densities. This suggests that an enhanced permeability to penicillin is a direct result of the ease of the formation of a thinner film with lower current densities (Adeloju & Moline 2001).

The application of current densities higher than 0.5 $mA/cm^2$ only resulted in slight improvements in the sensitivity, but a sharp decrease in the sensitivity was observed when a current density of 1.1 $mA/cm^2$ was applied. This is due to the over-oxidation of the inner layer, hence reducing its electroactive properties (Ghosh et al. 1998; Tamm et al. 2001). Other possible reasons include the increased thickness of the outer layer resulting from a more rapid polymerisation rate at the higher applied current density. The results show that lower sensitivities were obtained with the application of higher current densities. This implies that the amount of enzyme incorporated increases with

the applied current density which may be due to the tendency of PPy to form more rapidly at higher current densities (Adeloju & Moline 2001).

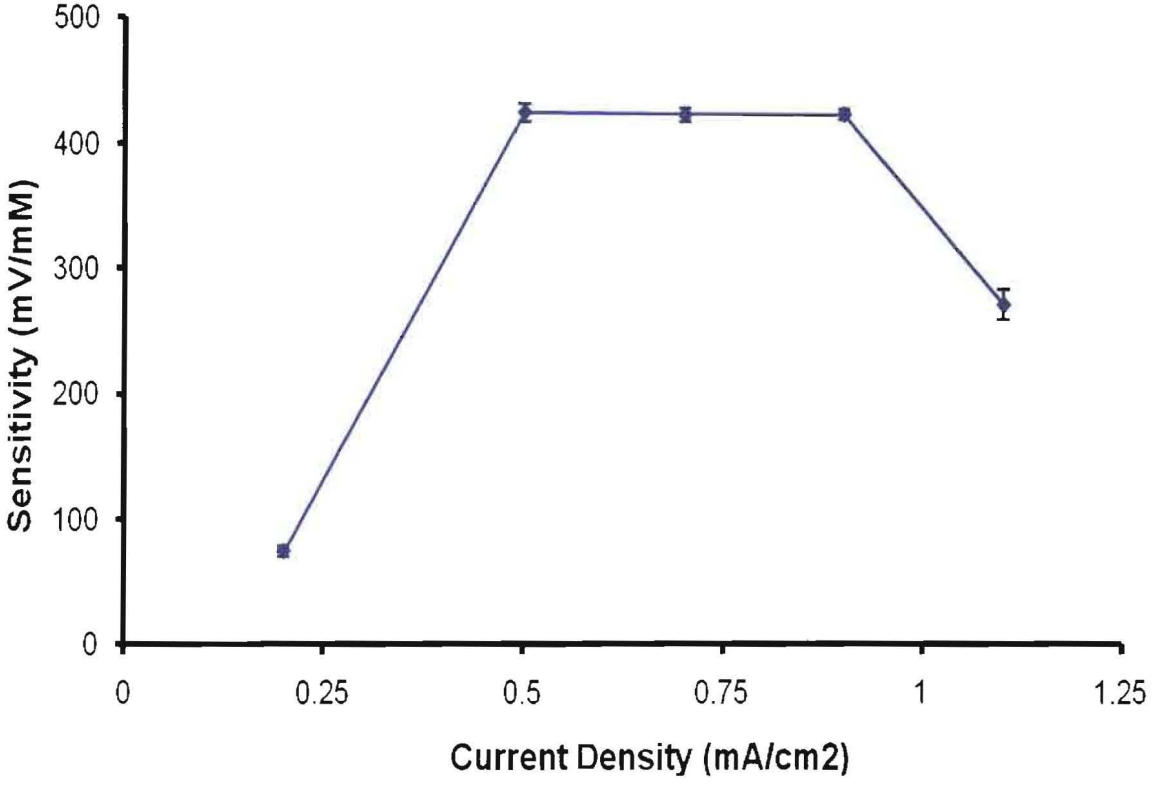



**Figure 3.8 Influence of the applied current density employed for the formation of the outer layer on the sensitivity of the penicillin response. The following conditions were kept constant: 0.04 M Py, 19 U/mL P'nase, 0.7 mM Pen, electropolymerisation time 40 s.**

The results support the dependency of the sensitivity of the electrode upon the applied current density applied during electropolymerisation. Hence, an applied current density of 0.5 - 0.9 mA/cm$^2$ can be applied for the formation of the outer layer. However, 0.5 mA/cm$^2$ was used for other investigations as the resulting film was slightly more responsive than those produced with the applied current densities of 0.7 and 0.9 mA/cm$^2$.

Figure 3.9 shows that the sensitivity of the penicillin response was greatly influenced by

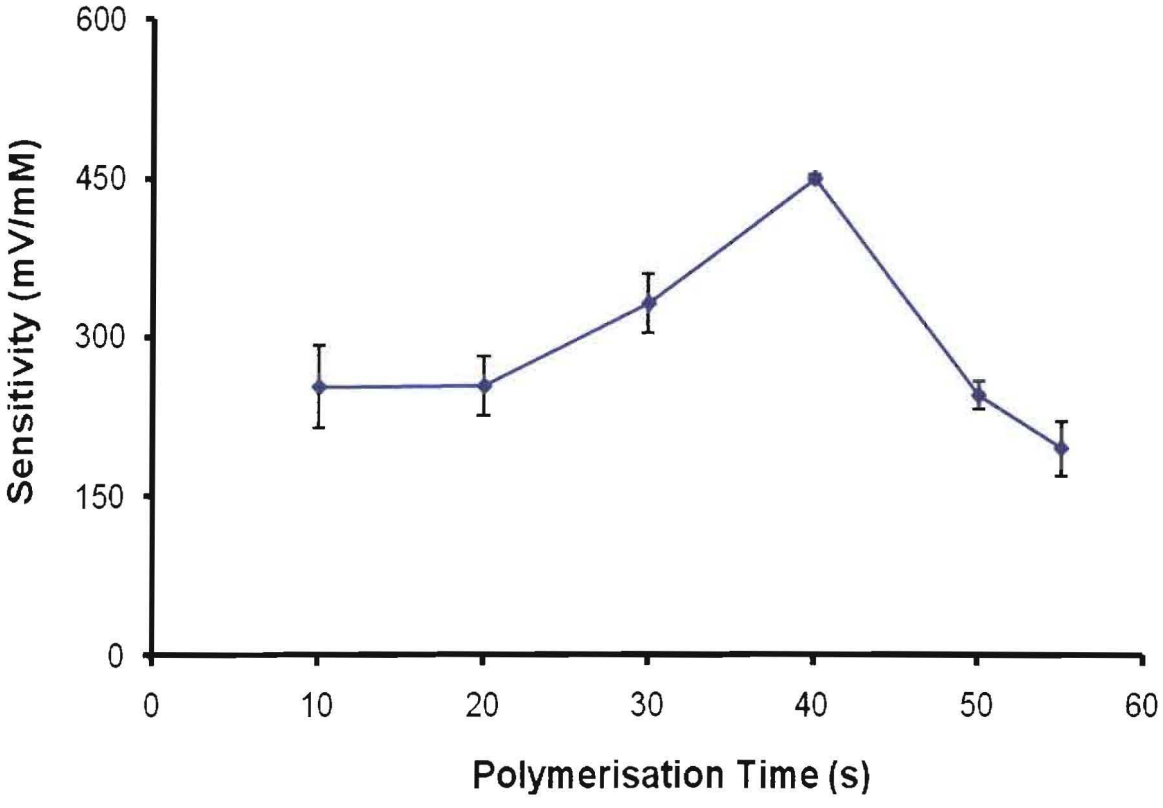



**Figure 3.9 Influence of the polymerisation time used for the formation of outer layer on the sensitivity of the penicillin response. The following conditions were kept constant: 0.04 M Py, 19 U/mL P'nase, 0.7 mM Pen, applied current density 0.5 mA/cm$^2$.**

the polymerisation time used for film growth. The response of the film did not change upon the application of the polymerisation times of 10 and 20 s. This is due to the resulting thinner film which results from the use of shorter polymerisation times (Li et al. 2004). However, a gradual increase in the sensitivity was observed with a polymerisation time of 30 s, reaching an optimum sensitivity at 40 s. The adequacy of the film thickness when the outer layer was polymerised for 40 s suggests that a sufficient amount of P'nase was incorporated into the film. Beyond this polymerisation time (40 s), the sensitivity of the film to penicillin decreased due to the formation of thicker films at higher polymerisation times (Li et al. 2004). This is attributable to the fact that thicker films such as those formed after 50 s or longer, hinders the ability of the enzymatic reaction products to reach the electrode surface (Adeloju & Moline 2001). The formation of the outer layer was therefore accomplished with a polymerisation time of 40 s.

### 3.3.9 Effect of the Buffer Concentration and Linear Concentration Range

The achievable linear concentration range and the sensitivity of a biosensor are influenced by the buffer concentration (Nilsson & Mosbach 1978; Van der Schoot & Schoot & Bergveld 1987). Figure 3.10 shows that the highest sensitivity was achieved with 0.3 mM buffer. A considerable decrease in the sensitivity of the biosensor was observed with buffer concentrations greater than 0.3 mM. At these higher concentrations, the increase in the ion concentration of the buffer had a negative effect on the sensitivity of the electrode such that the resultant change in the pH of the

enzymatic reaction is decreased with the increasing buffer capacity. This was also accompanied by a decrease in the sensitivity of the biosensor (Liu et al. 1998). On the other hand, concentrations lower than 0.3 mM showed lower electrode sensitivities, which is due to the decrease in the effective diffusion coefficient of protons. This translates into a lower electrode response as lower buffer concentrations also decrease the number of buffer molecules in the enzymatic membrane (Brand et al. 1990). For this reason, a buffer concentration of 0.3 mM was used for the potentiometric measurement of penicillin in all other studies.

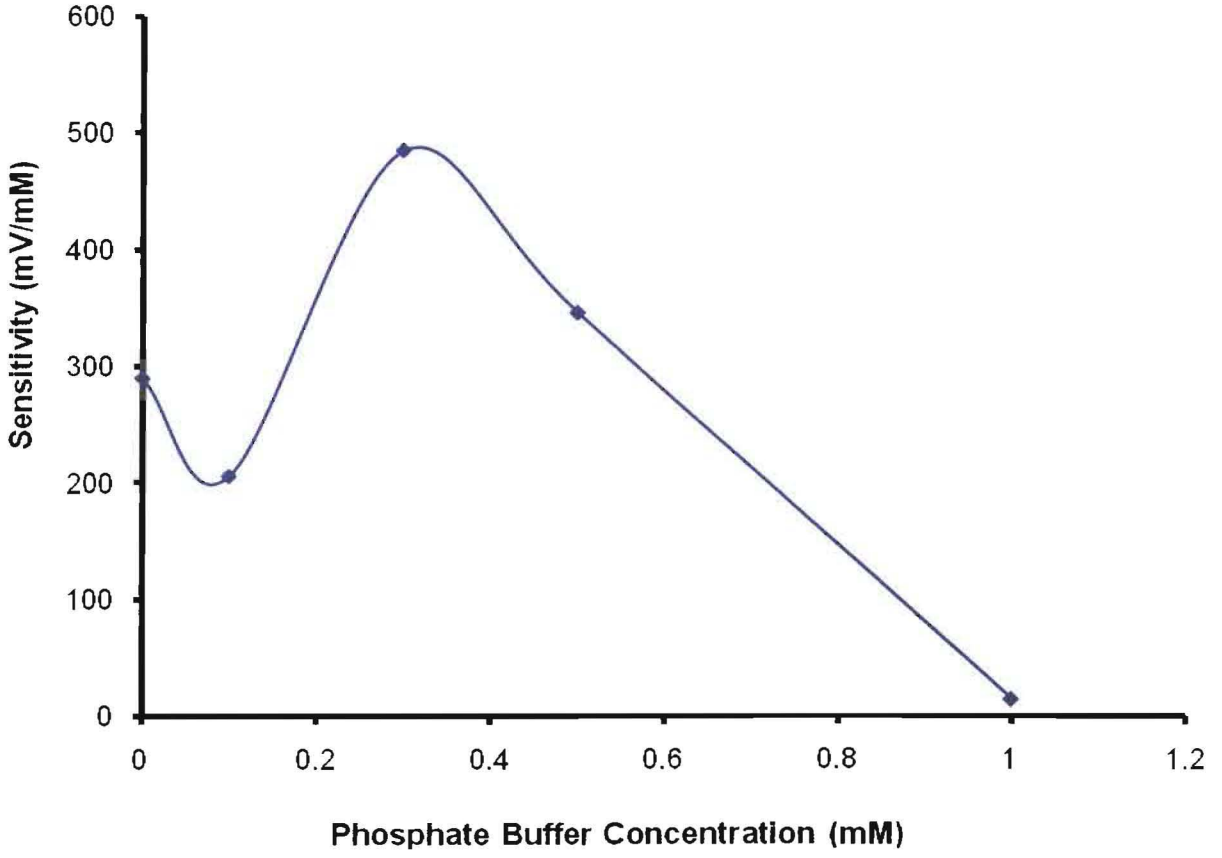



**Figure 3.10 Influence of the buffer concentration on the sensitivity of the penicillin response for the formation of outer layer. The following conditions were kept constant: 0.04 M Py, 19 U/mL P'nase, 0.7 mM Pen, applied current density 0.5 mA/cm$^2$, polymerisation time 40 s.**

A plot of potential versus the logarithmic of the penicillin concentration gave a slope of 87 mV/decade and a correlation coefficient of $R^2$=0.931 (where n=3). Figure 3.11 shows that the response to penicillin obtained with the bilayer configuration, is linear between 7.5 and 146 μM which is narrower than that obtained with the PPy single layer biosensor. This is because the single layer configuration used a lower buffer concentration (0.1 mM) than with the bilayer configuration (0.3 mM). The minimum detectable concentration obtained with the PPy bilayer biosensor was 0.3 μM.

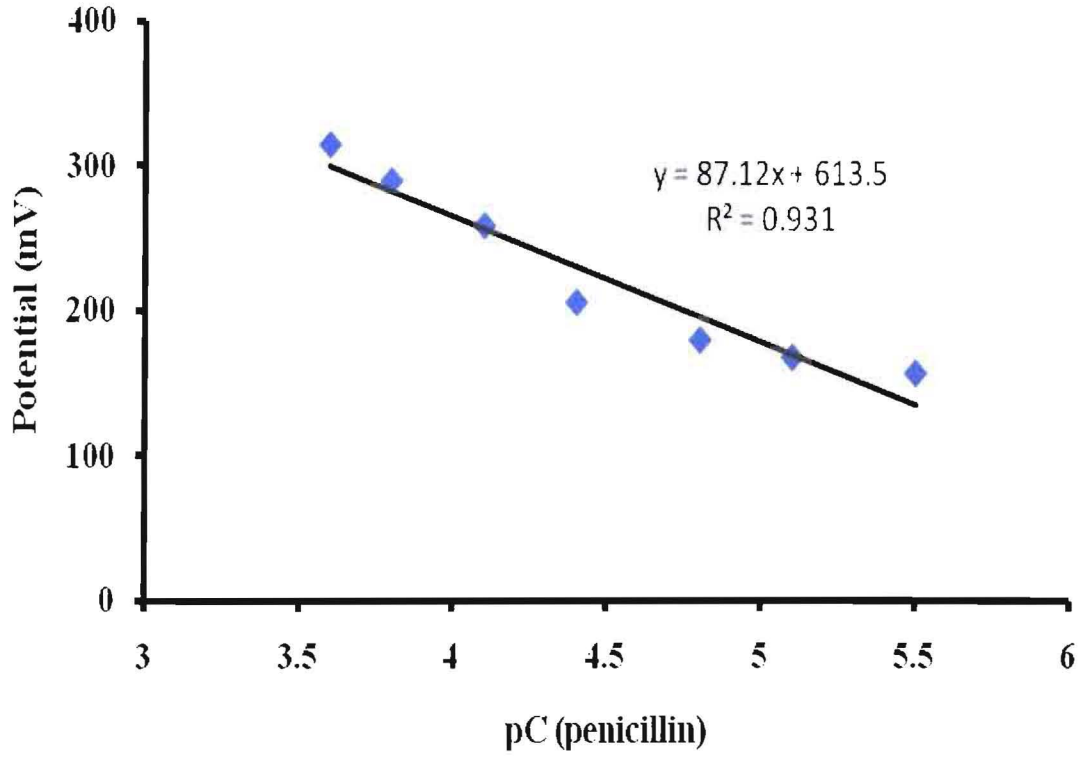



**Figure 3.11 Logarithmic concentration of penicillin response obtained with the PPy bilayer biosensor. The following film conditions were employed: 0.04 M Py, 19 U/mL P'nase, 0.7 mM Pen, applied current density 0.5 mA/cm$^2$, polymerisation time 40 s. Potentiometric biosensing was carried out in 0.3 mM phosphate buffer.**

This was determined by repeated penicillin additions until a response was obtained. To confirm the minimum detectable concentration, each experiment was repeated three times.

### 3.3.10 Determination of Penicillin in Amoxycillin Tablets

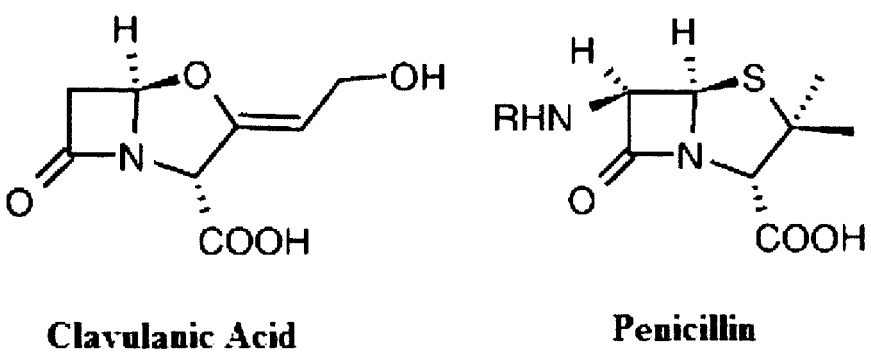
The dosages stated by the pharmaceutical company (GlaxoSmithKline, Melbourne) are 500 mg amoxycillin and 125 mg CA. Three amoxicillin tablets were prepared as described in section 3.2.3.

**Table 3.3 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Obtained with PPy Bilayer Biosensor**

| Amount Specified in Tablet (mg)[a] | Penicillin Concentration Obtained With Biosensor (mg) | % Recovery |
|---|---|---|
| Amoxycillin 500<br>Clavulanic Acid 125 | 512 ± 26 * | 102 ± 5 |
| Amoxycillin 500<br>Clavulanic Acid 125 | 567 ± 76 * | 113 ± 15 |
| Amoxycillin 500<br>Clavulanic Acid 125 | 622 ± 254 * | 124 ± 51 |
| **Average Values** | **567 ± 119** | **113 ± 24** |

[a] Dosage specified by pharmaceutical company
* Mean of 4 values obtained using biosensor with a 95% confidence limit

The results provided in Table 3.3 are expressed as a mean ± standard deviation and percentages with 95% confidence intervals. The percentage recovery of amoxycillin found in each tablet varied between 102 and 124%. It is possible that higher percentage recoveries are due to the sensitivity of the electrode to CA which has weak antibiotic properties and is also a beta-lactamase inhibitor (Parag et al. 2008). The structure of amoxycillin is analogous with the basic penicillins, where the sulfur atom is substituted with the oxygen atom (Parag et al. 2008), as shown below in Figure 3.12.




**Clavulanic Acid** **Penicillin**

**Figure 3.12 Structural representation of the similarities in the structures of clavulanic acid and penicillin. Reproduced from Parag et al. (2008).**

This might explain the ability of the electrode to detect CA which is contained in amoxycillin to act as an antibacterial formulation against β-lactamase bacteria (Yogev et al. 1981; Tsou et al. 1997) that are produced by the fermentation process of *Streptomyces clavuligerus.* The fact that CA contains a β-lactam ring means that the biosensor has the ability to detect it as well. However the biosensor is only able to detect some of the CA. It may be that once the electrode detected the amoxycillin,

enzymatic sites on the surface of the electrode were blocked by CA as it acts as a competitive inhibitor to β-lactamases such as P'nase (Aghazadeh & Kazemifard 2001). The average concentration of amoxycillin found was 567 mg with an average percentage recovery of 113%.

### 3.3.11 Titrimetric Determination of Penicillin in Tablets

This method produced adequate percentage recoveries when compared to the biosensor method. Table 3.4 shows the results obtained for the titration of amoxycillin 500 mg.

**Table 3.4 Titration Results**

| **Amount Specified in Tablet (mg)[a]** | **Volume of Titrant (mL)[b]** | **[Penicillin] Found (mg)** | **% Recovery** |
|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 2.5 ± 0.1 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 2.6 ± 0.2 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 2.4 ± 0.1 | 505 ± 20 | 101 ± 4 |
| **Average Values** | **2.5 ± 0.1** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 3 values

The average percentage recovery was found to be 105 ± 5% which is lower and more reproducible than that obtained using the PPy bilayer biosensor (113 ± 24 %). The variation in the results obtained with the biosensor is larger than that obtained with the titrimetric method. This variation is due to the high percentage recovery reported for the third batch of amoxycillin tablets which is attributed to the sensitivity of the biosensor towards compounds that have weak antibiotic properties such as CA. Even though the percentage recovery reported for the standard titrimetric analysis is lower than that reported for the bilayer biosensor, it can only be applied to the reliable determination of penicillins in vial form (Grime & Tan 1979). This is because interferents, such as sodium starch glycollate (Grime & Tan 1979), contained in amoxycillin tend to inflate percentage recoveries. These interferents need to be separated from the antibiotic preparations prior to undertaking titrimetric analysis (Grime & Tan 1979) which is time-consuming, unlike the biosensor method which is much less time consuming.

### 3.3.12 Determination of Penicillin G in Milk

Table 3.5 summarises the recovery values obtained with the bilayer configuration. Each sample was analysed 4 times. The data in Table 3.5 show that the percentage recoveries obtained for penicillin in milk ranged from 30 - 110%. It is interesting to note that the milk samples that contained higher penicillin concentrations gave lower percentage recoveries while milk samples that contained lower penicillin concentrations gave higher percentage recoveries. This may be explained by the fact that penicillins tend to bind to the hydrophobic sites of milk proteins (Grunwald & Petz 2003), specifically

whey proteins which are involved in non-specific binding (Gustavsson et al. 2002; Cacciatore et al. 2004). The amount of bound penicillins increases with the decreasing polarity of the side-chain of penicillins, i.e. ampicillin<penicillin<cloxacillin (Grunwald & Petz 2003). The potentiometric response of the biosensor seems to be affected by the presence of the milk proteins to which penicillins are bound.

**Table 3.5 Recovery of Penicillin G in Milk Samples**

| Concentration of Penicillin G in Milk (ppm) [a] | Penicillin Concentration Recovered With Biosensor (ppm) [b] | % Recovery |
|---|---|---|
| 5 | 5.5 ± 1.3 | 110 ± 26 |
| 10 | 6.0 ± 1.0 | 60 ± 10 |
| 20 | 6.0 ± 1.3 | 30 ± 7 |

[a] Spiked concentration of penicillin G in milk sample
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

The results show that samples that contained lower penicillin concentrations reported higher standard deviations than samples that contained higher penicillin concentrations. This suggests that the reproducibility of the response was better for higher penicillin concentrations than for lower penicillin concentrations.

## 3.4 Conclusion

The use of PPy for the immobilisation of P'nase in a bilayer configuration has been successfully demonstrated. The most favourable conditions for the formation of the inner PPy-P'nase-$NO_3$ layer were a current density of 0.9 mA/cm$^2$ for 40 s. This was achieved by using 19 U/mL of P'nase in the monomer solution for the outer layer. The optimum conditions established for the outer layer of the film included: 0.04 M Py and 0.7 mM Pen. The outer layer was polymerised using a current density 0.5 mA/cm$^2$ for 40 s. The incorporation of the enzyme in the outer layer proved to be advantageous in terms of enhancing the sensitivity of the biosensor. As well as this, the enzyme does not have to diffuse to the inner layer in order to reach the enzyme. This configuration produced a highly sensitive biosensor with a minimum detectable concentration of 0.3 μM and a linear concentration range between 7.5 - 146 μM.

The biosensor was applied to the determination of penicillin in amoxycillin, achieving a percentage recovery range of 102 - 124%. Recovery values that exceeded 100% are attributed to the sensitivity of the biosensor towards CA which has weak antibiotic properties as well as being a competitive β-lactamase inhibitor. On the other hand, the average recovery obtained from the titrimetric analysis (105 ± 5%) was lower than that obtained using the PPy bilayer biosensor (113 ± 24%). However the standard titrimetric method is sensitive to interferents present in the tablet and is more time consuming.

Penicillin residues in milk samples were also quantified and the results indicate that penicillin has the tendency to bind to the hydrophobic sites of the milk proteins. As a consequence, the electrode was only able to detect up to 5 ppm penicillin in milk. This indicates that the biosensor is adequate where penicillin residues in milk samples are

lower than 5 ppm which suggests that further optimisation of the parameters of the biosensor is required in order to detect higher concentrations of penicillin in milk.

# Chapter 4

## The Fabrication of a Penicillin Biosensor with Polytyramine

## 4.1 Introduction

Tyramine (Ty) or 4-(2-aminoethyl) phenol is a biogenic amine derivative of tyrosine which can be found naturally in food products, especially in fermented foods. It is a versatile monomer that has been used for the fabrication of biosensors. PTy produces sensitive biosensors with a rapid response time due to the self-limiting growth of non-conducting polymers. One of the methods used for the entrapment of enzymes is physical entrapment during the electropolymerisation of Ty. Figure 4.1 shows the formation of PTy which occurs in the ortho-position to the activating hydroxyl group (Dubois et al. 1981; Situmorang et al. 1998; Cole et al. 2007). The presence of the free amine groups on the polymer backbone allows the covalent attachment of an enzyme through the formation of a peptide bond (Situmorang et al. 1999).

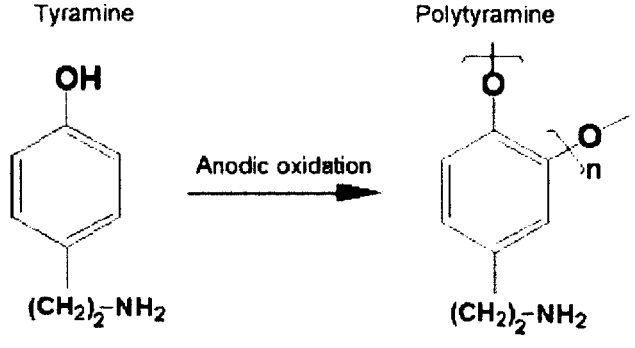



**Figure 4.1 Polymerisation process of tyramine. Reproduced from Losic et al. (2005).**

This behaviour promotes the formation of a thinner film with a more efficient diffusion rate (Nakabayashi 1998; Nakabayashi & Yoshikawa 2000). Typically, the thickness of a non-conducting layer is usually between 10 and 100 nm thick. As such, the diffusion of the

substrate to and from the membrane is rapid (Miao et al. 2005). This also means that the linear concentration range and the sensitivity of the biosensor is enhanced (Miao et al. 2005). PTy also has the added advantage of producing biosensors that are permselective and have the ability to prevent interferents from fouling the electrode surface (Miao et al. 2005). These characteristics are attractive for the fabrication of a potentiometric penicillin biosensor in this study. Very few studies have been conducted on the use of PTy for the entrapment of enzymes (Tsuji et al. 1990; Cooper & Schubert 1994; DeBenedetto et al. 1996; Palmisano et al. 1997; Situmorang et al. 1998).

Figure 4.2 shows a detailed mechanism for the polymerisation of Ty, a process that will be employed for the immobilisation of P'nase in this study. The first step of this process involves the formation of a radical-cation and dimerisation process involving 2 electrons and the loss of 2 protons. The linear chain polymerisation produces relatively short oligomers with little or no conductivity. This is followed by an increase in the size of the oligomer which is characterised by the formation of a smooth film. Oligomeric chains can be linked through the reactive sites which are denoted by asterisks in Figure 4.2. The polymerisation process goes through these sites and the oxidation of the monomer continues to produce a PTy film.

Other enzyme entrapment methods that can be undertaken with PTy include cross-linking, entrapment or covalent attachment. This is facilitated by the availability of the primary amine group (Situmorang et al. 1994; Tenreiro 2003; Tran 2003; Suprun 2004; Miao et al. 2005). For example, L-amino acid oxidase (Cooper & Schubert 1994), tyrosinase (Debenedetto et al. 1996), and lactate oxidase (Palmisano et al. 1997), have been cross-

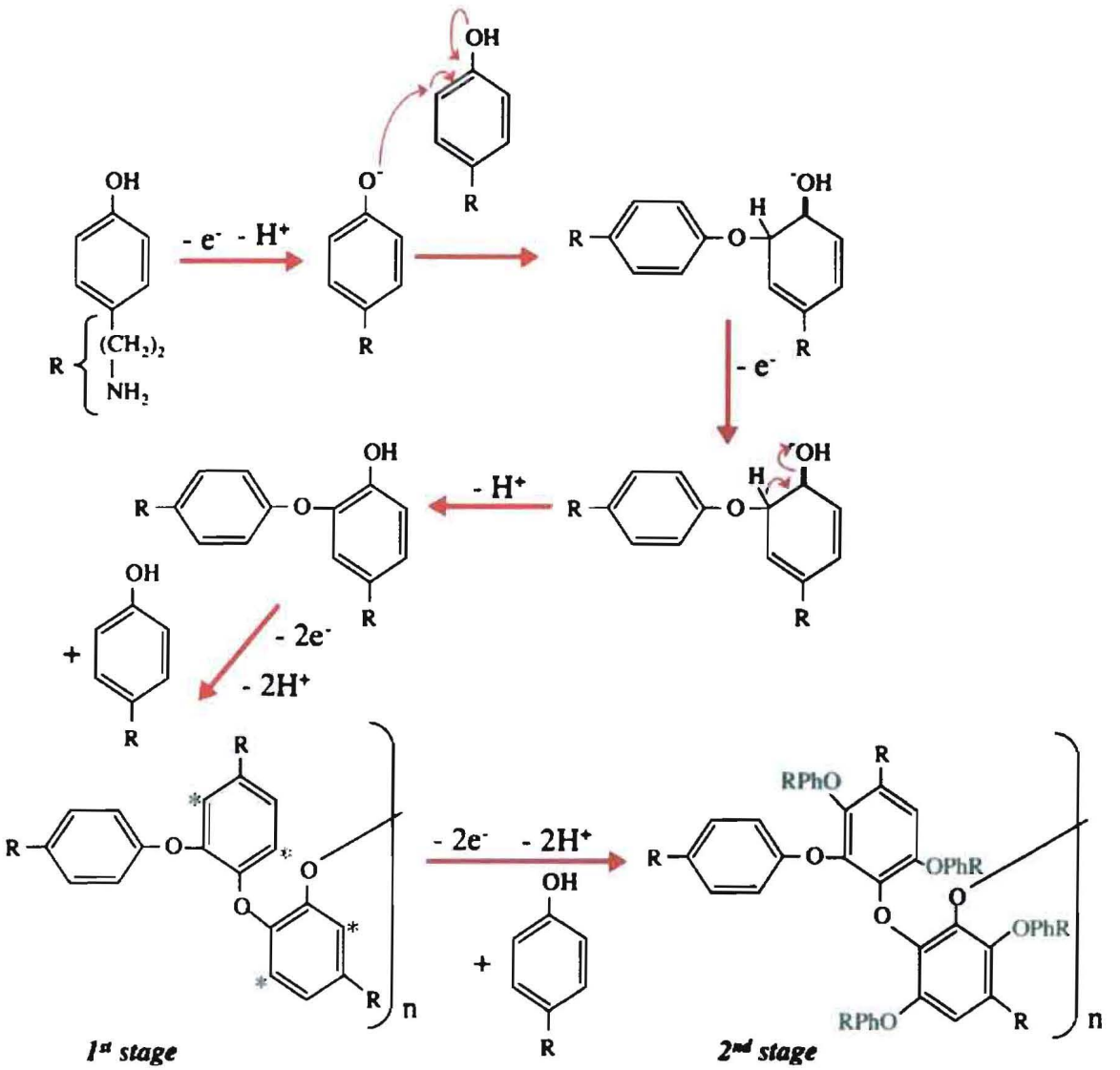



**Figure 4.2 Mechanism for the polymerisation of tyramine. Reproduced from Tenreiro et al. (2007).**

-linked to PTy with GLA. The covalent attachment of glucose oxidase to the free amine groups on the PTy film (Situmorang et al. 1998) as well as the attachment of sulfite oxidase, lactate oxidase and L-amino acid oxidase to the polymer chain, (Situmorang et al. 1999) has been reported.

Some of the methods reported for the fabrication of PTy biosensors include the modification of a gold electrode by electrochemical polymerisation into the defect sites of a hexadecanethiol monolayer (Losic et al. 2002). The covalent binding of the enzyme via carbodiimide coupling after electropolymerisation, is another method that has been described (Situmorang et al. 1999). Miscoria et al. (2006) reported the use of a glucose biosensor fabricated through the modification of a glassy carbon (GCE) and carbon screen printed (SPE) electrodes with rhodium, GOx and PTy. PTy was deposited on a graphite substrate to obtain a platinum-PTy composite (Spatura et al. 2009). However none of these methods have involved the fabrication of penicillin biosensors. Hence, the purpose of this study is to fabricate a PTy potentiometric penicillin biosensor. The aim of this chapter is to investigate the use of galvanostatic polymerisation for the formation of PTy and the entrapment of P'nase in a PTy layer. The effect of various parameters on the performance of the resulting biosensor will also be investigated. These will include the influence of the supporting electrolyte, P'nase concentration, Ty concentration, the applied current density, polymerisation time and buffer concentration. The biosensor will then be applied to the determination of penicillin in amoxycillin and in milk samples.

## 4.2 Experimental

The details of the experimental procedures were similar to those described in section 2.2 of chapter 2, except for the following variations.

### 4.2.1 Reagents and Chemicals

Ty (4-hydroxyphenethylamine) was purchased from Sigma Aldrich and was electropolymerised on a platinum electrode. 0.1 M Ty solution was prepared by dissolving 1.3272 g of Ty in 50 mL Milli-Q water. 1 mL orthophosphoric acid (85%) was then added and made up to 100 mL.

### 4.2.2 Electropolymerisation of Tyramine

The galvanostatic electropolymerisation of Ty was performed using a three-electrode cell, comprised of a platinum working electrode, pseudo reference and auxiliary electrode. Ty was electropolymerised from a solution which contained 0.1 M Ty, 19 U/mL of P'nase and 0.01 M Pen. A current density of 0.9 $mA/cm^2$ was applied for 40 s.

### 4.2.3 Preparation of Samples for XPS Analysis

PTy films were electropolymerised on aluminium coated electrodes (4 cm x 1 cm). The monomer solution contained 0.03 M Ty, 37 U/mL P'nase, 0.003 M Pen and 0.01 M $KNO_3$. The films were electropolymerised for 5 minutes at an applied current density of 0.9

$mA/cm^2$. The XPS conditions used were the same as specified in section 2.2.7 of chapter 2, unless otherwise indicated.

### 4.2.4 Preparation of Samples for SEM Analysis

The PTy films were prepared as indicated above in section 4.2.3. The SEM conditions were the same as those specified in chapter 2 in section 2.2.6.

## 4.3 Results and Discussion

### 4.3.1 Surface Characterisation using SEM and XPS

SEM and XPS analyses were performed in order to confirm the presence of the enzyme in the film, as well as to identify the composition of the PTy film. The peak assignments are listed in Table 4.1 along with the mean binding energies and standard deviations. Each XPS experiment was repeated 5 times.

The presence of P'nase in the film was also characterised by the presence of an S 2p peak at 168 eV (Adeloju et al. 1993). This peak confirms that the enzyme was incorporated in the film, as all enzymes are made up of amino acids that contain sulfur bonds.

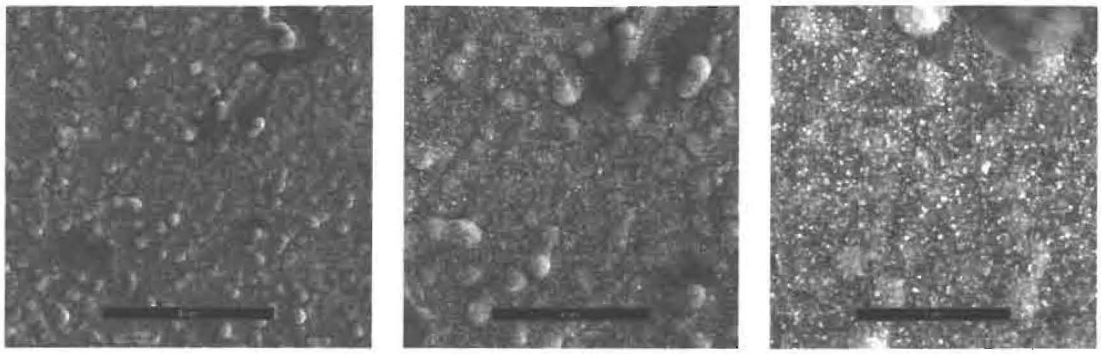
Table 4.1 indicates the presence of C 1s (1,2) at 285 eV which corresponds to the C-C bond of the reference carbon (Beamson & Briggs 1992; NIST XPS 2000). The N 1s peak at 398.3 eV corresponds to the N-H bond of PTy (Dubois et al. 1981). The C-O bonds of PTy are indicated at 286.5 eV. The N-C=O or C=O peaks at 288.3 eV pertain to the anodic oxidation of Ty (Losic et al. 2005).

**Table 4.1 Mean Binding Energy Values and Assignments for Photoelectron Peaks Detected for the PTy Electrode**

| Element | Mean Binding Energy (eV) | Standard Deviation (eV) | Peak Assignment |
|---|---|---|---|
| C 1s (1,2) | 285.0* | 0.0 | hydrocarbons $CH_x$ reference carbon |
| C 1s (3) | 286.5* | 0.1 | C-O, C-N |
| C 1s (4) | 288.3* | 0.0 | C=O |
| O 1s | 531.5* | 0.1 | C-O |
| N 1s | 398.3* | 0.1 | N-H |
| S 2p | 168.0* | 0.8 | Sulfur |

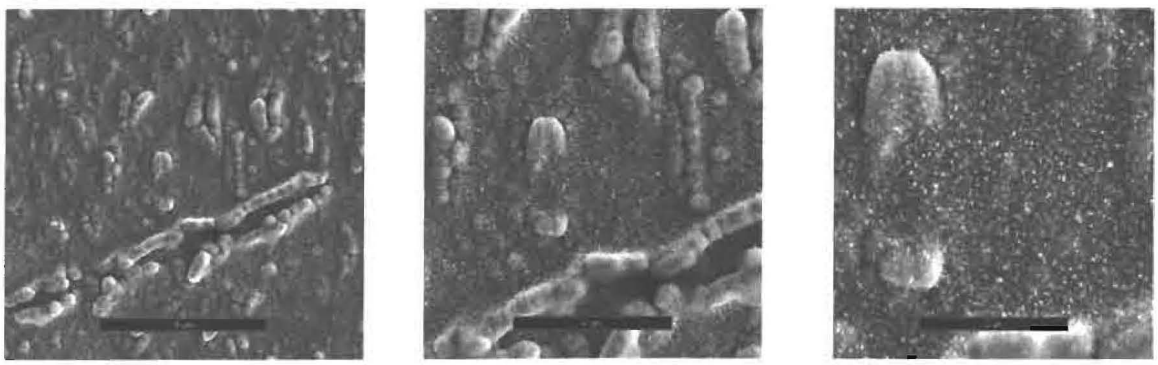
* Mean of 5 values

The SE micrographs shown in Figure 4.3 indicate that $KNO_3$ and Pen were both incorporated in the film in the absence of the enzyme. This is because Figure 4.3 shows more nodules than that shown in Figure 2.2 (page 50, Chapter 2) and Figure 3.1 (page 82, Chapter 3). Figures 2.2 and 3.1 only contained PPy and Pen but no $KNO_3$ and hence show a less nodular surface thus confirming the presence of $KNO_3$ in Figure 4.3. The surface appears to be more uniform due to the absence of P'nase.



(a) (b) (c)

**Figure 4.3 SE micrograph of PTy film in the absence of the enzyme. Monomer solution contained 0.03 M Ty, 0.003 M Pen and 0.01 M $KNO_3$ where (a) X5, 000 (b) X10, 000 (c) X20, 000. The films were electropolymerised using a current density of 0.9 mA/cm$^2$ for 5 minutes.**



(d) (e) (g)

**Figure 4.4 SE micrograph of PTy film in the presence of the enzyme. Monomer solution contained 0.03 M Ty, 37 U/mL P'nase, 0.003 M Pen and 0.01 M $KNO_3$ where (d) X5, 000 (e) X10, 000 (f) X20, 000. The films were electropolymerised using a current density of 0.9 mA/cm$^2$ for 5 minutes.**

Figure 4.4 shows the electron micrographs of the PTy film in the presence of the enzyme. These micrographs taken at 5, 000X, 10, 000X and 20, 000X showed nodular protrusions that appeared to be rod-like and chained together which is distinctly different from Figure 4.3. The nodules that appeared in the film containing the enzyme are larger than those exhibited in Figure 4.3 and, in comparison, the morphological differences shown in Figure 4.4 confirms the incorporation of the enzyme in the PTy film. This was further demonstrated by XPS measurements, as summarised in Table 4.1. All spectra were referenced by setting the hydrocarbon C 1s peak to 285.0 eV (Beamson & Briggs 1992) to compensate for the residual charging effects (Sharma et al. 2004).

### 4.3.2 Optimisation of the Tyramine Concentration

The influence of the Ty concentration on the sensitivity of the film was investigated by varying the concentration of Ty in the monomer solution. Figure 4.5 shows an increase in the sensitivity of the potentiometric response obtained for penicillin with increasing Ty concentration up to 0.03 M. Adequate coverage of the electrode was observed with this monomer concentration. The attainment of the optimum sensitivity with the PTy-P'nase film formed at this Ty concentration also indicates that a sufficient amount of P'nase was incorporated into the film.

The lowest electrode sensitivity was observed for films formed with 0.01 M Ty. This was due to the incorporation of less amounts of enzyme into the film. In addition, inadequate coverage of the electrode was also observed and provided further evidence for the low sensitivity. A similar observation was also made when 0.02 M Ty was used. The sensitivity

of the penicillin response declined when 0.04 M Ty was used due to an increase in the thickness of the film which also results in an increased diffusion barrier and decreased the sensitivity of the penicillin response.

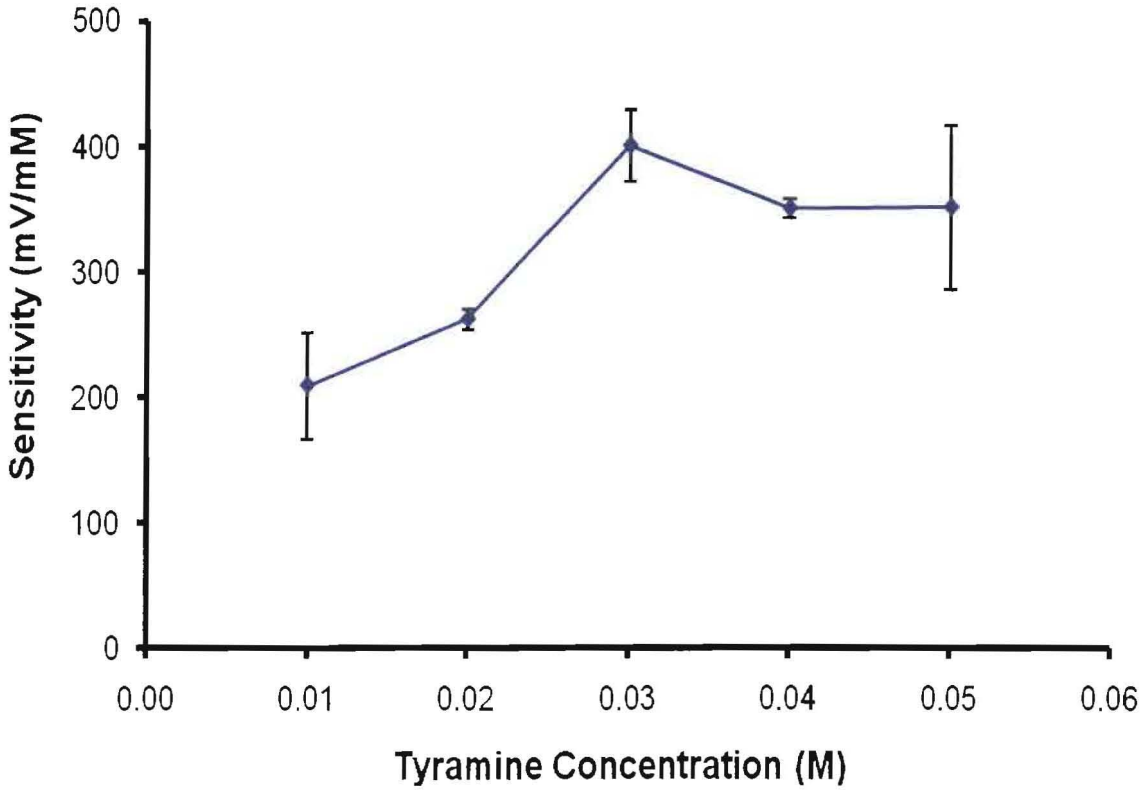



**Figure 4.5 Effect of the tyramine concentration used for the formation of the PTy-P'nase biosensor on the penicillin response. The following conditions were kept constant: 19 U/mL P'nase, 0.01 M Pen, applied current density 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

Films that were produced with 0.04 M and 0.05 M Ty appeared to be denser and less permeable than those produced with ≤ 0.03 M Ty. A similar observation was made when increases in the Py concentration were made (Adeloju & Moline 2001). As the optimum potentiometric response for penicillin was obtained with 0.03 M Ty, this monomer concentration was used for all other investigations.

### 4.3.3 Effect of Penicillinase Concentration

The immobilisation of the enzyme throughout the polymer layer influences the sensitivity of the biosensor (Hall et al. 1995; Situmorang et al. 1999). Figure 4.6 shows that there is a gradual increase in the sensitivity of penicillin response when the P'nase concentration in the monomer solution was doubled from 4.6 to 9.2 U/mL. A further increase in the sensitivity of the response was observed when the enzyme concentration was increased from 9.2 to 18.5 U/mL. The increase in the enzyme concentration is tantamount with increasing the relative catalytic activity of the sensor (Situmorang et al. 1999). When P'nase concentrations between 4.6 and 18.5 U/mL were added to the monomer solution, the corresponding sensitivity was also low due to the lower enzyme concentration in the film. Low enzyme concentrations also mean that the catalytic activity of the biosensor is low leading to kinetic limitations (Situmorang et al. 1999) as well as a decline in the electrode sensitivity.

The highest sensitivity was obtained when 37 U/mL of P'nase was used for the formation of the film. In fact the highest change in the electrode sensitivity was observed when the enzyme concentration was increased from 18.5 to 37 U/mL. Optimum sensitivity was

observed when 37 U/mL P'nase was incorporated into the film. Increases in the enzyme concentration added to the monomer solution increased the amounts of P'nase within the

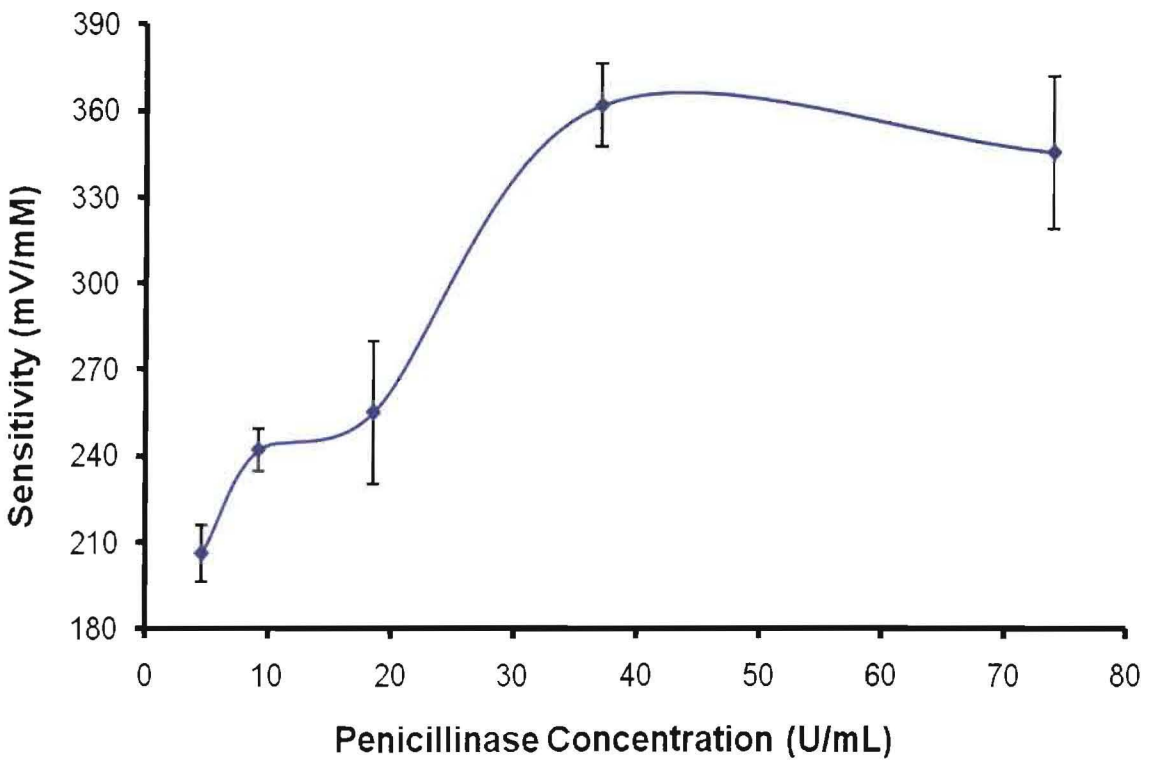



**Figure 4.6 Effect of the penicillinase concentration on the sensitivity of the PTy-P'nase electrode. The following conditions were kept constant: 0.03 M Ty, 0.01 M Pen, 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

polymer layer for the enzymatic reaction with penicillin resulting in an increase in the potentiometric response. The addition of >37 U/mL of enzyme decreased the electrode sensitivity due to the dense films produced resulting in an increased diffusion barrier and an associated decrease in the electrode response. The concentration of protons detected at the electrode decreased with such a thick film because of the increased distance for the reaction products to reach the electrode surface which resulted in the reduced electrode sensitivity (Situmorang et al. 1999). Consequently, 37 U/mL of P'nase was used in all other investigations.

### 4.3.4 Effect of Supporting Electrolyte

Supporting electrolytes are used to increase the electrical conductivity of monomer solutions and to improve the sensitivity of biosensors (Karyakina et al. 1994; Trojanowicz & Hitchman 1996; Guerrieriet al. 1998; Adeloju & Moline 2001). $KNO_3$ was used in this study to increase the solution conductivity as well as to enhance the conductivity of the PTy film. Figure 4.7 shows that the electrode sensitivity increased when 0.01 M $KNO_3$ was added to the monomer solution. However the response of the electrode decreased when 0.02 M $KNO_3$ was added to the monomer solution. Thus the usefulness of the supporting electrolyte was demonstrated with the use of 0.01 M $KNO_3$ for the formation of the PTy-P'nase film and was used in all further studies.

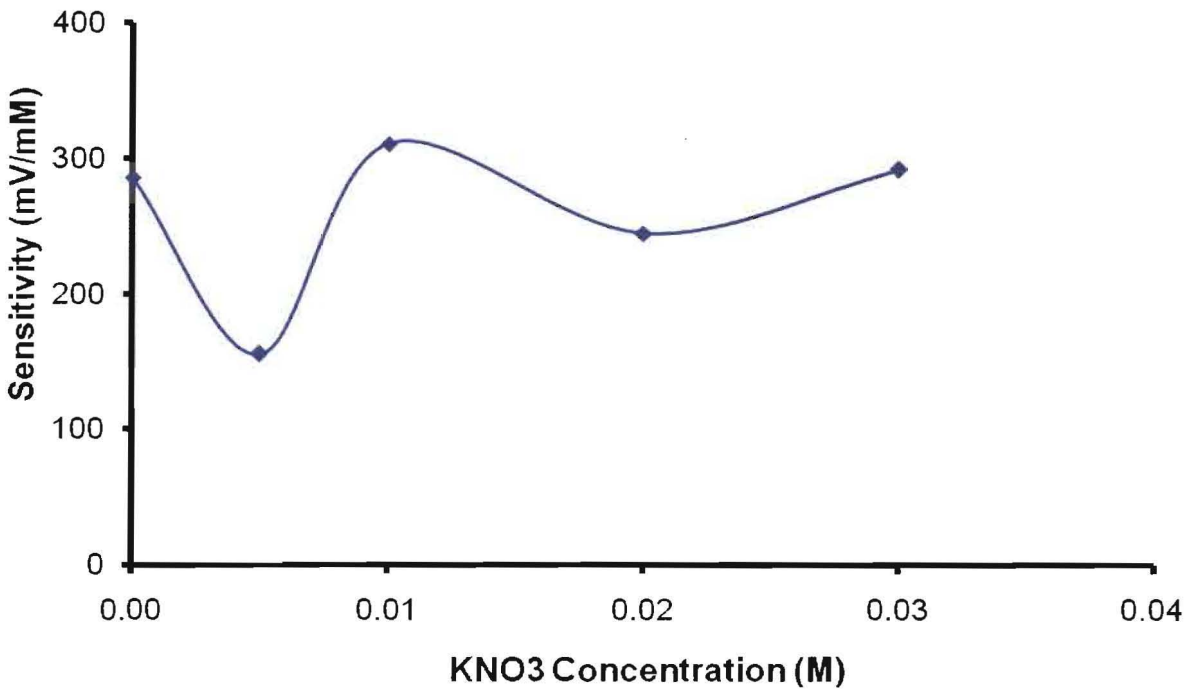



**Figure 4.7 Effect of $KNO_3$ concentration used for the formation of the PTy-P'nase electrode on the sensitivity of the penicillin response. The following conditions were kept constant: 0.03 M Ty, 37 U/mL P'nase, 0.01 M Pen, 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

### 4.3.5 Influence of Penicillin Concentration in the Monomer Solution

The presence of Pen in the monomer solution aids in the attainment of a steady state response as well as an enhanced electrode sensitivity. Figure 4.8 shows that the incorporation of Pen in the monomer solution had a significant influence on the

potentiometric response. In particular, the lowest electrode sensitivity was obtained in the absence of Pen in the monomer solution.

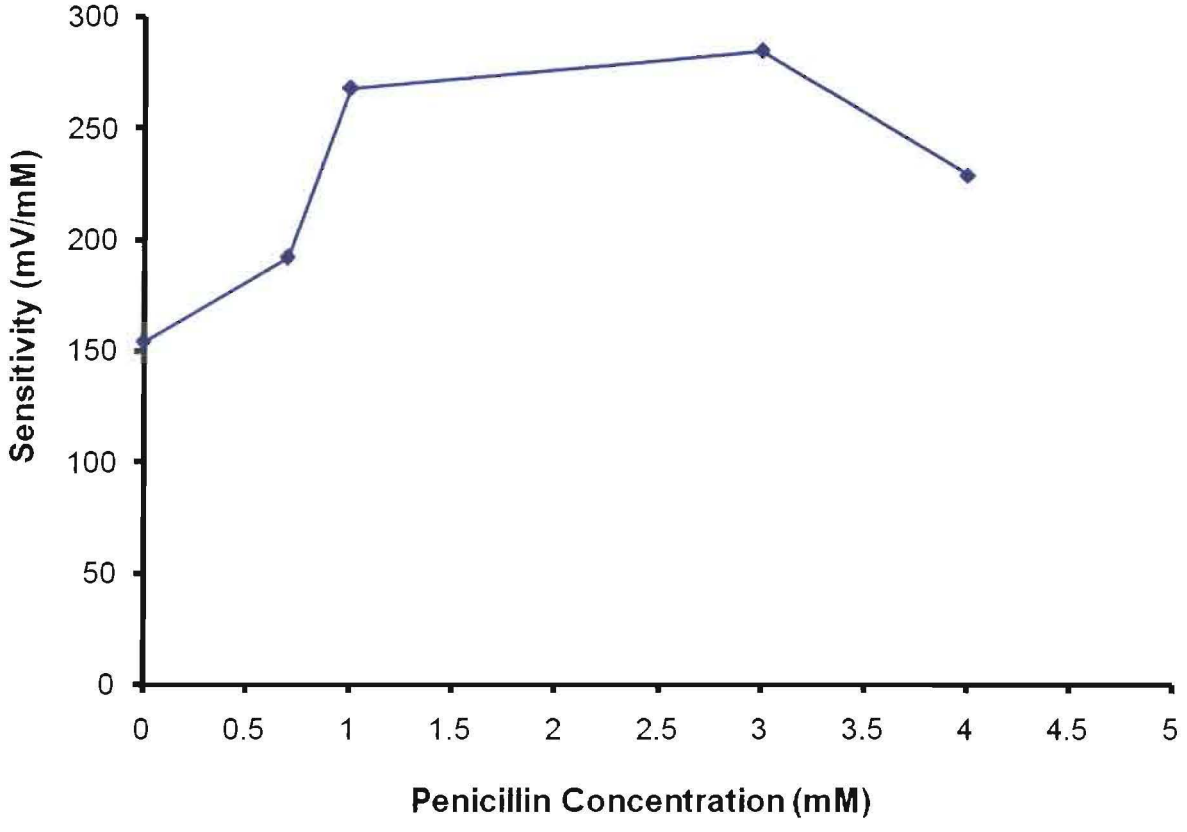



**Figure 4.8 Effect of the penicillin concentration used for the formation of the PTy-P'nase electrode on the penicillin response. The following conditions were kept constant: 0.03 M Ty, 37 U/mL P'nase, 0.01 M $KNO_3$, 0.9 mA/cm$^2$, electropolymerisation time 40 s.**

Interestingly, the sensitivity of the penicillin response increased with increasing penicillin concentration in the monomer solution. In fact the highest sensitivity that was achieved

with the PTy-P'nase film formed with the addition of 3 mM Pen in the monomer solution, is notably higher than the Pen concentration required for the outer layer of the PPy bilayer biosensor (0.7 mM Pen). This may be associated with the non-conducting nature of PTy and, thus, required a higher Pen concentration in the monomer solution. Other possible reasons include a decrease in the pH of the membrane due to the retention of penicilloate in the membrane. The charge on the membrane can have an electrostatic influence on the enzyme and substrate where the addition of an optimum Pen concentration (3 mM) allows the charge on the membrane to become positive. The more positive the charge on the membrane, the greater its ability to attract the substrate (Pen) resulting in an increase in the sensitivity of the biosensor. Beyond the Pen concentration of 3 mM, the sensitivity of the electrode decreased. Higher Pen concentrations resulted in a decline in the electrode sensitivity due to the saturation of the film with Pen. For this reason, 3 mM Pen was added to the monomer solution for the formation of PTy-P'nase film for further studies.

### 4.3.6 Effect of the Applied Current Density and Polymerisation Time

The current density used for the formation of the PTy-P'nase film and the resulting film thickness can significantly influence the electrode response. Figure 4.9 shows that the sensitivity of the penicillin response increased when increasing the applied current densities

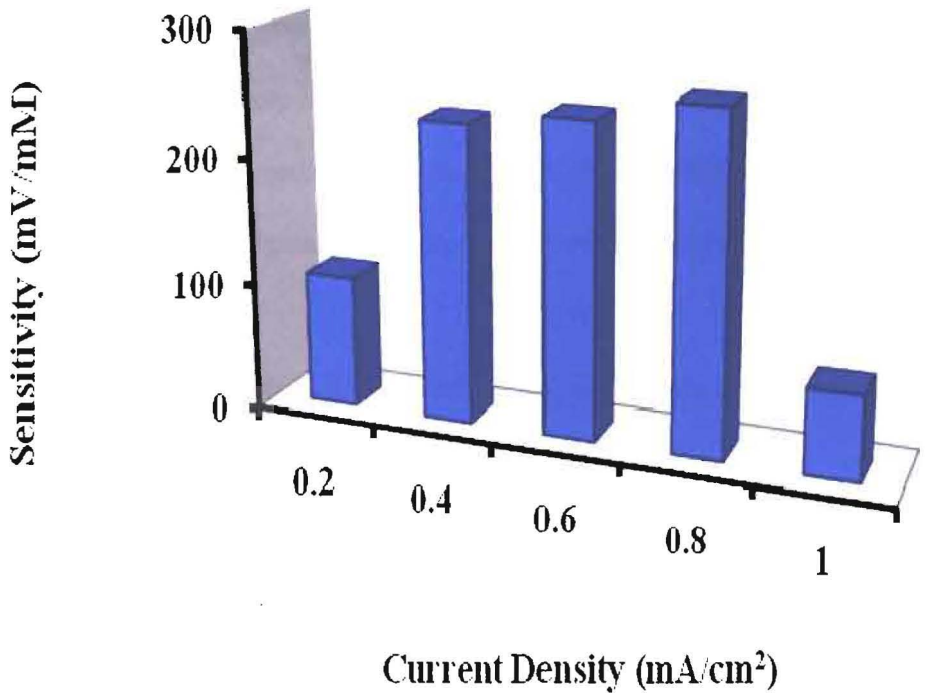



**Figure 4.9 Effect of the applied current density used for the formation of the PTy-P'nase film on the sensitivity of the penicillin response. The following conditions were kept constant: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, electropolymerisation time 40 s.**

and, thus, suggests that the amount of enzyme entrapped in the PTy layer increased with an increase in the applied current density. In fact the optimum sensitivity was obtained with the application of 0.8 mA/cm$^2$. Obviously the formation of PTy was more rapid at higher current densities ($\geq$0.4 mA/cm$^2$) than at 0.2 mA/cm$^2$. The slight variation in the sensitivities obtained with the films produced with applied current densities between 0.4 mA/cm$^2$ - 0.8 mA/cm$^2$, may be reflective of the slightly decreasing sensitivity which occurs as the film

approaches maximum thickness where the sensitivity of the penicillin response only increased slightly. However, when the applied current density was increased to 1 $mA/cm^2$, the sensitivity of the response was decreased substantially. This may be due to an increase in the diffusion barrier caused by an increase in the film thickness (Sohail & Adeloju 2008). Hence, all other investigations were conducted with films formed at a current density of 0.8 $mA/cm^2$.

Polymerisation time can also influence the amount of enzyme incorporated in the polymer film (Sohail & Adeloju 2008). The incorporation of sufficient amounts of P'nase is necessary to obtain a highly sensitive penicillin response. This was not the case when the film was polymerised for 10 s as the sensitivity of the film was very low. Obviously the use of the polymerisation time of 10 s did not enable the incorporation of a sufficient amount of P'nase. The minimum point that occurs between 20 and 30 s is due to a slight increase in the electrode sensitivity of 11 mV/mM. Hence both of these polymerisation times have the same effect, i.e. the sensitivities of both films are low.

Figure 4.10 shows that the sensitivity of penicillin increased with increasing polymerisation times for the formation of the PTy-P'nase electrode. The highest sensitivity was achieved when a polymerisation time of 40 s was used. At this polymerisation time, the coverage of the platinum electrode was adequate However, beyond this polymerisation time (>40 s), there was a significant decrease in the sensitivity of the penicillin response. This may be due to the increased film thickness and the associated effect of an increased diffusion barrier (Adeloju et al. 1993a). Evidently the increase in the diffusion barrier limits the ability of the catalytic product to reach the surface of the electrode. Hence, a polymerisation time of 40 s was used for further studies.

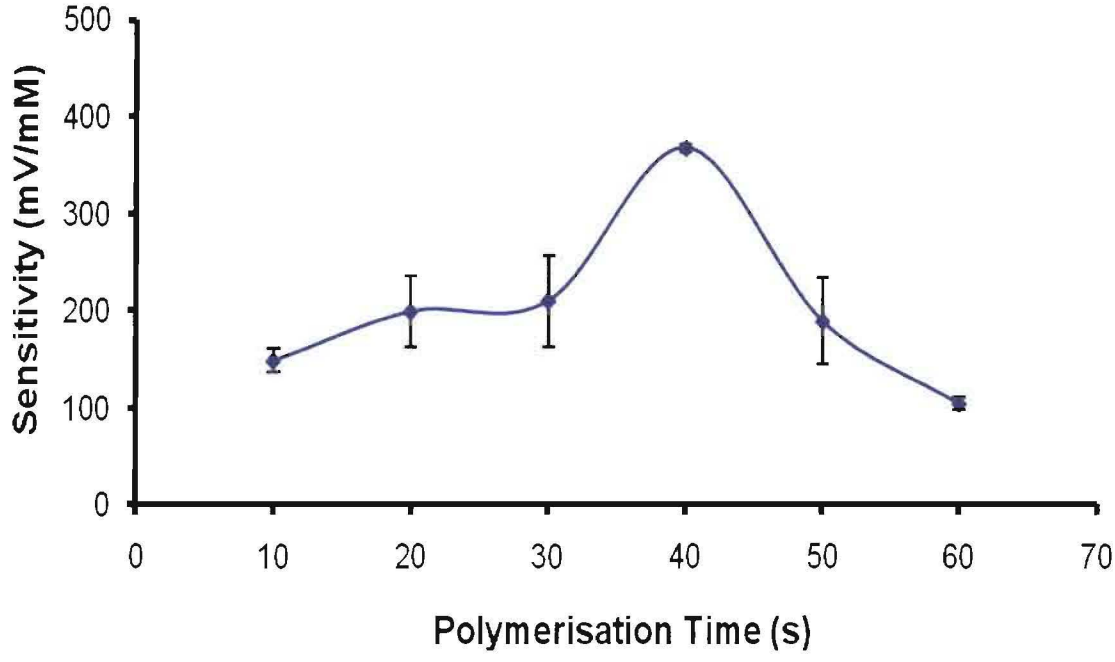



**Figure 4.10 Influence of the polymerisation time used for the formation of the PTy-P'nase film on the sensitivity of the penicillin response. The following conditions were kept constant: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, applied current density 0.8 mA/cm$^2$.**

## 4.3.7 Influence of the Buffer Concentration and Linear Concentration Range

The proper control of the buffer concentration is important for achieving an optimum potentiometric response, as well as an adequate linear concentration range for the biosensor

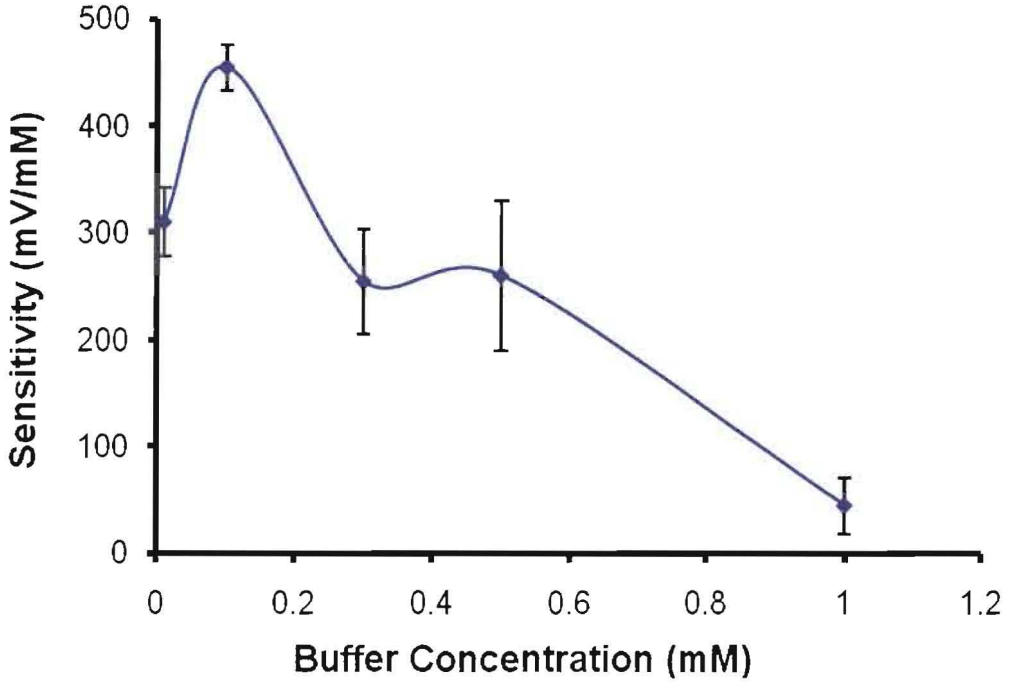



**Figure 4.11 Influence of the buffer concentration on the sensitivity of the penicillin response. The following conditions were kept constant: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, current density 0.8 mA/cm$^2$, electropolymerisation time 40 s.**

(Kulp et al. 1987). Figure 4.11 shows that an optimum potentiometric response was achieved when 0.1 mM buffer was used and an adequate linear range was obtained as shown in Figure 4.12. The maximum point between 0.4 and 0.8 mM is due to the slight increase in the electrode sensitivity when the buffer concentration is increased from 0.3 to 0.5 mM which is followed by a sharp decrease in the sensitivity when the buffer

concentration was increased from 0.5 to 1 mM. In contrast, a minimum potential change as well as the lowest sensitivity was obtained with the PTy-P'nase biosensor when 1 mM buffer was used. This observation indicates that 1 mM buffer was unsuitable because increasing the buffering capacity decreased the sensitivity of the electrode as the $H^+$ ions produced by the catalytic reaction was low (Chao & Lee 2000).

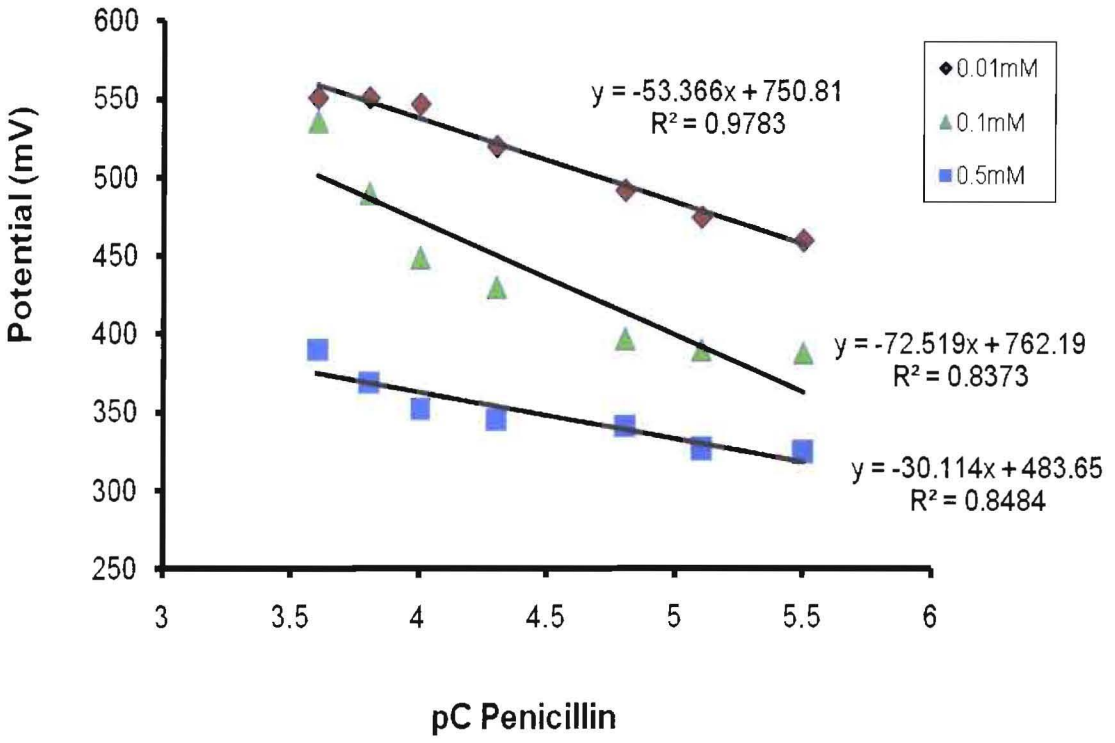



**Figure 4.12 Logarithmic concentration of the penicillin response obtained with the PTy-P'nase film in different buffer concentrations. The following film conditions were employed: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, current density 0.8 mA/cm$^2$, electropolymerisation time 40 s. Potentiometric biosensing was carried out in 0.1 mM buffer.**

The sensitivity of the electrode to the hydrogen ions produced from the dissociation of penicilloic acid decreased. This is due to the masking of the effect of the low hydrogen ions produced by stronger buffer concentrations. Higher buffer concentrations (≥0.1 mM) increased the effective diffusion coefficient of hydrogen ions, as well as shortening the response time for the detection of hydrogen ions (Chao & Lee 2000). For this reason, 0.1 mM buffer was used in further studies. The minimum detectable concentration achieved under this condition was 0.3 μM.

A plot of potential versus the logarithmic of penicillin concentration showed that the slope for 0.1 mM buffer was more sensitive than that obtained for 0.5 and 0.01 mM buffer as shown in Figure 4.12. The response of the PTy-P'nase electrode is linear between 3 and 283 μM with a correlation coefficient of $R^2$=0.9783. Beyond this, the response plateaus signifying saturation. The use of higher buffer concentrations, such as ≥ 0.5 mM, increased the upper limit of the linear range to 156 μM but also decreased the lower limit of the linear range (Chao & Lee 2000). On the other hand, lower buffer concentrations, such as 0.01 mM, decreased the upper limit of the linear concentration range and increased the lower limit of the linear range. This highlights the need to balance the importance of sensitive potentiometric measurement against the necessity to control the buffering capacity (Kulp et al. 1987). Overall the potentiometric response for the PTy electrode was found to be more positive (i.e. >500 mV) than that obtained with the PPy bilayer electrode (i.e. <300 mV). This may be due to the thinner film produced with PTy because of the self-limiting growth of non-conducting films, compared with the use of bilayer PPy film which is obviously thicker due to the bilayer configuration. Also because of the non-conducting nature of PTy,

change in conductivity caused by generation of $H^+$ may be more pronounced than with conducting polymer.

### 4.3.8 Determination of Penicillin in Amoxycillin Tablets

As stated in Chapter 3, the dosages stated by the pharmaceutical company are 500 mg amoxycillin and 125 mg CA. Three amoxycillin 500 mg tablets were prepared as described

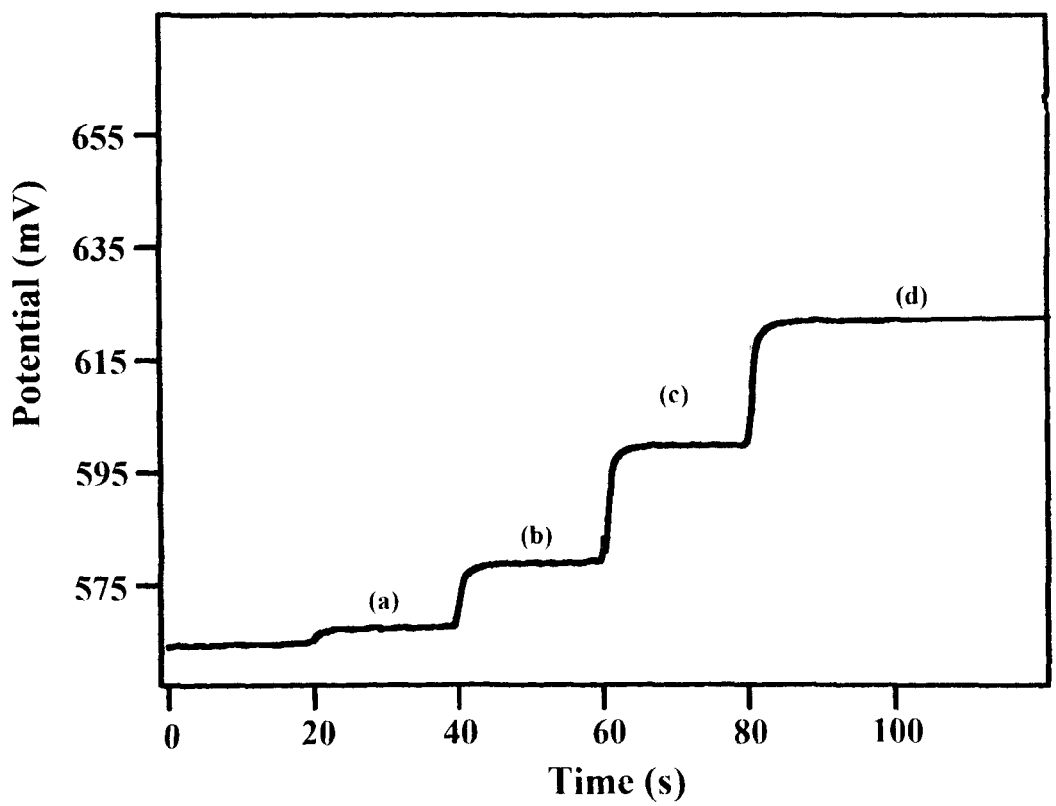



**Figure 4.13 Potentiometric response to amoxycillin 500 mg sample where (a) 30 µL amoxycillin sample (b) 100 µL penicillin standard (c) 200 µL penicillin standard (d) 800 µL penicillin standard.**

in section 3.2.3 of Chapter 3. Each sample was analysed 4 times with the PTy biosensor. Figure 4.13 shows a typical potentiometric response to penicillin in amoxycillin 500 mg obtained with the PTy-P'nase electrode. The mean and standard deviations, as well as the corresponding percentage recoveries based on the expected penicillin concentrations are provided in Table 4.2. The concentration of penicillin in the amoxycillin tablets was

**Table 4.2 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Obtained Using the PTy Biosensor**

| **Amount Specified in Tablet (mg)** [a] | **[Penicillin] Obtained With Biosensor (mg)** [b] | **% Recovery Obtained With Biosensor** | **[Penicillin] Obtained With Titration (mg)** | **% Recovery Obtained With Titration** |
|---|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 544 ± 43 | 109 ± 9 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 466 ± 32 | 93 ± 6 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 526 ± 15 | 105 ± 3 | 505 ± 20 | 101 ± 4 |
| **Average values** | **512 ± 30** | **102 ± 6** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

determined with the biosensor and the amount was compared with the results obtained with the standard titrimetric method. The achieved recoveries, obtained with the biosensor ranged from 93% to 109%. In some cases, recoveries greater than 100% were obtained and this can be attributed to the sensitivity of the electrode towards CA which has weak antibiotic properties. CA acid also acts as a competitive inhibitor despite being structurally similar to β-lactam antibiotics (Parag et al. 2008). The average recovery obtained with the biosensor (102 ± 6%) is in close agreement with the average recovery obtained with the standard titrimetric method (105 ± 5%). In fact it is slightly higher than the average recovery obtained with the biosensor. This may be ascribed to the presence of interferents, such as the sodium starch glycollate contained in amoxycillin.

The reproducibility of the standard titrimetric method seems to be the same as the biosensor method. However, the use of the biosensor was less time consuming and more reliable than the titrimetric method. Hence, the biosensor was more advantageous than the standard method.

### 4.3.9 Determination of Penicillin G in Milk

Table 4.3 summarises the recoveries obtained with the PTy-P'nase electrode. Four separate milk samples were prepared, as described in section 3.2.2 of chapter 3. Each sample was analysed 4 times with the PTy biosensor. The mean, standard deviations and the corresponding percentage recoveries based on the expected penicillin concentrations are provided in Table 4.3.

The results show that a higher percentage recovery was obtained for lower penicillin concentrations (1 ppm), while lower percentage recoveries were obtained for samples which contained higher penicillin concentrations. This behaviour may be attributed to the tendency of penicillins to bind to the hydrophobic sites of milk proteins (Grunwald & Petz 2003). Possible reasons for this may include the interference caused by milk proteins due to the non-specific binding that occurs with whey proteins (Cacciatore et al. 2004).

**Table 4.3 Recovery of Penicillin G in Milk Samples**

| Concentration of Penicillin G in Milk (ppm) [a] | Penicillin Concentration Obtained With Biosensor (ppm) [b] | % Recovery |
|---|---|---|
| 1 | 1.1 ± 0.8 | 110 ± 80 |
| 5 | 4.6 ± 1.6 | 92 ± 32 |
| 10 | 7.8 ± 0.8 | 78 ± 16 |
| 20 | 18 ± 2.7 | 90 ± 14 |

[a] Spiked concentration of penicillin G in milk sample

[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

The reproducibility of the recoveries obtained with the PTy biosensor was higher with lower penicillin concentrations (≥10 ppm). On the other hand, higher deviations were

observed for lower penicillin concentrations (≤5 ppm) and may be attributed to the non-selectivity of the biosensor at these concentrations.

## 4.4 Conclusion

The immobilisation of P'nase in PTy has been successfully employed for the fabrication of a PTy-P'nase biosensor. The optimum monomer concentration for the formation of the PTy-P'nase film was 0.03 M Ty. Higher monomer concentrations produced thicker films which caused a decrease in the sensitivity of the biosensor. It was interesting to note that the PTy film (401 mV/mM) was more sensitive than the bilayer PPy film (380 mV/mM). This is due to the self-limiting growth of non-conducting films such as PTy. 37 U/mL P'nase produced the most sensitive electrode (362 mV/mM). However this film was not as sensitive as the PPy bilayer film (448 mV/mM) which only required 19 U/mL P'nase in the outer layer for the enhancement of the sensitivity. This is because of the increase in the diffusion barrier resulting from the denser PTy film and the higher P'nase concentration required for optimum performance.

The conductivity of the film was improved with the incorporation of 0.01 M $KNO_3$ and 3 mM Pen in the monomer solution. A much higher concentration of Pen was required for the PTy film, compared with the PPy bilayer film which only required 0.7 mM Pen in the monomer solution. The difference in the behaviour of both films is attributed to the conducting nature of PPy and the non-conducting nature of PTy. The optimum buffer concentration for the PTy electrode was 0.1 mM and 0.3 mM for the PPy bilayer electrode indicating that lower buffer concentrations are better for non-conducting polymers. The

achievable minimum detectable concentration was 0.3 μM for both the PTy and PPy bilayer electrodes.

The biosensor was successfully applied to the determination of penicillin in the antibiotic preparation, achieving percentage recoveries ranging from 93 - 109% for amoxycillin. This percentage recovery range is smaller than that achieved for the PPy bilayer configuration (102 - 124%). In both cases recovery values exceeded 100% which may have been due to the presence of CA which has weak antibiotic properties. The results obtained with the PTy electrode were compared to those obtained with the standard titrimetric method and were found to be in close agreement with the recoveries obtained with the biosensor. However, the use of the biosensor was more advantageous than the standard titrimetric method because it was less time consuming and more reliable.

Penicillin in milk samples was also quantified with the PTy electrode and the results indicate that percentage recoveries of penicillin in milk were affected by the tendency of penicillin to bind to the hydrophobic sites of the milk proteins. Even though lower concentrations of penicillin were able to be recovered, the reproducibility was poor. This was also the case with the results obtained with the PPy bilayer electrode where both the PTy and PPy bilayer electrodes were able to detect $\leq$ 5ppm penicillin in milk. This suggests that further work is required to ensure reliable determination of penicillin in milk.

# Chapter 5

## Cross-linking Penicillinase with Glutaraldehyde and Bovine Serum Albumin for the Fabrication of a Penicillin Biosensor

## 5.1 Introduction

The enhancement of the sensitivity of the biosensor can be achieved by using good enzyme immobilisation methods. GLA coupling is one of the best methods to achieve this as the enzyme stability is improved with GLA coupling (Scouten et al. 1995). This method involves the creation of a protein membrane with the use of BSA and GLA (Liu et al. 1999) by cross-linking. The improvement in the stability of the enzyme may be attributed to the polymeric nature of GLA which provides a long lead attaching the protein to the matrix. This allows greater flexibility that is necessary for activity. In addition to this, the multipoint attachment that occurs with GLA prevents unfolding of the enzyme (Scouten et al. 1995).

BSA is an inert protein which facilitates the formation of a gel from solutions that only have oligomer forming capabilities as it increases the total protein concentration (Koudelka-Hep et al. 1997). This protein is also capable of alleviating steric interaction that is present throughout the three-dimensional cross-linked matrix (Oyama et al. 1988; Abe et al. 1991; Liu et al. 1999).

GLA or pentandial, is bifunctional by nature and, as such, has cross-linking capabilities. It has been used for a number of industrial applications, namely, for the production of β-lactam antibiotics (Perrachia & Mittler 1972), such as for the immobilisation of lactose on carbon by adsorption to produce 6-aminopenicillanic acid, which is a starting material for the production of β-lactam antibiotics. GLA exhibits complex chemical behaviour particularly when dissolved in water where linear monohydrates, dihydrates, cyclic hemiacetals and oligomers are formed as shown in Figure 5.1. The structures shown in Figure 5.1 are those that form when GLA is not stored at $0^0$ C and as a result can polymerise to form the aforementioned structures. The complexity of this behaviour is summed up by the fact that this practice still allows for efficient enzyme

immobilisation (Walt & Agayn 1994). On the other hand, higher temperatures increase free GLA and cyclic hemihydrate multimers can be formed under acidic conditions or in concentrated solutions (Walt & Agayn 1994). These polymeric materials can be converted to their respective monomers by dilution with distilled water. This highlights the need for specific storage requirements for GLA in order to increase the chances of GLA remaining in its monomeric form, as it contains polymers. These polymers are formed as a result of the abstraction of a proton from one monomer which forms an α carbanion. This condenses with a second monomer and the dimer that is formed, undergoes β elimination to form an α-β unsaturated ketone which is a reactive species. Further polymerisation may be prevented by the prior purification of the monomer (Scouten et al. 1995).

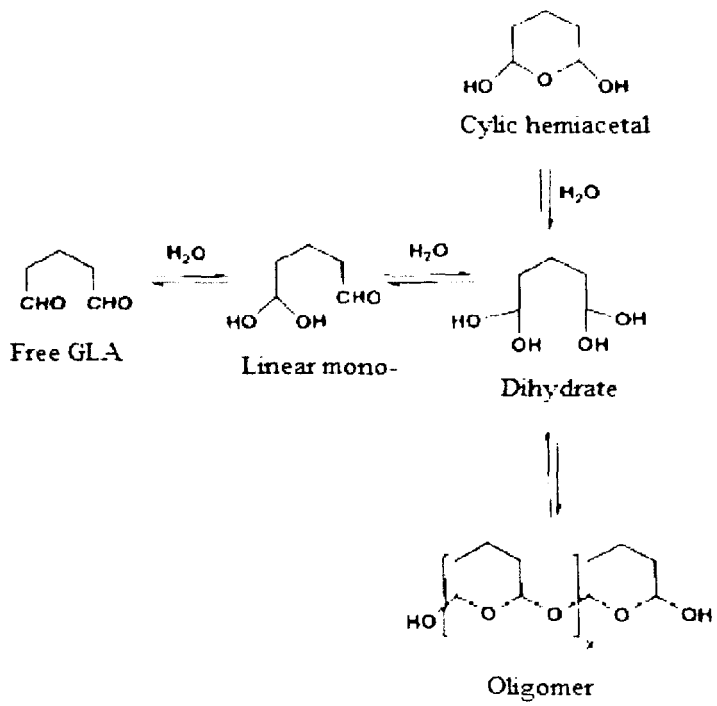



**Figure 5.1 Structure of GLA when dissolved in water. Reproduced from Walt (1994).**

BSA-GLA films have been used quite extensively for the fabrication of penicillin biosensors, as indicated in Table 5.1. These have been achieved with various detection modes including amperometric, potentiometric FET, FIA electrodes as well as potentiometric pH electrodes and thermistors (Li et al. 1995). Many of the potentiometric biosensors described in Table 5.1 have used different pH glass electrodes (Gnanasekaran & Mottola 1985; Olsson 1988; Meier & Tran-Minh 1992), while others have used pH sensitive membranes such as $AlO_2O_3/SiO_2$ (Seki et al. 1998). Reinhardt et al. (1990) also used potentiometry in conjunction with a bio-field-effect transistor (BioFET) which measures changes in the pH, a concept that has been used in all of the previously mentioned potentiometric biosensors. However, when $H^+$ are released or consumed, it is more sensitive to measure the potential change than the change in pH (Roy 1981; Gnanasekaran & Mottola 1985), as will be considered in this study.

The fabrication of a penicillin biosensor by cross-linking P'nase with BSA-GLA on a platinum electrode for the potentiometric measurement of penicillin will be examined. To our knowledge, BSA-GLA has not been used to develop a penicillin biosensor that monitors the potential change. This Chapter will use the method of cross-linking using BSA-GLA to enhance the sensitivity of the biosensor beyond that achieved with PPy, as demonstrated in Chapters 2 and 3, and PTy in Chapter 4. Cross-linking increases enzyme stability as it is improved with GLA coupling (Scouten et al. 1995) and hence the sensitivity of the biosensor. This study will test this hypothesis by investigating the influence of factors such as the BSA concentration, the GLA concentration, the effect of the enzyme concentration, the influence of the drying time and the effect of the buffer concentration. The possible use of the resulting biosensor for the determination of penicillin in milk samples and antibiotic preparations will also be undertaken.

**Table 5.1 The Use of BSA-GLA for the Fabrication of Penicillin Biosensors**

| Method of Immobilisation | Type of Electrode | Detection Limits | Reference |
|---|---|---|---|
| P'nase cross-linked with BSA and GLA on a $AlO_2O_3/SiO_2$ surface | Potentiometric light addressable | 5 - 25 mM | Seki et al. 1998 |
| P'nase G Acylase cross-linked with BSA and GLA. | $H^+$-ISFET | 0.5 - 0.8 mM | Liu et al. 1998 |
| P'nase cross-linked with BSA in saturated GLA vapour on a sensor chip. | ENFET | 0 - 15 mM | Soldatkin et al. 1997 |
| P'nase cross-linked with BSA in saturated GLA vapour | ISFET, ENFET | N/A* | Gorchkov et al. 1996 |
| GLA cross-linked between porous glass and P'nase | Potentiometric pH electrode | 0.1 - 20 mM | Olsson 1988 |
| GLA cross-linked between porous glass and P'nase | Potentiometric pH electrode | 0.05 - 0.5 mM | Gnanasekaran & Mottola 1985 |
| GLA cross-linked between P'nase and CPG | Thermistor | 0.1-10 (mg/mL) | Decristoforo & Danielesson 1984 |

*N/A not available

*Continued over the page...*

| Method of Immobilisation | Type of Electrode | Detection Limits | Reference |
|---|---|---|---|
| Covalent binding of P'nase directly to surface of pH detector | pH electrode | N/A* | Koncki et al. 1996 |
| P'nase cross-linked with BSA and GLA | Amperometric | 0 - 26 mM | Li et al. 1995 |
| β-Lactamase was immobilised in an enzyme column.<br><br>Penicillin V acylase was purified then immobilised on an enzyme column | Thermistor | 0.1 - 500 (for β-Lactamase) and 0.5 – 150 (for penicillin V acylase) | Rank et al. 1992 |
| P'nase was immobilised directly on the backside of a thermopile. | Calorimetric | 1 - 200 mM | Bataillard et al. 1992 |
| P'nase cross-linked with BSA and GLA | Potentiometric pH electrode | 2.5 - 1.5 mM | Meier & Tran-Minh 1992 |
| P'nase-amidase cross-linked with BSA and GLA | Potentiometric FET, FIA electrode | 0.5 - 10 mM | Reinhardt et al. 1990 |

*N/A not available

*Continued over the page...*

| Method of Immobilisation | Type of Electrode | Detection Limits | Reference |
|---|---|---|---|
| P'nase cross-linked with BSA in saturated GLA vapour on P'nase cross-linked with BSA in saturated GLA vapour on sensor chips | ENFET | N/A* | Soldatkin et al. 1997 |
| Hematein-adsorbed MWCNTs and P'nase on GCE by BSA-GLA cross-linking | Amperometric | 200 - 1000 μM | Chen et al. 2010 |
| P'nase immobilised on 3-aminopropyl-modified controlled-pore (APCPG) spread on one side to react with an aqueous GLA solution | pH electrode | 0.001 - 0.50 mM | Lapierre et al. 1999 |

## 5.2 Experimental

All other experimental conditions were the same as mentioned in section 2.2 of chapter 2 and section 3.2 of chapter 3, unless otherwise stated.

### 5.2.1 Chemicals and Reagents

GLA 25% v/v was purchased from Sigma Aldrich (USA) and from it a 2.5% v/v stock solution was prepared and refrigerated at 4°C. BSA was purchased from Sigma Aldrich (USA) and a 2.5% w/v solution was prepared. These stock solutions were further diluted with Milli-Q water to provide the required BSA and GLA concentrations. Various ratios were prepared by mechanically mixing the different concentrations of BSA-GLA and P'nase. Then 1 μL of this solution was applied to the electrode and air-dried for 10 minutes.

## 5.3 Results and Discussion

### 5.3.1 Influence of the BSA Concentration

BSA influences the activity of the enzyme and the behaviour on the film. This is because the BSA concentration is pivotal to the performance of the biosensor, as it facilitates the deposition process (Strike et al. 1995). In addition to this, the ratio of BSA to GLA is vital for the success of the coagulation process (Strike et al. 1995). The effect of the BSA concentration on the response of the electrode, was examined by fixing the GLA concentration, while varying the BSA concentration. Figure 5.2 illustrates the influence of increasing the BSA concentration on the sensitivity of the penicillin response. A slight increase in the sensitivity of the film is observed when the BSA concentration was increased from 0.125 - 0.25% w/v. This is because BSA acts as a spacer minimising close proximity problems that may occur when cross linking a single enzyme (Scheller et al. 1989; Bickerstaff 1997). The fact that BSA aids in the deposition process (Strike et al. 1995) may also contribute to the improvement in the electrode response. The maximum sensitivity was obtained when 0.22% w/v was used. This is then followed by a sharp decrease in sensitivity when BSA concentrations higher

than 0.22% w/v was used for the formation of the film. This is because an insufficient amount of BSA facilitates excess cross linking of the enzyme which also reduces the activity of the enzyme (Strike et al. 1995).

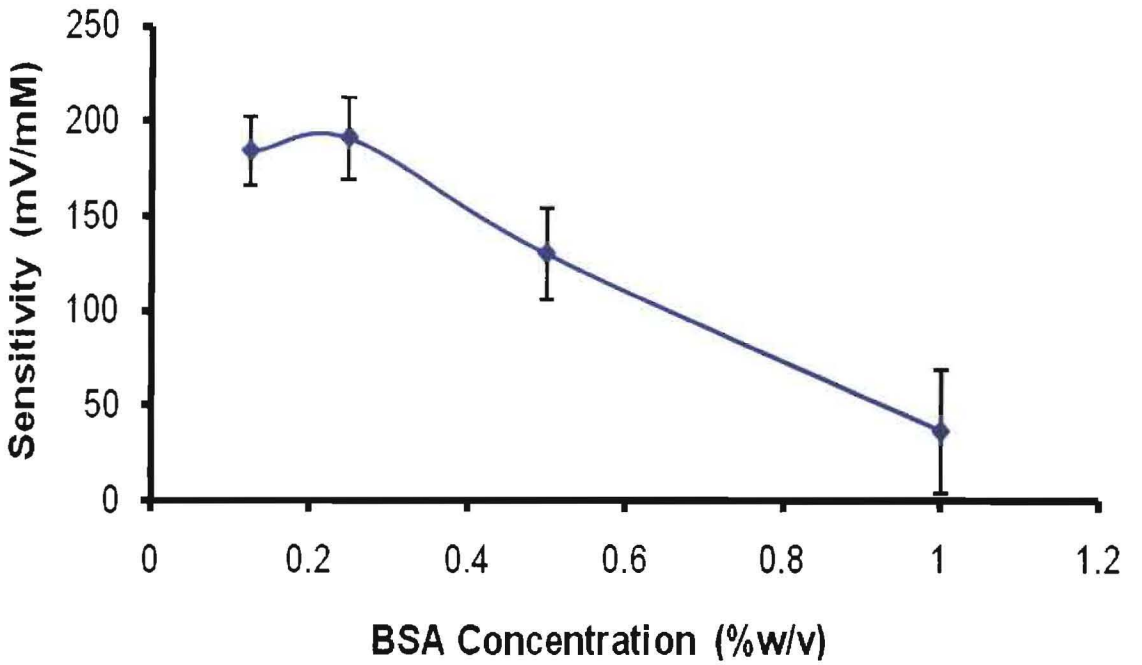



**Figure 5.2 Influence of the variation of the BSA concentration on the penicillin response. The following conditions were kept constant: 0.025% v/v GLA and 1 U/mL P'nase, drying time 10 minutes. Potentiometric biosensing was conducted in 0.1 mM buffer.**

On the other hand, the sensitivity of the penicillin response decreased in the presence of 0.5% w/v BSA. The significant amounts of negatively charged amino acid residues contained in the BSA membrane may have hindered the migration of hydrogen ions and, consequently, resulted in the decrease of the sensitivity of the electrode (Wu et al. 1997). In addition, the much lower responses obtained when higher BSA concentrations were used may have been caused by excessive spacing from the BSA which affects the

response mechanism. This is rather evident from the dramatic decrease in the sensitivity of the electrode in the presence of 0.5% w/v BSA. It appears that the increased spacing associated with higher BSA concentrations (Bickerstaff 1997) such as 1% w/v BSA, affected the ability of the catalytic products to be detected at the electrode. The highest sensitivity was achieved when 0.25% w/v BSA was used and was employed in further studies.

### 5.3.2 Effect of Glutaraldehyde Concentration

The concentration of GLA affects the cross-linking process (Chang et al. 1990; Polasek et al. 1991; Liu et al. 1998) and the response of the electrode. In terms of the mechanism, GLA couples with the amino groups of lysine in the enzyme (Gnanasekaran & Monttola 1985) and the polymerisation of GLA is catalysed by Knoevenagel type condensations (Walt & Agayn 1994). This is where the active hydrogen compound condenses with the aldehyde and is then catalysed by a weak base (Solomons 1988). Cross-linking with GLA is advantageous, as it allows strong chemical binding of the biomolecules (Scouten et al. 1995). As well as this, the activity of the immobilised enzyme is somewhat better than when it is free, hence the enhanced stability of the enzyme is further improved by GLA coupling (Scouten et al. 1995). This is consistent with the results presented in Figure 5.3 which shows that the highest electrode sensitivity was obtained when 0.012% v/v GLA was used. This GLA concentration appeared to enhance the activity of the enzyme and, hence, the response of the electrode. This behaviour is facilitated by the fact that the GLA is equipped with a long lead which attaches the enzyme to the matrix and hence allows greater flexibility.

This is necessary for protein conformational changes that are required for enhanced enzymatic activity. In addition to this, GLA also has a multi-point attachment that

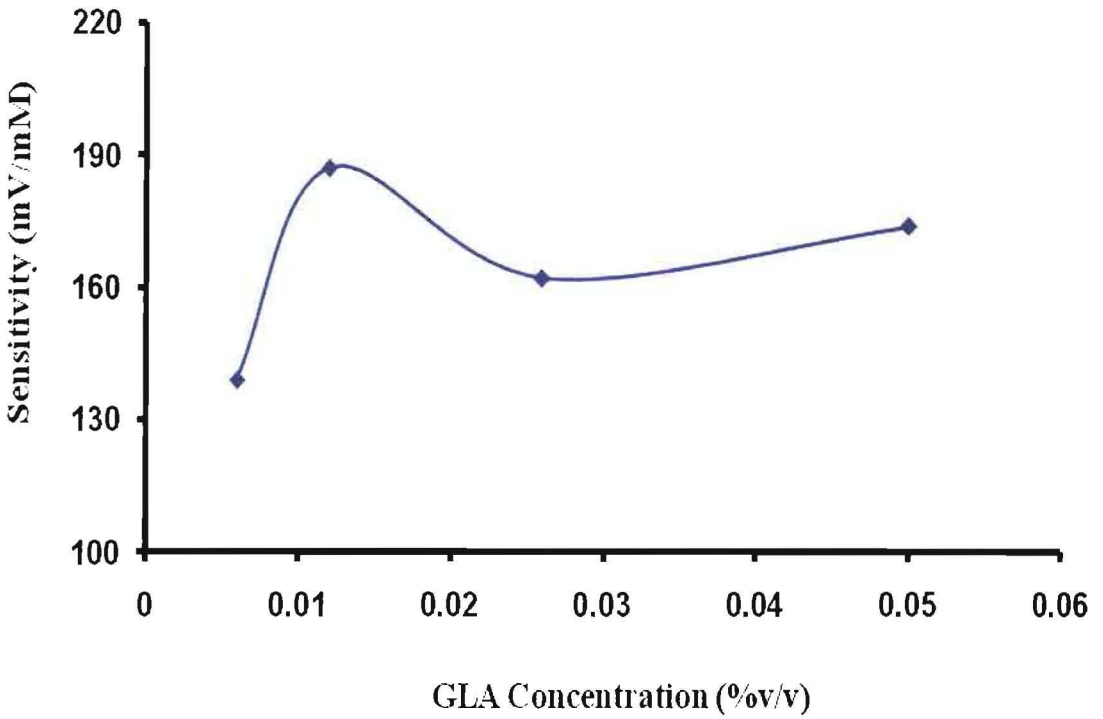



**Figure 5.3 Effect of the GLA concentration used to cross-link penicillinase on the penicillin response. The following conditions were kept constant: 0.25% w/v BSA, 1 U/mL P'nase, drying time 10 minutes. Potentiometric biosensing was conducted in 0.1 mM buffer.**

prevents unfolding of the enzyme (Scouten et al. 1995). The thickness of the film is another factor that may play a role in the response of the film. The optimum GLA concentration of 0.012% v/v, shown in Figure 5.3, may have also facilitated the movement of the substrate which enabled the detection of higher concentrations and hence explains the superior electrode response. GLA concentrations higher than 0.012%

v/v showed no further improvement in the response of the electrode. This is due to the ability of higher GLA concentrations to form thicker films which also creates diffusion limitations for the substrate (Fortier 1997). The electrode is then less able to detect higher analyte concentrations, which ultimately translates to a lower electrode sensitivity (Quiocho 1976; Walt 1994; Fortier 1997). GLA concentrations such as 0.026 and 0.05% v/v resulted in lower electrode sensitivities. It is possible that higher GLA concentrations may have denatured the enzyme (Strike et al. 1999). Other reasons for this behaviour include excess GLA concentrations in the monomeric form which may have polymerised the enzyme to which it cross-links and hence reduced the enzyme activity (Scouten et al. 1995) as well as the electrode sensitivity. The slight variation in the electrode sensitivity between 0.05 and 0.026% v/v is negligible. Both GLA concentrations seemed to be too high and may have had a negative effect on the activity of the enzyme (Khan & Wernet 1997) and on the sensitivity of the electrode.

The lowest sensitivity was obtained with lower GLA concentrations, such as 0.006% v/v, due to the incomplete cross-linking process with BSA. GLA facilitates the deposition of BSA (Strike et al. 1995) and this is demonstrated when the optimal GLA concentration 0.012% v/v produced the most sensitive electrode. Hence, 0.012% v/v GLA was employed in all subsequent experiments.

### 5.3.3 Optimisation of Penicillinase Concentration

The selectivity and sensitivity of the penicillin biosensor is very much dependent on the concentration of P'nase. The rate of the reaction is affected by the enzyme concentration which also impacts upon the response of the electrode (Bourdillon et al. 1985). Figure

5.4 shows that the sensitivity of the penicillin response increased with increasing P'nase concentration up to 8 U/mL.

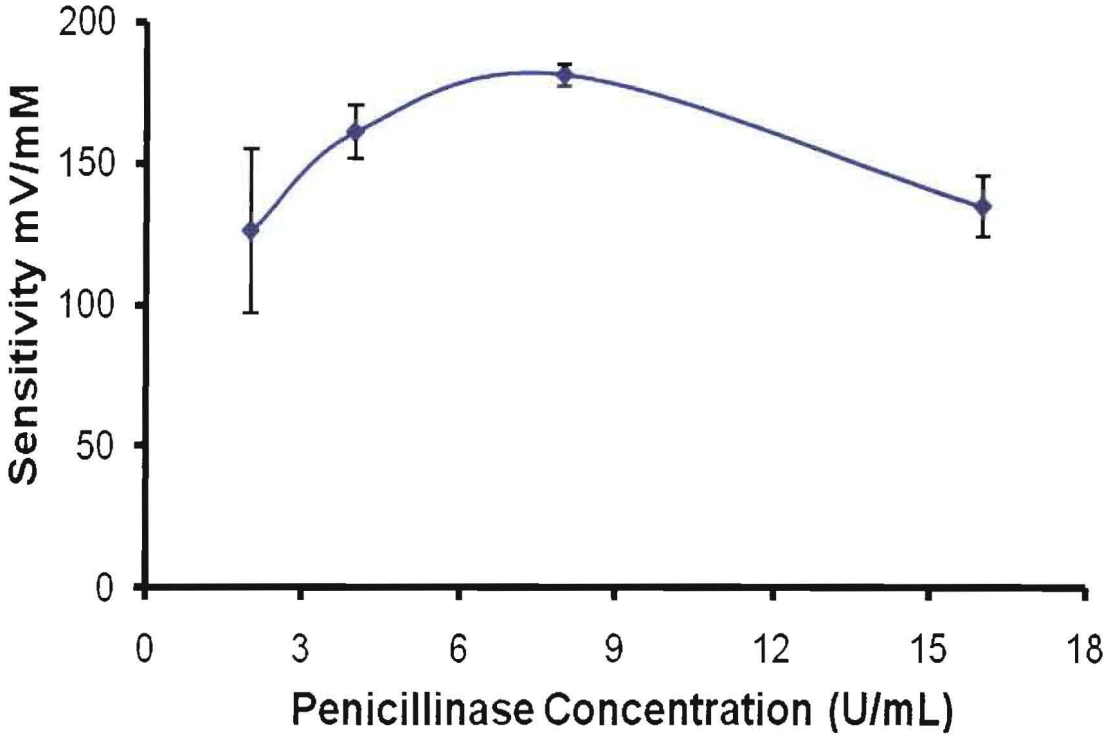



**Figure 5.4 Effect of the penicillinase concentration on the sensitivity of the penicillin response. The following conditions were kept constant: 0.012% v/v GLA, 0.25% w/v BSA, drying time 10 minutes. Potentiometric biosensing was conducted in 0.1 mM buffer.**

Optimum enzyme concentrations enable greater flexibility for conformational changes that are required for enhanced enzymatic activity (Scouten et al. 1995) which appears to be the case when 8 U/mL of P'nase was used due to the enhanced electrode sensitivity. In addition, the multi-point attachment that occurs with GLA prevents unfolding of the

protein and hence promotes the sensitivity of the electrode. This is facilitated by the long lead which attaches the protein to the matrix (Scouten et al. 1995).

At lower P'nase concentrations < 8 U/mL, a lower electrode sensitivity was obtained due to the lower enzyme concentrations in the BSA-GLA layer (Bourdillon et al. 1985). On the other hand, when higher P'nase concentrations (> 8 U/mL) were added to the monomer solution the sensitivity of the electrode declined due to the saturation of the BSA-GLA layer. Thus, 8 U/mL of P'nase was used to prepare all BSA-GLA-P'nase films for the potentiometric measurement of penicillin.

### 5.3.4 Influence of Penicillin Concentration

As mentioned in Chapter 4, the presence of Pen in the monomer solution is important as it aids in the attainment of a steady state response, as well as enhancing the sensitivity of the response. This is illustrated in Figure 5.5 where the absence of Pen in the monomer solution gave a lower electrode sensitivity than the film which had 0.9 mM Pen added to the monomer solution. The decline in the response of the electrode occurs when the pH of the membrane decreased and the charge on the membrane becomes negative as a result of the retention of penicilloate in the membrane. As a result, the loss and production rates of hydrogen ions are not balanced and a steady state response is not achieved (Kulp et al. 1987). This is also accompanied by a reduction in the electrode response, as well as a decrease in the sensitivity of the electrode. When 0.9 mM Pen was added to the monomer solution, the sensitivity of the electrode increased due to an increase in the response of the electrode, as well as the attainment of a steady state response.

A positive charge on the polymeric membrane attracts the substrate and promotes an increase in the potentiometric signal. The charge on the membrane appears to have an electrostatic influence on the charged substrate (Gorchkov et al. 1996) and explains the

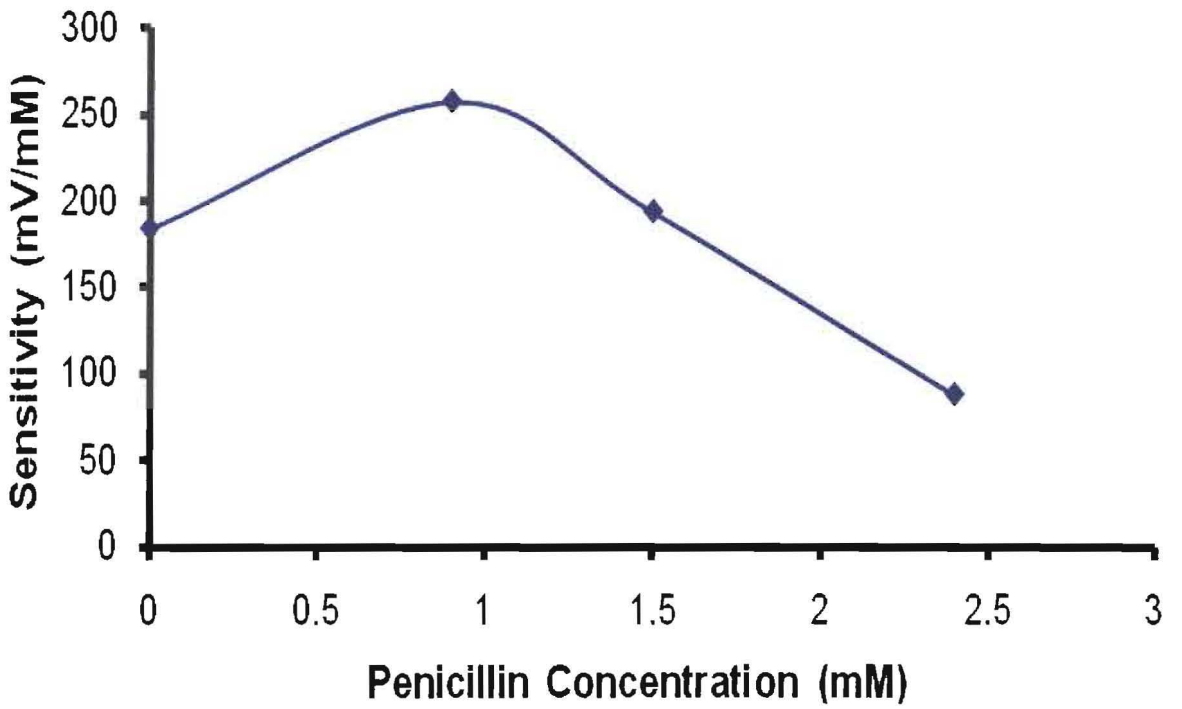



**Figure 5.5 Effect of the incorporation of penicillin in the BSA-GLA monomer solution on the penicillin response. The following conditions were kept constant: 8 U/mL P'nase, 0.25% w/v BSA, 0.012% v/v GLA, drying time 10 minutes. Potentiometric biosensing was conducted in 0.1 mM buffer.**

increase in the sensitivity of the electrode. The use of Pen concentrations greater than 0.9 mM decreased the sensitivity of the electrode. The inflexion point between 1 and 1.5 mM is due to the sharp decrease in the electrode sensitivity when Pen concentrations higher than 0.9 mM were incorporated in the film. In particular, the lowest electrode sensitivity was produced when 2.4 mM Pen was added to the monomer solution. This may be due to the saturation of the film with Pen which led to the decline in the potentiometric response and hence the electrode sensitivity. Hence, 0.9 mM Pen was used for the formation of the BSA-GLA-P'nase film for further studies.

## 5.3.5 Effect of Drying Time

The use of the ratio of BSA-GLA for the fabrication of the penicillin biosensor relies on the ability to form a gel-like layer on the platinum electrode. This also depends on how quickly the mixture dries on the surface of the electrode. The optimum drying time was achieved when the gelation process was complete and the biosensor provided the most sensitive response. This is confirmed in Figure 5.6 where the sensitivity of the penicillin response increased with increasing drying time up to 15 minutes. Beyond this drying time, the response of the penicillin electrode decreased considerably. This may have been due to the change in the porosity of the film with increased drying time (Walt 1994). The resistance resulting from this increase in the film porosity, means that less substrate is able to react at the surface of the electrode. This creates diffusional resistance which ultimately affects the sensitivity of the electrode (Walt 1994). Adequate diffusion properties are required for the liberal movement of the reaction products as well as the substrate in order to ensure the enhancement of the electrode sensitivity.

An incomplete gelation process was observed when a drying time of less than 7.5 minutes was used and no response was achieved. For this reason, the sensitivity is not shown in Figure 5.6. Seven and a half minutes did not appear to be sufficient for the polymerisation of GLA and the necessary incorporation of the enzyme which meant that

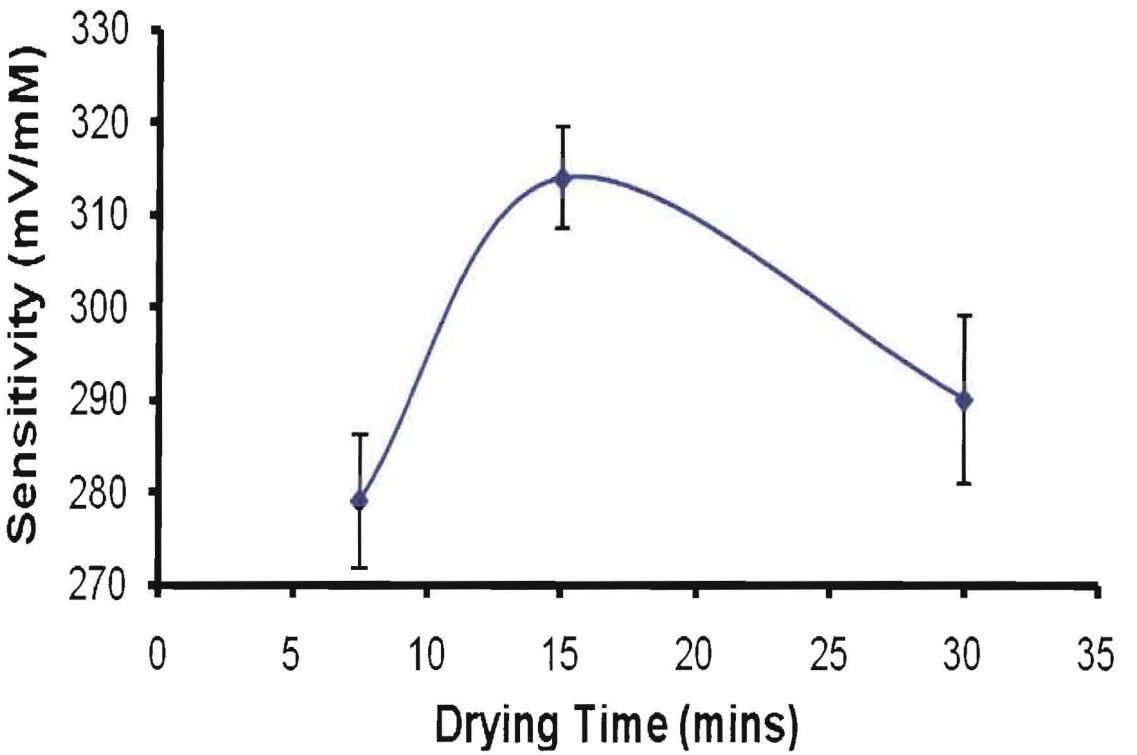



**Figure 5.6 Effect of the drying time on the sensitivity of the penicillin response of the BSA-GLA electrode. The following conditions were kept constant: 0.25% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase, 0.9 mM Pen. Potentiometric biosensing was carried out in 0.1 mM buffer.**

the cross-linking process was incomplete as the film appeared to be liquid like instead of a solidified gel and had not dried. The highest electrode sensitivity was achieved

when films were dried for 15 minutes. A solidified gel appeared on the surface of the electrode. This drying time facilitated the incorporation of a sufficient amount of enzyme in order to maximise the sensitivity of the electrode. All subsequent films were dried for fifteen minutes.

### 5.3.6 Influence of the Buffer Concentration and Linear Concentration Range

The working range of the biosensor is affected by the buffer concentration. In addition to this, the sensitivity of the biosensor is also affected by the concentration of the buffer. This is shown clearly in Figure 5.7 where the penicillin response increased with increa-

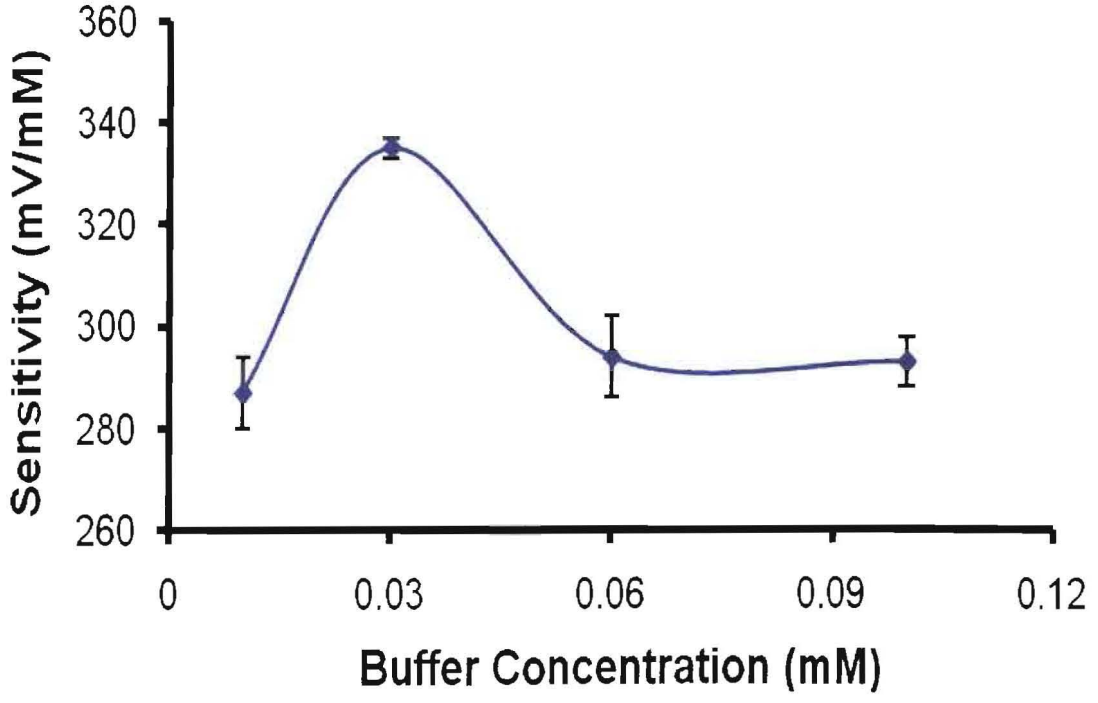



**Figure 5.7 Effect of the concentration of the buffer on the penicillin response of the BSA-GLA electrode. The following conditions were kept constant: 0.25% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase, 0.9 mM Pen, drying time 15 minutes.**

-sing buffer concentrations up to 0.03 mM. This is explained by the fact that higher buffer concentrations such as 0.03 mM enhance proton diffusion (Chao & Lee 2000). Increasing the buffering capacity beyond 0.03 mM decreased the response of the electrode (Chao & Lee 2000). Specifically, buffer concentrations such as 0.06 and 0.1 mM reduced the height of the potentiometric response, as well as the sensitivity of the electrode. This also affects the working range of the electrode. As expected, an increase in the buffer concentration will result in an increase in the buffering effect in the enzymatic membrane which buffers the protons produced by the enzymatic reaction (Brand et al. 1990). The magnitude of the signal is governed by the protolytic equilibria inside the membrane. This includes the products of the enzymatic reaction as well as the components of the buffer, hence the importance of the concentration of the buffer (Glab et al. 1991).

A lower buffer concentration, such as 0.01 mM, produced the lowest electrode sensitivity. This is due to the fact that decreasing the buffer concentration also decreases the effective diffusion coefficient of protons. This translates into a lower electrode response. That is the potentiometric response of the electrode also decreased as a result of the lower buffer concentrations such as 0.01 mM. This is because lower buffer concentrations, such as 0.01 mM, also decrease the number of molecules buffering the protons produced through the dissociation of penicilloic acid. It was interesting to note that, the difference between the use of 0.06 and 0.1 mM buffer was insignificant. In fact the difference between the sensitivities of 0.06 and 0.1 mM buffer is 1 mV/mM as shown in Figure 5.7. Higher buffer concentrations increase the number of buffer molecules in the enzymatic membrane (Brand et al. 1990). It is possible that this is what may have compromised the working ranges of the biosensor when 0.06 and 0.1 mM buffer were used. Both of these concentrations also proved to be unsuitable due to the

associated reduction in the electrode sensitivities. It seems that the required buffering capacity for optimum measurement for the BSA-GLA-P'nase electrode is somewhat lower than that reported for other films reported in previous chapters, specifically, 0.1 mM for the PPy single layer configuration, 0.3 mM for the bilayer PPy film and 0.1 mM for the PTy film. This may be attributed to the nature of the BSA-GLA film. Hence, 0.03 mM buffer was used in further studies. The minimum detectable concentration obtained with the BSA-GLA electrode was 0.3 μM.

Figure 5.8 shows that the linear concentration range that was obtained for penicillin was between 3 and 283 μM. A plot of potential versus the logarithmic concentration of penicillin

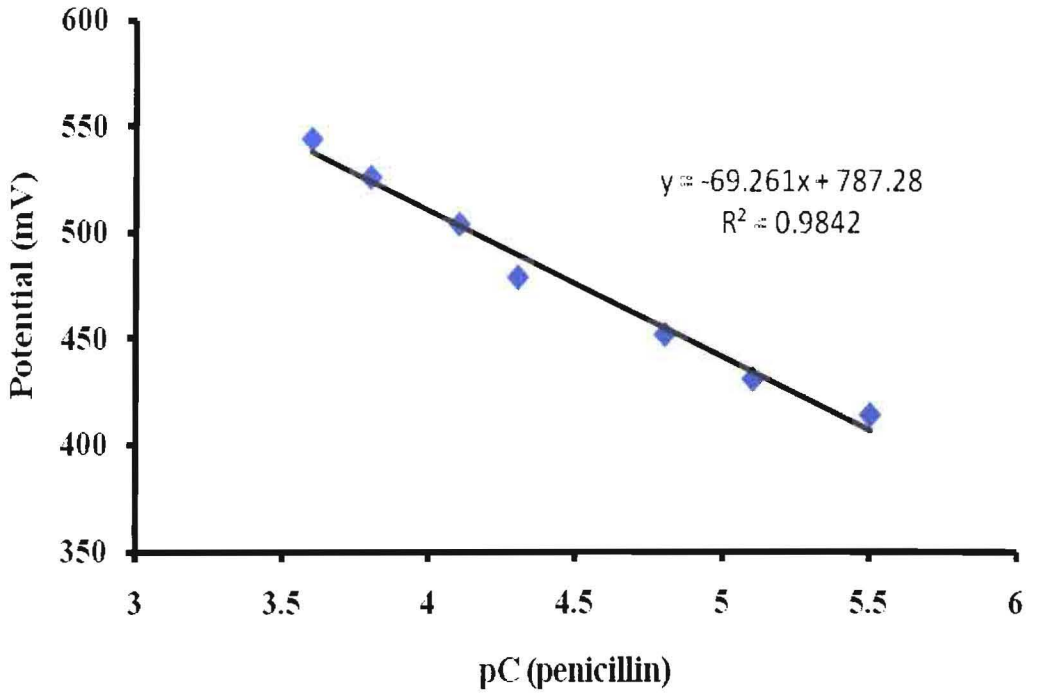



**Figure 5.8 Logarithmic concentration of the penicillin response obtained with the BSA-GLA electrode. The following film conditions were applied: 0.25% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase, 0.9 mM Pen. Potentiometric biosensing carried out in 0.03 mM buffer.**

gave a correlation coefficient of $R^2$=0.9842 and a slope of 69.3 mV/decade which is less sensitive than that obtained for the PTy electrode (72.5 mV/decade) and the PPy bilayer (87.1 mV/decade).

### 5.3.7 Determination of Penicillin in Amoxycillin Tablets

The application of the BSA-GLA biosensor to the determination of penicillin in commercially available antibiotics proved to be successful. Three amoxycillin 500 mg

**Table 5.2 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Obtained Using the BSA-GLA Biosensor**

| Amount Specified in Tablet (mg) [a] | Penicillin Concentration Obtained With Biosensor (mg) [b] | % Recovery Obtained With Biosensor | [Penicillin] Obtained With Titration (mg) | % Recovery Obtained With Titration |
|---|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 450 ± 25 | 90 ± 5 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 540 ± 21 | 108 ± 4 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 553 ± 25 | 111 ± 5 | 505 ± 20 | 101 ± 4 |
| **Average values** | **514 ± 24** | **103 ± 5** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

tablets were prepared as described in section 3.2.3 of chapter 3. Each sample was analysed 4 times with the BSA-GLA biosensor. The mean, standard deviations and

corresponding percentage recoveries are provided in Table 5.2. The percentage recoveries obtained using the BSA-GLA electrode obtained for amoxycillin ranged from 90 - 111%. The average amount of penicillin found in the amoxycillin tablet was 514 ± 24 mg and the average percentage recovery was 103 ± 5%. The results demonstrate that the accurate and sensitive determination of penicillin was achieved with the BSA-GLA electrode. The standard deviation values indicate that the reproducibility is relatively adequate.

Lower recovery values such as 450 ± 25 mg may be attributed to the inhibitory effects of the beta-lactamase inhibitor, CA, i.e. enzymatic sites on the surface of the electrode may have been blocked by CA and hence the electrode was unable to recover the 500 mg of the active constituent of the antibiotic. The limited ability of the electrode to detect CA is attributed to the fact that this β-lactam also acts as a competitive inhibitor. On the other hand, higher percentage recoveries may have been due to the ability of the electrode to detect CA which also has weak antibiotic properties. As mentioned previously, the fact that the BSA-GLA electrode has the ability to detect both amoxycillin and CA is advantageous as it means that the biosensor can detect compounds that have weak antibiotic properties.

The successful recovery of the active constituent in amoxycillin with the biosensor is characteristic of the behaviour of GLA which allows for conformational changes that are important for the activity of the enzyme. GLA allows greater flexibility for protein conformational changes which are necessary for the molecular rearrangement of the enzyme which must occur prior to its interaction with the antibiotic (Vazquez et al. 2007). Overall, the average percentage recovery of 103 ± 5% obtained with the BSA-GLA-P'nase-Pen biosensor, is indicative of a fairly desirable result in terms of the recovery of the active constituent present in amoxycillin. In addition to this, the result is

also in close agreement with the average percentage recovery (105 ± 5%) obtained with the standard titrimeric method. In fact the results obtained with the biosensor were more reliable, but the reproducibility may suggest that further sample preparation may be required.

### 5.3.8 Determination of Penicillin G in Milk Samples

Four separate milk samples were prepared as described in section 3.2.2 of chapter 3. Each sample was analysed 4 times with the BSA-GLA biosensor. The mean, standard deviations and the corresponding percentage recoveries based on the expected penicillin concentrations are provided in Table 5.3.

**Table 5.3 Recovery of Penicillin G in Milk Samples**

| Concentration of Penicillin G in Milk (ppm) [a] | Penicillin Concentration Recovered With Biosensor (ppm) [b] | % Recovery |
|---|---|---|
| 1 | 1.7 ± 0.4 | 170 ± 40 |
| 5 | 6.3 ± 1.9 | 126 ± 38 |
| 10 | 11.1 ± 2.6 | 111 ± 26 |
| 20 | 25.6 ± 1.8 | 128 ± 9 |

[a] Spiked concentration of penicillin G in milk sample
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

The results indicate that the sensitive determination of penicillin has been achieved using the BSA-GLA electrode. The reproducibility of the biosensor was also

satisfactory for penicillin concentrations of 5-20 ppm, as indicated by the standard deviations. The percentage recoveries obtained when penicillin concentrations were greater than 5 ppm were adequate in comparison to those obtained when the milk sample was spiked with 1 ppm penicillin. The elevation in the percentage recoveries is attributable to the non-specific binding that occurs with the hydrophobic sites of whey proteins (Cacciatore et al. 2004).

The results also indicate that the reproducibility of the electrode improves with increasing penicillin concentrations. In fact the standard deviation reported for the response obtained for 20 ppm penicillin was lower than for lower penicillin concentrations. It appears that the determination of penicillin in milk was somewhat more difficult at lower penicillin concentrations such as 1 ppm. This may be because the electrode cannot distinguish between the milk protein and the bound penicillin, resulting in an increased percentage recovery. This suggests that further sample preparation is required in order to avoid inflated percentage recoveries due to the non-specific binding of milk proteins to penicillin.

## 5.4 Conclusion

A BSA-GLA-P'nase biosensor was successfully developed for the determination of penicillin. The biosensor was also used for the determination of penicillin in milk and in amoxycillin 500 mg. A number of parameters were first optimised in order to achieve this. These included the concentrations of the enzyme, BSA and GLA amongst other parameters. The following optimum film conditions were established 0.25% w/v BSA, 0.012% v/v GLA, 0.9 mM Pen and 8 U/mL of P'nase.

The determination of penicillin in amoxycillin was also demonstrated where the average percentage recovery was 103 ± 5% and the mean concentration was 514 ± 24 mg which is in close agreement with the results obtained with the standard titrimetric method (105 ± 5 %). However the biosensor method was more reliable than the titrimetric method. The results obtained with the BSA-GLA electrode are also more adequate than the percentage recovery obtained with the PPy bilayer configuration (113 ± 24%). This may be due to the fact that GLA coupling enhances the behaviour of the electrode because of the long lead which attaches the protein to the matrix and allows greater flexibility for the conformational changes that are required for the enhanced activity of the enzyme.

The linear concentration range for the BSA-GLA-P'nase electrode was between 3 and 283 μM which was identical to that obtained with the PTy electrode and, thus, highlights the efficacy of non-conducting matrices.

# Chapter 6

## The Fabrication of a Penicillin Biosensor with Poly(Vinyl Alcohol) and Bovine Serum Albumin

## 6.1 Introduction

Poly (vinyl alcohol) (PVA) is a water-soluble polyhydroxy polymer that is a mechanically strong hydrogel containing a significant amount of water (Wu et al. 1997). It has been used for biomedical applications (Smith et al. 1988; Finch 1992; Gung et al. 1997; Wu et al. 1997), as well as for biosensing applications, as a solid support for optical sensors and for ion sensing (Dai & Barbari 1999). PVA is a biocompatible polymer (Minoura et al. 1993; Doretti et al. 1997) due to its biodegradability and permeability and is produced from poly(vinyl acetate) by controlled hydrolysis (Philp et al. 2003). It is easily prepared with excellent chemical resistance (Martien 1986; Kim et al. 2004). Chemically cross-linked PVA films are permeable and have received much attention in biomedical and biochemical applications (Yeom & Lee 1996; Muhlebach et al. 1997; Matsuyama et al. 1997; Kim et al. 2004). The entrapment of enzymes in PVA is an attractive immobilisation method as it preserves the quaternary structure of the protein and, most importantly, the enzyme activity (Doretti et al. 1997). This method is also regarded as a mild coupling method which provides a rationale for the utilisation of PVA in this study.

Several approaches have been used for the incorporation of various enzymes in a PVA network for the fabrication of biosensors. Formaldehyde and boric acid have previously been used to cross-link PVA (Zhang et al. 1987; Wu et al. 1992; Chen et al. 1994; Wu et al. 1997). Imai et al. (1986) immobilised enzymes in a PVA membrane that was cross-linked using UV light. Hajizadeh et al. (1991) immobilised lactate oxidase into a PVA network by cross-linking the reactive nucleophilic groups with aromatic tri-isocyanates. Chen et al. (1994) immobilised microorganisms with a phosphorylated PVA gel. Other researchers such as Hajizadeh et al. (1991) and

Galiatsatos et al. (1990) have incorporated glucose oxidase in a PVA matrix on platinised graphite electrodes by γ irradiation which produced an enzyme layer sandwiched between two polymer layers (Doretti et al. 1997). Another approach involved the immobilisation of poly(ethylene glycol) (PEG)-modified glucose oxidase in a PVA cryogel membrane which was produced using a cyclic process of freezing and thawing (Doretti et al. 1998). Although these studies demonstrate that PVA exhibits excellent properties as a biosensor matrix, its use has been quite limited (Davies et al. 1992) particularly in the area of penicillin biosensors. Wu et al. (1997) fabricated a penicillin biosensor by cross-linking a PVA membrane initiated by UV irradiation in the presence of benzoate/benzoic acid. The immobilisation of P'nase was carried out by activating the film with cyanuric chloride. However, this study concentrated on the pH change of the electrode instead of the change in the potential, which will be the focus of this study. To our knowledge, no other work has been conducted on the use of BSA-PVA for the fabrication of penicillin biosensors. Hence, this chapter will investigate the enhancement of the enzyme loading by cross-linking P'nase with PVA and BSA for the fabrication of a penicillin biosensor. More specifically, it will investigate the effect of the BSA concentration, PVA concentration, the P'nase concentration, the Pen concentration and the buffer concentration, on the performance of the biosensor. In addition, the biosensor will be applied to the determination of penicillin in milk and in amoxicillin tablets.

## 6.2 Experimental

All experimental conditions were the same as those prescribed in section 2.2 of chapter 2, unless otherwise indicated. A 2.5% v/v solution of PEG (molecular mass 6000) was prepared. A 10 % w/v PVA stock solution was prepared (average mw=22

000 gmol$^{-1}$, degree of hydrolysis 98%) in Milli-Q water at 100° C. The solution was then cooled to room temperature. The stock solution was further diluted to the required concentrations.

To determine the most sensitive immobilisation matrix, the following films were prepared: GLA: PVA, BSA: PVA, paraformaldehyde (PFA): PVA and PEG: PVA by mechanically mixing aliquots of GLA, PVA, BSA, PVA, PFA, PVA, PEG, PVA with P'nase and Pen. 1 μL of this solution was applied to the electrode and air-dried for 15 minutes. Following this, a number of BSA:PVA ratios were also prepared by mechanically mixing aliquots of BSA, PVA, P'nase and Pen. 1 μL of this solution was applied to the electrode and air-dried for 15 minutes.

## 6.3 Results and Discussion

### 6.3.1 Influence of the Different Immobilisation Matrices

GLA has been used extensively for cross-linking enzymes and other bioactive substances. However, under some conditions, GLA may deactivate the enzyme and reduce its activity as well as affecting the completion of the cross-linking process of BSA (Strike et al. 1999). This occurs when higher GLA concentrations are used (Strike et al. 1999) or when excess GLA concentrations in the monomeric form are used which can polymerise the enzyme to which it cross-links and thus reduce the activity of the enzyme (Scouten et al. 1995). For this reason, BSA is often added to minimise this effect. This study examined alternative cross-linking agents and provides a comparison of the sensitivities of films composed of GLA, PVA, PEG and PFA for the immobilisation of P'nase. PEG is a good polymer for enzyme immobilisation as the surface modification of an enzyme increases its structural and

catalytic stabilities (Bickerstaff 1997). PFA, like GLA is a condensation polymer (Joesten & Wood 1996) and will be used as a comparison with GLA. Figure 6.1 shows the results obtained for the sensitivities of the four cross-linking agents. The graph illustrates that the films formed with PVA produced the most sensitive response. This was followed by the responses of PEG, GLA and PFA, respectively. The highest sensitivities were obtained with the BSA-PVA-P'nase and BSA-PEG-P'nase electrodes. Generally, PVA and PEG are both hydrophilic polymers and were more sensitive than PFA and GLA. This is because the catalytic and structural stability of an enzyme increases with the modification of the enzyme surface when

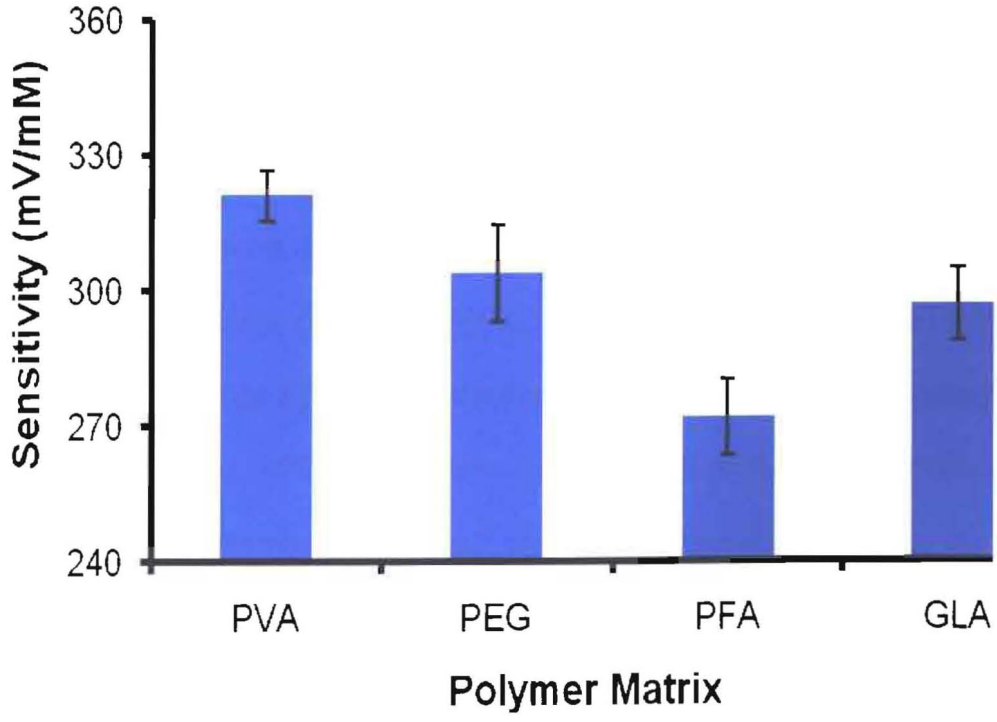



**Figure 6.1 The influence of the use of PVA, PEG, PFA and GLA on the sensitivity of the penicillin response. The following conditions were kept constant: 0.006% w/v BSA, 8 U/mL P'nase, 0.01 M Pen, drying time 15 minutes. Potentiometric biosensing was carried out in 0.03 mM buffer.**

immobilised in PEG (Bickerstaff 1997). This highlights the ability of PEG to act as a good polymer for enzyme immobilisation (Bickerstaff 1997) and may explain the increased sensitivity of the PEG film. However, the highest sensitivity was obtained with the use of the BSA-PVA-P'nase electrode. This is due to the fact that PVA is a hydrophilic polymer and has good film forming properties (Yu et al. 2002). PVA gel formation involves hydrogen bonding (Peppas & Stauffer 1991) highlighting the neutrality of PVA (Wu et al. 1997). Subsequently, the use of PVA was considered further in this study due to its film forming properties, biocompatibility (Minoura et al. 1997) and its ability to cross-link enzymes and other bioactive substances.

## 6.3.2 Optimisation of the BSA Concentration

The BSA concentration affects the sensitivity of the penicillin response. BSA relieves steric interaction (Oyama et al. 1988; Abe et al. 1991; Liu et al. 1999) and it helps to form a protein membrane (Liu et al. 1999). The adequate formation of the BSA-PVA film also determines the efficiency of the mass transfer of hydrogen ions through this membrane (Wu et al. 1997). This plays a pivotal role in the enhancement of the sensitivity of the electrode. Figure 6.2 shows the effect of increasing the BSA concentration on the sensitivity of the penicillin response. The response of the electrode increased up to a BSA concentration of 0.006% w/v. Beyond this concentration, the penicillin response decreased rapidly. This may have been due to the change in the porosity of the film which affects the sensitivity of the film (bourdillon et al. 1985). In addition to this, the BSA membrane may contain a significant amount of negatively charged amino acid residues which may have hindered the migration of protons and hence decreased the response of the electrode

(Wu et al. 1997). The highest electrode sensitivity was obtained when 0.005% w/v BSA was used with no further increase in the sensitivity. The lowest sensitivity was observed when 0.025 % w/v BSA was used for film formation. At this BSA concentration, the protein content is extremely high which increases the spacing of the enzyme molecules (Bickerstaff 1997) and may have reduced the activity of the film. High BSA concentrations also have a negative effect on the mechanical properties of the film. It is interesting to note that the BSA concentration required to achieve

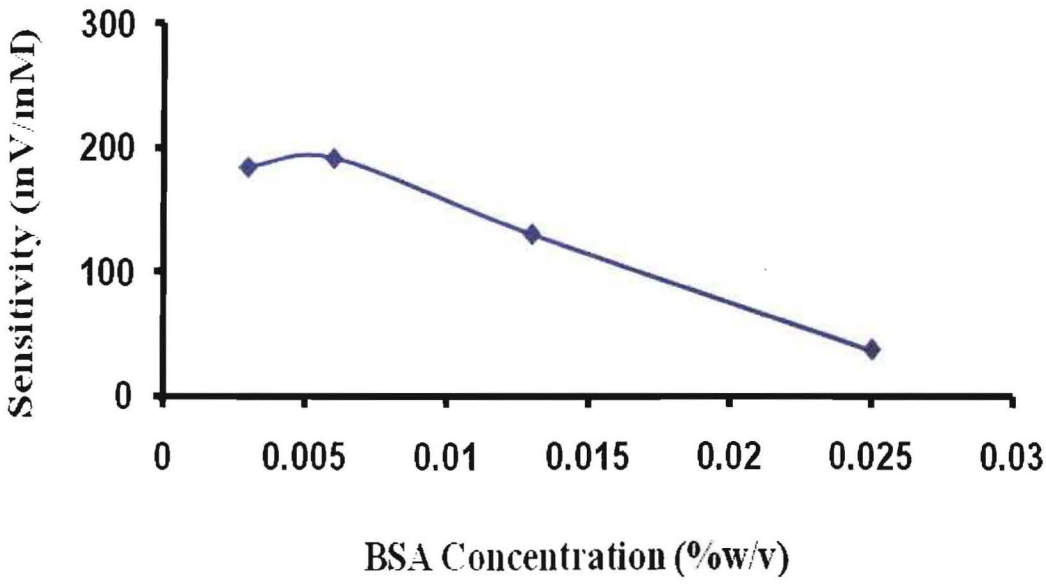



**Figure 6.2 Influence of the BSA concentration on the sensitivity of the penicillin response. The following conditions were kept constant: 2.5 % w/v PVA, 8 U/mL P'nase, 0.01 M Pen, drying time 15 minutes. Potentiometric biosensing was carried out in 0.03 mM buffer.**

maximum sensitivity was lower with the use of PVA than that required with the use of GLA (0.25% w/v BSA), as indicated in chapter 5. In the case of PVA, the highest electrode sensitivity was observed when 0.006 % w/v BSA was incorporated into the film and was used in further studies.

### 6.3.3 Influence of the PVA Concentration

The formation of a PVA gel is attributed to the formation of PVA crystallites which act as physical cross-linking sites (Hennink & Nostrum 2002). Figure 6.3 shows that the sensitivity of the electrode increased when the PVA concentration was increased from 1.25 - 2.5 % w/v. The presence of the hydroxyl groups and the water content in PVA (Doretti et al. 1997) promotes the hydrophilicity of the membrane which in turn increases the response of the electrode. The enhancement in the diffusion of the substrate towards the electrode also increased the sensitivity of the electrode. The highest electrode sensitivity was achieved with the use of 2.5 % w/v PVA. The improved porosity of the film may explain the behaviour of the film. In addition to this, the neutrality of PVA enhances the facilitation of the mass transfer of hydrogen ions through the membrane (Wu et al. 1997) which are produced through the dissociation of penicilloic acid. The structure of the matrix and the hydrophilicity of PVA provided ideal conditions for the enzyme which, in turn, increased the activity of the film. The maximum sensitivity was achieved when 2.45% w/v PVA was used and the use of higher concentrations led to a decrease in the electrode sensitivity. On the other hand, the sensitivity of the electrode declined with higher PVA concentrations. In particular, the rapid decline of the sensitivity when 5 % w/v PVA was incorporated into the film, which may have increased the thickness of the film and hence affected the electrode signal. Further to this, the sensitivity of the electrode decreased even

more when the concentration of PVA was increased to 10 % w/v. It seems that the effects of the cross-linking process affected the diffusion of penicillin to the electrode which had a negative effect on the response of the electrode. This increase in the PVA concentration may have increased the thickness of the film which also increased the

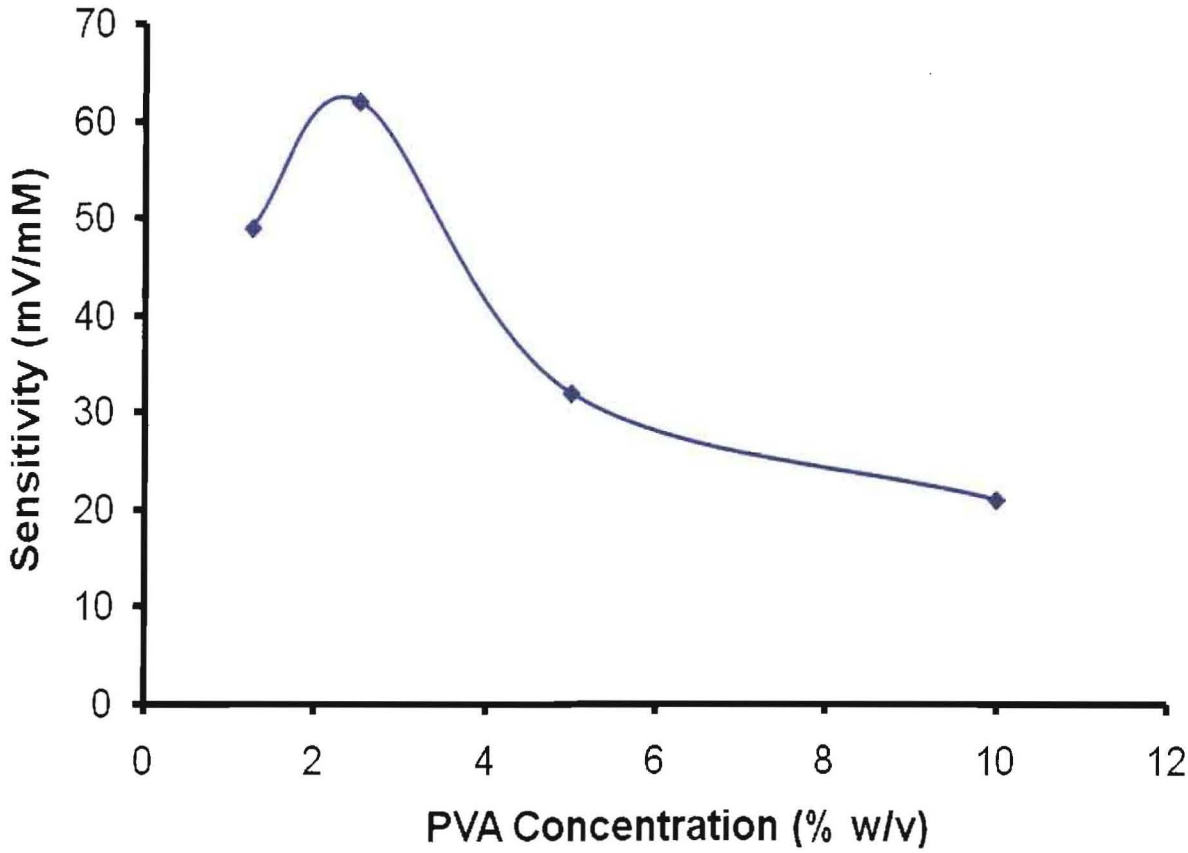



**Figure 6.3 The effect of the PVA concentration on the sensitivity of the penicillin response. The following conditions were kept constant: 0.006% w/v BSA, 8 U/mL P'nase, 0.01 M Pen, drying time 15 minutes. Potentiometric biosensing was carried out in 0.03 mM buffer.**

resistance of the mass-transport of the diffusion of the substrate. This is because the response of the electrode is influenced by the mass-transport through the membrane (Doretti et al. 1997). This was not the case when 2.5 % w/v PVA was used, as the sensitivity of the electrode was enhanced with this PVA concentration, hence providing a rationale for its use in further studies.

### 6.3.4 Influence of the Enzyme Concentration

The concentration of the enzyme is an important parameter that must be controlled carefully due to the fact that it influences the thickness of the film and the response of the electrode (Doretti et al. 1997). The feasibility of the use of PVA for the

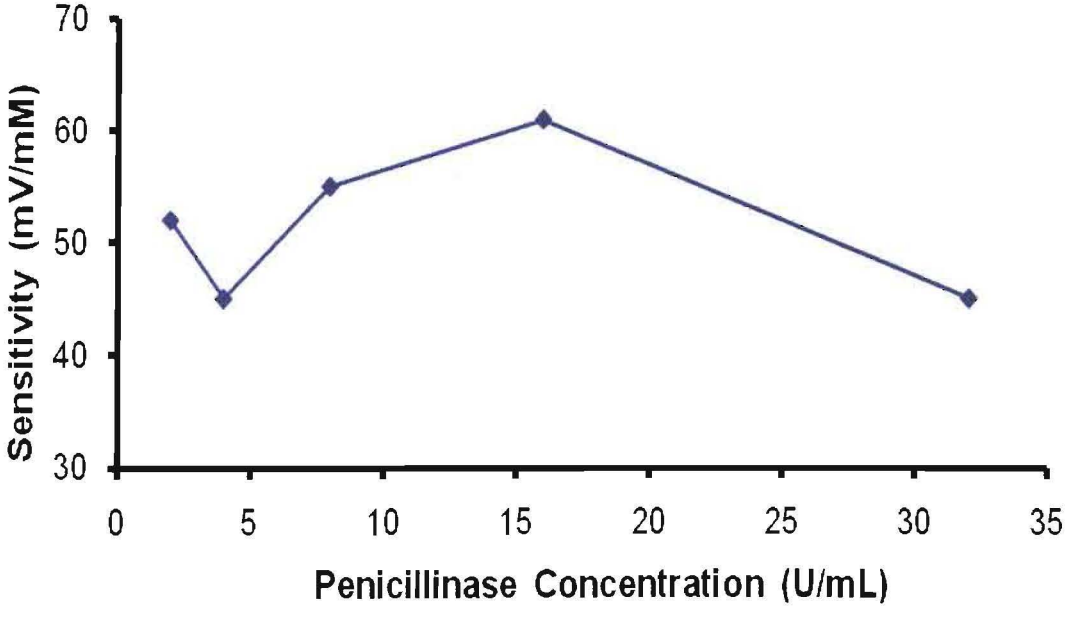



**Figure 6.4 The effect of the penicillinase concentration on the response of the electrode. The following conditions were kept constant: 0.006% w/v BSA, 2.5 % w/v PVA, 0.01 M Pen, drying time 15 minutes. Potentiometric biosensing was conducted in 0.03 mM buffer.**

entrapment of enzymes stems from its ability to preserve the activity of the enzyme as well as the quaternary structure of the protein (Doretti et al. 1997). Figure 6.4 shows that the highest electrode sensitivity is observed when 16 U/mL of P'nase was incorporated into the film. The presence of the hydroxyl groups and the high water content in PVA provides microenvironmental conditions that are ideal for the enzyme (Doretti et al. 1997). This enables the efficient diffusion of the substrate towards the electrode which improves the sensitivity of the electrode. Beyond this optimum concentration, the response of the electrode decreased. Plausible reasons for this may be that the diffusion of the substrate towards the active sites is affected by the conformational changes that occur within the enzyme as a result of the cross-linking process (Doretti et al. 1997). Specifically the minor modification in the tertiary structure of the enzyme, i.e. the distortion of amino acid residues that are involved in the catalysis process (Doretti et al. 1998), may account for this. This was not the case when 2 and 4 U/mL P'nase were incorporated into the film as the response shown is erratic and may be due to the lack of enzyme stability and the poor mechanical properties that are associated with cross-linking (Bickerstaff 1997).

The decline in the electrode response when 32 U/mL P'nase was incorporated in the film is due to the increase in the thickness of the film. The signal of the electrode is negatively impacted upon by the thickness of the film as the mass-transport is controlled through the membrane (Wu et al. 1997). Hence, a P'nase concentration of 16 U/mL was used in further studies.

### 6.3.5 Effect of the Penicillin Concentration in the Monomer Solution

The necessity of the incorporation of Pen in the monomer solution is depicted in Figure 6.5. Specifically, the incorporation of Pen in the monomer solution used for the

formation of BSA-PVA-P'nase film, resulted in an increase in the electrode response. Figure 6.5 shows that the response of the electrode increased with increasing Pen concentration up to 2.4 mM. Possible explanations for this include the fact that a

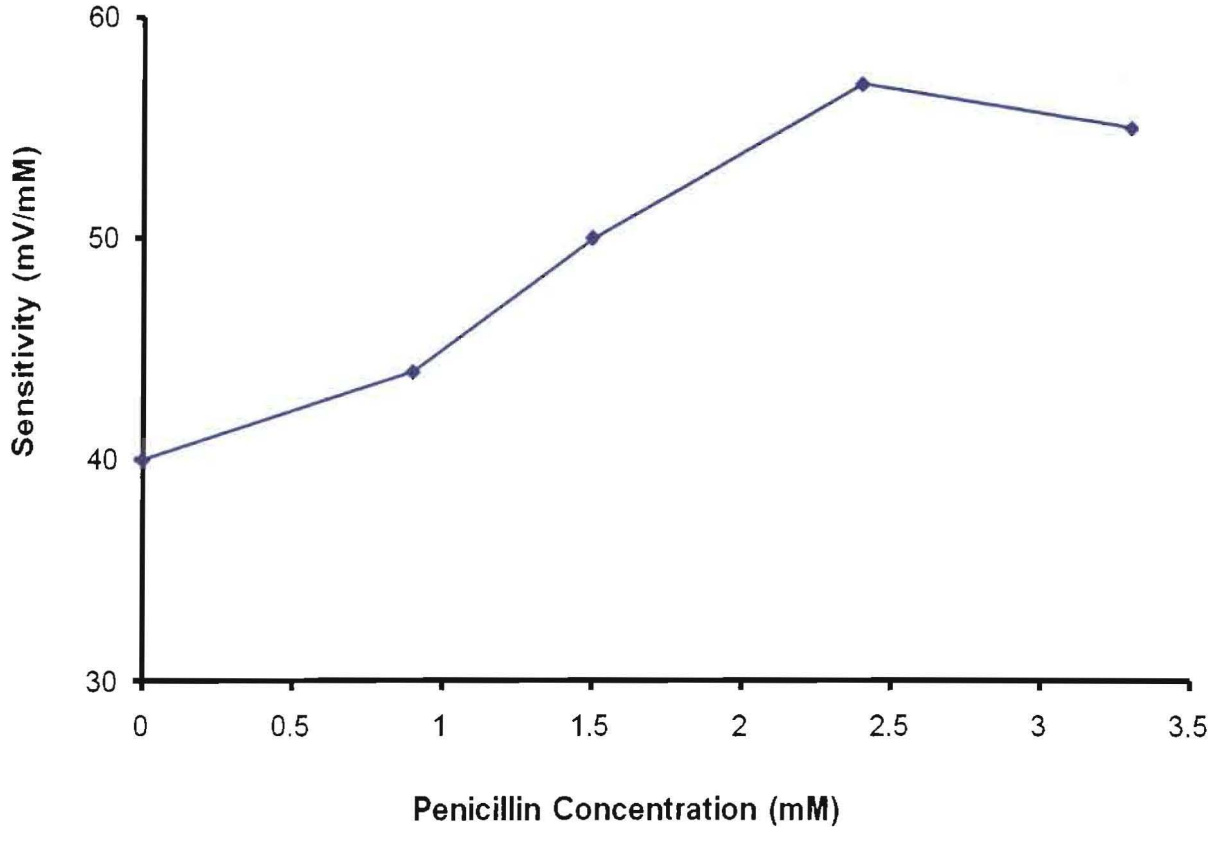



**Figure 6.5 The influence of the inclusion of penicillin in the monomer solution on the sensitivity of the penicillin response. The following conditions were kept constant: 0.006% w/v BSA, 2.5 % w/v PVA, 16 U/mL P'nase, drying time 15 minutes. Potentiometric biosensing was conducted in 0.03 mM buffer.**

positive charge on the polymeric membrane attracts the substrate which results in an increase in the response of the electrode, as well as a concomitant enhancement in the sensitivity of the response.

The decline in the response is shown when the Pen concentration exceeded 2.4 mM. This may be due to the saturation of the film with Pen which may contribute to diffusional limitations and subsequently affected the response of the film. Interestingly, the lowest sensitivity was obtained in the absence of Pen in the monomer solution. This can be ascribed to a decline in the potentiometric response due to the negative charge on the membrane which is due to the retention of penicilloate in the membrane which influenced the charge on the membrane (Gorchkov et al. 1996). This does not promote an increase in the potentiometric response and, hence, the reduced sensitivity of the biosensor. Also Nishizawa et al. (1992) claimed that the inclusion of Pen increases the conductivity of PPy films. Similarly, it seems that the presence of Pen in the BSA-PVA film enhanced its conductivity, as clearly shown in Figure 6.5, where 2.4 mM Pen exhibits the highest electrode sensitivity. Hence, 2.4 mM Pen was used for film formation for further studies.

### 6.3.6 Effect of Drying Time

The completion of the gelation process affects the performance of the biosensor. This process is dependent upon how quickly the film dries. An incomplete drying process was observed with films produced when drying times of less than 5 minutes were used. In addition, the appearance of these films was liquid like which is characteristic of the incompletion of the cross-linking process. Furthermore, drying times that were

less than 5 minutes were inadequate for the incorporation of a sufficient amount of enzyme which also explains why no response was produced with these films.

Figure 6.7 shows that the highest sensitivity was achieved when the film was dried for 10 or 15 minutes. In fact when drying times of 10 or 15 minutes were applied there was a 1 mV/mM difference between the sensitivities of both drying times signifying the adequacy of both drying times. However, a drying time of 15 minutes produced

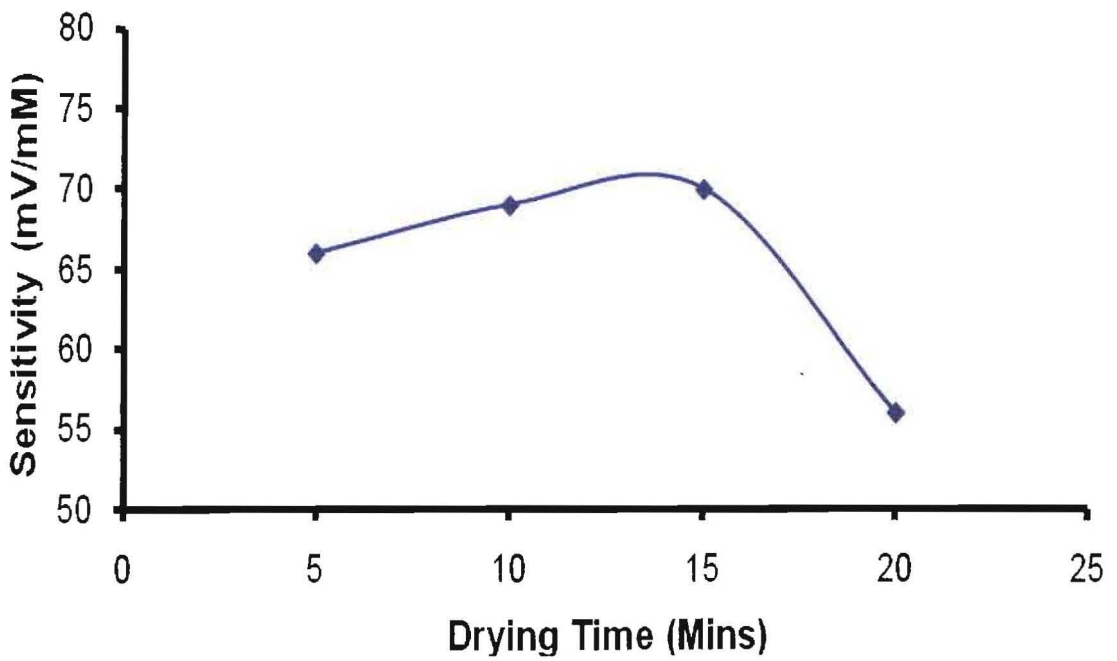



**Figure 6.6 The effect of the drying time on the sensitivity of the penicillin response. The following conditions were kept constant: 0.006% w/v BSA, 2.5 % w/v PVA, 16 U/mL P'nase, 2.4 mM Pen. Potentiometric biosensing was conducted in 0.03 mM buffer.**

the highest electrode sensitivity. Beyond this time, the sensitivity of the biosensor decreased due to the change in the porosity of the film. In particular, when the film was dried for 5 minutes, a lower electrode sensitivity was observed as this drying time did not provide satisfactory diffusion properties that indicated the completion of the cross-linking process. This is evident from the non-ideal response achieved with this electrode.

The lowest sensitivity is observed when the film was dried for 20 minutes. This is due to the fact that a less porous film may have been produced. The resulting diffusional resistance that accompanies non-ideal conditions, such as the decreased porosity, are pivotal to the lower electrode sensitivity, thus rendering this drying time as unsuitable. The highest electrode sensitivity was achieved when films were dried for 15 minutes and was used in further studies.

### 6.3.7 Influence of the Buffer Concentration

Figure 6.6 shows the influence of the buffer concentration on the penicillin response. The penicillin response increased with increasing buffer concentration up to 0.3 mM. Beyond this concentration, the response of the electrode decreased. In particular, lower buffer concentrations such as 0.01 and 0.1 mM decreased the diffusion coefficient of hydrogen ions through the membrane of the electrode (Wu et al. 1997) and hence produced a less sensitive response.

On the other hand, higher buffer concentrations such as 1 mM also exhibited lower responses due to a decrease in the diffusion coefficient of hydrogen ions. This is because higher buffer concentrations consume a high proportion of the protons produced from the dissociation of the penicilloic acid and, thus, suppressed its

response to penicillin. The lowest response was observed when 1mM buffer was used. This is because increases in the buffer concentration decrease the sensitivity of the electrode. The concentration gradient of phosphate ions contributes to the transfer of hydrogen ions (Wu et al. 1997) through the PVA membrane and affected the response

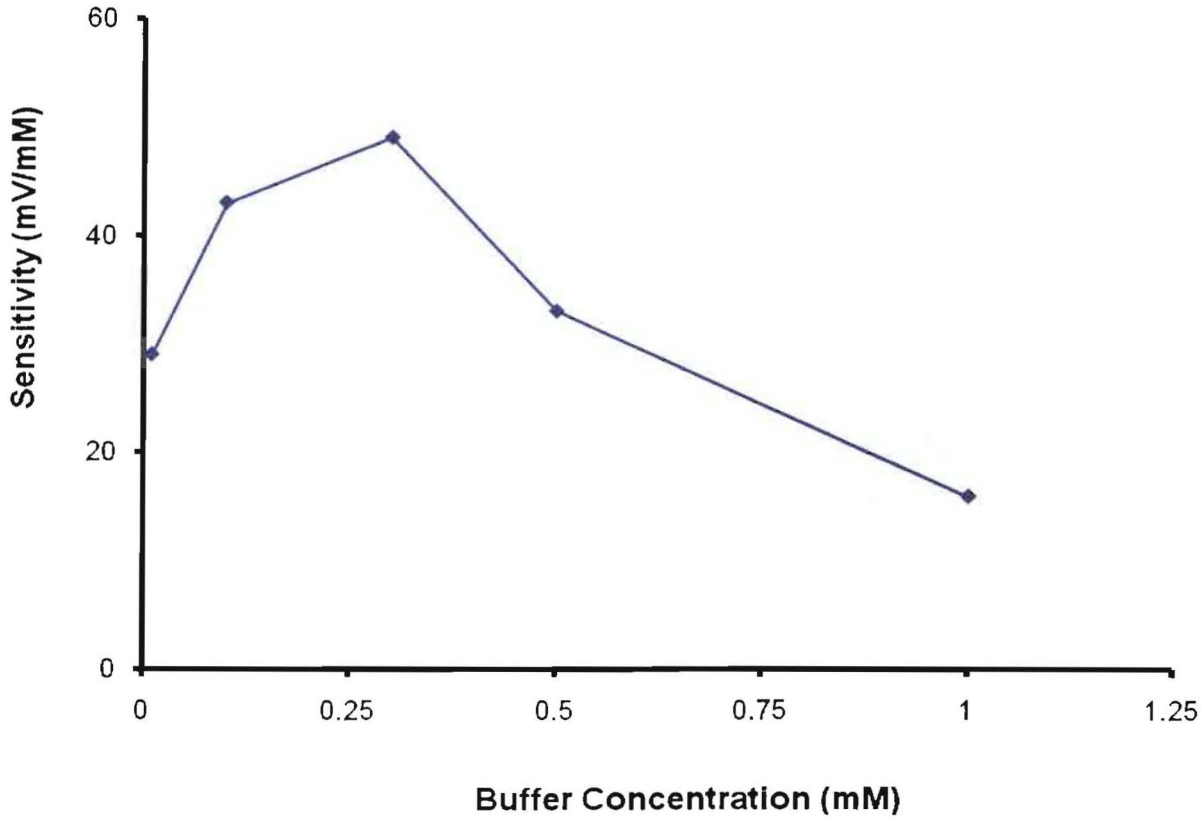



**Figure 6.7 The influence of the buffer concentration on the sensitivity of the penicillin response. The following conditions were kept constant: 0.006% w/v BSA, 2.5 % w/v PVA, 16 U/mL P'nase, 2.4 mM Pen, drying time 15 minutes.**

of the electrode. The highest sensitivity was achieved when 0.3 mM buffer was used. For this reason, this buffer concentration was used in all other experiments.

The linear concentration range is also influenced by the concentration of the buffer. A plot of potential versus the logarithmic of the penicillin concentration gave a correlation coefficient of $R^2$=0.9632 and a slope of 50.0 mV/decade which is less sensitive than that obtained with the BSA-GLA-P'nase electrode (69.3 mV/decade) and that obtained using the PTy-P'nase electrode (72.5 mV/decade). Figure 6.8 shows that the BSA- PVA-P'nase biosensor is linear between 7.5 - 283 µM. This linear range is slightly narrower than that achieved with the BSA-GLA-P'nase electrode (3 - 283 µM). This is attributed to the use of the higher buffer concentration (0.3 mM) for the BSA-GLA-P'nase electrode.

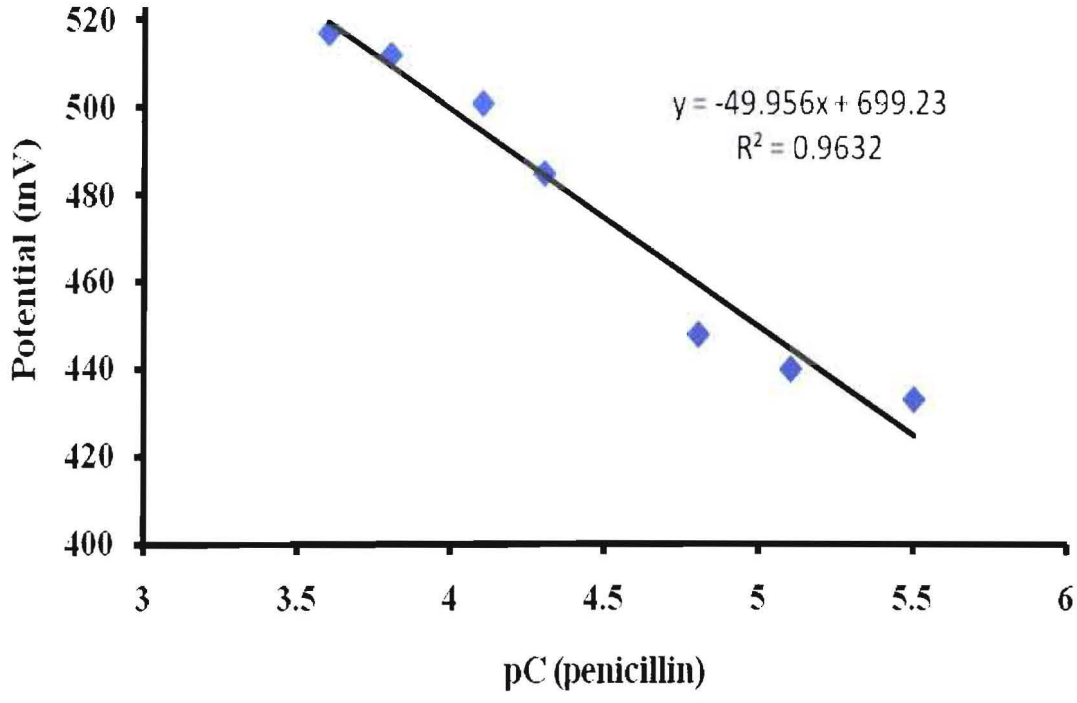



**Figure 6.8 Logarithmic concentration of the penicillin response obtained with the BSA-PVA-P'nase electrode. The following film conditions were applied: 0.006% w/v BSA, 2.5 % w/v PVA, 16 U/mL P'nase, 2.4 mM Pen, drying time 15 minutes. Potentiometric biosensing was carried out in 0.3 mM buffer.**

## 6.4 Determination of Penicillin in Amoxycillin

Table 6.1 shows the recoveries obtained with the BSA-PVA-P'nase electrode for the determination of penicillin in amoxycillin. Three amoxycillin 500 mg tablets were prepared as described in section 3.2.3 of chapter 3.

**Table 6.1 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Using the BSA-PVA Biosensor**

| Amount Specified in Tablet (mg) [a] | Measured Penicillin Concentration Obtained With Biosensor (mg) [b] | % Recovery | [Penicillin] Obtained With Titration (mg) | % Recovery Obtained With Titration |
|---|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 517 ± 35 | 103 ± 7 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 493 ± 29 | 99 ± 6 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 451 ± 109 | 90 ± 22 | 505 ± 20 | 101 ± 4 |
| **Average values** | **487 ± 58** | **97 ± 12** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

Each sample was analysed 4 times with the BSA-PVA-P'nase biosensor. The results were expressed as a mean ± standard deviation and percentages with 95% confidence

intervals. The average amount of penicillin detected with the biosensor was 487 ± 58 mg and the average percentage recovery was 97 ± 12%. The deviation values obtained for the first and the second batch of tablets are more reproducible than that obtained for the third batch of tablets (451 ± 109 mg). This suggests that the overall average recovery is affected by the third batch of tablets. This may be due to a variation in the original composition of the tablets. In addition, the variation in the standard deviation may be due to the presence of CA which is also a beta-lactamase inhibitor. The weak antibiotic properties of CA may also contribute to the higher standard deviation.

The average recovery value (487 ± 58 mg) obtained with this biosensor is actually lower than those reported in previous chapters. The titration results appear to be more reliable and reproducible than those obtained using the biosensor. This may be attributed to the porosity of the BSA-PVA film, which may have been affected by the CA which is a β-lactam, as well as acting as a competitive inhibitor (Miller 2002; Parag et al. 2008). That is the CA may have blocked enzymatic sites and contributed to lower percentage recoveries.

## 6.5 Determination of Penicillin G in Milk Samples

Table 6.2 shows the values obtained with the BSA-PVA-P'nase biosensor to determine penicillin in milk samples. The average percentage recovery for 10 ppm was 110 ± 30 % and 107 ± 15 % for 20 ppm. The results indicate that the percentage recovery obtained for the milk samples ranged from 107 - 110%. The fact that these values exceed 100% may be due to the non-specific binding that occurs with milk proteins (Cacciatore et al. 2004). In addition, penicillins bind to the hydrophobic sites of milk proteins (Grunwald & Petz 2003). Percentage recoveries for 1 ppm penicillin were not reported as the recovery values were too high due to the tendency of

penicillins to bind to the milk proteins. This may be responsible for the lower recovery values observed for lower penicillin concentrations (≤5ppm). The high deviation obtained for 10 ppm is attributed to the non-selectivity of the biosensor at these low concentrations. The results of this study, therefore, suggest that the BSA-PVA-P'nase biosensor can only be reliably used for the determination of penicillin in milk when ≤ 20 mg/L penicillin is present.

**Table 6.2 Recovery of Penicillin G in Milk Samples**

| Concentration of Penicillin G in Milk (ppm) [a] | Penicillin Concentration Recovered With Biosensor (ppm) [b] | % Recovery |
|---|---|---|
| 5 | 2.0 ± 0.9 | 40 ± 18 |
| 10 | 11.0 ± 3.0 | 110 ± 30 |
| 20 | 21.0 ± 3.0 | 107 ± 15 |

[a] Spiked concentration of penicillin G in milk sample
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

## 6.6 Conclusion

The immobilisation of P'nase in a BSA-PVA film, for the fabrication of a penicillin biosensor, has been demonstrated. The optimum conditions for this biosensor included: 2.5% w/v PVA, 16 U/mL P'nase and 0.006% w/v BSA. The presence of 2.4 mM Pen in the monomer solution was necessary to produce a more sensitive electrode

and a drying time of 15 minutes was required. The optimum buffer concentration required for the potentiometric measurement of penicillin with the BSA-PVA-P'nase biosensor was 0.3 mM. Higher buffer concentrations proved to be inadequate as they consume the protons generated from the dissociation of penicilloic acid and decrease the sensitivity of the biosensor. However, the linear concentration range is also affected by the choice of the buffer concentration. The linear concentration range achieved with the BSA-PVA-P'nase biosensor was between 7.5 - 283 μM which is narrower than the linear concentration ranges reported with other configurations, particularly the PTy-P'nase electrode.

The BSA-PVA-P'nase biosensor was applied to the determination of penicillin in amoxycillin. The average percentage recovery of penicillin detected with the biosensor was 97 ± 12% and the average amount recovered was 487 ± 58 mg. The variation in the values may be attributed to the porosity of the membrane as well as the presence of CA which has antibiotic properties as well as being a β-lactamase inhibitor. The results obtained with the titrimetric analysis were more reproducible and reliable than those obtained using the biosensor. Again this may have been due to the porosity of the BSA-PVA film allowing the inhibition of enzymatic sites with CA. Percentage recoveries obtained for milk samples ranged from 107 - 110% for 10 to 20 ppm penicillin. Lower concentrations such as 5 ppm penicillin were not fully recovered due to the tendency of penicillins to bind to the hydrophobic sites of milk proteins.

# Chapter 7

## The Development of a Penicillin Biosensor: A Comparative Study of PPy/BSA-PVA and PTy/BSA-GLA Films

## 7.1 Introduction

Polymer bilayers have been used in a number of studies to improve the sensitivity of biosensors by increasing the efficiency of the transfer of electrons between the enzyme and the electrode (Vidal et al. 1999). Bilayers maximise the analytical scope of the biosensor by increasing the enzyme loading of the biosensor (Vidal et al. 1999). In some cases, this is achieved by immobilising the enzyme in both layers, as will be done in this study. One method of constructing a bilayer arrangement involves the use of a conducting and a non-conducting polymer. The non-conducting polymer is used as a protective coating to improve the selectivity of the biosensor (Vidal et al. 1999). The use of a non-conducting component can reduce the current response of the bilayer system, but this is often balanced by the presence of a conductive polymer, such as PPy.

The properties of a biosensor can be modified by using non-conducting polymers to limit the permeability of smaller molecules (Miao et al. 2004; Miao et al. 2005). The choice of the components used in the bilayer system, is based on the separation levels of the two chosen polymers (Hillman & Mallen 1990). This can be achieved by using a conducting polymer as the inner layer in order to preserve a high level of conductivity enabling the growth of the outer layer i.e. the non-conducting polymer. This helps produce a film of low thickness (Vidal et al. 1999) as the growth of non-conducting polymers is self-limited. This approach will be considered in this study. The production of a film of low thickness enables the fabrication of a highly sensitive electrode, as the thickness of the biosensor is pivotal to the enhancement of the sensitivity of the biosensor. Although some have reported that the immobilisation of the enzyme in both layers is not advantageous

(Vidal et al. 1998), it is believed to be beneficial only where the arrangement of the polymers is controlled as mentioned before. In some cases, the thickness of the film can be controlled by cross-linking the outer layer which uses lower enzyme concentrations. This is an approach that will also be explored in this chapter.

Other methods of controlling the film thickness include the use of non-conducting polymers as one of the components of the bilayer system. Over-oxidised PPy poly(o-phenylenediamine) and composite (PPy ox-PPD) have been used rather extensively (Malitesta et al. 1990; Centonze et al. 1992a; Centonze et al. 1992b; Palmisano et al. 1993; Tatsuma et al. 1993; Centonze et al. 1994; Palmisano et al. 1995; De Benedetto et al. 1996). Vidal et al. (1999) reported the use of an inner conducting PPy layer and a non-conducting poly (o-phenylenediamine) (oPPD) outer layer. These methods demonstrated spatial control and an efficient method of entrapping enzymes. Palmisano et al. (1997) used the non-conducting polymer, PTy, in the outer layer and a conducting polymer, PPy, as the inner layer. However, many of these approaches have not used methods that minimise the enzyme concentration in the outer layer in order to minimise the thickness of the film and hence maximise the sensitivity of the biosensor. This chapter will investigate this hypothesis, specifically, the use of the conducting polymer, namely, PPy, as the inner layer and bovine serum albumin-poly (vinyl alcohol), BSA-PVA, as the outer layer. This arrangement will ensure that a high level of conductivity is maintained to enable the growth of a non-conducting polymer in the outer layer. The sensitivity of the system will be enhanced by minimising the thickness of the film as well as entrapping the enzyme in both layers. The second hypothesis will look at the use of the non-conducting polymer, PTy in the inner layer and BSA-GLA in the outer layer. The use of PTy will

ensure a thinner film and hence maximise the sensitivity of the biosensor, particularly with the entrapment of P'nase in both layers. To our knowledge, both of these methods have not been used for the fabrication of a penicillin biosensor. This chapter aims to demonstrate the application of the biosensor for the detection of penicillin in pharmaceutical preparations, as well as in milk samples. Prior to this, relevant parameters that pertain to the effective operation of the biosensor will be optimised. Such parameters include the choice of the bilayer configuration and the buffer concentration.

## 7.2 Experimental

The experimental procedures used for the two films chosen for this study were similar to those described in section 2.2 of chapter 2 and section 6.2 of chapter 6 except for the following variations:

The inner layer for the PPy/BSA-PVA film was formed using the following: 0.1 M Py, 19 U/mL P'nase and 0.1 M $KNO_3$ The applied current density was 0.9 mA/cm$^2$ for 40 s.

The outer layer was formed using the following: 0.006% w/v BSA, 2.5% w/v PVA, 16 U/mL P'nase and mechanically stirred with a pipette tip. 1 µL of this solution was applied to the electrode and air-dried for 15 minutes. Potentiometric biosensing was carried out in 0.3 mM buffer.

The inner layer for the PTy/BSA-GLA film was formed using the following: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen and 0.01 M $KNO_3$ The applied current density was 0.9

$mA/cm^2$ for 40 s. The outer layer was formed using the following: 0.006% w/v BSA, 0.012% w/v GLA, 8 U/mL P'nase and mechanically stirred with a pipette tip. 1 μL of this solution was applied to the electrode and air-dried for 15 minutes. Potentiometric biosensing was conducted in 0.03 mM buffer.

The experimental conditions used for the other two films were:
The inner layer for the PTy/BSA-PVA film was formed using the following: 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen and 0.01 M $KNO_3$ The applied current density was 0.9 $mA/cm^2$ for 40 s.

The outer layer was formed using the following: 0.006% w/v BSA, 2.5 % w/v PVA, 16 U/mL P'nase and mechanically stirred with a pipette tip. 1 μL of this solution was applied to the electrode and air-dried for 15 minutes. Potentiometric biosensing was conducted in 0.3 mM buffer.

The inner layer for the PPy/BSA-GLA film was formed using the following: 0.1 M Py, 19 U/mL P'nase and 0.1 M $KNO_3$ The applied current density was 0.9 $mA/cm^2$ for 40 s. The outer layer was formed using the following: 0.006% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase and mechanically stirred with a pipette tip. 1 μL of this solution was applied to the electrode and air-dried for 15 minutes. Potentiometric biosensing was carried out in 0.03 mM buffer.

## 7.3 Results and Discussion

### 7.3.1 Comparison of the Bi-layer Configurations

Two PPy and two PTy bi-layer arrangements were considered in this study to determine the best conditions for obtaining the optimum response for penicillin based on the sensitivity of the electrode. Figure 7.1 shows the variation in the response of several bilayer configurations, namely:

1. polytyramine/bovine serum albumin/glutaraldehyde (PTy/BSA-GLA),
2. polytyramine/bovine serum albumin/poly(vinyl alcohol) (PTy/BSA-PVA),

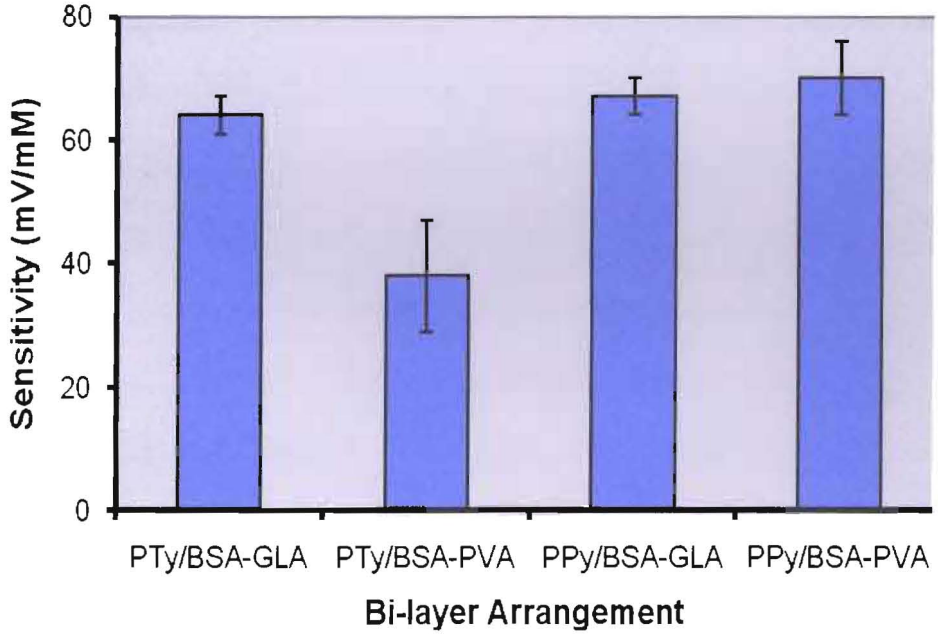



**Figure 7.1 Influence of the PTy/BSA-GLA, PTy/BSA-PVA, PPy/BSA-GLA and PPy/BSA-PVA electrodes on the sensitivity of the penicillin response.**

3. polypyrrole/bovine serum albumin/glutaraldehyde (PPy/BSA-GLA), and
4. polypyrrole/bovine serum albumin/poly(vinyl alcohol) (PPy/BSA-PVA)

The most sensitive response was obtained with the PPy/BSA-PVA electrode which produced the highest sensitivity (70 mV). This may have been due to the nature of the outer layer which in this case was BSA-PVA. PVA promotes the mass transfer of hydrogen ions through the PVA-BSA membrane. This is due to the neutrality of the PVA membrane which is enhanced through hydrogen bonding that is facilitated through the interaction of the hydroxyl groups (Wu et al. 1997). This was not the case with the electrodes that were comprised of BSA-GLA in the outer layer (i.e. PTy/BSA-GLA and PPy/BSA-GLA). There are several reasons that may explain this behaviour. These include higher concentrations of GLA which may denature the enzyme and as a result the activity of the enzyme is destroyed (Strike et al. 1999). Even though GLA facilitates the deposition of the protein, it is also responsible for steric hindrance within the three-dimensional matrix (Oyama et al. 1988: Abe et al. 1991; Liu et al. 1999) which causes a dramatic increase in the sensitivity of the electrode. In addition to this, GLA has the tendency to polymerise upon exposure to the protein to which it cross-links (Walt & Agayn 1994). The result of this may be a thicker film which had diffusional implications and a concomitant decline in the response of the electrode. The sensitivities shown for the PTy/BSA-GLA and PPy/BSA-GLA electrodes are similar as they both use BSA-GLA films for the outer layer. The difference between the responses of these electrodes is negligible. Even though this is the case, it is worth noting that the variation between the responses of those electrodes may be due to the conductivity of the PPy layer. The PPy/BSA-GLA film produced the second highest response followed

by the PTy/BSA-GLA electrode. Interestingly, the electrode that used PTy as the inner layer was less sensitive.

The electrode that used PPy in the inner layer was more sensitive which highlights the advantage of incorporating a conducting film in the inner layer. PPy/BSA-PVA was chosen due to the fact that this film was the most sensitive due to the conducting nature of the inner layer as well as the nature of the PVA membrane. The permeability of the BSA-PVA membrane enhances the diffusion of protons which ultimately promotes the sensitivity of the biosensor (Wu et al. 1997). Hence, the PPy/BSA-PVA and PTy/BSA-GLA configurations were used in further investigations for comparative purposes.

### 7.3.2 Influence of the Buffer Concentration and Linear Concentration Range

Figure 7.2 shows the influence of the buffer concentration on the sensitivity of the electrode. The lowest buffer concentration of 0.1 mM produced a less sensitive response to penicillin. Higher buffer concentrations such as 0.5 mM produced a slightly higher sensitivity, specifically, 39 mV/mM. The fact that higher buffer capacities increase the effective diffusion coefficient of hydrogen ions, may explain the increase in the sensitivity of the electrode (Wu et al. 1997). This was further demonstrated by the highest sensitivity which was achieved when 0.3 mM buffer was used. It was obvious that the concentration gradient of phosphate ions facilitates the transfer of hydrogen ions. Higher buffer concentrations, such as 0.5 and 1 mM, mask the majority of the hydrogen ions produced through the dissociation of penicilloic acid and decreases the sensitivity of the biosensor.

This explains the decreased sensitivity of the electrode when 1 mM buffer was used. The determination of the potentiometric properties of PPy bilayers involves the mobility of the ions in the outer layer (Wojda 1998). The influence of the inner layer is related to the permeability of the outer layer and the sensitivity of the inner layer (Alumaa et al. 2004).

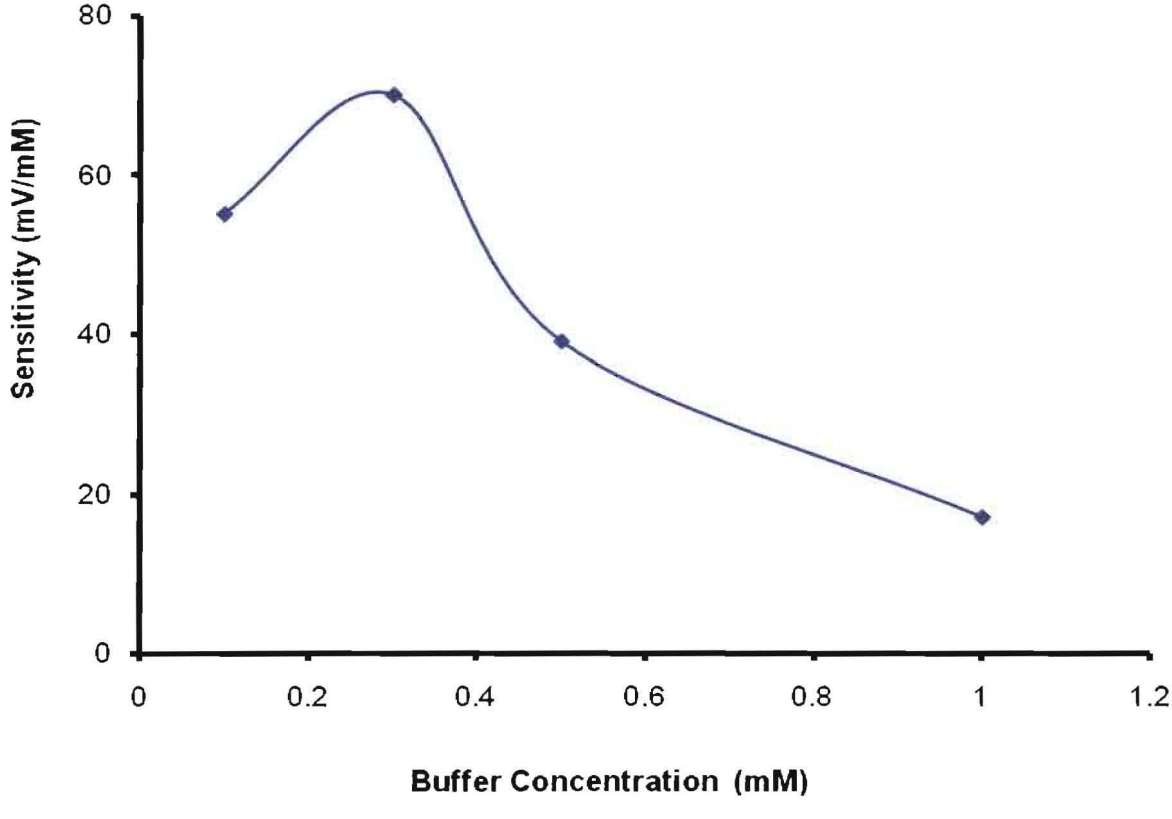



**Figure 7.2 The influence of the buffer concentration on the sensitivity of the PPy/BSA-PVA-P'nase bilayer biosensor. The following conditions were kept constant: inner layer: 0.1 M Py, 19 U/mL P'nase, 0.1 M $KNO_3$, applied current density 0.9 mA/cm$^2$, polymerisation time 40 s. Outer layer: 0.006% w/v BSA, 2.5% w/v PVA, 16 U/mL P'nase, drying time 15 minutes.**

Figure 7.3 shows the influence of the buffer concentration on the PTy/BSA-GLA electrode where the lowest buffer concentration (0.1 mM) gave the highest sensitivity of 80 mV/mM which is in contrast to the PPy/BSA-PVA film which required a buffer concentration of 0.3 mM and produced a sensitivity of 70 mV/mM. As can be seen, the film which contained

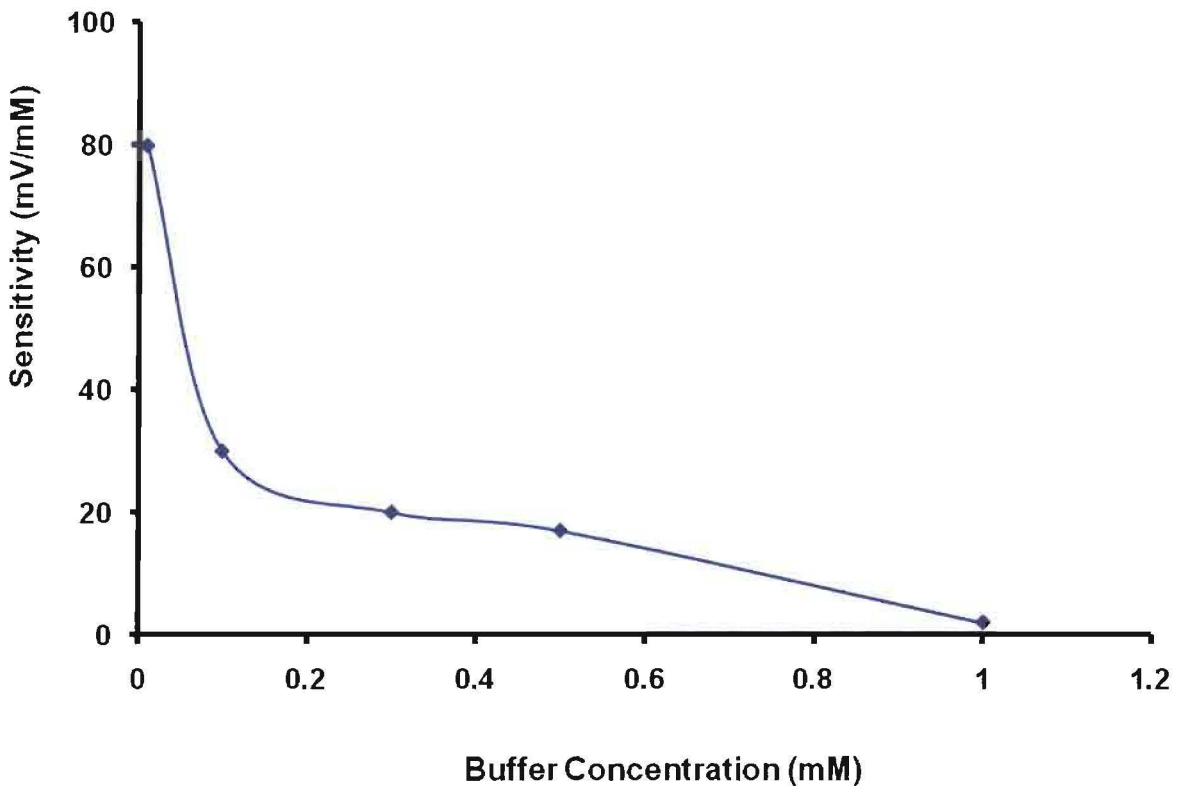



**Figure 7.3 The influence of the buffer concentration on the sensitivity of the PTy/BSA-GLA biosensor. The following conditions were kept constant: inner layer; 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, applied current density 0.9 mA/cm$^2$ polymerisation time 40 s. Outer layer: 0.006% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase, drying time 15 minutes.**

the conducting polymer as the inner layer and BSA-PVA as the outer layer, was more sensitive than the non-conducting electrode. Also the concentration gradient of phosphate

ions may contribute to the mass transfer of hydrogen ions and may explain this result. Figure 7.3 shows that the lowest sensitivity was obtained with the highest buffer concentration (1 mM). A sharp decrease in the sensitivity is observed when the buffer concentration increased from 0.01 to 0.1 mM. A further decrease in the sensitivity was apparent when the buffering concentration was increased from 0.1 to 0.3 mM. Higher

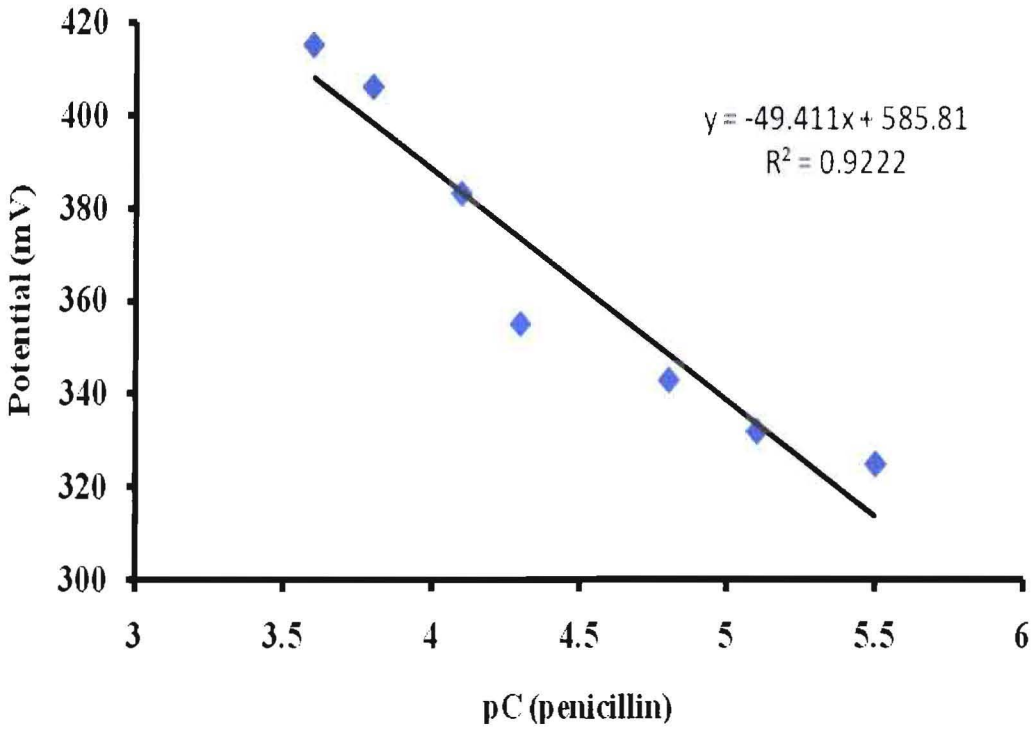



**Figure 7.4 Logarithmic concentration of the penicillin response obtained with PPy/BSA-PVA-P'nase biosensor. The following film conditions were applied: inner layer: 0.1 M Py, 19 U/mL P'nase, 0.1 M $KNO_3$, applied current density 0.9 mA/cm$^2$, polymerisation time 40 s. Outer layer: 0.006% w/v BSA, 2.5% w/v PVA, 16 U/mL P'nase, drying time 15 minutes. Potentiometric biosensing carried out in 0.3 mM buffer.**

buffering capacities consume most of the hydrogen ions generated by the catalytic reaction which also decreases the sensitivity of the electrode. This explains the low yet negligible difference between the sensitivities of the electrode when 0.3 and 0.5 mM buffer solutions were used respectively. Hence, the PPy/BSA-PVA electrode is more responsive than the PTy/BSA-GLA electrode. The PPy/BSA-PVA was more sensitive in a higher buffer concentration (0.3 mM) than the PTy/BSA-GLA electrode which was most responsive in 0.01 mM buffer.

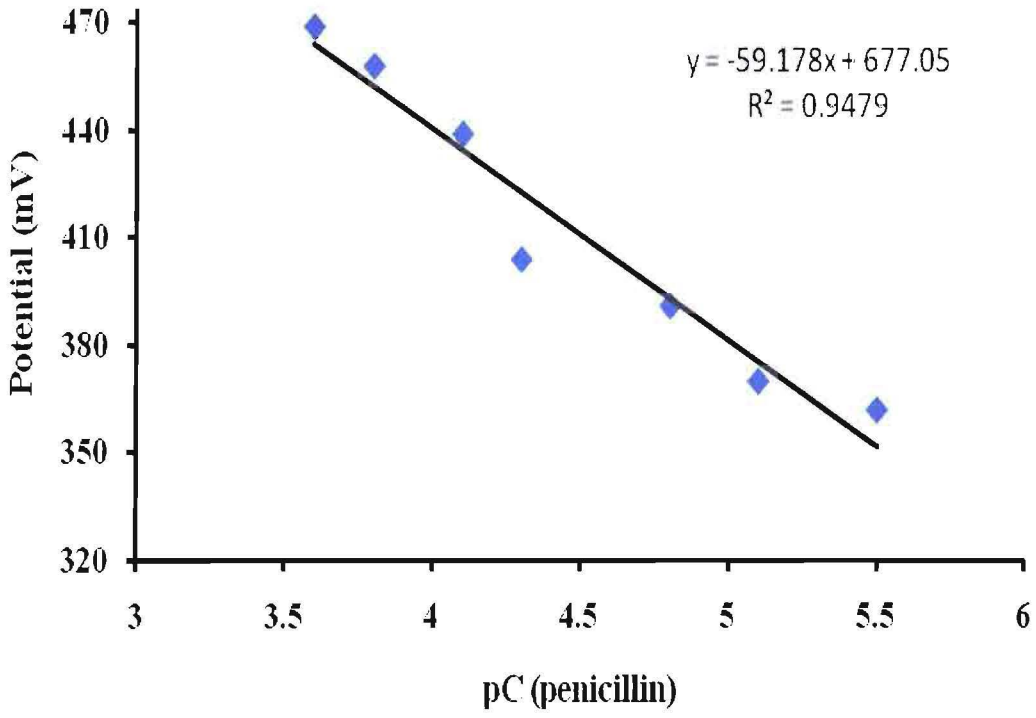



**Figure 7.5 Logarithmic concentration of the penicillin response obtained with PTy/BSA-GLA biosensor. The following conditions were applied: inner layer; 0.03 M Ty, 37 U/mL P'nase, 3 mM Pen, 0.01 M $KNO_3$, applied current density 0.9 mA/cm$^2$ polymerisation time 40 s. Outer layer: 0.006% w/v BSA, 0.012% v/v GLA, 8 U/mL P'nase, drying time 15 minutes. Potentiometric biosensing carried out in 0.01 mM buffer.**

A plot of potential versus the logarithmic of penicillin concentration gave a slope of 49.4 mV/decade and a correlation coefficient of $R^2$=0.9222. Figure 7.4 shows the linear concentration range for the PPy/BSA-PVA electrode where the achievable linear concentration range is 7.5 - 89 μM. This is narrower than the linear concentration range obtained for the PTy/BSA-GLA electrode where the linear concentration range is 7.5 – 283 μM, as shown in Figure 7.5, where the plot of potential versus the logarithmic penicillin concentration gave a slope of 59.1 mV/decade and a correlation coefficient of $R^2$=0.9479. The wider linear range is attributable to the use of a lower buffer concentration (0.01 mM buffer) for the PTy/BSA-GLA electrode while the narrower linear range was obtained with the use of 0.01 mM buffer. Higher buffer concentrations, such as 0.3 mM, consume most of the protons generated from the enzymatic membrane which also decreases the electrode sensitivity, as well as the linear concentration range. In addition, the saturation level of the PTy/BSA-GLA electrode is higher than that obtained for the PPy/BSA-GLA electrode. This can be explained by the differences in the buffer concentrations that were used for each electrode.

## 7.4 Determination of Penicillin in Amoxycillin

The PPy/BSA-PVA biosensor was applied to the determination of penicillin in amoxycillin. Three amoxycillin 500 mg tablets were prepared as described in section 3.2.3 of chapter 3. Each sample was analysed 4 times with the PPy/BSA-PVA biosensor. The results are expressed as means ± standard deviations and percentages with 95% confidence intervals. The results obtained for the determination of penicillin in amoxycillin are shown in Table 7.1. The average amount of amoxycillin that was detected with the PPy/BSA-

PVA electrode was 513 ± 74 mg with an overall average percentage recovery of 102 ± 15%. This indicates that the electrode is able to recover the active constituent successfully. However, the standard deviation values indicate that the reproducibility of the PPy/BSA-PVA membrane is inadequate. The average percentage recovery obtained with the standard

**Table 7.1 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Obtained with the (PPy/BSA-PVA) Electrode**

| Amount Specified in Tablet (mg) [a] | Penicillin Concentration Obtained With Biosensor (mg)[b] | % Recovery Obtained With Biosensor | [Penicillin] Obtained With Titration (mg) | % Recovery Obtained With Titration |
|---|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 597 ± 78 | 119 ± 16 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 536 ± 57 | 107 ± 11 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 407 ± 88 | 81 ± 18 | 505 ± 20 | 101 ± 4 |
| **Average values** | **513 ± 74** | **102 ± 15** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

titrimetric method, (105 ± 5 mg), is less reliable, but more reproducible than that obtained using the biosensor (513 ± 74 mg). The results obtained with the biosensor may be attributed to the porosity of the outer BSA- PVA layer as enzymatic sites on the surface of the electrode may have been inhibited by CA (Aghazadeh & Kazemifard 2001). This has contributed to a larger variation in the results. The third batch of antibiotics

**Table 7.2 Percentage Recoveries of Penicillin in Amoxycillin 500 mg Obtained with the (PTy/BSA-GLA) Electrode**

| Amount Specified in Tablet (mg)[a] | Penicillin Concentration Obtained With Biosensor (mg)[b] | % Recovery | [Penicillin] Obtained With Titration (mg) | % Recovery Obtained With Titration |
|---|---|---|---|---|
| Amoxycillin 500 Clavulanic Acid 125 | 558 ± 89 | 117 ± 18 | 525 ± 20 | 105 ± 4 |
| Amoxycillin 500 Clavulanic Acid 125 | 430 ± 99 | 86 ± 20 | 545 ± 40 | 109 ± 8 |
| Amoxycillin 500 Clavulanic Acid 125 | 512 ± 101 | 102 ± 20 | 505 ± 20 | 101 ± 4 |
| **Average values** | **500 ± 96** | **100 ± 19** | **525 ± 27** | **105 ± 5** |

[a] Dosage specified by pharmaceutical company
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

recorded a lower recovery (407 ± 88 mg) and the average percentage recovery obtained was 81 ± 18%. The recovery is lower and the standard deviation is higher than that obtained for the two previous batches which may indicate a problem with the third batch of antibiotics. The recovery values obtained with the PTy/BSA-GLA electrode are summarised in Table 7.2. The average recovery was 500 ± 96 mg and the average percentage recovery was 100 ± 19% which is close to the value that was reported for the PPy/BSA-PVA electrode (102 ± 15%). The average recovery value obtained with the PPy/BSA-PVA electrode (513 mg) is relatively the same as that obtained with the PTy/BSA-GLA electrode (500 mg). The reproducibility of the PTy/BSA-GLA electrode is slightly lower than that shown for the PPy/BSA-PVA electrode. This may be attributable to the porosity of the outer BSA-PVA layer as enzymatic sites on the surface of the electrode may have been inhibited by the CA (Aghazadeh & Kazemifard 2001).

Percentage recoveries that were greater than 100% may have been due to the presence of CA which is a β-lactam and is an analog of the basic penicillins (Parag et al. 2008). CA acts as a competitive inhibitor as well as having weak antibiotic properties (Townsend 2002) and may explain the variation in the results. However, the recoveries obtained using the biosensor were more reliable but not as reproducible as the results obtained using the standard titrimetric method. This may be ascribed to the nature of the bilayer configuration as the reproducibility of both bilayer electrodes was notably low. This was also the case with the PPy bilayer configuration, (in chapter 3), where the average recovery of penicillin in amoxycillin was 567 ± 119 mg and the average percentage recovery was 113 ± 24%.

## 7.5 Determination of Penicillin G in Milk

Table 7.3 shows the recoveries obtained with PPy/BSA-PVA electrode. In some cases percentage recoveries greater than 100% were obtained, such as the percentage recovery for 10 ppm penicillin (143 ± 10%). The inflated percentage recovery is due to the tendency of penicillin to bind to the hydrophobic sites of milk proteins (Grunwald & Petz 2003). The ability of the electrode to detect the penicillin, as well as the milk protein to which it is bound to, contributed to the variation in the results.

Lower percentage recoveries, such as 43% and 84%, can be attributed to the inhibition of enzymatic sites by milk proteins. The standard deviation values for lower percentage recoveries, is higher and the reproducibility of these values is lower than those shown for the higher percentage recovery of 10 ppm.

**Table 7.3 Recovery of Penicillin G in Milk Samples Using the PPy/BSA-PVA Biosensor**

| Concentration of Penicillin G in Milk (ppm)[a] | Penicillin Concentration Recovered With Biosensor (ppm)[b] | % Recovery |
|---|---|---|
| 5 | 2.2 ± 1 | 43 ± 20 |
| 10 | 14 ± 1 | 143 ± 10 |
| 20 | 17 ± 6 | 84 ± 30 |

[a] Spiked concentration of penicillin G in milk sample
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

Table 7.4 shows the recovery values obtained with the PTy/BSA-GLA electrode. Similar trends were observed with the PPy/BSA-PVA electrode. In this case, values greater than

**Table 7.4 Recovery of Penicillin G in Milk Samples Using the PTy/BSA-GLA Biosensor**

| Concentration of Penicillin G in Milk (ppm)[a] | Penicillin Concentration Recovered With Biosensor (ppm)[b] | % Recovery |
|---|---|---|
| 5 | 2 ± 1.0 | 40 ± 20 |
| 10 | 11 ± 0.3 | 114 ± 3 |
| 20 | 15 ± 4.0 | 76 ± 20 |

[a] Spiked concentration of penicillin G in milk sample
[b] Mean of 4 values obtained using biosensor with a 95% confidence limit

100% and lower than 100%, were obtained both due to the tendency of penicillin to bind to the hydrophobic sites of the milk proteins (Grunwald & Petz 2003). Similar to the PPy/BSA-PVA electrode, higher standard deviations are shown for 5 and 20 ppm penicillin, indicating that the responses for these penicillin concentrations are not as reproducible as they are for 10 ppm. The percentage recovery shown for 10 ppm penicillin with the PTy/BSA-GLA biosensor is 114 ± 3%, which is similar to the recovery obtained for 10 ppm with the PPy/BSA-PVA film.

## 7.6 Conclusion

The comparative study of PPy/BSA-PVA and the PTy/BSA-GLA electrodes generated interesting results, especially for the recovery of penicillin G in milk. The behaviour of both electrodes was similar in terms of the percentage recoveries and the reproducibility of the electrode. The PTy/BSA-GLA electrode was more reproducible and the PPy/BSA-PVA electrode was slightly more responsive for the recovery of penicillin in milk. These results were obtained using optimum film conditions. These included the PPy/BSA-PVA configuration which produced the highest response because of the nature of the outer layer which promotes the mass transfer of hydrogen ions through the BSA-PVA membrane. Configurations such as PTy/PVA-BSA were not chosen due to the resultant low response.

The choice of the buffer concentration is crucial to the performance of the biosensor and it was interesting to note that the PPy/BSA-PVA electrode was more sensitive in a higher buffer concentration (0.3 mM). On the other hand, the PTy/BSA-GLA electrode was more responsive in a lower buffer concentration such as 0.01 mM buffer. The minimum detectable concentration was 3.3 μM for both electrodes. The linear concentration range for the PPy/BSA-PVA electrode was 7.5 - 89 μM, which was narrower than that exhibited for the PTy/BSA-GLA electrode where the linear concentration range is between 7.5 - 283 μM.

The average percentage recovery for amoxycillin determined with the PPy/BSA-PVA electrode was 102 ± 15%, which is similar to that obtained with the PTy/BSA-GLA electrode (100 ± 19%). The reproducibility of both electrodes seems to be compromised in comparison to the standard titrimetric method (105 ± 5%).

The detection of 10 ppm penicillin G in milk proved to be more reliable and reproducible with both the PPy/BSA-PVA and PTy/BSA-GLA electrodes. However, there was a certain level of difficulty associated with the detection of 5 and 20 ppm of penicillin G in milk with both electrodes. Again, this may be due to the nature of the bilayer configuration, as this was not the case with single layer configurations, such as the BSA-GLA electrode reported in chapter 5.

# Chapter 8

## Conclusions and Future Recommendations

## 8.1 Conclusion

This study has examined a number of novel aspects such as the immobilisation of P'nase in a number of matrices, such as PPy, PTy, BSA-GLA, BSA-PVA and a number of bilayer configurations, namely, PPy bilayer, PPy/BSA-PVA and PTy/BSA-GLA and has been demonstrated successfully for the fabrication of a penicillin biosensor. This study also focussed upon the change in the potential rather than the change in the pH which has been reported quite extensively in the literature.

The physical entrapment of P'nase in a single layer PPy film was the first approach to be successfully applied. The following conditions were established for the potentiometric measurement of penicillin: polymerisation time: 40 s, applied current density of 0.9 $mA/cm^2$, 50 U/mL P'nase, 0.03 M PPy and 0.1 mM buffer. However, the resulting potentiometric response was unstable due to the charge on the membrane which becomes negative as the result of the retention of the products of the enzymatic reaction, namely, penicilloate. In order to attract the substrate, penicillin, the charge on the membrane should be positive and was necessary for an increase in the potentiometric signal, hence 0.01 M Pen was added to the monomer solution.

The PPy-P'nase-Pen electrode was successfully applied to raw milk samples which were spiked with penicillin G. However, the response achieved for the milk samples indicated that sample preparation required further optimisation in order to ensure reliable quantification of penicillin. The electrode was also applied to pharmaceutical preparations, namely, abocillin 125 mg, flucoxacillin 500 mg and amoxycillin 875 mg. However, quantification was not possible at this stage as sample preparation required further optimisation. Quantitative analysis was only applied to pharmaceutical

preparations and milk samples after sample preparation methods were further optimized and film conditions were established.

Some of the most important parameters in the establishment of film conditions include the monomer concentration and the resulting polymer matrix. The effects of such are summarised below in Table 8.1.

**Table 8.1 The Influence of the Nature of the Polymer Matrix on the Electrode Sensitivity**

| Configuration | Matrix | Monomer Concentration (M) | Sensitivity(mV/mM) |
|---|---|---|---|
| Single layer | PPy | 0.03 | 380 |
| Bilayer | PPy | 0.04 (in outer layer) | 380 |
| Single layer | PTy | 0.03 | 401 |

The growth of non-conducting films is self-limiting hence producing thinner films. This is demonstrated in Table 8.1 with the highest sensitivity obtained with the PTy electrode. The thickness of the film contributed to an enhanced diffusion barrier for the catalytic reaction products. The growth of non-conducting polymers differentiates them from conducting polymers, as these polymers are able to be grown at varying thicknesses. As a non-conducting film forms over the electrode surface, passivation occurs and this prevents further polymerisation. The formation of successive layers in the case of bilayer

films produces electrical resistance which inhibits the diffusion barriers across the layers that exist. Lower sensitivities were recorded for both PPy configurations due to diffusional limitations caused by thicker films. Interestingly, the sensitivity for both the single layer and bilayer films were also identical.

The sensitivity of the film is also influenced by the immobilisation methods that were used for the entrapment of P'nase. The methods investigated in this study include physical entrapment and cross-linking. Table 8.2 summarises the influence of the immobilisation method on the sensitivity of the biosensor. The data in the Table provides the optimum enzyme concentration with the resultant sensitivity for each film. The most responsive electrode was the PPy bilayer configuration which achieved the highest sensitivity of 448 mV/mM with 19 U/mL of P'nase incorporated in the outer layer of the film. The fact that the PPy bilayer configuration was the most sensitive may be due to the presence of the enzyme in both layers which in turn would have increased the sensitivity of the electrode. This parameter is governed not only by the permeability of the outer layer but also by the sensitivity of the inner layer which is also influenced by the presence of the enzyme. The results support the literature in proving that physical entrapment is an effective method in terms of producing a more sensitive electrode. In fact physical entrapment is a rapid method that does not require chemical bonds which aids in the retention of the activity of P'nase and protects it from chemical and physical stress. This enhances the stability of the enzyme as well as the sensitivity of the biosensor. The self-limiting nature of the PTy film may have affected the sensitivity of the electrode and may explain why the highest sensitivity was obtained with the PPy bilayer biosensor. It was

interesting to note that the sensitivity of the PPy single layer was lower than that for the PPy bilayer and the PTy biosensors.

**Table 8.2 Influence of Immobilisation Method on the Response of the Electrode**

| Immobilisation Method | Enzyme Concentration (U/mL) | Sensitivity (mV/mM) |
|---|---|---|
| Physical entrapment using PPy single layer | 50 | 147 |
| Physical entrapment using PPy bilayer | 19 (in outer layer)* | 448 |
| Physical entrapment using PTy single layer | 37 | 362 |
| Cross-linking with BSA-GLA | 8 | 181 |
| Cross-linking with BSA-PVA | 16 | 61 |

* The inner layer contained 19 U/mL of P'nase

This may be due to the incorporation of a higher enzyme concentration which meant that there was an increase in the thickness of the film, as well as a decrease in the sensitivity of the electrode. In contrast, the BSA-PVA and BSA-GLA electrodes were the least sensitive. Both of these biosensors used cross-linking to incorporate the enzyme.

However, the method suffered from poor stability and a general lack of mechanical properties. The optimum enzyme concentration for both of these films was lower than those for films that used physical entrapment to incorporate the enzyme which may also correspond to the lower sensitivities obtained with these films. Specifically, the BSA-PVA film was less sensitive than the BSA-GLA film. This may be a direct result of the multi-point attachment that occurs with GLA which prevents unfolding of the protein and enhances the sensitivity of the electrode. Overall, the results support physical entrapment as a more effective method. This is because the porosity of the film is tight which prevents leakage of the enzyme. Despite this, the movement of the substrate is not limited which enhances the sensitivity of the electrode.

The applied current density is another parameter that affects the sensitivity of the electrode. Table 8.3 outlines the effect of the current density on the amount of enzyme incorporated in the film and on the electrode sensitivity. The highest sensitivity was obtained with the application of the lowest current density (0.5 $mA/cm^2$). Implicit with this observation is the fact the applied current density controls the film thickness particularly in a bilayer system, where the porosity of the outer layer determines the sensitivity of the biosensor. Thus, the application of a lower current density to the outer layer enables the formation of a thinner outer layer and possibly a more sensitive film. Inversely, the highest applied current density (0.9 $mA/cm^2$) produced the least sensitive film. The incorporation of a higher enzyme concentration may have contributed to a thicker film and a reduced diffusion barrier. Lower current densities also mean that the polymerisation rate is slower and produced a thinner film, which are more sensitive.

**Table 8.3 The Effect of the Applied Current Density on the Electrode Sensitivity**

| Sensor Configuration | Applied Current Density ($mA/cm^2$) | Enzyme Concentration (U/mL) | Sensitivity (mV/mM) |
|---|---|---|---|
| PPy bilayer | 0.5* | 19 | 424 |
| PTy single layer | 0.8 | 37 | 264 |
| PPy single layer | 0.9 | 50 | 117 |

* Current density applied to the outer layer

The concentration of the buffer is important for the successful operation of the biosensor. Table 8.4 summarises the influence of the buffer concentration on the response of the electrode. The results show that conducting polymers required higher buffer concentrations while non-conducting polymers required lower buffer concentrations. The BSA-PVA electrode required a slightly higher buffer concentration than the other non-conducting polymers such as PTy and BSA-GLA. This may be attributable to the hydrophilicity of PVA which promotes the mass transfer of hydrogen ions generated through the dissociation of penicilloic acid and required higher buffer concentrations in order to enhance the effective diffusion coefficient of hydrogen ions.

Bilayer configurations such as the PPy bilayer and the PPy/BSA-PVA electrodes were more responsive in 0.3 mM buffer concentration, whereas the PTy/BSA-GLA electrode

was more responsive in a lower buffer concentration, specifically, 0.01 mM. The concentration of protons produced using this configuration, required lower buffer concentrations.

**Table 8.4 The Influence of the Buffer Concentration on the Electrode Response**

| Polymer Matrix | Configuration | Buffer Concentration (mM) | Sensitivity (mV/Mm) | Linear Concentration Range (μM) |
|---|---|---|---|---|
| PPy | Single layer | 0.1 | 56 | 45 – 283 |
| PPy | Bilayer | 0.3 | 87 | 7.5 – 146 |
| PTy | Single layer | 0.1 | 24 | 3 – 283 |
| BSA-GLA | Single layer | 0.03 | 69 | 3 – 283 |
| BSA-PVA | Single layer | 0.3 | 50 | 7.5 – 283 |
| PPy/BSA-PVA | Bilayer | 0.3 | 49 | 7.5 – 89 |
| PTy/BSA-GLA | Bilayer | 0.01 | 59 | 7.5 – 283 |

The widest linear concentration range was obtained with the PTy-P'nase and BSA-GLA-P'nase electrodes. On the other hand, a narrower linear concentration range was obtained with the PPy/BSA-PVA electrode. This is attributed to the higher buffer concentration

which was used for the PPy/BSA-PVA electrode which in turn affected the response of the electrode and the linear concentration range of the biosensor. The lower buffer concentrations, such as 0.1 and 0.03 mM, contributed to wider linear concentration ranges.

This study has also examined the response of the electrode to a number of pharmaceutical preparations, including abocillin 125 mg, flucoxacillin 500 mg and amoxycillin 875 mg with the PPy single layer electrode. The potential change produced by each of the antibiotic samples reflected the different concentrations of the active constituent present in each tablet or capsule. That is, the highest potential change was produced by amoxycillin 875 mg sample, while the lowest potential change was produced by abocillin 125 mg sample. This proved the ability of the electrode to distinguish between the β-lactam antibiotics and the varying penicillin concentrations present in the pharmaceutical formulations. However, further sample preparation was required in order to quantify the active constituent in the tablets.

The quantitative study focussed on amoxycillin 500 mg. The recovery of the active constituent in amoxycillin 500 mg was performed successfully with the various biosensor configurations and the results are summarised in Table 8.5. The most reliable percentage recoveries were obtained with the PTy-P'nase electrode (102 ± 6%) and the BSA-GLA-P'nase (103 ± 5%) electrodes. Both of these values were in close agreement with the average percentage recovery obtained using the standard titrimetric method (105 ± 5%). However, the titrimetric method was more time consuming than the biosensor method hence highlighting the advantages of the biosensor.

Satisfactory percentage recoveries obtained with the PTy-P'nase electrode was due to the enhanced sensitivity of the film due to the resulting thinner film because of the self-limiting growth of these films. In the case of the BSA-GLA-P'nase electrode, the more adequate percentage recovery may be attributed to GLA coupling as a method. GLA enables greater flexibility for protein conformational changes that are required for the activity of the enzyme. This is important as the molecular rearrangement of the enzyme must occur prior to its interaction with the antibiotic. Both of these characteristics proved to be apt in producing satisfactory average percentage recoveries as well as reducing the uncertainty in the results. This is consistent with the fact that GLA coupling is one of the best methods because of the stability of the enzyme which interestingly seems to be enhanced when immobilised.

Relatively good percentage recoveries were reported for both the BSA-PVA-P'nase and the PPy/BSA-PVA electrodes. This is due to the presence of PVA in both films which provides favourable conditions for the enzyme to move freely. This means that the enzyme is able to change its conformation prior to interacting with the antibiotic. PVA also preserves the quaternary structure of the enzyme which may have contributed to the adequate percentage recoveries. However, the reproducibility of the results was lower. This may have been due to the porosity of the PVA membrane which is common to both electrodes. The porosity of the electrode may have also been affected by the presence of CA which is a β-lactam antibiotic, as well as, a beta-lactamase inhibitor. CA may have inhibited enzymatic sites which may explain the lower reproducibility obtained with this configuration.

**Table 8.5 Average Recovery Values for Amoxycillin 500 mg obtained with Various Biosensor Configurations**

| Sensor Configuration | Average Penicillin Concentration Recovered (mg) | Average % Recovery |
|---|---|---|
| PPy bilayer | 567 ± 119 | 113 ± 24 |
| PTy single layer | 512 ± 30 | 102 ± 6 |
| Single layer BSA-GLA | 514 ± 24 | 103 ± 5 |
| Single layer BSA-PVA | 487 ± 58 | 97 ± 12 |
| Bilayer PPy/BSA-PVA | 513 ± 74 | 102 ± 15 |
| Bilayer PTy/BSA-GLA | 500 ± 96 | 100 ± 19 |

Table 8.5 shows that all bilayer configurations exhibit large standard deviations hence the inadequacy of the reproducibility of this configuration. Specifically, a higher percentage recovery was obtained with the PPy bilayer electrode (113 ± 24%) for the determination of penicillin in amoxycillin. Elevated percentage recoveries may also mean that the biosensor is able to detect CA due to the fact that that it has weak antibiotic properties. This may also highlight the efficacy of the biosensor towards the different types of antibiotics. However, once detected CA may also block enzymatic sites as it is also a β-lactamase inhibitor.

The determination of penicillin in milk was also undertaken. Table 8.6 summarises the average recoveries obtained with the various configurations. Evidently, adequate percentage recovery values were obtained for 5 ppm penicillin with the PPy bilayer biosensor (110 ± 26 %). Both BSA-GLA (111 ± 26%) and BSA-PVA (110 ± 30%) configurations gave satisfactory recoveries for 10 ppm penicillin in milk. BSA-PVA also reported an adequate percentage recovery (107 ± 15%) for 20 ppm penicillin in milk, which highlights the efficacy of the BSA-PVA biosensor for the determination of penicillin in milk for penicillin concentrations 10-20 ppm. However, the reproducibility of the electrodes was quite low. All adequate percentage recoveries were over 100% which is due to the tendency of penicillins to bind to the hydrophobic sites of the milk proteins. Overall, the performance of the BSA-PVA electrode seemed to be better than the other electrodes. This may be attributed to the nature of PVA which allows the enzyme to act as if it were free as opposed to GLA which reduces the activity of the enzyme and in turn affects the ability of the electrode to recover penicillin residues in milk. However, the BSA-PVA electrode was not able to recover lower penicillin concentrations such as 5 ppm, where the percentage recovery was 40 ± 18%. Blockage of enzymatic sites on the electrode surface by milk proteins may have caused this. This was also the case with the PTy/BSA-GLA electrode, however the reproducibility of the BSA-PVA biosensor was slightly better than that obtained with the PTy/BSA-GLA biosensor. Inadequate percentage recoveries were also achieved for 10 ppm penicillin (60 ± 10%) and 20 ppm penicillin (30 ± 7%) with the PPy bilayer configuration. Extremely high percentage recoveries were obtained with the BSA-GLA-P'nase electrode (126 ± 38%)

**Table 8.6 Average Recovery Values Obtained for Milk**

| Sensor Configuration | Concentration of Penicillin in Milk (ppm) | Average [Penicillin] Found (ppm) | Average % Recovery |
|---|---|---|---|
| BSA-GLA | 5 | 6.3 ± 1.9 | 126 ± 38 |
| PPy bilayer | 5 | 5.5 ± 1.3 | 110 ± 26 |
| PTy single layer | 5 | 4.0 ± 1.6 | 92 ± 32 |
| PPy/BSA-PVA | 5 | 2.2 ± 1.0 | 43 ± 20 |
| BSA-PVA | 5 | 2.0 ± 0.9 | 40 ± 18 |
| PTy/BSA-GLA | 5 | 2.0 ± 1.0 | 40 ± 20 |
| PPy/BSA-PVA | 10 | 14.0 ± 1.0 | 143 ± 10 |
| PTy/BSA-GLA | 10 | 11.0 ± 0.3 | 114 ± 3 |
| BSA-GLA | 10 | 11.1 ± 2.6 | 111 ± 26 |
| BSA-PVA | 10 | 11.0 ± 3.0 | 110 ± 30 |
| PTy single layer | 10 | 7.8 ± 0.8 | 78 ± 16 |
| PPy bilayer | 10 | 6.0 ± 1.0 | 60 ± 10 |
| BSA-GLA | 20 | 25.6 ± 1.8 | 128 ± 9 |
| BSA-PVA | 20 | 21.0 ± 3.0 | 107 ± 15 |
| PTy single layer | 20 | 18.0 ± 2.7 | 90 ± 14 |
| PPy/BSA-PVA | 20 | 17.0 ± 6.0 | 84 ± 30 |
| PTy/BSA-GLA | 20 | 15.0 ± 4.0 | 76 ± 20 |
| PPy bilayer | 20 | 6.0 ± 1.3 | 30 ± 7 |

for 5 ppm penicillin, PPy/BSA-PVA biosensor (143 ± 10%) for 10 ppm penicillin and 128 ± 9% BSA-GLA-P'nase electrode for 20 ppm penicillin.

Overall, the fabrication of a potentiometric penicillin biosensor was useful for the determination of penicillin in amoxycillin with the PTy-P'nase ($R^2$= 0.992) and BSA-GLA-P'nase ($R^2$= 0.984) electrodes. Wider linear concentration ranges were obtained with both of these films and were adequate for the determination of penicillin in amoxycillin 500 mg. Specifically, the average percentage recovery obtained with the PTy-P'nase electrode was 102 ± 6% and the average percentage recovery achieved with the BSA-GLA-P'nase electrode was 103 ± 5%. Both recoveries were in close agreement with the percentage recovery (105 ± 5%) achieved with the standard titrimetric method. In fact the percentage recoveries obtained with the PTy-P'nase and BSA-GLA-P'nase electrodes were more reliable and reproducible than that obtained with the standard titrimetric method.

Recommendations for future work include further sample preparation of antibiotic samples to avoid inhibition of the enzymatic sites by CA. In addition, the whey fraction of milk samples also requires further sample preparation in order to avoid non-specific binding and to ensure reliable quantification of penicillin in milk.

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