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Towards the development of an electrochemical
immunosensor for the identification of transient ischemic
attack via the labelless detection of biomedical markers.

Supervisor: Professor S.P.J. Higson

This degree is submitted in partial fulfilment of the requirements for the degree of Doctor
of Philosophy

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For my mum, Margaret Elizabeth Barton.

Declaration

This is a declaration to certify that except as stated below, the contents of this thesis have not been submitted for any other academic or professional award or published in any other form and the contents of this thesis represents entirely my own work.

The results detailed in Chapter 4 have contributed to a joint publication in volume 20 of *Biosensors and Bioelectronics*, (2004), pages 328-337; this paper is included in the appendix of this thesis.

Andrew. C. Barton, 2006.

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Abstract.

The thesis describes the development of generic platforms for labelless transduction of binding events. A particular application of the project is the labelless detection of biochemical markers for stroke, specifically transient ischemic attack, to facilitate rapid diagnosis and subsequent introduction of appropriate therapy within the required three-hour time frame. Novel biocomponents are under development for detection of these biochemical markers, and further demonstration of the generic nature of the technologies being developed will be realised via the use of a library of monoclonal antibodies as capture biocomponents.

This work forms part of an investigation into labelless affinity immunosensor detection via impedimetric transduction utilising the technique of electrochemical impedance spectroscopy (EIS). Novel microelectrode arrays were fabricated by the sonochemical ablation of a homogeneous insulating layer of poly(*o*-phenylenediamine) exposing discrete areas of an underlying screen-printed conductive substrate. The receptor biocomponents are then subsequently coupled to the transducer by either electropolymerised entrapment or non-covalent affinity within and/or at the surface of conducting polymer 'mushroom' shaped protrusions for site-specific immobilisation.

Upon exposure to a range of concentrations of antigen analyte solution, complex plane impedance analyses are used to relate the differing redox states of the polymer to possible charge transfer and / or related mechanisms between the immobilised antibody / antigen analyte and the polymer. Results show that signal differentiations were obtained, for a number of antibody / antigen species over a range of concentrations of clinical relevance (ng-pg/ml⁻¹), suggesting that the techniques employed offer a viable approach towards the labelless quantification of biochemical markers of transient ischemic attack (TIA).

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Nomenclature

A	geometric electrode area
A	piezo-electrically active area,
Ab	antibody
$[Ab]$	molar concentrations (mol^{-1}) of unoccupied binding sites on an antibody
$Ab-Ag$	antibody-antigen stable immunocomplex
$[Ab-Ag]$	the molar concentration of an antibody-antigen complex (mol^{-1})
Ag	antigen
$[Ag]$	molar concentrations (mol^{-1}) of unoccupied binding sites on an antigen
ABSA	anti-bovine serum albumin monoclonal antibody
AC	alternating current
AFM	atomic force microscopy
$Ag/AgCl$	silver/ silver chloride
AIgG	anti-IgG antibody
AMI	acute myocardial infarction
ANSE	anti- neuron specific enolase monoclonal antibody
APSA	anti-prostate specific antigen monoclonal antibody
AS-100	anti-S100 monoclonal antibody
BMBD	biochemical markers of brain disease
BSA	bovine serum albumin
PSA	prostate specific antigen
CA125	carcinoma antigen 125 (tumor marker)
C_{Au}	constant capacitance of an unmodified polycrystalline Au electrode, typically $40\text{-}60\mu\text{Fcm}^{-2}$.
C_{dl}	double layer capacitance
C_{mod}	variable component of C_{dl} , C_{mod} , controlled by the modifier layer
CNS	central nervous system

C_o	bulk concentration of oxidant
<i>CPE</i>	conductive polymer entrapment
CPE	constant phase element
CSF	cerebrospinal fluid
CT scan	computed tomography scan
D-dimer	degradation fragment of cross-linked fibrin due to action of plasmin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
c-DNA	complementary deoxyribonucleic acid
D_o	diffusion coefficients for the oxidant (cm^2s^{-1})
D_R	diffusion coefficients for the reductant (cm^2s^{-1})
E	applied potential
E_{eq}	equilibrium potential
E_p^a	anodic peak potential
E_p^c	cathodic peak potential
EB	emeraldine base
$E(CE)_n$	mechanism in which monomer oxidation is an electrochemical process (E), radical-radical coupling is a chemical process (C), and chain propagation is another electrochemical process (E). The last two processes repeated indefinitely
ECG	electrocardiogram
EIS	electrochemical impedance spectroscopy
EIA	enzyme immunometric assay kit
ELISA	enzyme-linked immunosorbent assay
ES	emeraldine salt
Δf	measured frequency shift
f_0	resonant frequency of the fundamental mode of the crystal
f	excitation frequency (Hz)
f_u	resonant frequency of the unloaded crystal,
F	Faraday constant $9.65 \times 10^4 \text{Cmol}^{-1}$
F(ab)	fragment antigen-binding portion of an antibody

F(c)	constant region of an antibody encompassing heavy and light chains
F(v)	variable region of an antibody encompassing heavy and light chains
Fc(CO ₂ H)	ferrocenemonocarboxylic acid
FEG-SEM	field emission gun scanning electron microscopy
FIA	flow-injection analysis
FRA	frequency response analyser
GFAP	glial fibrillary acid protein
h	Plank's constant ($6.26 \times 10^{-34} \text{Js}^{-1}$)
HA	hyaluronic acid
HABA	2-(4-Hydroxyphenylazo) benzoic acid
HCl	Hydrochloric acid
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulphuric acid
IHP	inner helmholtz plane
i	net observed current
i_0	standard exchange current
i_p^a	peak anodic current
i_p^c	peak cathodic current
I_F	current contribution from the faradaic process
I_C	current contribution from the double layer charging
$I(j\omega)$	system current phasor
IgG	immunoglobulin G
in-situ	in biology, in-situ means to examine the phenomenon exactly in place where it occurs
KA	affinity constant for antigen binding (mol^{-1})
KCl	potassium chloride
Fe(CN) ₆ ^{3-/4-}	ferri/ferrocyanide reversible redox species
Fe(CO ₂ H) ^{2+/3+}	ferrocenemonocarboxylic acid reversible redox species
K ₂ HPO ₄	dipotassium phosphate

k_b	backwards electron transfer rate constant (s^{-1})
k_{et}	the electron transfer rate constant for the applied redox probe derived from the electron transfer resistance R_{et}
k_f	forward electron transfer rate constant (s^{-1})
Δm	mass change per unit area (g/cm^2),
MAb	monoclonal antibody
MIPS	molecularly imprinted polymers
MMP-9	matrix metalloproteinase 9 acute phase reactant
MRI	magnetic resonance imaging
MW	molecular weight
NaCl	sodium chloride
$ng\ ml^{-1}$	nano grams per millilitre
n	number of electrons transferred
Na_2CO_3	disodium carbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NaH_2PO_4	dihydrogen orthophosphate
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
NSE	neuron specific enolase
(O)	oxidized species
OHP	outer helmholtz plane
<i>o</i> PD	<i>o</i> -phenylenediamine
PANI	polyaniline
PBS	phosphate buffered saline
$pg\ ml^{-1}$	pico grams per millilitre
PNS	peripheral nervous system
PPD	poly(<i>o</i> -phenylenediamine)
PSA	prostate specific antigen
Pt	platinum
QCM	quartz crystal microbalance

R	molar gas constant $8.314 \text{ Jmol}^{-1}\text{K}^{-1}$
(R)	reduced species
R_{et}	electron transfer resistance also termed charge transfer resistance, R_{ct}
R_{mod}	the variable component of R_{et} corresponding to the different modifier states
R_{p}	polarization resistance
R_{s}	solution resistance
ΔR_{et}	change in electron transfer resistance
RSD	relative standard deviation
rt-PA	recombinant tissue plasminogen activator
$\text{Ru}(\text{NH}_3)_6\text{Cl}_3$	hexamine ruthenium trichloride
$\text{Ru}(\text{NH}_3)_6^{3+/2+}$	hexammineruthenium reversible redox species
$[\text{S}]$	corresponds to the bulk concentration of the redox probe (mol cm^{-3})
SCE	standard calomel electrode
SEM	scanning electron microscope
S-100 protein	highly acidic calcium-binding protein found in large concentration in the brain and believed to be glial in origin, also found in other organs in the body
ScFv	single-chain variable fragment
S/N ratio	signal-to-noise ratio
$S(t)$	ac signal obtained from the cell
T	absolute temperature (in Kelvins)
TIA	transient ischemic attack
TM	thrombomodulin
t-PA	tissue plasminogen activator
tris	tris (hydroxymethyl) methylamine
$U(j\omega)$	system voltage phasor
UV-VIS	ultraviolet-visible spectroscopy
v	the speed of light ($2.998 \times 10^8 \text{ ms}^{-1}$)
VEGF	vascular endothelial growth factor

V_H	aminoterminal portion of the heavy chain, characterised by a highly variable amino-acid composition
V_L	aminoterminal portion of the light chain, characterised by a highly variable amino-acid composition
W	Warburg resistance
$Z(\omega)$	impedance at a given frequency
$Z'(\text{re})$	real component of impedance
$-Z''(\text{im})$	imaginary component of impedance with a complex representation
Z_w	Warburg impedance
α	transfer coefficient
ϵ_0	8.85Fm^{-1} , the dielectric constant of the vacuum
ϵ_{dl}	dielectric permittivity introduced into the double-charged layer molecules
ϵ_p	is the effective dielectric constant of layer separating the ionic charges and the electrode surface
ρ_L	density of liquid in contact with the crystal
ρ_q	density of quartz, 2.648 g/cm^3
μ_q	shear modulus of quartz, $2.947 \times 10^{11}\text{ g/cm} \times \text{s}^2$
δ	the thickness of the separating layer
η	overpotential
η_L	viscosity of liquid in contact with the crystal
λ	wavelength
θ	phase angle
τ_{rxn}	time constant of the electrochemical reaction = $R_p.C_{dl} = 1/\omega_{\text{max}} = 1/(2\pi f_{\text{max}})$
ω	phase angle in $\text{rad s}^{-1} = 2\pi f$
ω_0	characteristic frequency
$\cos(\omega t)$	reference signal
$\sin(\omega t)$	reference signal

Chapter 1: Rationale for research.

1.0 Rationale for research.

Currently there are no rapid analytical approaches for the early diagnosis of transient ischemic attack (TIA). The short-term transient attacks are often a warning for subsequent ischemic strokes of greater severity. Rapid diagnosis and introduction of thrombolytic therapy can prevent much of the damage caused by the TIAs from occurring and can prevent any further attacks of greater magnitude.

Immediate diagnosis and care of a patient-experiencing stroke can be critical. For example tissue plasminogen activator (t-PA), given within three hours of symptom onset in ischemic stroke, is beneficial for selected acute stroke patients.

Ischemic stroke patients may also benefit from anticoagulants (e.g. heparin) if they are not candidates for t-PA therapy. In contrast, thrombolytics and anticoagulants are strongly contraindicated in hemorrhagic stroke. Thus, early differentiation of ischemic events from hemorrhagic events is imperative. Moreover, delays in the confirmation of stroke diagnosis and the identification of stroke type limit the number of patients that may benefit from early intervention therapy.

As part of a Framework Five funded quality of life project (QLRT-2001-02583), an international collaboration of ten laboratories was established with the aim of developing a range of generic platforms for the labelless transduction of binding events, focused towards the detection and quantification of biochemical markers of brain disease (BMBDs). In particular, investigations are aimed at markers critical to the diagnosis of transient ischemic attack or stroke events.

In the United Kingdom stroke is the third highest leading cause of death after heart disease and all cancers (Wolfe *et al*, 1996). Each year in England and Wales over 130,000 people suffer a stroke each year, 53,700 of which are recurrent attacks, and this is equivalent to one person having a stroke every five minutes (Office of National Statistics Health Statistics, 2001). Each year in the United Kingdom 65,000 strokes result

in the death of the patient (Peterson *et al*, 2005). Strokes are also the leading cause of serious, long-term disability and cause a greater range of disabilities than any other condition in the United Kingdom (Adamson *et al*, 2004). At least 450,000 people are severely disabled as a result of stroke in England (Department of Health, 2005).

There are currently no rapid diagnostic methods that can identify a TIA, or predict delayed neurological deficits which are often detected at a time after the onset of the attack, that are concurrent with the presentation of symptoms. Accordingly, there is at present need for a rapid, sensitive and specific diagnostic assay for stroke and TIA that can differentiate the stroke type and identify those individuals at risk for delayed neurological deficits. Such a diagnostic assay would greatly increase the number of patients that can receive beneficial stroke treatment and therapy, whilst also reducing the costs associated with incorrect stroke diagnosis.

Tissue plasminogen activator is currently the only approved drug in the treatment of acute stroke, through thrombolytic therapy, which offers a three-hour treatment window leading to a 30% more likely chance of recovery from the brain attack with little or no disability (National Institute of Neurological Disorders and Stroke, 1995; Kwiatkowski *et al*, 1999; Marler *et al*, 1999; Marler *et al*, 2000).

The particular application of this project is to develop an individually addressable array of labelless affinity biosensors for the rapid detection of biochemical markers for stroke, facilitating the rapid diagnosis essential for the introduction of thrombolytic therapy.

Labelless affinity sensors address the problem associated with rapid diagnosis of stroke by allowing cost effective, rapid (in minutes), specific (using novel monoclonal antibodies) and sensitive (ng-pg/ml) detection of stroke markers for use at point of care.

A range of novel biomedical markers for brain disease (BMBD) have been identified in recent research (Massaro *et al*, 1990; Fassbender *et al*, 1997; Hill *et al*, 2000; Reynolds *et al*, 2003) most importantly S-100 β protein (Aurell *et al*, 1991; Kim *et al*, 1996; Abraha *et al*,

al, 1997; Buttner *et al*, 1997; Ingebrigsten *et al*, 1997; Missler *et al*, 1997; Martens *et al*, 1998; Missler & Wiesmann, 1998; Hermann *et al*, 1999; Raabe *et al*, 1999; Wunderlich *et al*, 1999; Hermann *et al*, 2000) and neuron specific enolase (NSE) (Hatfield & McKernan, 1992; Barone *et al*, 1993; Schaarschmidt *et al*, 1994; Butterworth *et al*, 1996; Cunningham *et al*, 1996; Missler *et al*, 1997; Martens *et al*, 1998; Hermann *et al*, 1999; Schoerhuber *et al*, 1999; Wunderlich *et al*, 1999). Canag Diagnostics have been responsible within this programme for producing, developing and characterising monoclonal antibodies against possible novel markers. These will be produced by using conventional immunisation and hybridoma technology, c-DNA immunisation in combination with hybridoma technology and establishment of scFv phage display libraries that would allow high throughput screening of binders.

The antibodies during this programme have been characterised with respect to detailed epitope specificity by pep-scan analysis peptide phage display libraries.

It is the detection of biomedical markers of stroke such as those mentioned above that facilitate the rapid diagnosis essential for the introduction of the appropriate thrombolytic therapy.

The monoclonal antibodies will act as the capture biocomponents for the BMBDs and have been supplied for impedimetric investigations into binding events and any other associated processes, to obtain affinity binding and signal transduction processing for electrode platform assemblies.

Antibodies have been immobilised at the varying sensor transducer platforms via a range of approaches including co-entrapment within in-situ polymerised conducting polymers such as polyaniline.

Initially, novel microelectrode arrays were formed at screen-printed carbon working electrodes insulated by poly (*o*-phenylenediamine) thin-films. Acoustic cavitation processes ablate micron dimension pores across the insulated working electrode surfaces,

enhancing the sensors' electrochemical characteristics. Site-specific immobilisation of biomolecules is known to occur within and at the surface of conducting polymers during their electrogeneration (Bartlett & Cooper, 1993; Cosnier, 1999; Sargent & Sadik, 1999; Sargent *et al*, 1999; Korri-Youssoufi *et al*, 2001; Richad *et al*, 2001). As polyaniline is deposited from a solution of monomer containing the biomolecule at the microelectrode arrays, 'mushroom-like' protrusions are formed from the sub-micron areas of the underlying carbon substrate simultaneously immobilising the bio-receptor via entrapment. Non-covalent affinity immobilisation procedures will also be investigated to provide a spatially controlled, orientation-specific alternative providing a uniformed monolayer of bioreceptor at the PANI protrusion surface (Katz *et al*, 1998; Cosnier *et al*, 1998; Turkova, 1999; Cosnier *et al*, 1999; Cui *et al*, 2003; Ionescu *et al*, 2004; Cosnier, 2005; Hleli *et al*, 2005; Zacco *et al*, 2005). Both sensor platform approaches will be interrogated, compared, evaluated and reported.

Complex plane impedance analyses will be used to relate the differing redox states of the polymer to possible charge transfer / and or association mechanisms between the antibody, antigen and the polymer at the electrode sensor assemblies.

Data obtained will examine how these factors may affect and thus be used to modulate the impedance of the polymer-modified electrodes, leading to the quantification of antigen analyte in test solution.

The fundamental components of the design include the sensor array with the liquid and the (bio)-chemical interface, the digital interface where the signal processing is performed and the user interface for the control and interpretation of the sensing mechanism. The final integrated microsystem with microfluidic sample handling and labelless biosensor detection have, within this programme, been subjected to reliability and failure analysis studies and tested in standard and 'real' samples. The overall objective of the project is to develop a viable labelless immunosensor for the quantification of a range of biochemical markers for TIA.

The results reported herein so far have at the time of writing led to the publication of two papers detailing the fabrication of novel sensor electrode platforms and their potential widespread applicability as biosensors. AC impedance work has also produced a separate AC impedance interrogation protocol. Both papers are included in the Appendix of this thesis. Further publications will follow the completion of the project and publication of this thesis.

Chapter 2: Introduction and literature review.

2.0 Introduction and literature review.

2.1 Introduction to biosensors.

A Biosensor can be defined as “a device incorporating a biological sensing element coupled in close proximity with a transducer”, (Turner *et al*, 1987; Law *et al*, 2002), and is depicted in Figure 2.1.

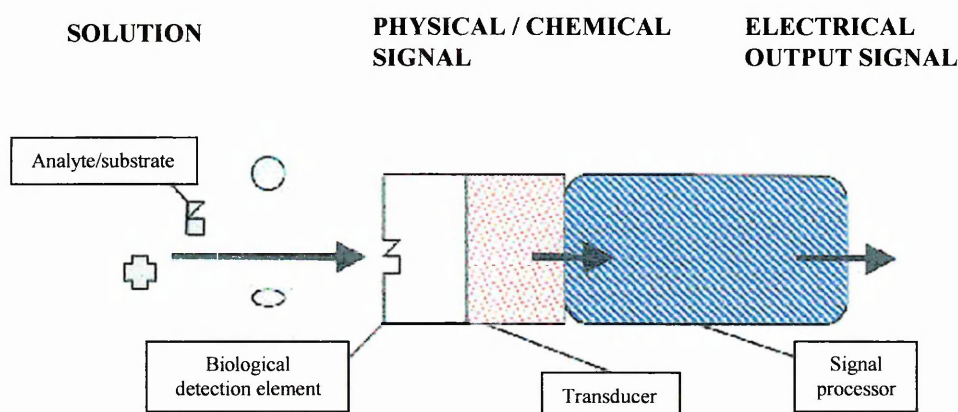


Fig 2.1: Schematic Layout of a Biosensor.

Biosensors are generally concerned with sensing and measuring particular chemical species, which may or may not be biological in nature themselves. These substances are referred to as “analytes”. In a basic biosensor, the analyte and any co-reactants diffuse through an outer protective membrane, which may also have the function of selectively eliminating any interfering species. A reaction then takes place with the biological material and the product is subsequently measured. In some cases, this product is passed through a second, selectively permeable membrane and is detected at the transducer. The signal-processing equipment then converts the transducer signal into a suitable (e.g. digital) display.

The recognition entity is biologically sensitive and can be anything from an enzyme or multienzyme system, to an antigen or antibody. In simpler terms, it is a molecule that is capable of either bonding to the analyte / substrate or catalysing a reaction that involves

the analyte / substrate. This entity is immobilised to the transducer by a suitable method such as covalent attachment or physical entrapment.

The transducer can also take a number of different forms, but basically converts an observed change (physical or chemical) into a measurable signal, (usually electronic) whose magnitude is proportional to the concentration of a specific chemical or set of chemicals. The biosensor combines the specificity and sensitivity of biological systems with the computing power of the microprocessor (Eggins, 1996(a)).

Biosensors have proved to be very useful for a number of medical and industrial applications. The main commercial market for biosensors in the health-care sector has been for the monitoring of blood glucose levels in diabetics. The sensor may be in the form of a pen-shaped dipstick device or a larger hand-held device. Such biosensors are very successful as the patient can use them at home as opposed to a visit to a doctor or clinic for monitoring.

2.2 Biosensors and Immunosensors.

A biosensor is an analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. The biological element can be a layer of molecules qualified for bio-recognition, such as enzymes, receptors, peptides, single-stranded DNA or even living cells (Luppa *et al*, 2001). If antibodies or antibody fragments are applied as biological elements, the device is termed an Immunosensor (Gizeli & Lowe, 1996). Compared to conventional analytical instruments, biosensors are characterised by an integrated structure of these components. Many devices are connected with a flow-through cell, enabling a flow-injection analysis (FIA) mode of operation. Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems.

There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor frequently uses enzymes as the biological component. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies. Recently antibody-related substances, for example, aptamers or molecular imprinted polymers, have also been exploited for biomolecular recognition (Yano & Karube, 1999; Brody & Gold, 2000; Dickert *et al*, 2000; Luzi *et al*, 2003; McCauley *et al*, 2003; Huang *et al*, 2004; Kawde *et al*, 2005; Mahony *et al*, 2005; Bossi *et al*, 2006; Cai *et al*, 2006; Kim *et al*, 2006; Schlecht *et al*, 2006; Wei *et al*, 2006; Yoshida *et al*, 2006).

Biosensors are extensively used as diagnostic tools, and often for point-of-care testing. Probably the most successful commercialisation of biosensors to date is the Medisense glucose pen device, providing discrete self-testing of blood glucose levels (Turner *et al*, 1999).

2.2.1 Immunosensor Principles.

A general immunosensor design is depicted in Figure 2.2. There are four types of immunosensor detection devices: electrochemical (potentiometric, amperometric or conductimetric / capacitive), optical, microgravimetric, and thermometric. All types of device can either be run as direct (non-labelled) or as indirect (labelled) immunosensors. The direct sensors are able to detect the physical changes during the immune complex formation, whereas the latter sensors use signal-generating labels, which allow for more sensitive and versatile detection modes.

Although indirect immunosensors are highly sensitive due to the analytical characteristics of the label applied; the concept of a direct sensor device is still fascinating and represents a true alternative development to immunoassay systems. The potential simplicity of the device holds multiple advantages, making immunosensors an emerging technology.

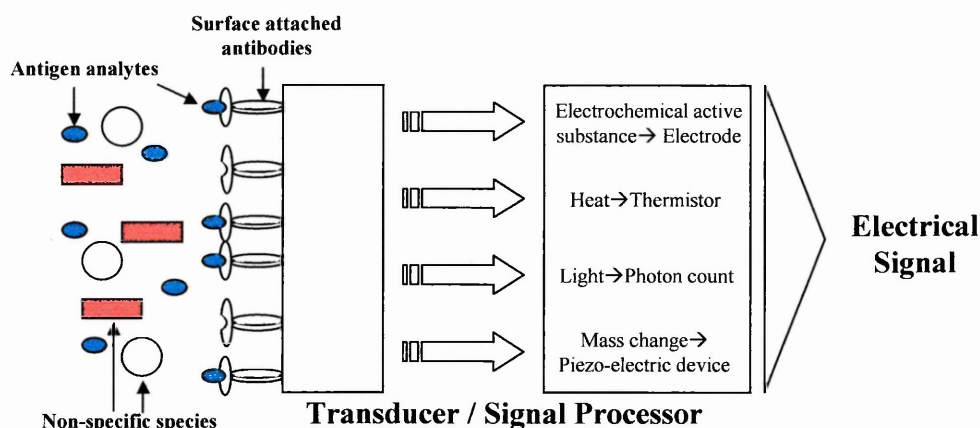


Fig 2.2: Schematic of general immuosensor design depicting the intimate integration of immunological recognition at the solid-state surface and the signal transduction.

2.2.2 Immunosensors – Principles and applications to clinical chemistry.

Immunosensors are affinity-based biosensor solid-state devices in which the immunochemical reaction is coupled to a transducer (Luppa *et al*, 2001). The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex. This is similar to the immunoassay methodology. Immunosensors can be categorised based on the detection principle applied. In contrast to immunoassay, modern transducer technology enables the label-free detection and quantification of the immune complex.

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and immunosensors on solid-state interfaces. The underlying fundamental concept of these analytical methods as ligand assays is based upon the observation of the product(s) of the ligand-binding reaction between the target analyte and a highly specific binding reagent (Ekins, 1999).

The immunosensor is now considered as a major development in the immunochemical field. However, to date, there are only a few applications of immunosensors in clinical diagnostics.

The reasons for this are, in part, due to unresolved fundamental questions relating to immobilisation, orientation and specific properties of the antibodies or antibody-related reagents on the transducer surface.

2.3 Antibodies as bioaffinity interface for both immunoassays and immunosensors.

It should first be clarified that the specificity for the measurement of analyte(s) in all immunosensor systems, as in the case of immunoassays, is dependent on the application of binding molecules. This pivotal feature is shared by both technologies. New developments in protein engineering for immunoglobulins (antibody fragments, chimeric antibodies, etc.) or in substituting antibodies by alternative binding components (e.g. aptamers) or structures (molecular imprinting) will, therefore, be applicable to either technology.

In particular, the possibilities in antibody engineering will enable changes in the affinity and fine specificity of antibodies, (Soderland *et al*, 1999; Borrebaeck, 2000), as well as the expression of fragments as fusion proteins coupled to reporter molecules (Casadei *et al*, 1990).

The regeneration of the binding sites of the antibodies, bound to the immunosensor surface, requires stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilised antibodies and insidious drift problems.

The highly specific recognition of the antigen is a kinetic controlled process due to the complexity of the conformational changes in the fragment antigen-binding (F(ab)) portion of the antibody upon binding of the antigen.

2.3.1 Antibody Structure & Function.

The basic principle of immunochemical techniques is that a specific antibody will combine with its specific antigen to yield an antibody-antigen complex. Antibodies are immune system-related proteins called immunoglobulins. As shown in Figure 2.3, each antibody consists of four polypeptides, consisting of two heavy chains and two light chains, joined to form a "Y" shaped molecule.

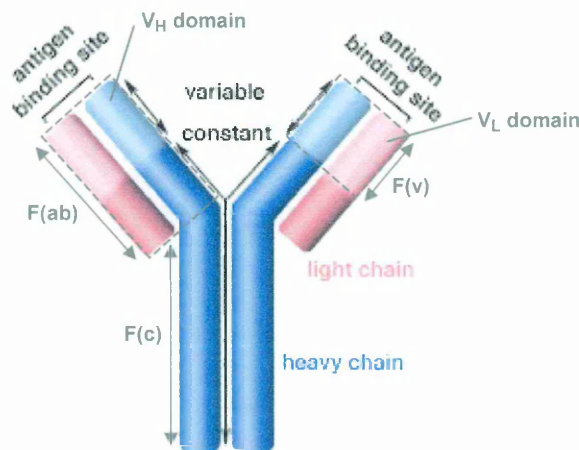


Fig 2.3: General antibody structure showing four polypeptides two heavy chains and two light chains joined to form a "Y" shaped molecule (adapted from Alberts *et al*, 2003).

The amino acid sequence in the tips of the "Y" varies greatly among different antibodies. This variable region, composed of 110-130 amino acids, give the antibody its specificity for binding antigen. The variable region includes the ends of the light and heavy chains. Treating the antibody with a protease can cleave this region, producing F_(ab) or fragment antigen binding that include the variable ends of an antibody.

The constant region, F_(c), determines the mechanism used to destroy the antigen. Antibodies are divided into five major classes, IgM, IgG, IgA, IgD and IgE, based on their constant region structure and immune function.

An antibody is defined as “an immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible animal” (Killard *et al*, 1995). Antibodies are produced in response to the invasion of foreign molecules in the body.

They exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each Y unit contains two identical copies of a “heavy” chain, and two identical copies of a “light” chain, named as such by their relative molecular weights.

Antibodies can be divided into the five classes based on the number of Y units and the type of heavy chain. The light chains of any antibody can be classified as either a kappa (k) or lambda (l) type (a description of molecular characteristics of the polypeptide); however, the heavy chain determines the subclass of each antibody.

Heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as gamma, mu, alpha, delta, and epsilon, respectively. The most commonly used antibody is IgG, which can be cleaved into three parts, two F(ab) regions and one F(c), by the proteolytic enzyme papain, or into two parts, one F(ab')₂ (both F(ab) regions conjoined at the hinge region) and one F(c) by the proteolytic enzyme pepsin. The F(ab) regions comprise the “arms” of the antibody, which are critical for antigen binding. The F(c) region comprises the “tail” of the antibody and plays a role in immune response, as well as serving as a useful “handle” for manipulating the antibody during some immunochemical procedures. The number of F(ab) regions on the antibody, corresponds with its subclass, and determines the “valency” of the antibody (loosely stated, the number of “arms” with which the antibody may bind its antigen).

The two F(v) variable domains of the heavy and light chain together form the binding site. Antibodies vary in sequence and shape in this domain and more precisely, in the loops that contact the antigen. Immediately adjacent to them are the constant domains. Together with the variable domains, they form the F(ab) fragment. The F(ab) fragment is *not* the smallest antigen-binding fragment; this is the F(v) fragment. This fragment contains all residues necessary for antigen recognition. The F(ab) fragment can be obtained by proteolysis, while this is close to impossible for the F(v) fragment.

The bottom part of the antibody is the F(c) fragment, which activates complement F(c) receptors and / or binds to these F(c) receptors. It is not needed for antigen binding, but of

course for physiological activity of the antibody. This part is glycosylated (in the CH₂ domains), and this glycosylation is important for maintaining the structure and thus activity. It is worth noting that glycosylation is not needed for antigen binding.

An antibody has two obvious binding sites for its antigen. This is for a very good reason. Usually the antibody is supposed to dock to a virus or a cell - for example a microbe. The binding to a surface is much tighter with two binding sites than with one. In order to dock to a surface it is of great advantage that the two binding sites are connected via a flexible hinge to the constant region.

Hydrogen bonds stabilize the antibody-antigen interaction. In addition to hydrogen bonds, other weak interactions such as Van der Waal's forces, hydrophobic interactions and electrostatic forces improve the binding specificity between antibody and antigen. These interactions occur over large and sometimes discontinuous regions of the molecules so improving binding affinity.

2.3.2 Antibody-Antigen interactions.

The bonding between antigens and antibodies is dependent on hydrogen bonds, hydrophobic bonds, electrostatic forces, and Van der Waal's forces. These are all bonds of a weak, non-covalent nature, yet some of the associations between antigen and antibody can be quite strong.

Like antibodies, antigens can be multivalent, either through multiple copies of the same epitope, or through the presence of multiple epitopes, which are recognized by multiple antibodies. Interactions involving multivalency can produce more stabilized complexes; however multivalency can also result in steric difficulties, thus reducing the possibility for binding. All antigen-antibody binding is reversible, however, and follows the basic thermodynamic principles of any reversible bimolecular interaction.

Equation 2.1

$$KA = [Ab-Ag]/[Ab]*[Ag]$$

The principles are given by Equation 2.1 where KA is the affinity constant (mol^{-1}), [Ab] and [Ag] are the molar concentrations (mol^{-1}) of unoccupied binding sites on the antibody or antigen respectively, and [Ab-Ag] is the molar concentration of the antibody-antigen complex (mol^{-1}).

The time taken to reach equilibrium is dependent on the rate of diffusion and the affinity of the antibody for the antigen, and can vary widely. The affinity constant for antibody-antigen binding can span a wide range, extending from below 10^5 mol^{-1} to above 10^{12} mol^{-1} (Kelley & O'Connell, 1993; Malmborg & Borrebaeck, 1995; Nieba *et al*, 1996; Ober *et al*, 2001; Gonzales *et al*, 2002; Juraj *et al*, 2003). Affinity constants can be affected by temperature, pH and solvent species. Affinity constants can be determined for monoclonal antibodies, but not for polyclonal antibodies, since multiple bondings take place between polyclonal antibodies and their antigens (Eggins, 1996(b)). Avidity is a measure of the overall stability of the antibody-antigen complex. It is controlled by three major factors; the affinity of the antibody for the epitope, the valency of the antigen and antibody, and the structural arrangement of the interacting parts.

Immunochemical techniques capitalize upon the extreme specificity, at the molecular level, of each immunoglobulin for its antigen, even in the presence of high levels of contaminating molecules. The multivalency of most antigens and antibodies enables them to interact to form a precipitate. Examples of experimental applications, which use antibodies, are Western Blot, Immunohistochemistry and Immunocytochemistry, Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoprecipitation.

2.4 Stroke, Transient Ischemic Attack and the Biochemical Markers for Brain Disease.

Stroke or “brain attack” is clinically defined as a rapidly developing syndrome of vascular origin that manifests itself in focal loss of cerebral function. In more severe situations, the loss of cerebral function is global. A stroke occurs when the blood supply to the part of the brain is suddenly interrupted (ischemic) or when a blood vessel in the brain bursts, spilling blood into the spaces surrounding the brain cells (hemorrhagic). The symptoms of stroke are relatively easy to spot: sudden numbness or weakness, especially on one side of the body; sudden confusion or trouble speaking or understanding speech; sudden trouble seeing in one or both eyes; sudden trouble walking; dizziness or loss of balance or coordination (National Institute of Neurological Disorders and Stroke, 2001). Stroke is a manifestation of vascular injury to the brain, which is commonly secondary to atherosclerosis or hypertension. Stroke is the most common devastating neurological disease, the second most common cause of neurological disability (Adamson *et al*, 2004) and the third leading cause of death in the United Kingdom after heart disease and all cancers (Wolfe *et al*, 1996; Office of National Statistics Health Statistics, 2001). Despite recent progress understanding stroke mechanisms, stroke management is still not optimal for a number of reasons.

The importance of promptly diagnosing stroke after symptoms appear cannot be overstated. Delays in diagnosis and medical intervention beyond three hours after stroke onset may contribute to clinical deterioration and disability. An early diagnosis enables doctors to more effectively choose the emergency intervention such as anti-platelet and / or neuroprotective therapy and also facilitates better prognoses of disease outcome. Successful treatment of stroke requires rapid state diagnosis. The delay in achieving an accurate and certain diagnosis wastes the limited amount of time available in which the brain can respond to reperfusion and this significantly increases the risk of haemorrhage even after most of the permanent injury has occurred (National Institute of Neurological Disorders and Stroke, 1995; Kwiatkowski *et al*, 1999; Marler *et al*, 1999; Marler *et al*, 2000).

Unfortunately, however, many people who have a stroke either fail to seek immediate medical care - or suffer from delays in medical care even in countries where stroke care is advanced, such as the United States and Europe (Alberts *et al*, 2000). Several clinical criteria can be employed to diagnose whether a patient is having a stroke, however, even this does not always allow one to differentiate the episode from other disorders, such as epilepsy, syncope and migraine (Toole, 1999). Moreover, progressing stroke is only partially predictable based on the clinical and neuroimaging data that is currently available to neurologists.

TIA is a short-lived episode of focal neurological deficit which often precedes the cerebral infarction of a stroke. It occurs when the blood supply to part of the brain is briefly interrupted, and is typically accompanied by permanent brain damage - albeit normally less severely than results from a stroke. TIA symptoms, which usually occur suddenly, are similar to those of stroke but do not last for as long. Most symptoms of a TIA disappear within an hour, although they may persist for up to 24 hours.

Symptoms can include: numbness or weakness in the face, arm or leg, especially on one side of the body; confusion or difficulty in talking or understanding speech; trouble seeing in one or both eyes; and difficulty with walking, dizziness, or loss of balance and coordination (National Institute of Neurological Disorders and Stroke). Patients who have suffered a TIA have 9.5 times greater risk of having a future stroke than those who have not had a TIA and about one third of patients who suffer a TIA will typically have an acute stroke in the future (Office of National Statistics Health Statistics, 2001). However, because symptoms of TIA are short term, many patients do not recognise the event as a TIA and fail to perceive the event as warning of a potentially impending stroke.

TIA is therefore a very interesting aspect of investigation for the scientist in terms of an obvious need for identifying the underlying systemic changes in the brain and spinal cord throughout the TIA. A precise understanding also enables the development of varying diagnostics that permit and validate the effective intervention of the appropriate thrombolytic therapy so as to prevent subsequent damage. This can significantly increase

the recovery of a patient enabling little or no disability provided there is the close monitoring of patients identified as high risk (National Institute of Neurological Disorders and Stroke, 1995; Kwiatkowski *et al*, 1999; Marler *et al*, 1999; Marler *et al*, 2000).

Standard treatments to reduce the risk of future stroke include the use of antiplatelet agents - particularly aspirin. People with atrial fibrillation (irregular beating of the heart) may be prescribed anticoagulants. The most important treatable factors linked to TIAs and stroke are high blood pressure, cigarette smoking, heart disease, carotid artery disease, diabetes and heavy consumption of alcohol. Lifestyle changes can often be implemented to reduce these factors. However, it is necessary to diagnose the TIA as a warning sign of impending stroke before such treatments can be administered. Therefore, a laboratory blood test to detect TIA or stroke, or the risk of suffering a TIA or stroke in the future, would be of tremendous benefit.

Stroke can be categorised into two broad types “ischemic stroke” and “hemorrhagic stroke”. Additionally a patient may experience transient ischemic attacks, which are in turn a high risk factor for the future development of a more severe episode.

Ischemic stroke encompasses thrombotic, embolic, lacunar and hypoperfusion types of strokes. Thrombi are occlusions of arteries created in situ within the brain, while emboli are occlusions caused by material from a distant source, such as the heart and major vessels, often dislodged due to myocardial infarct or atrial fibrillation. Less frequently, thrombi may also result from vascular inflammation due to disorders such as meningitis.

Thrombi or emboli can result from atherosclerosis or other disorders, for example arteritis, and lead to physical obstruction of arterial blood supply to the brain. Lacunar stroke refers to an infarct within non-cortical regions of the brain. Hypoperfusion embodies diffuse injury caused by non-localised cerebral injury, typically caused by myocardial infarction and arrhythmia.

The onset of ischemic stroke is often abrupt, and can become an “evolving stroke” manifested by neurological deficits that worsen over a 24-48 hour period. In evolving stroke, “stroke-associated symptom(s)” commonly include unilateral neurological dysfunction, which extends progressively, without producing headache or fever. Evolving stroke may also become a “completed stroke”, in which symptoms develop rapidly and are maximal within a few minutes.

Hemorrhagic stroke is caused by intracerebral or subarachnoid hemorrhage, i.e., bleeding into brain tissue, following blood vessel rupture within the brain. Intracerebral and subarachnoid hemorrhages are subsets of a broader category of hemorrhage referred to as intracranial hemorrhage.

Intracerebral hemorrhage is typically due to chronic hypertension, and as a resulting rupture of an arteriosclerotic vessel. Stroke-associated symptoms of intracerebral hemorrhage are abrupt, with the onset of headache and steadily increasing neurological deficits. Nausea, vomiting, delirium, seizures and loss of consciousness are additional common stroke-associated symptoms.

In contrast, most subarachnoid hemorrhage is caused by head trauma or aneurysm rupture, which is accompanied by high-pressure blood release, which also causes direct cellular trauma. Prior to rupture, aneurysms may be asymptomatic, or occasionally associated with tension or migraine headaches. However, headache typically becomes acute and severe upon rupture, and may be accompanied by varying degrees of neurological deficit, vomiting, dizziness and altered pulse and respiratory rates.

TIAs have a sudden onset and brief duration, typically around 2-30 minutes. Most TIAs are due to emboli from atherosclerotic plaques, often originating in the arteries of the neck, and can result from brief interruptions of blood flow. The symptoms of TIAs are identical to those of stroke, but are only transient. Concomitant with underlying risk factors, patients experiencing TIAs are at a markedly increased risk for stroke.

Current diagnostic methods for stroke include costly and time-consuming procedures such as noncontrast-computed tomography (CT) scan, electrocardiogram, magnetic resonance imaging (MRI) and angiography. Determining the immediate cause of stroke and differentiating ischemic from hemorrhagic stroke is difficult.

CT scans can detect parenchymal bleeding greater than 1cm and also 95% of all subarachnoid hemorrhages. CT scans often cannot detect ischemic strokes until 6 hours from onset, depending on the infarct size. MRI may be more effective than CT scans in the early detection of ischemic stroke, but is less accurate at differentiating ischemic from hemorrhagic stroke, and is not widely available.

An electrocardiogram (ECG) can be used in certain circumstances to identify a cardiac cause of stroke. Angiography is a definitive test to identify stenosis or occlusion of large and small cranial blood vessels and can locate the cause of subarachnoid hemorrhages, define aneurysms and detect cerebral vasospasm. It is, however, an invasive procedure that is also limited by cost and availability. Coagulation studies can also be used to rule out a coagulation disorder (coagulopathy) as a cause of hemorrhagic stroke (BBC Health; Royal College of Physicians of London, 2004; The Stroke Association).

Immediate diagnosis and care of a patient-experiencing stroke can be critical. For example, tissue plasminogen activator (t-PA) given within three hours of symptom onset in ischemic stroke is beneficial for selected acute stroke patients. Alternatively, ischemic stroke patients may benefit from anticoagulants (e.g. heparin) if they are not candidates for t-PA therapy. In contrast, thrombolytics and anticoagulants are strongly contraindicated in hemorrhagic stroke (National Institute of Neurological Disorders and Stroke, 1995; Kwiatkowski *et al*, 1999). Thus, early differentiation of ischemic events from hemorrhagic events is imperative. Moreover, delays in the confirmation of stroke diagnosis and the identification of stroke type limit the number of patients that may benefit from early intervention therapy (Marler *et al*, 1999; Marler *et al*, 2000).

Local intra-arterial thrombolysis of acutely occluded blood vessels has potential for revascularisation of ischemic cerebral territories. Rapid diagnosis and initiation of the therapy are crucial in stroke patients to reduce brain tissue necrosis. Therefore, patients presenting with acute neurological deterioration undergo a full neurological evaluation and a battery of blood tests. A cerebral angiogram is performed to map out and identify the region in the brain suffering from the occlusion, any collateral circulation and the size and the extent of the occlusion. If the patient “passes” these tests, then thrombolytic therapy can be initiated.

Thrombolytic therapy involves directing a microcatheter to the site of the occlusion and releasing thrombolytic agents directly into the clot. The treatment is highly variable; i.e., differing agents, infusion rates and dosages are used at the attending physician’s discretion.

Clots in the vascular system can be classified in one of two ways. Either they are calcified (hard) or are fibrous (soft). This simple classification obscures the typical situation, as in most cases a clot will have some of both character. In particular, the older the clot the more calcified it becomes. In any case, thrombolytics are effective in dissolving the fibrous part of the clot.

The optimal treatment plan for thrombolytic therapy has yet to be determined. One particular thrombolytic agent is rt-PA (recombinant tissue plasminogen activator). Currently, the most widely recommended dosage is 0.9mg/Kg of body weight (National Institute of Neurological Disorders and Stroke, 1995). Once injected, the thrombolytic agent acts upon the circulating plasma proenzyme, plasminogen, cleaving it to form the enzyme, plasmin. Plasmin binds directly to cross-linked fibrin (and also fibrinogen and non-crosslinked fibrin) and breaks it down. Only cross-linked fibrin (which forms the “web” of soft clots) due to its covalent bonding side-to-side and end-to-end of the fibrin chains, produce the degradation fragment called D-dimer.

There are currently no rapid diagnostic methods that can differentiate and identify a TIA, or predict delayed neurological deficits which are often detected at a time after the onset concurrent with the presentation of symptoms.

Accordingly, there is a present need in the area for a rapid, sensitive and specific diagnostic assay for stroke and TIA that can differentiate the stroke type and identify those individuals at risk for delayed neurological deficits. Such a diagnostic assay would greatly increase the number of patients that can receive beneficial stroke treatment and therapy, and reduce the costs associated with incorrect stroke diagnosis. The main focus of recent research relates to the identification and use of diagnostic markers for stroke and cerebral injury.

The term “marker” refers to proteins or polypeptides used as targets for screening test samples obtained from subjects and include any fragments thereof, in particular, immunologically detectable fragments. Proteins that are released by cells of the central nervous system, which become damaged during a cerebral attack, could become degraded or cleaved into such fragments. Additionally, certain markers are synthesised in an inactive form, which may be subsequently activated, e.g. by proteolysis.

The term “specific marker of cerebral injury” refers to proteins or polypeptides that are associated with brain tissue and neural cells, and which can be correlated with cerebral injury, but are not correlated with other types of injury. Such specific markers of cerebral injury include adenylate kinase, brain-derived neurotrophic factor, calbindin-D, creatine kinase-BB, glial fibrillary acidic protein, lactate dehydrogenase, myelin basic protein, neural cell adhesion molecule, neuron-specific enolase, neurotrophin-3, proteolipid protein, S-100 [β], thrombomodulin, protein kinase C gamma and similar.

The term “non-specific marker of cerebral injury” refers to proteins or polypeptides that are elevated in the event of cerebral injury, but may also be elevated due to non-cerebral events. Such markers may be typically proteins related to coagulation and hemostasis or acute phase reactants. Factors in the activation of platelets and the

mechanisms of coagulation include $[\beta]$ -thromboglobulin, D-dimer, fibrinopeptide A, plasmin- $[\alpha]$ -2 antiplasmin complex, platelet factor 4, prothrombin factor 1+2, thrombin-antithrombin III complex, tissue factor and von Willebrand factor.

Other non-specific markers include: adrenomedullin, cardiac troponin I, head activator, hemoglobin- $[\alpha]$ 2 chain, caspase-3, vascular endothelial growth factor (VEGF), one or more endothelins (e.g endothelin-1, endothelin-2, endothelin-3), interleukin-8, A-type natriuretic peptide, B-type natriuretic peptide and C-type natriuretic peptide.

The term “acute phase reactants” refers to proteins whose concentrations are elevated in response to stressful or inflammatory states that occur during various insults that include infection, injury, surgery, trauma and tissue necrosis. Acute phase reactant expression and serum concentration elevations are not specific for the type of process, but rather as a part of the homeostatic response to the insult. All acute phase reactants are produced in response to insult, and it is possible that this is in order to handle extensive injury, even though some components may not be needed. Examples of classical acute phase proteins include C-reactive protein, ceruloplasmin, fibrinogen, $[\alpha]$ 1-acid glycoprotein, $[\alpha]$ 1-antitrypsin and haptoglobin.

Various cytokines and related molecules such as insulin-like growth factor-1, interleukin-1 $[\beta]$, interleukin-1 receptor antagonist, interleukin-6, transforming growth factor $[\beta]$, and tumor necrosis factor $[\alpha]$ are components of the inflammatory response that are also intimately involved in the acute phase reaction. Such cytokines are released into the bloodstream from the site of injury and are capable of inducing expression of other acute phase proteins. Other acute phase reactants include E-selectin, intercellular adhesion molecule-1, matrix metalloproteinases (e.g., matrix metalloproteinase 9 (MMP-9)), monocyte chemotactic protein-1 and vascular cell adhesion molecule.

Stroke is a pathological condition with acute onset that is caused by the occlusion or rupture of a vessel supplying blood, and thus oxygen and nutrients, to the brain. The immediate area of injury is referred to as the “core”, which contains brain cells that have

died as a result of ischemia or physical damage. The “penumbra” is composed of brain cells that are neurologically or chemically connected to cells in the core. Cells within the penumbra are injured, but still have the ability to completely recover following removal of the insult caused during stroke. However, as ischemia or bleeding from hemorrhage continues, the core of dead cells can expand from the site of insult, resulting in a concurrent expansion of cells in the penumbra. The initial volume and rate of core expansion is related to the severity of the stroke and, in most cases, neurological outcome.

The brain contains two major types of cells; neurons and glial cells. Neurons are the most important cells in the brain, and are responsible for maintaining communication within the brain via electrical and chemical signalling. Glial cells function mainly as structural components of the brain and they are approximately ten times more abundant than neurons.

Glial cells of the Central Nervous System (CNS) are astrocytes and oligodendrocytes. Astrocytes are the major interstitial cells of the brain, and they extend cellular processes that are intertwined with and surround neurons, isolating them from other neurons. Astrocytes can also form “end feet” at the end of their processes that surround capillaries. Oligodendrocytes are cells that form myelin sheathes around axons in the CNS. Each oligodendrocyte has the ability to ensheath up to 50 axons (signal carrying appendages of the nerve cells). Schwann cells are glial cells of the Peripheral Nervous System (PNS). Schwann cells form myelin sheathes around axons in the periphery, and each Schwann cell ensheathes a single axon.

Cell death during stroke occurs as a result of ischemia or physical damage to the cells of the CNS. During ischemic stroke, an infarct occurs, greatly reducing or stopping blood flow beyond the site of infarction. The zone immediately beyond the infarct soon lacks suitable blood concentrations of the nutrients essential for cell survival. Cells that lack nutrients essential for maintenance of important functions like metabolism soon perish.

Hemorrhagic stroke can induce cell death by direct trauma, elevation in intracranial pressure, and the release of damaging biochemical substances in blood. When cells die, they release their cytosolic contents into the extracellular milieu.

The barrier action of tight junctions between the capillary endothelial cells of the central nervous system is referred to as the “blood-brain barrier”. This barrier is normally impermeable to proteins and other molecules, both large and small. In other tissues such as skeletal, cardiac and smooth muscle, the junctions between endothelial cells are loose enough to allow passage of most molecules, but not proteins.

Substances that are secreted by the neurons and glial cells (intracellular brain compartment) of the Central nervous system (CNS) can freely pass into the extracellular milieu (extracellular brain compartment).

Likewise, substances from the extracellular brain compartment can pass into the intracellular brain compartment. The passage of substances between the intracellular and extracellular brain compartments are restricted by the normal cellular mechanisms that regulate substance entry and exit. Substances that are found in the extracellular brain compartment also are able to pass freely into the cerebrospinal fluid, and vice versa; this movement is controlled by diffusion.

The movement of substances between the vasculature and the CNS is restricted by the blood-brain barrier. This restriction can be circumvented by facilitated transport mechanisms in the endothelial cells that transport, among other substances, nutrients like glucose and amino acids across the barrier for consumption by the cells of the CNS. Furthermore, lipid-soluble drugs or narcotics can freely diffuse across the blood-brain barrier.

Depending upon their size, specific markers of cerebral injury that are released from injured brain cells during stroke or other neuropathies will only be found in peripheral

blood when CNS injury is coupled with or followed by an increase in the permeability of the blood-brain barrier. This is particularly true of larger molecules.

Smaller molecules may appear in the peripheral blood as a result of passive diffusion, active transport, or an increase in the permeability of the blood-brain barrier. Increases in blood-brain barrier permeability can arise as a result of physical disruption in cases such as tumor invasion and extravasation or vascular rupture, or as a result of endothelial cell death due to ischemia. During stroke, the blood-brain barrier is compromised by endothelial cell death, and any cytosolic components of dead cells that are present within the local extracellular milieu can enter the bloodstream. Therefore, specific markers of cerebral injury may also be found in the blood or in blood components such as serum and plasma, as well as the CSF of a patient experiencing stroke or TIAs. Furthermore, clearance of the obstructing object in ischemic stroke can cause injury from oxidative insult during reperfusion, and patients with ischemic stroke can sometimes experience hemorrhagic transformation as a result of reperfusion or thrombolytic therapy.

Additionally, injury can be caused by vasospasm, which is a focal or diffuse narrowing of the large capacity arteries at the base of the brain following hemorrhage. The increase in blood-brain barrier permeability is related to the insult severity, and its integrity is re-established following the resolution of insult.

Specific markers of cerebral injury will only be present in peripheral blood if there has been a sufficient increase in the permeability of the blood-brain barrier that allows these large molecules to diffuse across. In this regard, most specific markers of cerebral injury can be found in cerebrospinal fluid following stroke or any other neuropathy that affects the CNS. Furthermore, many investigations of coagulation or fibrinolysis markers in stroke are performed using cerebrospinal fluid (BBC Health; Royal College of Physicians of London, 2004; The Stroke Association).

Within this thesis, work is focussed towards the labelless and reversible detection of markers for transient ischemic attack and is directed towards the specific markers for

brain injury, namely NSE, S-100 β , Thrombomodulin and GFAP. Accordingly each of these proteins will be discussed in turn.

Enolase is a 78 kDa homo- or heterodimeric cytosolic protein produced from $[\alpha]$, $[\beta]$, and $[\gamma]$ subunits. It catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate in the glycolytic pathway. Enolase can be present as $[\alpha][\alpha]$, $[\beta][\beta]$, $[\alpha][\gamma]$, and $[\gamma][\gamma]$ isoforms. The $[\alpha]$ subunit is found in glial cells and most other tissues, the $[\beta]$ subunit is found in muscle tissue, and the $[\gamma]$ subunit is found mainly in neuronal and neuroendocrine cells (Quinn *et al*, 1994). The $[\gamma][\gamma]$ enolase isoform is most specific for neurons, and is referred to as neuron-specific enolase (NSE). NSE, found predominantly in neurons and neuroendocrine cells, and is also present in platelets and erythrocytes. The normal serum concentration of NSE is $<12.5 \text{ ng ml}^{-1}$ (160 pM). NSE is made up of two subunits; thus, the most feasible immunological assay used to detect NSE concentrations would be one that is directed against one of the subunits. In this case, the $[\gamma]$ subunit would be the ideal choice. However, the $[\gamma]$ subunit alone is not as specific for cerebral tissue as the $[\gamma][\gamma]$ isoform, since a measurement of the $[\gamma]$ subunit alone would detect both the $[\alpha][\gamma]$ and $[\gamma][\gamma]$ isoforms. In this regard, the best immunoassay for NSE would be a two-site assay that could specifically detect the $[\gamma][\gamma]$ isoform. Serum NSE is reportedly elevated after all stroke types, including TIAs, which are cerebral in origin and are thought to predispose an individual to having a more severe stroke at a later date (Isgro *et al*, 1997).

Elevations of NSE in serum can be attributed to cerebral injury due to physical damage or ischemia caused by infarction or cerebral hemorrhage, coupled with increased permeability of the blood brain barrier. The serum concentration of NSE has also been reported to correlate with the extent of damage (infarct volume) and neurological outcome (Martens *et al*, 1998). Additionally, a secondary elevation of serum NSE concentration may be an indicator of delayed neuronal injury resulting from cerebral vasospasm (Laskowitz *et al*, 1998). NSE, which has a biological half-life of 48 hours and is normally detected in serum at an upper limit of 12.5 ng ml^{-1} (160 pM), is elevated after stroke and cerebral injury. Serum NSE is elevated after 4 hours from stroke onset, with

concentrations reaching a maximum between 1-3 days after onset (Missler *et al*, 1997). After the serum concentration reaches its maximum, which can exceed 300 ng ml⁻¹ (3.9 nM), it gradually decreases to normal concentrations over approximately one week.

Because the severity of damage has a direct effect on the release of NSE, it will affect the release kinetics by influencing the length of time that NSE is elevated in the serum. NSE will be present in the serum for a longer period of time as the severity of injury increases. The release of NSE into the serum of patients with head injury follows different kinetics as observed with stroke, with the maximum serum concentration being reached within 1-6 hours after injury, often returning to baseline within 24 hours (Skogseid *et al*, 1992).

NSE is a specific marker for cerebral injury, specifically, injury to neuronal cells due to cell death caused by ischemia or physical damage. Neurons are about 10-fold less abundant in the brain than glial cells, so any cerebral injury coupled with increased permeability of the blood-brain barrier will have to occur in a region that has a significant regional population of neurons to significantly increase the serum NSE concentration. In addition, elevated serum concentrations of NSE can also indicate complications related to cerebral injury after acute myocardial infarction (AMI) and cardiac surgery. Elevations in the serum concentration of NSE correlate with the severity of damage and the neurological outcome of the individual. NSE can be used as a marker of all stroke types, including TIAs. However, NSE cannot be used to differentiate ischemic and hemorrhagic stroke, and it is elevated in the population of individuals having tumours with neuroendocrine features.

S-100 is a 21 kDa homo - or heterodimeric cytosolic Calcium-binding protein produced from [α] and [β] subunits. It is thought to participate in the activation of cellular processes along the Ca²⁺-dependent signal transduction pathway (Bonfrer *et al*, 1998). S-100ao ([α][α] isoform) is found in striated muscles, heart and kidney, S-100a ([α][β] isoform) is found in glial cells, but not in Schwann cells, and S-100b ([β][β] isoform) is found in high concentrations in glial cells and Schwann cells, where it is a major cytosolic component.

The $[\beta]$ subunit is specific to the nervous system, predominantly the CNS, under normal physiological conditions and, in fact, accounts for approximately 96% of the total S-100 protein found in the brain (Jensen *et al*, 1985). In addition, S-100 $[\beta]$ can be found in tumors of neuroendocrine origin, such as gliomas, melanomas, Schwannomas, neurofibromas, and highly differentiated neuroblastomas, like ganglioneuroblastoma and ganglioneuroma (Perrson *et al*, 1987).

The normal serum concentration of S-100 $[\beta]$ is $<0.2 \text{ ng ml}^{-1}$ (19 pM), which is the detection limit of the immunological detection assays used. Serum S-100 $[\beta]$ is elevated after all stroke types, including TIAs. Elevations of S-100 $[\beta]$ in serum can be attributed to cerebral injury due to physical damage or ischemia caused by infarction or cerebral hemorrhage, coupled with increased permeability of the blood-brain barrier. The serum concentration of S-100 $[\beta]$ has also been shown to correlate with the extent of damage (infarct volume) and neurological outcome (Martens *et al*, 1998; Missler *et al*, 1997).

S-100 $[\beta]$ has a biological half-life of 2 hours and is not normally detected in serum, but is elevated after stroke and cerebral injury. Serum S-100 $[\beta]$ is elevated after 4 hours from stroke onset, with concentrations reaching a maximum 2-3 days after onset. After the serum concentration reaches its maximum, which can approach 20 ng ml^{-1} (1.9 mM), it gradually decreases back to normal levels over approximately one week. Because the severity of damage has a direct effect on the release of S-100 $[\beta]$, it will affect the release kinetics by influencing the length of time that S-100 $[\beta]$ is elevated in the serum. S-100 $[\beta]$ will be present in the serum for a longer period of time as the severity of injury increases.

The release of S-100 $[\beta]$ into the serum of patients with head injury seems to follow somewhat similar kinetics as reported with stroke, with the only exception being that serum S-100 $[\beta]$ can be detected within 2.5 hours of onset and the maximum serum concentration is reached approximately 1 day after onset (Woertgen *et al*, 1997). S-100 $[\beta]$ is a specific marker for cerebral injury and specifically, injury to glial cells due to cell death caused by ischemia or physical damage.

Glial cells are about 10 times more abundant in the brain than neurons, so any cerebral injury coupled with increased permeability of the blood-brain barrier will likely produce elevations of serum S-100[β]. Furthermore, elevated serum concentrations of S-100[β] can indicate complications related to cerebral injury after AMI and cardiac surgery. S-100[β] has been virtually undetectable in normal individuals, and elevations in its serum concentration correlate with the severity of damage and the neurological outcome of the individual. S-100[β] can be used as a marker of all stroke types, including TIAs. However, S-100[β] cannot be used to differentiate ischemic and hemorrhagic stroke, and is known to be elevated in the population of individuals having neuroendocrine tumours - usually in advanced stages.

Thrombomodulin (TM) is a 70 kDa single chain integral membrane glycoprotein found on the surface of vascular endothelial cells. TM demonstrates anticoagulant activity by changing the substrate specificity of thrombin. The formation of a 1:1 stoichiometric complex between thrombin and TM changes thrombin function from procoagulant to anticoagulant. This change is facilitated by a change in thrombin substrate specificity that causes thrombin to activate protein C (an inactivator of factor Va and factor VIIIa), but not to cleave fibrinogen or activate other coagulation factors (Davie *et al*, 1991).

The normal serum concentration of TM is 25-60 ng ml⁻¹ (350-850 pM). Current reports describing serum TM concentration alterations following ischemic stroke are mixed, reporting no changes or significant increases (Seki *et al*, 1997). Serum elevations of TM concentration reflect endothelial cell injury and would not indicate coagulation or fibrinolysis activation.

Glial fibrillary acidic protein (GFAP) is a 55 kDa cytosolic protein that is a major structural component of astroglial filaments and is the major intermediate filament protein in astrocytes. GFAP is specific to astrocytes, which are interstitial cells located in the CNS and can be found near the blood-brain barrier. GFAP is not normally detected in serum. Serum GFAP is elevated following ischemic stroke (Niebroj-Dobosz *et al*, 1994).

Current reports investigating serum GFAP elevations associated with stroke are severely limited, and much further investigation is needed to establish GFAP as a serum marker for all stroke types. Most studies investigating GFAP as a stroke marker have been performed using cerebrospinal fluid. Elevations of GFAP in serum can be attributed to cerebral injury due to ischemia, coupled with increased permeability of the blood brain barrier.

No correlation of the serum concentration of GFAP with the extent of damage (infarct volume) or neurological outcome has been established. GFAP is elevated in cerebrospinal fluid of individuals with various neuropathies affecting the CNS, but there are no reports currently available describing the release of GFAP into the serum of individuals with diseases other than stroke (Albrechtsen *et al*, 1985). Serum concentrations of GFAP appear to be elevated soon after the onset of stroke and continuously increase and persist for an amount of time (weeks) suggesting that there may yet be a correlation with the severity of damage. However, GFAP appears to a very specific marker for severe CNS injury and specifically, injury to astrocytes due to cell death caused by ischemia or physical damage.

Within the subsequent results chapters we report the development of electrochemical immunosensors designed for the detection and quantification of such markers of stroke as those already mentioned. The target analyte will be recognised by specific receptor biocomponents immobilised within intimate proximity of the transducer. Monoclonal antibodies specific to the target analyte are selected for their high selectivity and specificity of interaction as the recognition component.

Changes at the electrode - test solution interface will be monitored by electrochemical impedance spectroscopy and related to the quantity of antigen present following exposure of the sensor surface to known standards of antigen analyte. Calibration profiles obtained will enable the development of software for prototype diagnostic devices encompassing a range of antibody-antigen systems relevant to the differentiation of stroke type and the diagnosis of TIA.

Ultimately, it is the aim to develop a rapid, labelless and reversible electrochemical impedance immunosensor capable of detection and quantification of generic markers of stroke (TIA) at concentration levels of clinical significance to address the urgent demand in the medical field.

2.5 Microelectrodes and ultra-sonic ablation.

The immunosensor electrode platform is based on the development of microarray electrode assemblies via polymerisation and sonication. The modification of a planar electrode dramatically alters the electrochemical behaviour of the transducer.

Electrochemistry involves the study of the interrelationship of electrical potentials and/or currents associated with either chemical systems and/or the chemistry they may undergo. Electrochemistry may be used as a fundamental analytical tool as well as having numerous industrial applications. To avoid regurgitation a thorough theoretical overview of the subject can be found elsewhere (Greef *et al* 1985; Bard & Faulkner, 2001). Where appropriate electrochemical theory will be introduced and briefly discussed.

In general conditions planar diffusion occurs to a flat macroscopic electrode surface. This requires diffusion of reactants in a perpendicular direction to the electrode surface. Under these conditions, convection controlled mass transport would be faster than diffusion controlled mass transport and so diffusion controlled mass transport is the rate determining step.

This will produce a diffusion-controlled voltammogram, exhibiting redox-type behaviour as shown in Figure 2.4.

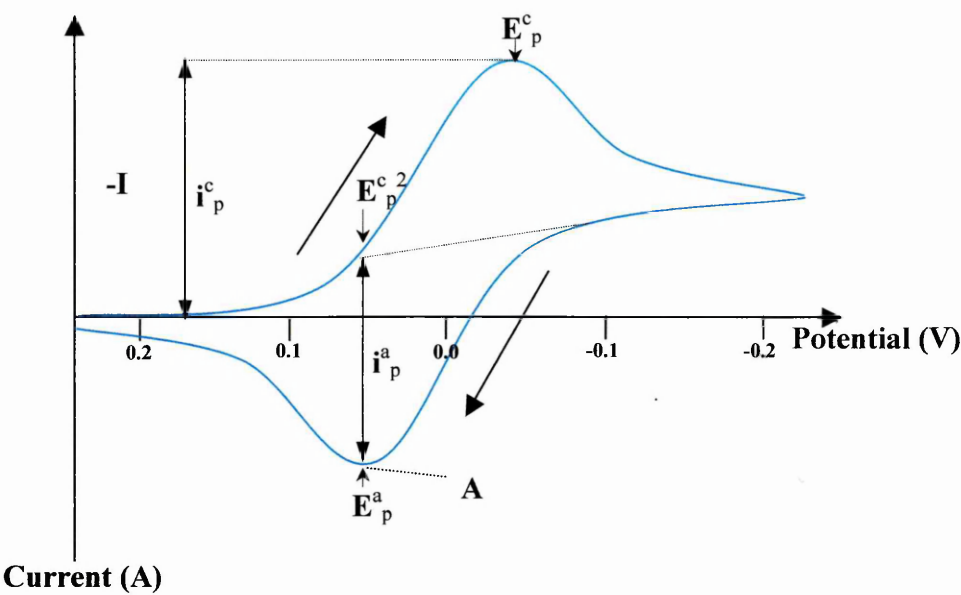


Fig 2.4: An example of a cyclic voltammogram for a planar electrode.

The down turn of measured current observed at point A in Figure 2.4 is a consequence of the diffusion gradients created by planar diffusion, Figure 2.5.

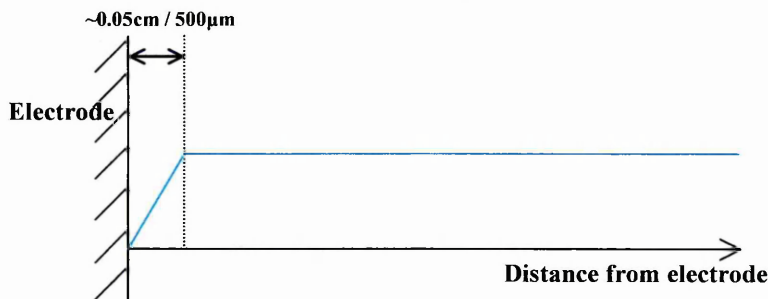


Fig 2.5: Concentration profile gradient approaching working electrode.

It can be assumed that the concentration of analyte decreases as the surface of the electrode is approached (at an approximate distance of 0.05cm) and falls to zero as the electrode surface is reached.

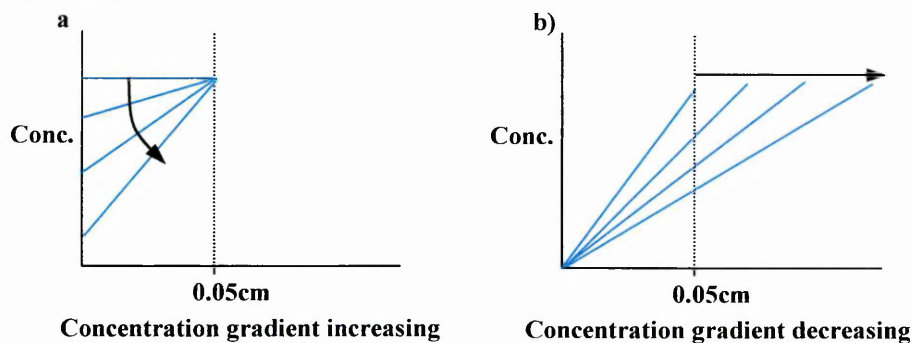


Fig 2.6: Concentration gradient approaching a working electrode during use.

The measured current size is proportional to the rate of delivery of reactant to the surface, and the rate of diffusion is proportional to the concentration gradient. The concentration gradient is as steep as possible when the diffusion layer reaches 0.05cm and the concentration is zero at the electrode surface, (Figure 2.6(a)). At this point, the first peak on Figure 2.4 is reached and current begins to fall as the diffuse layer extends into solution, (Figure 2.6(b)).

If the working electrode is reduced in size to approximately 10µm diameter or below then hemispherical diffusion can provide diffusional mass transport through a 180° half sphere. Consequently, the diffusion rate becomes greater than that of convection controlled mass-transport.

With convectional mass transport no longer the rate-determining step, the electrode is rendered stir-independent, illustrated schematically in Figure 2.7.

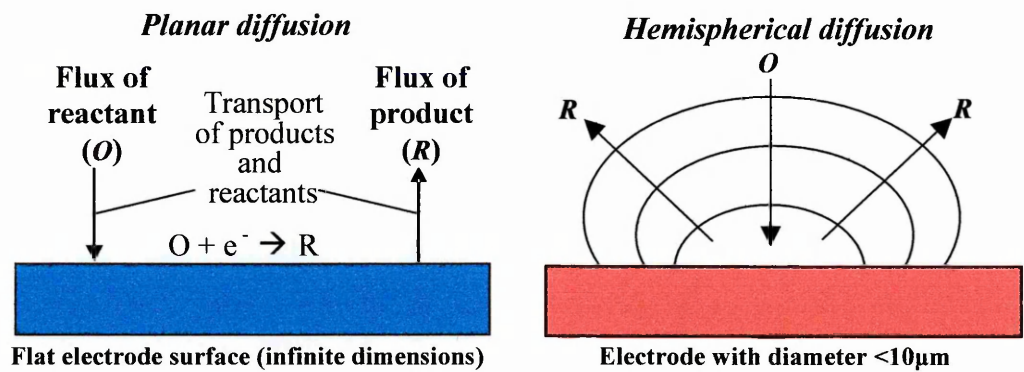


Fig 2.7: Schematic comparison of diffusion profiles.

Accordingly, the cyclic voltammogram obtained when diffusional mass transport is the rate-limiting step differs in appearance illustrated by Figure 2.8, (dotted line). In order to compensate for the much smaller signal achieved from such a small micron-dimension area, thousands of microelectrodes need to be formed to produce a microelectrode array and are then electrically connected together to provide a combined cumulative signal.

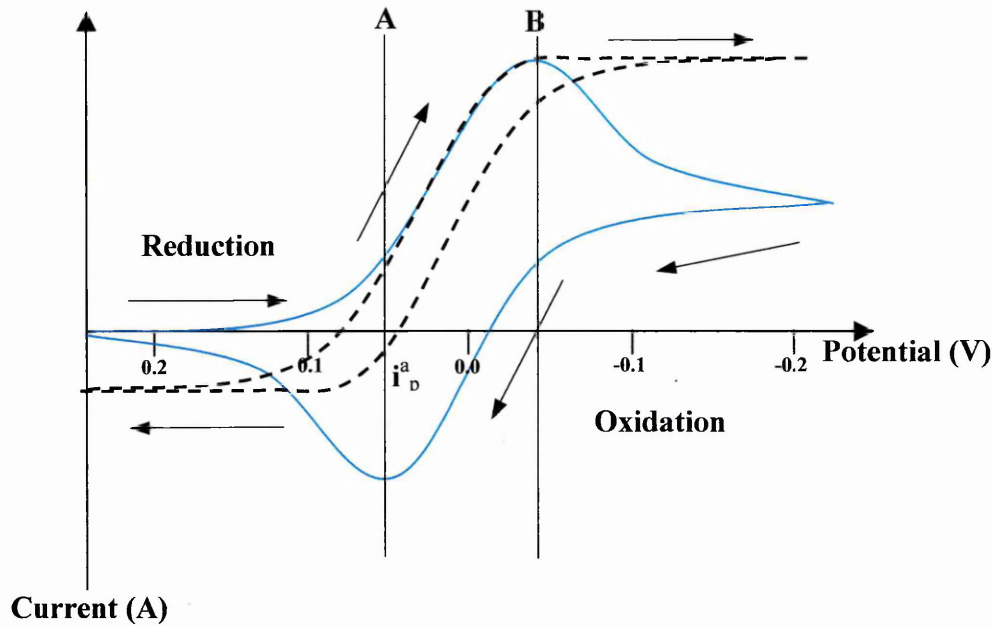


Fig 2.8: An example of a diffusion-controlled cyclic voltammogram.

Current methods of forming microelectrode arrays include photolithography (Cunningham *et al*, 2001) and laser ablation (Stulik *et al*, 2000; Fofonoff *et al*, 2004). These methods, however, incur high costs and are therefore not suitable for the commercial mass production of disposable sensors.

Ultra-sonic activity has been shown (Higson, 1996, Myler, 2000; Gornall, 2005; Barton *et al*, 2004) to successfully form microelectrode arrays through ablation of a thin, soft insulating polymer layer, and can allow for a suitable approach for low-cost commercial production (Mills, 2005).

Sound waves produced at frequencies above 20 kHz are inaudible to the human ear, yet can be highly energetic. As sound passes through a water medium, it naturally stretches and compresses the molecular spacing of the molecules. If during the rarefaction (stretch) cycle, the intermolecular spacing increases sufficiently then the liquid can break down and voids are created - hence the description 'ultra-sonic cavitation' (Frederick, 1965).

Although theoretical calculations indicate a negative pressure of 10,000 atmospheres is required to cause cavitation in pure water, this is reduced by an order of magnitude as the bubbles are filled with evaporated liquid vapour. However, in practice cavitation occurs at much lower negative pressures as weak spots in the solution reduce its tensile strength. Examples of these include dissolved gas, tiny suspended bubbles, suspended particulates and dissolved solutes. The cavitation bubbles, initiated during a rarefaction cycle, may grow over a period of a few cycles as they take up vapour and reach temperatures of thousands of degrees (Suslick *et al*, 1986). However, these localised hot spots are soon cooled down by the surrounding bulk medium and collapse in microseconds to create micro-jets of water travelling at velocities of hundreds of metres per second (Suslick, 1990). These jets are able to puncture a soft polymer layer and these punctured areas become sites for further nucleation until a maximum pore size of around 10 microns is generated (Barton *et al*, 2004).

2.5.1 Acoustic cavitation.

Cavitation may be said to occur whenever a new surface, or cavity, is created within a liquid, with a cavity being defined as any bounded volume, whether empty or containing gas or vapour, with at least part of the boundary being liquid. This broad definition includes such phenomena as boiling and effervescence, which involve only expansion of the gas phase.

In the presence of a sound field, not only formation and expansion, but also contraction of cavities will generally occur. The term acoustic cavitation is therefore usually restricted to cases involving both expansion and contraction of cavities or bubble nuclei. It is worth noting that in the literature the term *cavitation* is often also loosely applied to the effects, as well as the formation and motion, of cavities in liquids (Mason *et al*, 1989; Suslick *et al*, 1999; Mason, 2003; Suslick, 2005).

2.5.2 Types of cavitation.

In generating acoustic cavitation a time varying, generally sinusoidal, pressure is superimposed on the steady ambient pressure. All normal liquids contain small gas bubbles in suspension or gas entrained in colloidal solids. Their response to the applied sound field may be violent or rather gentle, depending on the pressure levels and other ambient conditions. These two types of cavitation are called *transient* and *stable*. Stable cavities are simply bubbles that oscillate, often non-linearly, around some equilibrium size. They are relatively permanent and may continue oscillating for many cycles of the acoustic pressure. On the other hand, transient cavities usually exist for less than one cycle. If the acoustic pressure is great enough for the liquid to go into tension during the negative half-cycle, cavities will expand rapidly, often to many times their original size. Following this, on the compression half-cycle, they collapse violently (implode) often breaking up into many smaller bubbles. It is during this collapse phase that most of the well-known disruptive effects of transient cavitation occur (Neppiras, 1984).

Either type of cavity may contain non-condensable gas (generally air) or liquid vapour. With a transient cavity, events occur so rapidly that it is usual to assume that there is no time for any mass flow, by diffusion, of permanent gas into or out of the cavity, whereas exchange of vapour may occur relatively freely. So, for example, a collapsing gas-filled transient cavity will retain most of its gas content over its life cycle, while a vaporous cavity will contain only vapour, which may remain at or near its equilibrium pressure, evaporating and condensing freely at the cavity wall.

The implosion of a vaporous cavity may therefore be more violent than a gas-filled one as there is no residual permanent gas to cushion the collapse. With stable cavities though, the time-scale of events is typically so long that diffusion of permanent gas, as well as thermal diffusion and vapour-exchange, can occur, resulting in significant long-term effects.

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2.5.4 Cavitation fields.

The simple model for all the early theoretical treatments of acoustic cavitation was a spherical bubble in radial motion, which remained spherical indefinitely. In practice, violent radial motion may initiate surface oscillations and instabilities. These non-radial oscillations are important in some practical applications. Also, in practice, bubbles are rarely produced in isolation. We have to contend with cavitation fields consisting of many bubbles interacting with one another and undergoing translational motions directed by steady forces generated by the field. Forming a macroscopic cavity in a liquid is equivalent to fracturing it, so the pressure threshold for the onset of cavitation equates with the effective dynamic tensile strength of the liquid. All normal liquids contain minute gas nuclei stabilized against dissolution in various ways, so that, unless very carefully prepared, liquids never exhibit their inherent tensile strength. The largest existing bubble or cavity determines its strength (Mason, 2003(b)).

2.5.5 Preparation of microarray electrodes by acoustic cavitation.

A novel procedure has been developed for preparing arrays of microelectrodes based on sonochemical ablation of thin insulating polymer films on electrode surfaces. Films are formed via the oxidative electropolymerisation of *o*-phenylenediamine and micron-diameter holes are subsequently ‘punched’ through films by interfacial acoustic cavitation (Higson, 1996; Myler, 2000; Law, 2002; Barton *et al*, 2004; Gornall, 2004; Mills, 2005). Electrodes prepared in this way exhibit steady-state currents in quiescent solution and yield lower detection limits than bare electrodes offering hemispherical diffusion profiles.

The method takes advantage of the extreme transient temperatures and pressures generated during acoustic cavitation (Suslick, 1990). This process involves the nucleated formation, growth and collapse of vapour-filled bubbles due to the imposition of a sonic field through a liquid in the kilohertz frequency range (Mason *et al*, 1989; Mason & Lorrimer, 2002; Mason, 2003(a); Mason, 2003(b)). Bubbles which form at extended

surfaces collapse in an asymmetric manner, generating microjets of fluid with velocities of hundreds of metres per second (Suslick, 1990; Suslick *et al*, 1990 Suslick *et al*, 1999; Suslick, 2004; Suslick, 2005). The combined effects of these jets with hot spot formation (Suslick *et al*, 1986) are exploited to ablate an insulating polymer from an electrode surface in a controlled way to create an array of microelectrodes (Higson, 1996; Myler, 2000; Law, 2002; Barton *et al*, 2004; Gornall, 2004; Mills, 2005).

2.6 Polyaniline synthesis.

Polyanilines are most commonly prepared through the chemical or electrochemical oxidative polymerisation of the respective aniline monomers in acidic solution. However, a range of polymerisation techniques have now been developed, including;

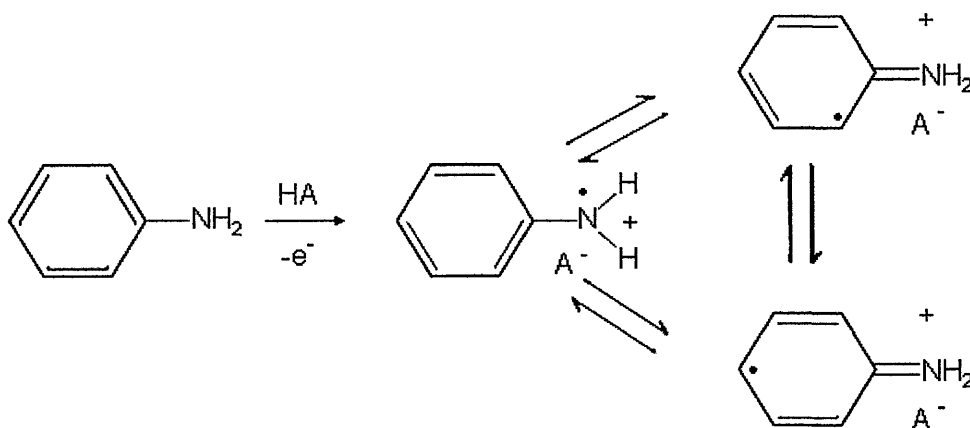
- Electrochemical polymerisation
- Chemical polymerisation
- Photochemically initiated polymerisation
- Enzyme-catalysed polymerisation
- Polymerisation employing electron acceptors

The polymerisation of aniline via conventional electrochemical techniques is utilised within this investigation using potentiodynamic electrodeposition from an acidic aqueous solution of aniline monomer. Electrochemical polymerisation is routinely carried out in an acidic aqueous solution of aniline. This low pH is required to solubilise the monomer and to generate the emeraldine salt, the most conductive form of polyaniline. Constant potential or potentiodynamic techniques are generally employed because the overoxidation potential for polyaniline is very close to that required for monomer oxidation (Genies *et al*, 1988). The appearance of defects due to overoxidation of the polymer have been proposed but the exact nature of the over oxidation is not known. One theory is that crosslinking occurs, (Kobayashi *et al*, 1984) while another describes the opening of the chain after the formation of a paraquinone (Hyodo & Oomae, 1990). A short term increase of the applied potential to 0.9-1.1V (vs. Ag/AgCl) during potentiostatic deposition has been reported to give more adherent films (Barbero *et al*, 1993).

2.6.1 Mechanism of electrochemical polymerisation (PANI).

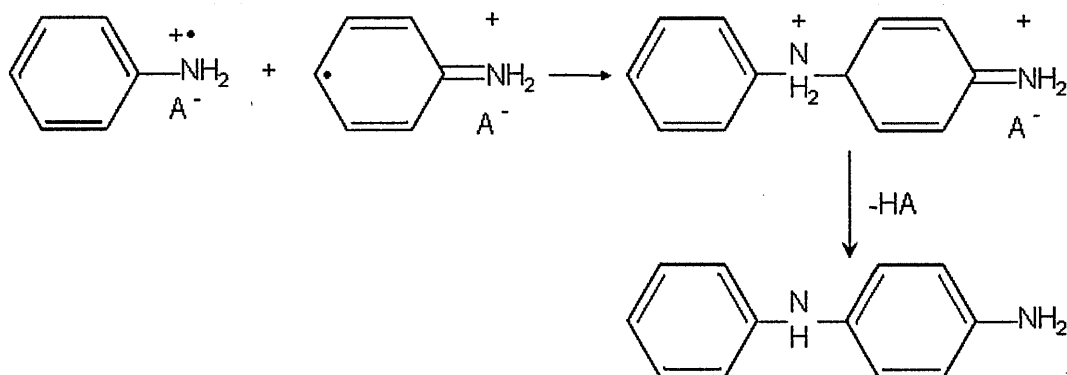
The generally accepted mechanism for the electropolymerisation of aniline is an E(CE)_n process, as presented in Figure 2.9 (Chiou *et al* 1997; Mu *et al*, 1997; Innis & Wallace, 2002).

Step 1. Oxidation of monomer.



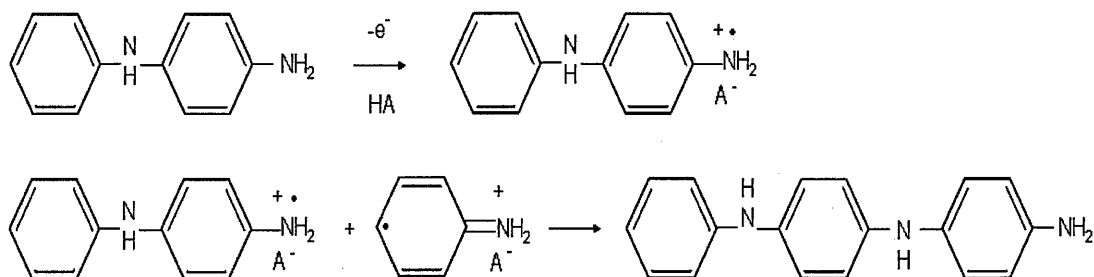
Formation of the radical cation of aniline oxidation on the electrode surface (step 1) is considered to be the rate-determining step.

Step 2. Radical coupling and rearomatization.



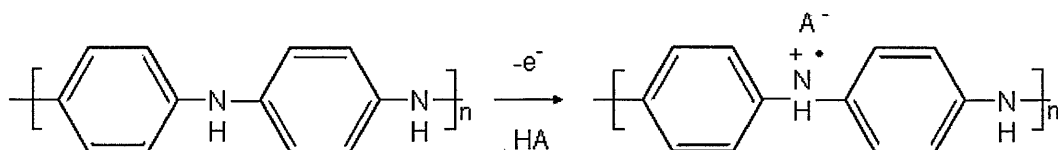
This is followed by coupling of radicals, mainly *N*- and *para*-forms, and elimination of two protons. The dimer (oligomer) formed then undergoes oxidation on the electrode surface along with aniline.

Step 3. Chain propagation.



The radical cation of the oligomer couples with an aniline radical cation, resulting in propagation of the chain.

Step 4. Oxidation and doping of polymer.



The formed polymer is doped by the acid (HA) present in solution (step 4).

Fig 2.9: Electropolymerisation of aniline (Genies *et al*, 1985(a); Genies *et al*, 1988).

The growth of polyaniline has been found to be self-catalysing; (Genies *et al*, 1985(b); Zotti *et al*, 1988) the more polymer deposited, the higher the rate of polymer formation. A mechanism for this has been proposed, involving adsorption of the anilinium ion onto the most oxidised form of polyaniline, followed by electron transfer to form the radical

cation and subsequent reoxidation of the polymer to its most oxidised state (Zotti *et al*, 1988).

2.7 Biomolecule immobilisation at electrode surfaces by electrochemical approaches.

Since the first demonstration by Clark and Lyons (Clark & Lyons, 1962) that an enzyme could be integrated into an electrode to form a biosensor, the development of such electrochemical devices has made considerable progress (Turner *et al*, 1987; Kaufmann & Guilbault, 1992). The advantages associated with these devices are their high selectivity and simple use in complex media, as well as the possibility of developing compact and portable analysers. In recent years, there has been a growing interest in electrochemical microdevices owing to their potential application in monitoring and diagnostic tests in clinical laboratories. In particular, the development of microfabricated biosensors constitutes an attractive avenue for the *ex vivo* and *in vivo* measurements of metabolites, for example, for glucose, hormones, neurotransmitters, antibodies and antigens (Willner *et al*, 2000). The stable immobilisation of macromolecular biomolecules on conductive microsurfaces with complete retention of their biological recognition properties is a crucial problem for the commercial development of miniaturised biosensors. Effectively most of the conventional procedures of biomolecule immobilisation such as cross-linking, covalent binding and entrapment in gels or membranes suffer from a low reproducibility and a poor spatially controlled deposition.

Apart from these conventional methods, the immobilisation of biomolecules in electropolymerised films is gaining importance (Bartlett & Cooper, 1993; Schuhmann, 1995; Trojanowicz *et al*, 1995; Cosnier, 1997).

Effectively, the electrochemical formation of polymer layers of controlled thickness constitutes a reproducible and non-manual procedure of biosensor fabrication. This approach has received considerable attention due to increase demand for miniaturised biosensors (Matsue *et al*, 1991; Bartlett & Caruana, 1992; Schuhmann *et al*, 1993).

The electrochemical method involves the entrapment of biomolecules in organic polymers during their electrogeneration on an electrode surface.

The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers and biomolecules. Recently, a more sophisticated approach involving the adsorption of amphiphilic monomers and biomolecules before the polymerisation step has been described (Cosnier, 1997).

Another electrochemical method involves, initially, the electropolymerisation of functionalised conducting polymers. Then the attachment of biomolecules to the polymers surfaces can be obtained by chemical grafting or by affinity of the biomolecule at the functional group (Schuhmann *et al*, 1990; Korri-Youssoufi *et al*, 1997; Cosnier *et al*, 1998; Cosnier & Gondran, 1999; Kranz *et al*, 1998).

In comparison to the physical entrapment of biomolecules within polymer films such as polypyrrole, polythiophene, polyacetylene or polyaniline, this approach preserves a better access of substrate to the immobilised receptors and facilitates macromolecular interactions.

However, the amount of immobilised biomolecule is restricted to a monolayer at the interface of polymer and solution.

Initially within this work biomolecules (antibody receptors) are electrochemically entrapped in the organic polymer polyaniline, as the main immobilisation technique performed. Subsequently more sophisticated approaches will be utilised to gain greater control over the reproducibility of the transducer surface yielding the most effective biomolecule-loading and enabling optimum detection criteria.

The advantage of electrochemical polymerisation is that films can be prepared easily in a rapid one-step procedure. Furthermore, this method enables exact control of the thickness of the polymer layer based on the measurement of the electrical charge passed during

electrochemical polymerisation. One major advantage of electrochemical deposition procedures over more conventional methods is the possibility to precisely electrogenerate a polymer coating over small electrode surfaces of complex geometry.

Most of the electrochemically deposited polymer films used for the biomolecule immobilisation are conducting polymers for example, polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole, (Bartlett & Cooper, 1993; Pandey, 1988; Shinohara, 1988).

Owing to their conductivity, the thickness of the polymer film can be easily varied and is not restricted to very thin films, contrary to non conducting organic polymers.

Nevertheless, a few biosensors based on insulating electropolymerised films like polyphenols, poly (ortho-phenylenediamine), poly (dichlorophenolindophenol) and overoxidised polypyrrole have been elaborated (Bartlett & Caruana, 1992; Malitesta *et al*, 1990; Groom & Luong, 1993). Since the growth of the polymer film is self-limiting, the resulting biosensors may exhibit fast response times.

In addition, the insulating films confer permselective and antifouling properties on the biosensors (Centonze *et al*, 1992; Lowry *et al*, 1994; Palmisano *et al* 1995).

Among the conducting polymers, polypyrrole and its derivatives play the leading role due to their versatile applicability and the wide variety of molecular (redox) species that may be covalently linked to a pyrrole group (Bidan, 1992; Deronzier & Moutet, 1994).

Coenzymes, antibodies, cells and biological tissue have all been immobilised within conducting polymer matrices illustrating the wide potentialities of this method for biomolecule retention (Yabuki *et al*, 1990; Deschpande & Hall, 1990; Battaglini *et al*, 1991; John *et al* 1991; Uchiyama *et al*, 1997).

2.8 Electrochemical Impedance Spectroscopy (EIS).

An electrochemical reaction at the electrode–electrolyte interface cannot be fully understood by using traditional electrochemical measurements. Those methods provide only currents comprising of both faradaic and non-faradaic components. A complete description requires impedance measurements made over a broad frequency range at various potentials and determination of all the electrical characteristics of the interface, which can be thought of as a thin capacitor that forms between the charged electrode and the counter ions lined up parallel to it (Bard & Faulkner, 2001; Schmickler, 1996).

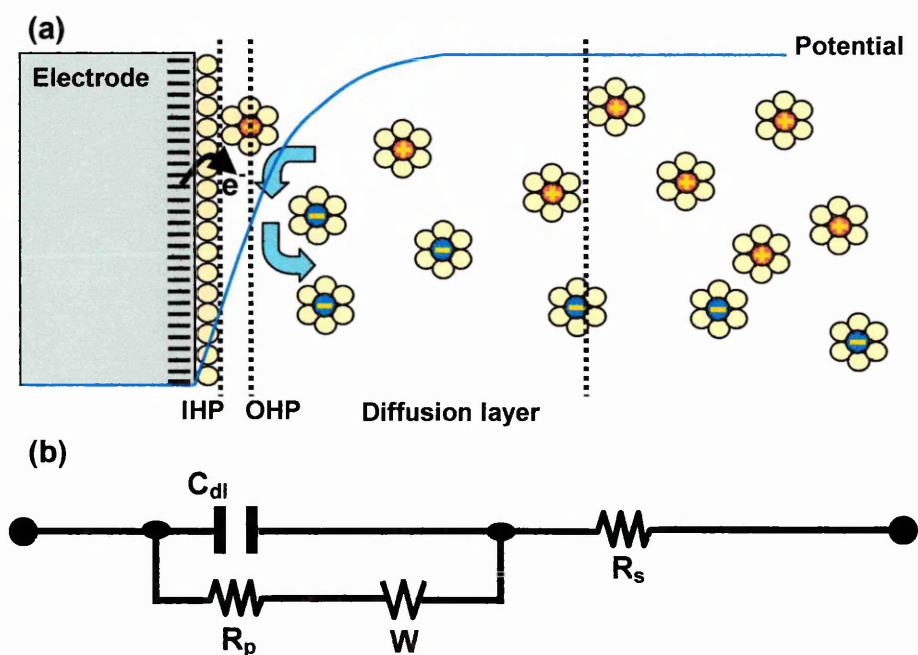


Fig 2.10: A simple electrified interface, in which the vertical dotted lines in (a) are represented by the electronic components in (b).

- (a) The oxidants (red) with a positive charge diffuse toward the negatively charged electrode, accept electrons from the electrode at the interface, become the reductants (blue), and diffuse to the bulk of the solution. The oxidant is also a counterion to the electrode. No specific adsorption is considered at the interface. IHP and OHP are the inner and outer Helmholtz planes, respectively.
- (b) An equivalent circuit representing each component at the interface and in the solution during an electrochemical reaction is shown for comparison with the physical components. C_{dl} , double layer capacitor; R_p , polarization resistor; W , Warburg resistor; R_s , solution resistor.

Representations of the electrified interface have gradually evolved from repeated modifications (Figure 2.10(a)) of the model first proposed by Helmholtz (Helmholtz, 1853; Parsons, 1990). A comprehensive review has been given by Brett and co workers (Brett *et al*, 1993). In a simple case, the interface can be modelled by an equivalent circuit (Figure 2.10(b)), also called a Randles circuit (Randles, 1947; Randles, 1948; Randles, 1952; Randles & Somerton, 1952), made of a double-layer capacitor in parallel with a polarization resistor (also known as a charge transfer resistor with certain constraints) and a Warburg impedance, connected in series with a resistor that measures the resistance of the electrolyte solution. Depending on the types of electrochemical reactions involved at the interface, the equivalent circuit can be much more complicated.

The model in Figure 2.10(b) predicts that a faradaic current, which is due to the redox reactions, is always coupled with a capacitive component. Capacitance is regarded as noise, and considerable efforts have been made to eliminate or minimize it, resulting in test (sampling), normal pulse, and differential pulse voltammetry (Bard & Faulkner, 2001). However, reducing or minimizing the effects of non-faradaic currents is considered a passive way to solve the problem. A more active and direct approach would be to determine and separate the faradaic currents from the non-faradaic signals (Singhal *et al*, 1997), which is possible with EIS because it provides all the information about the interface and the electrolyte solution.

2.8.1 Definition of impedance.

Impedance is a totally complex resistance encountered when a current flows through a circuit made of resistors, capacitors, or inductors, or any combination of these. Depending on how the electronic components are configured, both the magnitude and the phase shift of an ac can be determined. Because an inductive effect is not usually encountered in electrochemistry, we consider only the simple equivalent circuit shown in Figure 2.10(b) in which no inductor is present.

First consider an experiment in which a series of increasing dc potentials (a ramp) are applied to a working electrode in an electrochemical cell containing an electroactive species.

A current – potential curve (Figure 2.11, solid line) is obtained, which is described by the Butler–Volmer equation (Equation 2.2) (Butler, 1924; Erdey-Gruz & Volmer, 1930).

Equation 2.2

$$i = i_0 \left\{ e^{\frac{-\alpha n F (E - E_{eq})}{RT}} - e^{\frac{(1-\alpha) n F (E - E_{eq})}{RT}} \right\}$$

Here η is the overpotential defined as $E - E_{eq}$, with E and E_{eq} representing the applied and equilibrium potentials, respectively; i_0 is the exchange current at $\eta = 0$; n is the number of electrons transferred; F is the Faraday constant ($9.65 \times 10^4 \text{ Cmol}^{-1}$); R is the gas constant ($8.314 \text{ Jmol}^{-1}\text{K}^{-1}$); T is the absolute temperature (K); and α is the transfer coefficient for electron transfer.

The α term reflects the sensitivity of the transition state to the applied potential. This term is also known as the symmetry factor. If $\alpha = 0$, then the transition state shows no dependence on potential. If α is close to zero, then the transition state is thought to resemble the reactants in its potential dependence, whereas when the value approaches unity, the transition state behaves more like the product. Typically, however, the α term tends to approximate to 0.5. This means that the transition state responds to potential in an intermediate behaviour between the reactants and the products response.

The faradaic current i is limited by the mass transport (Figure 2.11, dashed line curving to the right) when the rate of electron transfer becomes sufficiently large. At a given overpotential η_{bias} , the slope of the curves, $di/d\eta_{\text{bias}}$, is $1/R_p$, in which R_p is the polarization resistance.

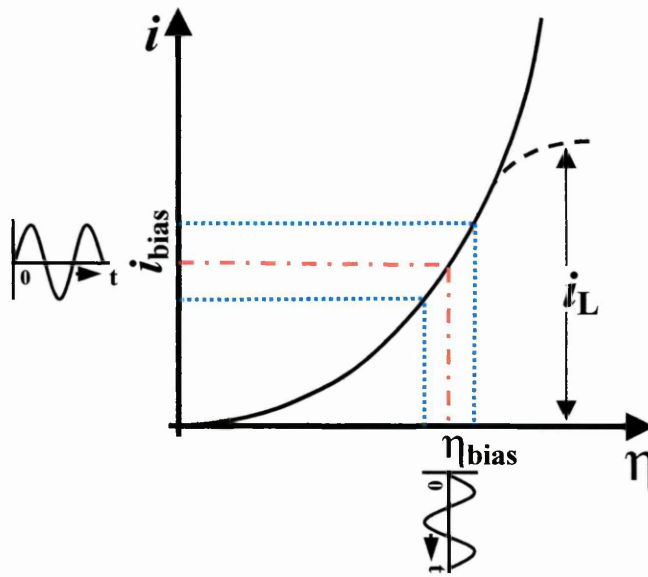


Fig 2.11: The dc plotted as a function of overpotential according to the Butler–Volmer equation (solid line), which is limited by mass transport at large overpotentials (dashed line curving to the right), an ac voltage (broken blue line) superimposed on the dc bias potential, η_{bias} (dot-dashed line), shown on the i axis [$\eta_{\text{bias}} + \Delta\eta \sin(\omega t)$], and the resulting ac superimposed on the dc on the i axis [$i_{\text{bias}} + \Delta i \sin(\omega t + \theta)$]. R_p is obtained by taking $\Delta\eta/\Delta i$, in which i is obtained after applying the ac voltage wave at a given η .

When a small ac voltage wave of frequency ω at η_{bias} (Figure 2.11) is superimposed, the ac of the same frequency will be flowing on top of the dc. Because the interface has resistors and a capacitor (Figure 2.10(b)), the ac current will experience a phase shift, expressed as i_{bias} , caused by the ac wave perturbation.

For an equivalent circuit (Figure 2.10(b)), a straightforward impedance expression can be derived by applying Ohm's law to two components connected in parallel (Equation 2.3). One of these is R_p (the polarisation resistance, ohms), and the other is $1/(j\omega C_{dl})$, in which C_{dl} is the double-layer capacitance measured in farads. R_s is the solution resistance, ohms).

Equation 2.3

$$Z(\omega) = R_s + \frac{R_p}{1 + j\omega R_p C_{dl}} =$$

$$R_s + \frac{R_p}{1 + \omega^2 R_p^2 C_{dl}^2} - \frac{j\omega^2 R_p^2 C_{dl}}{1 + \omega^2 R_p^2 C_{dl}^2} = Z' + jZ''$$

To make the derivation of the equation and its interpretation straightforward, the contribution of the Warburg component is neglected. Thus, the impedance of the interface at a given frequency ($Z(\omega)$) consists of two parts, a real number Z' (re) and an imaginary number $-Z''$ (im) with a complex representation $Z(\omega) = Z'(\omega) + jZ''(\omega)$ with $\theta = \tan^{-1} [Z''(\omega)/Z'(\omega)]$, (θ is the phase angle, °). $\omega = 2\pi f$, the frequency in hertz translated into radians. Although the capacitance is relatively constant over the potential at a given electrode, the R_p varies as a function of η_{bias} applied to the electrode.

At a given dc bias potential, a series of $Z(\omega)$ data are obtained in a range of frequencies, typically 100 kHz – 1×10^{-4} Hz. The impedance varies, depending on frequencies, and is often plotted in different ways as a function of frequency (making it a spectroscopic technique), hence, the name EIS (Macdonald, 1987; Bard & Faulkner, 2001; Barsoukov & Macdonald, 2005).

However, this is a misnomer because the frequencies do not represent energy levels and thus the impedance has nothing to do with quantum states in spectroscopic transitions.

By treating the impedance data in such a frequency range, system characteristics for an electrochemical reaction (i.e., R_s , R_p , and C_{dl}) can be obtained. R_p is a function of potential; however, at $\eta = 0$, it becomes the charge-transfer resistance, R_{ct} , also termed the electron-transfer resistance, Ret . Two convenient ways of treating the impedance data are the *Nyquist* plot, in which imaginary numbers $Z''(\omega)$ are plotted against real numbers

$Z'(\omega)$, and the *Bode* plot, in which absolute values of impedance or phase angle are plotted against the frequency. Extraction of the system characteristics requires interpreting the *Nyquist* plot (Figure 2.12(a)) according to Equation 2.3. At high frequencies, the frequency-dependent term of Equation 2.3 vanishes, resulting in $Z(\omega) = Z'(\omega) = R_s$, which is an intercept on the $Z'(\omega)$ axis on the high-frequency side ($\theta = 0$ or $Z''(\omega) = 0$).

For $\omega \rightarrow 0$, Equation 2.3 becomes $Z(\omega) = R_s + R_p$, which is an intercept on the $Z'(\omega)$ axis on the low-frequency side. At the frequency where a maximum $Z''(\omega)$ is observed, the straightforward relationship $R_p \cdot C_{dl} = 1/\omega_{\max} = 1/(2\pi f_{\max}) = \tau_{\text{rxn}}$, which is the time constant of the electrochemical reaction, can be shown and indicates how fast the reaction takes place. Also, if $R_p \cdot C_{dl}$ is known, C_{dl} can be obtained because R_p is already known from the low-frequency intercept on the $Z'(\omega)$ axis. The *Nyquist* plot gives all the necessary information about the electrode-electrolyte interface and the reaction. The data shown in Figure 2.12 (a) and (b), were generated using Equation 2.5 from arbitrarily picked values of R_p , represent the impedance results regardless of the measurement methods used.

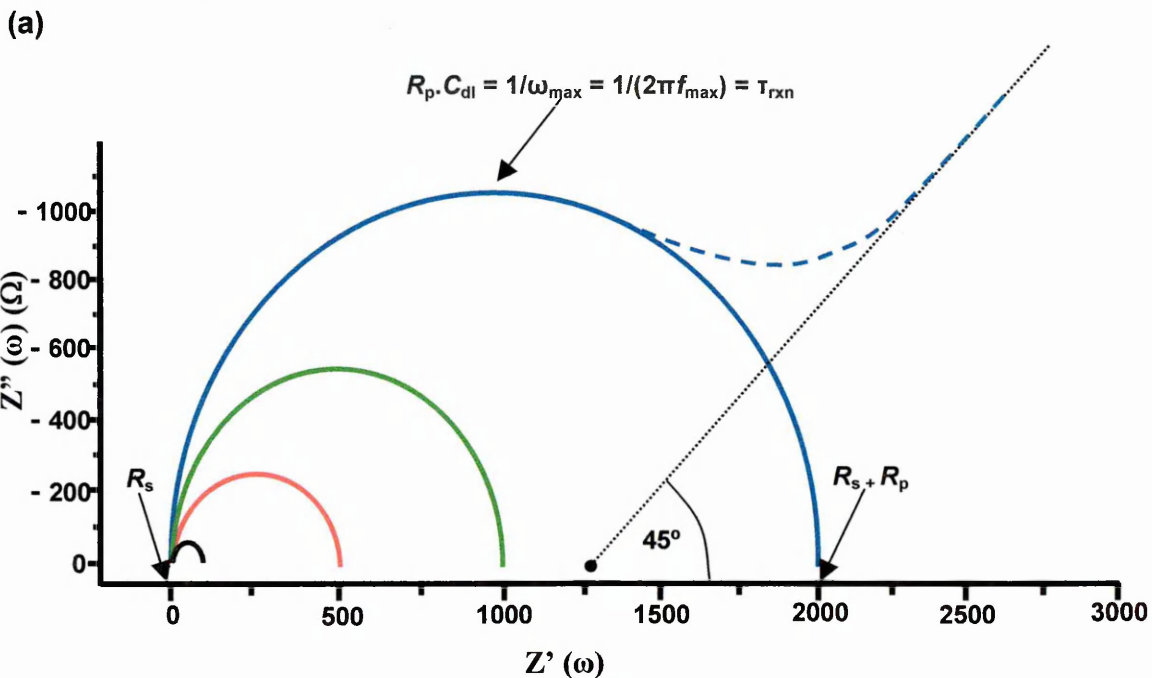


Fig 2.12: (a) Nyquist plot.

The contribution from the Warburg impedance (dashed blue line) is shown only for one case ($R_p = 2\text{ k}\Omega$) for both plots. (Figure 2.12 (b), top). It should be noted that the amplitude axis is in logarithmic scale at amplitudes $>3\text{ k}\Omega$ in the amplitude vs. frequency plot to accommodate the large Warburg impedance values. Note that the phase angle in both Figures 2.12 (a) and (b) approaches 45° as the Warburg contribution becomes dominant. The black dashed line in Figure 2.12 (a) represents Warburg components with its intercept at $R_s + R_p - R_p^2 \lambda^2 C_{dl}$, from which $\lambda = 3.08$ is obtained; therefore, $k_f = 4.1 \times 10^{-3}\text{ cm s}^{-1}$ for a diffusion coefficient of $1.8 \times 10^{-6}\text{ cm}^2\text{ s}^{-1}$. R_s , $10\text{ }\Omega$; C_{dl} , $20\text{ }\mu\text{F}$; R_p black, $100\text{ }\Omega$; R_p red, $500\text{ }\Omega$; R_p green, $1\text{ k}\Omega$; R_p blue, $2\text{ k}\Omega$.

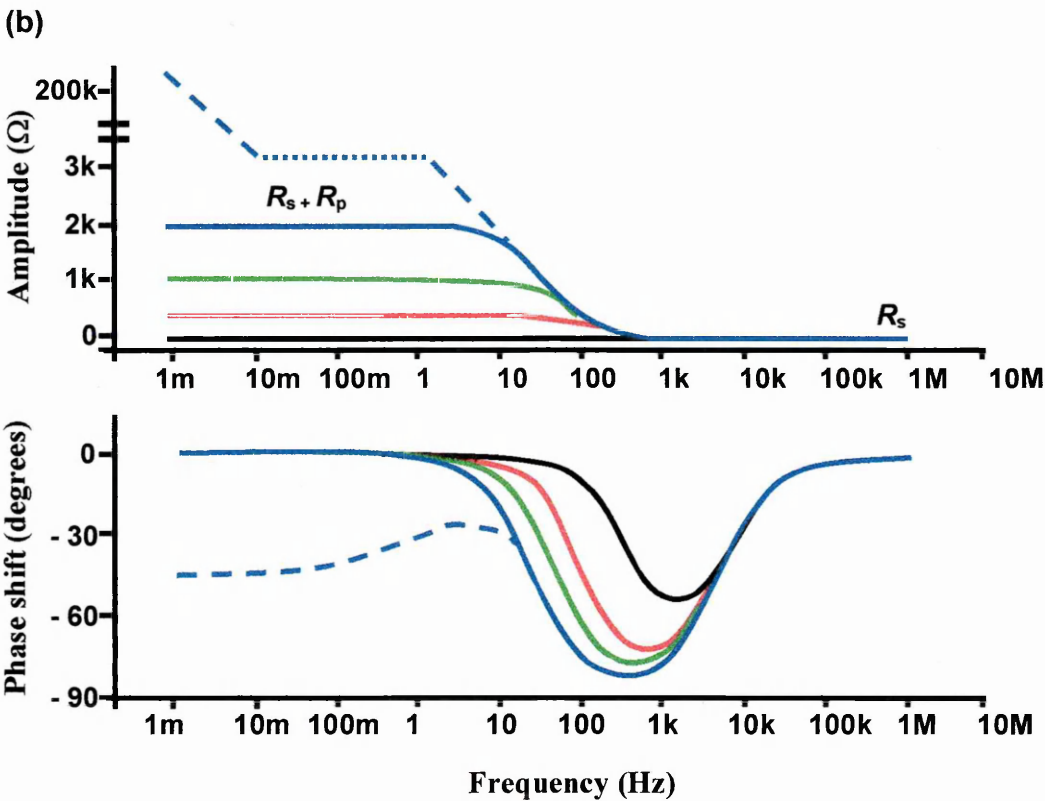


Fig 2.12: (b) Bode plot.

Similar information is obtained by examining the *Bode* diagram (Figure 2.12(b)) using Equation 2.3. $\log R_s$ and $\log (R_p + R_s)$ are obtained straightforwardly from the $Z(\omega)$ versus $\log \omega$ plot at high and low frequencies from the same argument as the *Nyquist* plot. In the intermediate frequency region, an almost straight line with a slope of ~ -1.0

can be seen. The equation for this line is obtained by ignoring the frequency-independent terms, R_s and 1 in the denominator of Equation 2.3 to yield Equation 2.4.

Equation 2.4

$$Z(\omega) = R_s + \frac{R_p}{1 + j\omega R_p C_{dl}}$$

Taking the logarithm on both sides of the resulting equation yields $\log Z(\omega) = -\log \omega - \log C_{dl}$, which equates to $\log |Z(\omega)|$ versus $\log \omega$ having a slope of -1 , and C_{dl} can be obtained from the intercept of this line with the $Z(\omega)$ axis when $-\log \omega = 0$ at $\omega = 1$. Thus, the *Bode* plot provides the same information as the *Nyquist* plot.

The θ versus $\log \omega$ plot shows that the impedance responses are resistive, primarily at high and low frequencies, as indicated by practically no change in phase shifts, whereas at intermediate frequencies, they are mostly capacitive as their phase shifts get closer to 90° . Thus far, we have discussed the equivalent circuit without considering the effect of the Warburg impedance; however, its contribution can be important at low frequencies because the mass transport of the electroactive species may limit the electron-transfer process. The Warburg impedance (Sluyters-Rehback *et al*, 1970) is imparted by mass transfer and can be derived as in Equation 2.5.

Equation 2.5

$$Z(\omega) = R_s + R_p \left[1 + \frac{\lambda}{\sqrt{2\omega}} \right] - R_p^2 \lambda^2 C_{dl} - \frac{jR_p \lambda}{\sqrt{2\omega}}$$

in which $\lambda = k_f / \sqrt{D_O} + k_b / \sqrt{D_R}$, and k_f and k_b are the forward and backward electron-transfer rate constants (s^{-1}), respectively, and D_O and D_R ($cm^2 s^{-1}$) are the diffusion coefficients for the oxidant, O , and the reductant, R , respectively, for the reaction $O + ne \rightarrow R$.

Here the frequency-dependent terms $\lambda/\sqrt{2\omega}$, appearing in both the real and the imaginary terms in Equation 2.5, are called the Warburg impedance. When $Z''(\omega)$ is plotted against $Z'(\omega)$ with the Warburg component included, the high- and low-frequency intercepts on the $Z'(\omega)$ axis are still R_s and $R_s + R_p$, as was the case without the Warburg impedance, and the plot of the Warburg components becomes a straight line with a slope of unity and an intercept of $R_s + R_p - R_p^2 \lambda^2 C_{dl}$ (blue dashed line in Figure 2.12(a)).

Thus we can calculate λ , and thereby the diffusion coefficient from λ when k_f is known, and k_f can be obtained from $i_f = nFAk_fC_O$, in which C_O is the bulk concentration of oxidant and A is the area. In other words, we now have a complete description of the electrochemical system.

2.8.2 Measuring impedance.

The principle shown in Figure 2.11 is the basis on which impedance is measured: A small ac wave, typically 5–10 mV (peak-to-peak) of a given frequency, is superimposed on the dc η_{bias} , and the resulting ac and its phase shift i_{bias} are measured.

These measurements may be made in various ways (Armstrong *et al*, 1968; deLevie & Husovsky, 1969; Armstrong & Henderson, 1972) however, the frequency response analyser (FRA) presented in Figure 2.13 has become the industry standard in electrochemical instrumentation in recent years.

The reference ac wave of frequency ω superimposed on a given dc bias potential is applied to a working electrode in the electrochemical cell. The ac signal $S(t)$ obtained from the cell is then multiplied by the reference cosine or sine wave and integrated to obtain Equation 2.6 and Equation 2.7, respectively.

Equation 2.6

$$Z'(\omega) = \frac{1}{NT} \int_0^{NT} S(t) \cdot \cos(\omega t) dt$$

Equation 2.7

$$Z''(\omega) = \frac{1}{NT} \int_0^{NT} S(t) \cdot \sin(\omega t) dt$$

Here T is the period ($1/f$) of the ac wave, N is the number of cycles included in the integration, and $\cos(\omega t)$ and $\sin(\omega t)$ are the reference signals. Higher harmonics that may occur during the testing are removed by digital filtering. As in any other measurements, the S/N is enhanced by $N^{1/2}$, in which N is the number of cycles included in the integration.

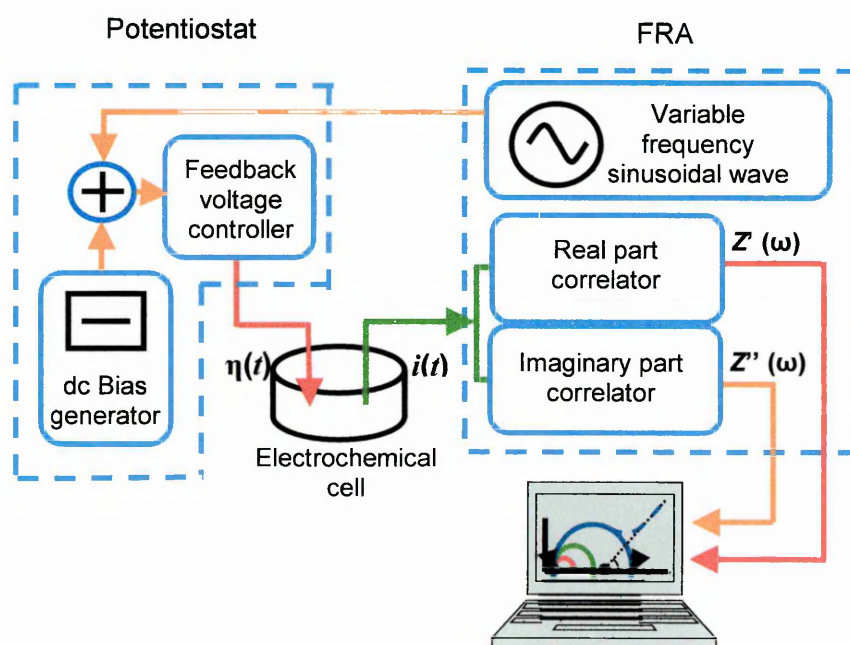


Fig 2.13: Concept of the frequency response analyser (FRA).

Although the FRA is state of the art, it has a few shortcomings. The entire frequency range must be scanned by generating ac voltage waves of 10 – 30 representative frequencies per decade to make measurements. Often, measurements of more than a few ac cycles are necessary at a given frequency to obtain a reasonable signal to noise ratio, and the instrument usually waits for the system to reach a constant (or equilibrium) current after a step to a desired dc bias potential before it takes measurements. Thus, this method is viable only for an electrochemically stable system in equilibrium. All these operations often take longer than required, and the system can change significantly by the time the measurements are made, particularly when the electrochemical reaction is not reversible or involves a series of subsequent reactions such as adsorption–desorption and/or deposition–dissolution processes after the initial electron transfer.

Early investigators questioned the validity of impedance measurements (Sluyters-Rehback *et al*, 1979; Popkirov & Schindler, 1993) and postulated that instantaneous impedance measurements of the non-stationary systems were needed because traditional methods provide impedance data only for the stationary systems. Stoynov and Savoya-Stoynov presented some mathematical expressions to extract instantaneous impedance values for non-stationary systems from the results obtained on stationary systems (Stoynov & Savoya-Stoynov, 1985; Stoynov, 1992; Stoynov & Savoya-Stoynov, 1992; Stoynov, 1993). Urquidi-Macdonald *et al* also presented requirements for “true” impedance signals; the stability and linearity of a system can be demonstrated only when imaginary and real components are interconvertible via Kramers–Kronig transforms (Urquidi-Macdonald *et al*, 1990).

2.8.3 Applications and prospectives.

It is worth discussing the usefulness of the technique to electrochemists, and also to consider future enhancements to the techniques. With impedance data, a complete description of an electrochemical system can be made using an equivalent circuit.

The Randles circuit can be worked out by fitting the experimentally obtained impedance data to the theoretical behaviour of the postulated equivalent circuit; the fitting provides all the related parameters.

Although impedance responses for a simple system have been described, few systems can be represented as simply as shown in Figure 2.10, and thus the actual responses are not always represented by those shown in Figure 2.12. In many cases, components with properties somewhere between the discrete electronic components, called the constant-phase elements, are needed to describe the impedance behaviours (Macdonald, 1987). As a result, it could be very difficult to simulate the impedance response with discrete components such as a resistor, capacitor, or Warburg impedance. For this reason, many impedance data do not fit the theoretical behaviour predicted by the equivalent circuit made of ideally behaving components. For such a system, constant-phase elements are used instead and the resulting interpretations are not always straightforward.

Nonetheless, impedance measurements are most useful for studying complex systems such as anodic behaviours of metals and other composite materials (Keddam *et al*, 1981; Schmidt *et al*, 1986; Cai & Park, 1996; Cha & Park, 1997), corrosion (Lay, 1985), states of electrodes during charging/discharging cycles of batteries (Karunathilaka, 1983; Cha & Park, 1998; Piao *et al*, 1999; Kim & Park, 2001), surface characterization of polymer-modified electrodes (Gabrielli *et al*, 1987; Johnson & Park, 1996; Lee & Park, 2000), and many other complex electrochemical phenomena.

In most complex systems, the impedance responses are made of more than one semicircle. The second or third semicircles in lower frequency domains, when present, represent the electrochemical reactions of slower rise times. When the interface is complex because of adsorption or chemical reactions, more than one semicircle can be observed.

The $i-\eta$ curve (Figure 2.11) follows the Butler–Volmer equation (solid line) until the current is limited by mass transfer when η becomes large, resulting in a limiting current

as shown by the dashed line curving to the right. Although the Nyquist and Bode plots are very useful for extracting various parameters for the electron transfer reaction, the $i-\eta$ curve constructed from the impedance data can provide information not available from the impedance results alone. The current at a given η can be obtained from Equation 2.8 and the electrokinetic parameters such as i_0 and α are obtained by fitting the data to the Butler-Volmer equation, Equation 2.2.

Equation 2.8

$$i(\eta) = \int \frac{1}{R_p} d\eta$$

Impedance measurements have changed the way electrochemists interpret the electrode–electrolyte interface. The technique will offer the most powerful analysis on the status of electrodes during charging–discharging processes of batteries and fuel cells as well as during corrosion experiments.

More importantly, this technique may be used to monitor and probe many different processes that occur during electrochemical experiments, including adsorption of reactants and products as well as various reactions that either precede or follow the experiments, thereby changing the electrical characteristics of electrode–electrolyte interfaces.

2.8.4 Probing Biomolecular Interactions at Conductive and Semiconductive surfaces by Impedance Spectroscopy.

Impedance Spectroscopy is a rapidly developing electrochemical technique for the characterisation of biomaterial-functionalised electrodes and biocatalytic transformations at electrode surfaces, and such as for the transduction of biosensing events at electrodes or field effect transistor devices.

The immobilisation of biomaterials, e.g. enzymes, antigens/antibodies or DNA at electrodes or semiconductor surfaces alters the capacitance and interfacial electron transfer resistance of the conductive or semiconductive electrodes.

Impedance spectroscopy allows analysis of interfacial changes originating from bio recognition events at electrode surfaces. Kinetics and mechanisms of electron transfer processes corresponding to biocatalytic reactions occurring at modified electrodes can be also be derived from Faradaic impedance spectroscopy.

Bioelectronics is a rapidly progressing field at the junction of chemistry, biochemistry, physics and materials science (Willner, 2002). The basic feature of a bioelectronic device is the interaction of a biomaterial with a conductive or semiconductive support, and the electronic transduction of the biological functions associated with the biological matrices.

Biomaterials that can be interacted with electronic transducers include proteins, including enzymes (Willner & Katz, 2000; Willner *et al*, 2000; Heller, 1990), receptors (Gopel & Heiduschka, 1995; Zhong & Porter, 1995) antibodies or antigens (Bourdillon *et al*, 1993; Bourdillon *et al*, 1994; Bourdillon *et al*, 1996; Rogers, 2000), oligonucleotides or DNA fragments (Yang *et al*, 1997; Campbell *et al*, 2002; Willner & Katz, 2002), saccharides interacting with lectins (Willner *et al*, 1993) and low molecular weight molecules exhibiting affinity interactions with other biomaterials such as cofactors, namely, NAD(P)⁺ (Bardea *et al*, 1997) and biotin (Anzai *et al*, 1998).

Many different electronic methods can be employed to transduce the biological functions occurring at the electronic supports (Sethi, 1994). These include electrical transduction such as current (Bartlett *et al*, 1991), potential changes (Ianniello *et al*, 1982; Ghindilis & Kurochkin, 1994), piezoelectric transduction (Lion-Dagan *et al*, 1997; Ben-Dov *et al*, 1997; Bardea *et al* 1998; O'Sullivan *et al*, 1999), field effect transistor transduction (Kazanskaya *et al*, 1996; Zayats *et al* 2000), photoelectrochemical transduction (Pardo-Yissar *et al*, 2003) and other methodologies (Amador *et al*, 1993;

Hobara *et al*, 1994; Liedberg *et al*, 1995; Jordan & Corn, 1997; Raitman *et al*, 2002(a); Raitman *et al*, 2002(b)).

Many of the biosensor devices involve the formation of a recognition complex between the analyte and the sensing biomaterial in the form of a monolayer or thin film configuration on the electronic transducer (Kharitonov *et al*, 2000). Formation of the complex on a conductive or semiconductive surface alters the capacitance and the resistance at the surface-electrolyte interface.

Furthermore, the build-up of the sensing biomaterial film on the conductive or semiconductive support alters the capacitance and resistance properties of the solid support-electrolyte interface.

Impedance spectroscopy is a very powerful tool for the analysis of interfacial properties associated with biorecognition events occurring at the modified surfaces.

Impedance measurements provide detailed information on capacitance / resistance changes occurring at conductive or semiconductive surfaces. Thus impedance spectroscopy (Athey *et al*, 1995; Nahir & Bowden, 1996), including non-Faradaic impedance measurements resulting in capacitance sensing (Bresler *et al*, 1992), is becoming an attractive electrochemical tool to characterise biomaterial films associated with electronic elements, therefore allowing transduction of biorecognition events at the respective surfaces.

Faradaic impedance spectroscopy allows analysis of kinetics and mechanisms of bioelectrocatalytic reactions at the modified electrode surfaces providing important information for the development of amperometric biosensors and biofuel cells.

2.8.5 Immunosensors Based on Impedance Spectroscopy.

Among the biomaterial-based sensing devices, immunosensors are anticipated to be an important class of sensing systems in, for example, clinical diagnosis, food quality control, environmental analysis, detection of pathogens or toxins, and even the detection of explosives or drugs (Pravda *et al*, 2002; Sapsford *et al*, 2002; Luppá *et al*, 2001). Several methodologies for the electrochemical transduction of the formation of antigen-antibody complexes on the electrode surface have been reported (O'Sullivan *et al*, 1999; Mullett *et al*, 2000).

Impedance Spectroscopy (including Faradaic impedance in the presence of a redox probe and non-Faradaic - capacitance measurements) represents an important electronic transduction approach for antigen-antibody binding interactions.

2.8.6 Electrochemical Impedance Spectroscopy (EIS) – Theoretical background.

Impedance spectroscopy is an effective method for probing the features of surface-modified electrodes (Bard & Faulkner, 1980; Stoynev *et al*, 1991). A small-amplitude perturbing sinusoidal voltage signal is applied to the electrochemical cell, and the resulting current response is measured. The impedance is calculated as the ratio between the system voltage phasor, $U(j\omega)$, and the current phasor, $I(j\omega)$, which are generated by a frequency response analyser during the experiment. The inclusion of the theoretical background is important to understand the analytical applications of impedance measurements.

The complex impedance can be presented as the sum of the real, $Z_{re}(\omega)$ (or $Z'(re)$), and imaginary, $Z_{im}(\omega)$ (or $Z''(im)$) components that originate mainly from the resistance and capacitance of the cell, respectively.

Equation 2.9

$$Z(j\omega) = \frac{U(j\omega)}{I(j\omega)} = Z_{re}(\omega) + jZ_{im}(\omega); \text{ where } \omega = 2\pi f$$

Where $j = \sqrt{-1}$ and ω and f (excitation frequency) have units of rad.s^{-1} and Hz, respectively.

Electrochemical transformations occurring at the electrode / electrolyte interface can be modelled by extracting components of the electronic equivalence circuits that correspond to the experimental impedance spectra.

A general electronic equivalent circuit (Randles and Ershler model), (Ershler, 1947; Randles, 1947; Randles, 1947; Randles, 1952; Randles & Somerton, 1952) which is very often used to model interfacial phenomena, includes the ohmic resistance of the electrolyte solution, R_s , the Warburg Impedance, Z_w , resulting from the diffusion of ions from the bulk electrolyte to the electrode interface, the double layer capacitance, C_{dl} , and electron transfer resistance, R_{et} , that exists if a redox probe is present in the electrolyte solution, Figure 2.14.

Varying equivalence circuits for impedance spectroscopy

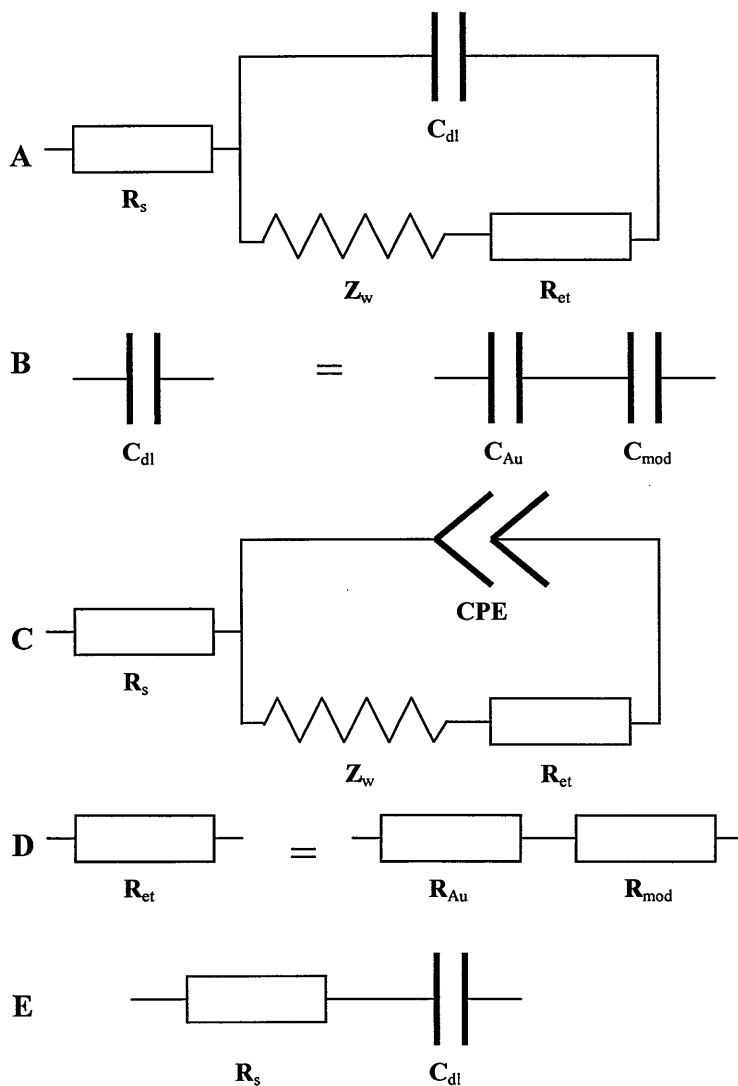


Fig 2.14: Varying equivalence circuits for impedance spectroscopy.

- A) General equivalence circuit for impedance spectroscopy measurements in an electrochemical cell.
- B) Equivalent circuit corresponding to the double-layer capacitance, C_{dl} , that includes variable component, C_{mod} , controlled by the modifier layer. C_{Au} = constant capacitance of an unmodified polycrystalline Au electrode.
- C) General equivalent circuit for the impedance spectroscopy with the constant phase element (CPE) depending on the roughness at the electrode surface.
- D) Equivalent circuit for the electron transfer resistance, R_{et} , which includes the variable component, R_{mod} , corresponding to the different modifier states.
- E) Equivalent circuit for non-Faradaic impedance spectroscopy measurements in the absence of the redox probe.

The parallel elements (C_{dl} and $Z_w + R_{et}$) of the equivalent circuit are introduced since the total current through the working interface is the sum of distinct contributions from the Faradaic process, I_F , and the double-layer charging, I_C . Since all of the current must pass through the uncompensated resistance of the electrolyte solution, R_s , is inserted as a series element in the circuit. The two components of the electronic scheme, R_s and Z_w , represent bulk properties of the electrolyte solution and diffusion features of the redox probe in solution. Therefore, these parameters are not affected by chemical transformations occurring at the electrode surface.

The other two components in the scheme, C_{dl} and R_{et} , depend on the dielectric and insulating features at the electrode/electrolyte interface. The double-layer capacitance depends on the dielectric permittivity introduced into the double-charged layer molecules, ϵ_{dl} , and for the less polar molecules the capacitance should be smaller, Equation 2.10, where $\epsilon_o = 8.85\text{Fm}^{-1}$ is the dielectric constant of the vacuum, ϵ_p is the effective dielectric constant of layer separating the ionic charges and the electrode surface. A is the electrode area, and δ is the thickness of the separating layer.

Equation 2.10

$$C_{dl} = \frac{\epsilon_{dl} A}{\delta} ; \text{ where } \epsilon_{dl} = \epsilon_o \epsilon_p$$

In the equivalent electronic circuit the double layer capacitance, C_{dl} , can be represented as the sum of a constant capacitance of an unmodified electrode (e.g. for a polycrystalline Au electrode, $C_{Au} \approx 40\text{-}60\mu\text{Fcm}^{-2}$), depending on the applied potential (Champagne *et al*, 1989) and a variable capacitance originating from the electrode surface modifier. C_{mod} , connected as series elements, Figure 2.14B. Any electrode modifier of insulating features decreases the double-layer capacitance as compared to the pure metal electrode. Thus, the double-layer capacitance could be expressed by Equation 2.11.

Equation 2.11

$$\frac{1}{C_{dl}} = \frac{1}{C_{Au}} + \frac{1}{C_{mod}}$$

Sometimes, particularly when the electrode surface is rough (Savitri & Mitra, 1999), the electronic properties of the interface cannot be described sufficiently well with a capacitive element, and a constant phase element (CPE), Equation 2.12 should be introduced instead of C_{dl} , Figure 2.14C.

Equation 2.12

$$CPE = A^{-1} (j\omega)^{-n}$$

The constant phase element reflects non-homogeneity of the layer, and the extent of the deviation from the Randles and Ershler model is controlled by the parameter n in Equation 2.12.

Introduction of a CPE element into an equivalent electronic circuit instead of a simple capacitance element was shown to be important for the modelling of primary protein layers on an electrode surface, and the parameter n was found to be ca. 0.97-0.98 (Bardea *et al*, 2000).

The CPE has meaning of capacitance and the coefficient A becomes equal to the C_{dl} when $n=1$.

The electronic transfer resistance, R_{et} , controls the electron transfer kinetics of the redox probe at the electrode interface. Thus, the insulating modifier on the electrode is expected to retard the interfacial electron transfer kinetics and to increase the electron transfer resistance. Equation 2.13 gives the electron transfer resistance at the electrode.

Equation 2.13

$$R_{et} = R_{Au} + R_{mod}$$

Where R_{Au} and R_{mod} are the constant electron transfer resistance of the unmodified electrode and the variable electron transfer resistance introduced by the modifier, respectively, in the presence of the dissolved redox probe. These resistances are also connected as series elements in the equivalent electronic circuit, Figure 2.14D.

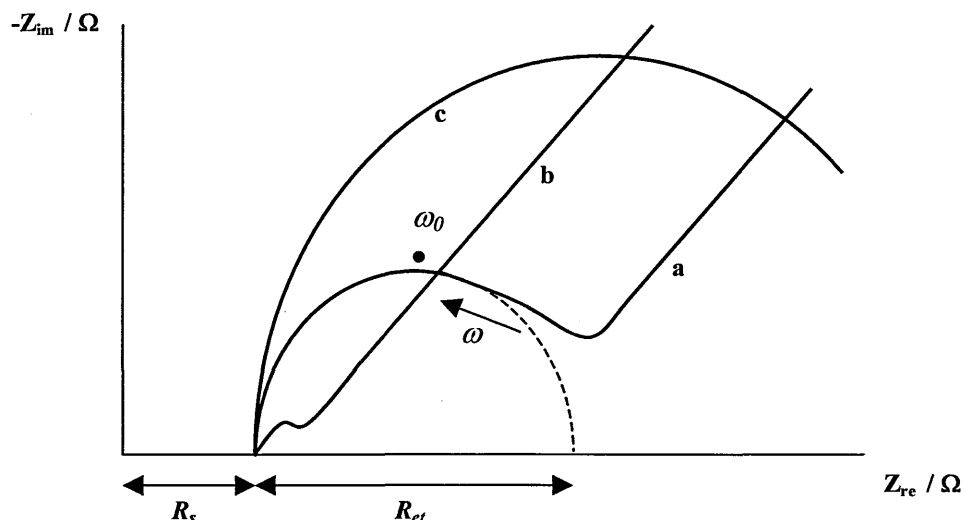


Fig 2.15: Schematic Faradaic impedance spectra presented in the form of a Nyquist plot.

- A modified electrode where the impedance is controlled by diffusion of the redox probe (low frequencies) and by the interfacial electron transfer (high frequencies).
- A modified electrode where the impedance is mainly controlled by diffusion of the redox probe.
- A modified electrode where the impedance is controlled by the interfacial electron transfer within the entire range of the applied frequencies.

The arrow shows the direction of the frequency increase. Resistance of the electrolyte solution, R_s and electron transfer resistance, R_{et} are shown.

A typical shape of a Faradaic impedance spectrum (presented in the form of an impedance complex plane diagram - a Nyquist plot) includes a semicircle region lying on the axis followed by a straight line, Figure 2.15, curve a. The semicircle portion, observed at higher frequencies, corresponds to the electron transfer-limited process, whereas the linear part is characteristic of the lower frequencies range and represents the

diffusionally limited electrochemical process. In the case of very fast electron transfer processes, the impedance spectrum could include only the linear part, curve **b**, whereas a very slow electron-transfer step results in a large semicircle region that is not accompanied by a straight line, curve **c**. The electron transfer kinetics and diffusional characteristics can be extracted from the spectra. The semicircle diameter equals to the electron transfer resistance R_{et} . The intercept of the semicircle with the Z_{re} -axis at high frequencies ($\omega \rightarrow \infty$) is equal to the solution resistance; R_s .

Extrapolation of the circle to lower frequencies yields an intercept corresponding to $R_s + R_{et}$. The characteristic frequency, ω_0 , given by Equation 2.14, has the meaning of the reciprocal of the time constant of the equivalent circuit. The maximum value of the imaginary impedance in the semicircle part corresponds to $Z_{im} = 0.5R_{et}$ and is achieved at the characteristic frequency, ω_0 .

Equation 2.14

$$\omega_0 = (C_{dl}R_{et})^{-1}$$

One of the most important parameters governing the technique is the applied frequency. At low frequencies ($f < 1\text{mHz}$) the impedance value is basically determined by the DC-conductivity of the electrolyte solution.

At very high frequencies ($f > 100\text{kHz}$) inductance of the electrochemical cell and connecting wires could contribute to the impedance spectra. Thus, the analytically meaningful impedance spectra are usually recorded at frequencies where they are mainly controlled by the interfacial properties of the modified electrodes ($10\text{mHz} < f < 100\text{kHz}$).

The electron transfer resistance can be translated into the exchange current under equilibrium, I_0 , Equation 2.15, and then the heterogeneous electron transfer rate constant, k_{et} , can be evaluated, Equation 2.16, where R ($8.314 \text{ Jmol}^{-1}\text{K}^{-1}$) is the gas constant, T is the temperature (K), F ($9.65 \times 10^4 \text{ Cmol}^{-1}$) is the Faraday constant, A is the electrode area

(cm⁻²), [S] corresponds to the bulk concentration of the redox probe (mol cm⁻³), and n is the number of electrons transferred per molecule of the redox probe.

Equation 2.15

$$R_{\text{et}} = RT (nFI_o)^{-1}$$

Equation 2.16

$$I_o = nFAk_{\text{et}}[S]$$

In the absence of any redox component in the electrolyte solution, only non-Faradaic impedance is operative. The electron transfer characterising parameters, R_{et} and Z_w , become infinite and the equivalent circuit can be simplified, as shown in Figure 2.14E. The variable component in the circuit is presented by C_{mod} , and it affects the imaginary part of the impedance Z_{im} , Equation 2.17. Considering only the dense part of the double-charged layer:

Equation 2.17

$$Z_{\text{im}} = \frac{1}{\omega(C_{\text{Au}} + C_{\text{mod}})}$$

The experimental results can be analysed graphically (Bard & Faulkner, 1980; Stoyanov *et al*, 1991) (in the case of Faradaic impedance spectra usually by the use of the Nyquist coordinates Z_{im} vs Z_{re}) in the frame of the theoretical model (Ershler, 1947; Randles, 1947; Randles, 1948; Randles, 1952; Randles & Somerton, 1952).

A computer fitting of the experimental data to a theoretical model represented by an equivalent circuit is usually performed. All electronic characteristics of the equivalent circuit and the corresponding physical parameters of the real electrochemical system can be extracted from such analysis. Since the variable parameters of the system represent the functions of the modifying layer and its composition, they can be used to quantitatively

characterise the layer. Analysis of the $Z_{im}(\omega)$ values observed at different frequencies allows the calculation of the following important parameters: a) the double layer capacitance, C_{dl} and its variable component, C_{mod} ; b) the electron transfer resistance, R_{et} , and its variable component, R_{mod} ; c) the electron transfer rate constant, k_{et} , for the applied redox probe derived from the electron transfer resistance R_{et} . Thus, the impedance spectroscopy represents not only a suitable transduction technique to follow the interfacial interactions of biomolecules, but it also provides a very powerful method for the characterisation of the structural features of the sensing interface and for explaining mechanisms of chemical processes occurring at the electrode / solution interface (Janata, 2002).

For brevity the author refers to several thorough reviews detailing electrochemical approaches and techniques relevant to the following investigations (Greef *et al*, 1985; Macdonald, 1987; Turner *et al*, 1987; Bard & Faulkner, 2001; Park & Yoo, 2003; Barsoukov & Macdonald, 2005; University of Bath, 2005).

The aims and objectives identified in this work, specifically to create a rapid, labelless and reversible impedimetric immunosensor for the detection of stroke (TIA), will be achieved via the development and fabrication of novel sensor platforms, reported in Chapter 4, in which unique approaches towards the site-specific immobilisation of biological recognition entities will be described (Chapter 5).

Fabricated immunosensors will be interrogated by electrochemical impedance spectroscopy (EIS) and the results obtained via the differing immobilisation procedures will be presented and discussed in the final results chapters (Chapter 6 and Chapter 7). Comparisons will be made and conclusions will be drawn in terms of suitability for the application as a TIA immunosensor. A clear direction for further investigations will be identified and laid out.

Chapter 3: Materials and methods.

3.0 Materials and methods:

3.1 Reagents.

Orthophosphoric acid (GPR grade), sodium di-hydrogen orthophosphate 1-hydrate, di-sodium hydrogen orthophosphate 12-hydrate, sodium chloride, sodium carbonate, potassium chloride and tris (hydroxymethyl) methylamine (all analytical grade) were purchased from BDH lab supplies (Poole, Dorset, UK).

o-phenylenediamine dihydrochloride (1,2-diaminobenzenedihydrochloride), ferrocene carboxylic acid, potassium ferricyanide (III) reagent, potassium ferricyanide (IV) reagent, Luminol, human serum albumin (HSA), albumin serum bovine (BSA) and anti-bovine serum albumin (ABSA developed in rabbit), aniline, aniline hydrochloride and biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt (all analytical grade) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

Immunoprobe™ biotinylation kits containing 0.1M sodium phosphate buffer, 0.01M phosphate buffered saline (NaCl 138mM; KCl 27mM; pH 7.4 at 25°C), biotinamidohexanoic acid sulfo-N-hydroxysuccinimide (BAC-Sulfo-NHS), pronase, affinity purified avidin, dimethylsulfoxide and 10mM 2-(4-Hydroxyphenylazo) benzoic acid (HABA) in 10mM NaOH were also purchased from Sigma-Aldrich (Gillingham, Dorset, UK). All chemicals were supplied as analytical grade and therefore were used without the need for further purification.

Magnesium sulphate, sodium hydroxide, potassium chloride, potassium hydroxide, potassium hydrogen phosphate and hydrochloric acid were purchased from Fisher Scientific (Loughborough, Leicester, UK).

Potassium gold (I) cyanide was obtained from Avocado Research Chemicals, (Alpha Aesar, Heysham, Lancashire, UK).

Monoclonal antibodies against Neuron Specific Enolase (NSE), S-100 β protein, Prostate specific antigen (PSA) and CA125 (tumor marker) were developed and supplied by Canag Diagnostics, Ltd. (Gothenburg, Sweden).

NSE, S-100, PSA and CA125 antigen were also developed and supplied by Canag Diagnostics, Ltd. (Gothenburg, Sweden).

All antibodies and antigen were supplied with sodium azide as preservative.

3.2 Buffers and solutions.

Unless otherwise stated, all solutions were prepared using deionised water ($\geq 18 \text{ M}\Omega\text{cm}^{-1}$ resistivity) prepared via an Elga Purelab UHQ-II Water System (Vivendi Water Systems, High Wycombe, Buckinghamshire, UK).

A pH 7.8 buffer comprising 31.8mM disodium hydrogen orthophosphate 12-hydrate (Na_2HPO_4), 4.35mM dihydrogen orthophosphate 1-hydrate (NaH_2PO_4) and 100mM potassium chloride was prepared. The pH was adjusted to pH 7.4 with hydrochloric acid diluted to 10mM from a 10M stock solution.

pH 7.4 buffers comprising 52.8 mM disodium hydrogen orthophosphate 12-hydrate, 13mM sodium dihydrogen orthophosphate 1-hydrate and with 5.1mM sodium chloride were prepared.

pH 4.0 buffers comprising 9mM orthophosphoric acid, 90mM sodium dihydrogen orthophosphate 1-hydrate and 500mM potassium chloride were prepared.

pH 1.0 buffers comprising 300mM HCl and 500mM KCl were prepared.

pH 6.8 buffers comprising 50mM tris (hydroxymethyl) methylamine and 6.5 mg ml^{-1} magnesium sulfate were prepared using distilled deionised water. The pH was adjusted to pH 7.4 with sodium hydroxide diluted to 10mM from a 10M stock solution.

1mM ferrocene carboxylic acid solutions were prepared in pH 7.8 phosphate buffer for further electrochemical characterisation experiments on carbon electrodes throughout sensor fabrication.

1mM potassium ferricyanide (III) reagent solutions were prepared in pH 7.8 phosphate buffer for electrochemical characterisation experiments on both gold and carbon electrodes throughout sensor fabrication.

5mM *o*-phenylenediamine monomer solutions were prepared in pH 7.4 phosphate buffer for the electropolymerisation of *o*-phenylenediamine and insulation of planar carbon electrodes.

0.2M aniline hydrochloride monomer solutions were prepared in pH 4.0 phosphate buffer for the electrogeneration of polyaniline and stable immobilisation of antibody via entrapment.

0.5mg ml⁻¹ antibody was suspended in 0.2M aniline hydrochloride monomer solution (pH 4.0) for electrodeposition and the subsequent immobilisation via co-entrapment.

0.2M aniline monomer solutions were prepared in pH 1.0 chloride buffer for the electrogeneration of polyaniline and stable immobilisation of antibody via non-covalent affinity techniques.

Antibodies against BSA, NSE, PSA and CA125 were supplied with sodium azide preservative and reconstituted in pH 7.4 phosphate buffer. These were stored at -20°C in working aliquots prior to their co-entrapment during the polymerization of PANI or their biotinylation for non covalent affinity attachment to the PANI-modified working electrode. Prior to biotinylation sodium azide is removed via a de-salting column since the presence of the preservative can impair the efficiency of the biotinylation process.

1mg ml⁻¹ antibody, reconstituted in pH 7.4 phosphate buffer, was used for the biotinylation process.

All solutions used for biotinylation of antibody and determination of extent of biotinylation were prepared following the instructions provided with the Immunoprobe biotinylation kit.

Biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt solutions were prepared for the biotinylation of polyaniline modified (pH 1.0 chloride monomer solution) carbon microelectrode array sensors at a concentration of 1mg ml^{-1} .

Neutravidin biotin-binding protein solutions were prepared in distilled deionised water at $10\mu\text{g ml}^{-1}$.

HSA blocking solution was prepared at a concentration of 10^{-6}M in de-ionised water. In this study, the effect on the impedimetric data of adding 10^{-6}M of human serum albumin (HSA) to the electrode surface during fabrication was examined. Albumin was used as an interfering agent in an attempt to reduce non-specific binding (Ahluwalia *et al*, 1995). HSA is quantitatively the most important protein in plasma and is commonly used in immunoassays as a blocking agent and for conjugating with haptens. (Mariani, & Bianchi, 1982).

Neuron specific enolase antigen test solutions were prepared in pH 6.8 Tris buffer and stored in working aliquots at -20°C .

All other antigen test solutions were prepared in pH 7.4 phosphate buffer and stored at -20°C . PSA antigen, however, was stored at -70°C as recommended.

For the recording of all impedance spectroscopy traces 10mM potassium ferricyanide (III) and 10mM ferrocyanide (IV) were added to the appropriate buffer test solution. The resultant pH of these solutions was always 7.4.

For analysis of the ultrasound process finely ground Luminol was dissolved within a 15mM Na_2CO_3 solution with deionised water. The final concentration of Luminol in the ultrasonic tank was 0.05mM , pH 7.0.

For imaging of microelectrode arrays 2.0g of K_2HPO_4 was dissolved in 200mL of de-ionised water. The pH was adjusted to approximately 10.5 with potassium hydroxide (8N). 0.16g of KAuCN (Alfa Aesar, Premion, 99.99%, Lancashire, UK) was dissolved in 20.0mL of the K_2HPO_4 / de-ionised water solution. Finally 202.5mg Na_2CO_3 was dissolved into 20.0mL of the prepared KAuCN solution.

A hot 'Piranha' solution, 1:3 mixture of 30% H_2O_2 / concentrated H_2SO_4 , was used to clean the quartz crystal chips in between experiments. (*Caution! Piranha solution reacts violently with almost any organic materials and should be handled with extreme care!*)

3.3 Materials.

Menzel[®] 'Superfrost' frosted microscope slides (76 x 26 mm) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and were used for the construction of gold-sputter coated working and counter electrodes.

Epoxy resin (Araldite[®]) and Acheson Electrodag[®] (1415M) silver conductive paint were purchased from AGAR Scientific Ltd. (Stansted, Essex, UK) and was used for the construction of gold sputter-coated working and counter electrodes, as well as for the preparation of electron microscopy samples.

Aluminium pin stubs (12.5 mm diameter, 8 mm pin length) and carbon adhesive discs (12 mm diameter) were purchased from AGAR Scientific Ltd. (Stansted, Essex, UK) and used for the preparation of electron microscopy samples.

Multicore copper wire was purchased from Maplin Electronics (Luton, Bedfordshire, UK) was used for the construction of gold sputter-coated working and counter electrodes.

Stainless steel gauze ('20 mesh, plain weave') was obtained from Fine Mesh Metals (Brierley Hill, West Midlands, UK) and was used as a counter electrode for the polymerisation cell.

Perforated stainless steel type 304 sheeting (hole diameter 3 mm hexagonal arrangement, pitch 5 mm, thickness 1 mm) was purchased from RM Perforating Ltd. (Warrington, UK) and was used for ultrasound baffling during sonocation.

Figure 3.1 depicts a typical Microarray sensor, used in this investigation. Microarray (Manchester, UK) manufactures the sensor electrode platforms via screen-printing techniques and was the supplier of all screen-printed electrodes used during this work.

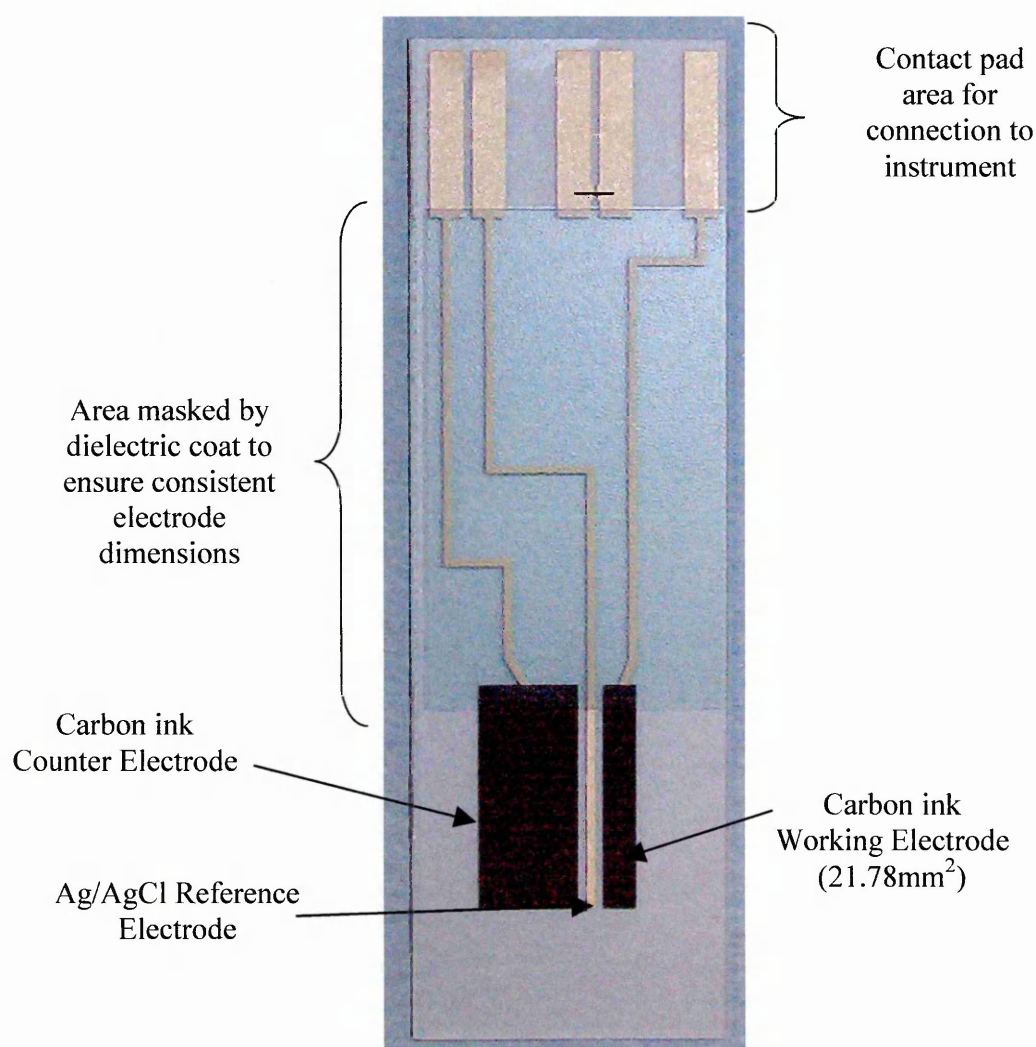


Fig 3.1: Single screen-printed carbon/graphite electrode.

3.4 Experimental apparatus.

3.4.1 Gold sputter coating

An Agar B7341 Automatic Sputter Coater (Agar Scientific Ltd., Essex, UK), in conjunction with a Pfeiffer Rotary Vane Vacuum Pump (Pfeiffer Vacuum Ltd., Newport Pagnell, UK), was used for the preparation of gold sputter-coated working and counter electrodes and samples for investigation under SEM (Figure 3.2).



Figure 3.2: Agar scientific gold sputter coater.

3.4.2 Potentiostats.

A Sycopel Analytical Electrochemical Workstation Model AEW2-10 (Figure 3.3) potentiostat (Sycopel Scientific Ltd., Washington, Tyne & Wear, UK) controlled by a PC equipped with an ECProg3 (version 3.60) software program was used for all laboratory scale electrochemical studies.



Figure 3.3: Sycopel AEW2-10 potentiostat.

3.4.3 Frequency response analysers.

AC Impedance measurements were performed using an ACM (Grange-over-Sands, Cumbria, UK) Auto AC DSP frequency response analyser (Figure 3.4), 10^4 to 10^{-4} Hz a.c., 5mV amplitude perturbation, and potentiostat linked to an IBM compatible PC utilising ACM software (version 4.10) for data acquisition.

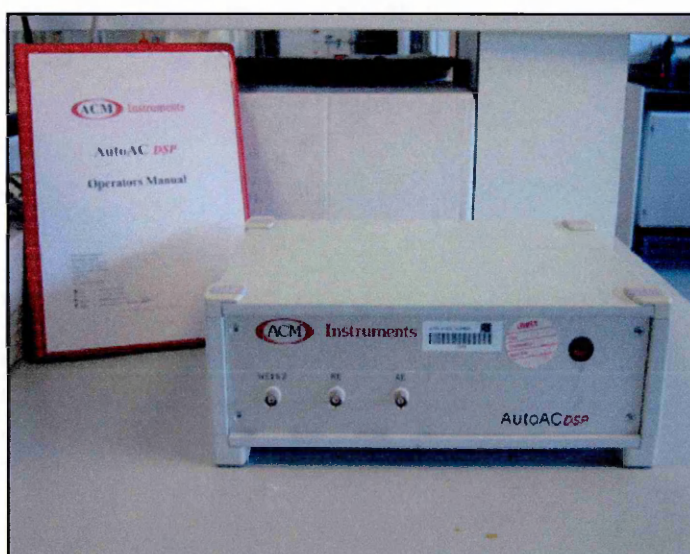


Fig 3.4: ACM DSP Auto Frequency response analyser.

3.4.4 Ultrasonic tanks.

Sonication experiments were performed using a custom built 2 kW, 25.1 kHz ultrasound tank (Figure 3.5) with internal dimensions of 750 x 750 x 600 mm (working volume 750 x 750 x 500 mm) (Ultrawave Ltd., Cardiff, UK) unless otherwise stated. The tank incorporates a frequency sweep of ± 1 kHz in 100 Hz steps three times per second, and a temperature control system allowing the temperature of the water to be maintained at 25°C. Software incorporated within the power generation system allows sonication times to be varied between 1 and 99 seconds and for the power to be varied between 50 and 100 % of the maximum power output. The software also supports a square wave sonic pulse (50% duty cycle) to facilitate de-gassing of the water, which was carried out for 60 minutes prior to use.



Fig 3.5: Custom built 2kW ultrasound tank.

As the purity of the water is critical to the efficiency of the cavitation process, ultrasound tank water is continually purified via a 100 μm particulate filter, a ‘CC9’ carbon filter and a ‘DC9’ deioniser (Purite Ltd., Thame, Oxfordshire, UK), producing water with a conductivity of $<0.1 \mu\text{S}.\text{cm}^{-1}$ ($>18 \text{ M}\Omega\text{cm}^{-1}$), confirmed by an integrated conductivity meter (Figure 3.6).

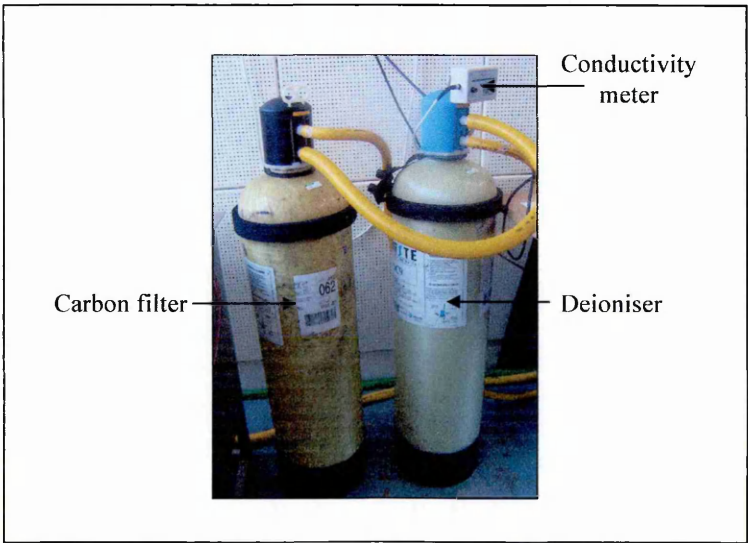


Figure 3.6: Water purification system for 2kW ultrasound tank.

A perforated stainless steel baffle was also custom designed and built to reduce the formation of standing waves and wave reflections from the tank walls, thus improving ultrasound homogeneity (Figure 3.7). Baffle sides were set at a distance of 50 mm from tank walls, including the base which was separated by vibration damping rubber feet. The baffling was employed during all sonocation procedures unless otherwise stated.

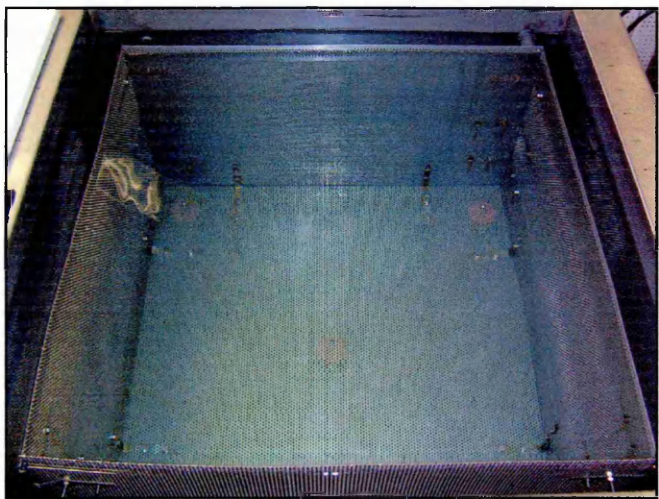


Figure 3.7: Ultrasound tank baffling.

3.4.5 Microscopy.

An Olympus SZX12 bifocal microscope (max 90x magnification) incorporating a Schott KL1500 LCD light source, and a KPM1E/K CCD camera with Leutron Vision PicPort Colour image frame grabber was used for all light microscopy analysis (Figure 3.8).

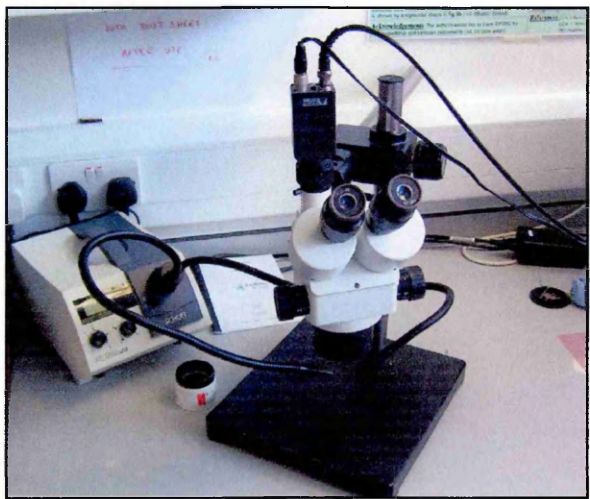


Figure 3.8: Olympus SZX12 microscope with Schott KL1500 light source.

A Philips XL30 FEG-SEM was used for all scanning electron microscopy (Guildford, Surrey, UK). The specimen was mounted on an aluminium stage, pre-coated with a gold sputtered layer and adhered with a silver dag conductive paint (Maplins, Luton, UK).

Ultraviolet spectroscopy was performed on a Shimadzu scientific instruments UVPC spectrophotometer (Columbia, U.S.A.). Quartz cuvettes were used to confirm the presence of protein at 280nm after biotinylation to create an elution profile and to determine the extent of biotinylation at 500nm utilising the avidin-HABA assay.

3.4.6 Quartz Crystal Microbalance.

A Maxtek, Inc (California, U.S.A.) quartz crystal microbalance research system (RQCM) that can measure up to three crystals simultaneously on separate channels (Fig 3.9) was used for the real-time monitoring of mass change at the electrode surface using a static cell arrangement that achieved a stabilisation frequency over 24 hours prior to experimental analysis. The electrical design of this system is based on that published by Bruckenstein (Bruckenstein & Shay, 1985).



Fig 3.9: RQCM Real-Time Crystal Frequency, Mass and Conductance Measurement Analyser, research crystals and crystal holder.

3.5 Experimental Procedure.

3.5.1 Carbon microelectrode-array sensor fabrication.

3.5.1.1 Electrochemical insulation of the Carbon working electrode.

Prior to the immersion of the sheet of carbon electrodes, the monomer solution used was thoroughly purged with N₂ for 20 minutes in a sealed cell to provide an oxygen free atmosphere. An initial 1-second blast of ultrasound was also applied to a submerged sheet of planar electrodes causing passivation at the electrodes, which displaces bubbles trapped at the surface. The trapped gas bubbles are due to the inherent hydrophobicity of carbon. A passivated carbon electrode surface provides a more hydrophilic surface and permits better wet up throughout the polymerisation process and ensures a homogeneous topography. The cycle number, potentiodynamic sweep rates and the time taken to achieve homogeneous insulation have been thoroughly investigated and optimised (Gornall, 2004; Mills, 2005).

Homogenous insulation of a planar carbon electrode was achieved by sequentially scanning the working electrode potential from 0mV through to +1000mV (vs. Ag/AgCl) and back to the starting potential at a scan rate of 50mVs⁻¹ for 20 sweeps. A 5mM *o*-phenylenediamine dichloride solution was prepared in pH 7.4 phosphate buffer for the electropolymerisation of *o*-phenylenediamine films.

3.5.1.2 The sonochemical ablation of insulating polymer films at screen-printed carbon electrodes to form microelectrode arrays.

Up to 2×10^5 microelectrodes per cm^2 on each carbon sensor were created through the sonochemical ablation of insulated planar carbon electrodes (Higson, 1996; Myler, 2000; Barton *et al*, 2004, Gornall, 2005; Mills, 2005). Low wavelength penetration of the insulating thin film polymer exposes discrete micron dimension pores of the underlying electrode using ultrasound kinetics.

Ultrasound is generated in the tank by 10 sonic horn transducers geometrically arranged and bonded to the base of the stainless steel tank housing. The water in the tank is subject to rigorous cleaning regimes. Organics and ions were removed through a two-stage ion exchange resin filter cartridge system. The water was thermostated at 25°C, via in-built heating elements within the tank for consistency during the sonication process.

To achieve a homogenous ultrasound energy map and to aid in the effective distribution of cavitation a number of approaches have been utilised. A crystal-based microphone transducer in conjunction with an oscilloscope has been used to allow for tank profiling recording the sound waves on digital storage. This method, coupled with foil testing, has allowed for the modification of tank baffling, thus further improving the homogeneity of the ultrasound within the tank (Mills, 2005).

The stainless steel baffling has been designed to disrupt standing waves (nodes and antinodes) – again profiled using the transducer system above (Mills, 2005).

An enhanced frequency modulation sweep has also been incorporated to further disrupt the nodes / antinodes formation (i.e. standing waves). The sweep is $\pm 1\text{KHz}$, swept at 3 times a second.

Ultrasound was applied at a frequency of 25 KHz for 10 seconds duration. Discrete micron dimension pores were created via thin film penetration of the insulating polymer through a cavitation process due to the application of ultrasound in the liquid medium.

The ultra-sound gives rise to thermal agitation of water molecules leading to superheated vapour bubble formation, asymmetric growth and collapse. The collapse gives rise to micro-jets of fluid at velocities up to several hundred ms^{-1} . (Higson, 1996; Suslick *et al*, 1986; Suslick, 1990; Myler, 2000; Barton *et al*, 2004)

A mechanical movement system rotated a sheet batch of 100 insulated planar carbon electrodes throughout the 10 seconds sonication time to achieve an evenly distributed proportion of caviatation across the sheet. This further reduced variability of the fabricated microelectrode sensors and also helped to further remove any standing wave effects (i.e. node and anti-node regions) (Gornall, 2005; Mills, 2005).

The effects of sonochemistry principally derive from the formation, growth and asymmetric collapse of bubbles within solvents (Neppiras, 1984). Extremely high temperatures and pressures are generated in such processes which may give rise to a range of effects such as surface cavitation of solids or, for example, the thermal excitation of chemical intermediates.

The intensity of heating in a sonochemical process may be controlled by a number of physical parameters, such as the thermal conductivity of the dissolved gasses, the solvent vapour pressure inside the bubble, the ambient pressure, and of course the frequency and intensity of the ultra-sound itself (Frederick, 1965).

3.5.2 Characterisation of ultrasonic profile within the tank and electrochemical confirmation of microelectrode behaviour.

3.5.2.1 Electrochemical characterisation of microelectrode behaviour.

A sheet batch of 100 carbon electrodes was subjected to insulation and sonochemical ablation. Confirmation of microelectrode behavior was achieved by performing cyclic voltammetry in the presence of a redox couple, such as potassium ferricyanide, on a selected number of electrodes at different positions on the fabrication sheet. Enhanced characterisation of electrode behaviour has been obtained through investigations using couples reported to show enhanced redox activity, in terms of electrochemical reversibility, for screen printed carbon electrodes, i.e. hexaammineruthenium (III) chloride and ferrocene carboxylic acid.

3.5.2.2 Visual assessment of the distribution of cavitation sites within the sonication tank via sonoluminescence.

Light output from multibubble sonoluminescence has been investigated as a technique to predict the conditions under which the desired ablation effects will be optimum. The cavitation sites can be identified in the tank in the presence of Luminol.

Discrete micron dimension pores are created via thin film penetration of the insulating polymer (*o*-phenylenediamine) through a cavitation process due to the application of ultrasound. The ultrasound gives rise to thermal agitation of water molecules leading to superheated vapour bubble formation, asymmetric growth and collapse. The collapse gives rise to micro-jets of fluid at velocities up to several hundred ms^{-1} .

Faint fluorescence in the blue light region is given off as the water molecules undergo thermal agitation from the applied ultrasound and the OH^- ions react in an alkaline biased environment (Suslick *et al*, 1990; Suslick, 2004).

A visual assessment can be made and photographic techniques are utilised to record the distribution of sites for cavitation. Luminescent emission is therefore used to characterise the system. Figure 3.10 shows the experimental set up for recording the fluorescent images.

The fluorescent energy map under differing baffling regimes reflects the ultrasound profile within the tank and can aid in the distribution refinement to increase the homogeneity of the ultrasound process.

Finely ground luminol was added to deionised water containing 1.5×10^{-2} M Na_2CO_3 , followed by stirring to dissolve. The final concentration of luminol in the tank was 5×10^{-5} M.



Fig 3.10: Experimental set-up for the photographic recording of sonoluminescence.

Various photographic conditions, exposure times and film types were investigated under blackout conditions in the ultrasonic fabrication tank facility.

3.5.2.3 Visual assessment of pore density and distribution.

Gold cyanide labelling can be used for the definition of pore distribution and in pore density calculations utilising light microscopy as visual confirmation of insulating film perforation, following electrochemical deposition of the gold cyanide.

Several electrochemical methods can be employed. The gold cyanide solution was prepared as in section 3.2. For purposes of deposition control, a constant potential method was employed. A polarisation potential of -1.2V (vs. SCE) was applied. Deposition was performed at room temperature (25°C). A Pt wire was used as the counter electrode. The total charge passed was recorded to follow the deposition. Sensors were rinsed with distilled water and allowed to air dry after the deposition process.

3.5.3 Antibody immobilisation at microelectrode array sensor platforms.

3.5.3.1 Antibody immobilization via electropolymerisation and simultaneous co-entrapment within PANI, emeraldine, ‘mushroom-like’ protrusions.

The potentiodynamic electrodeposition of PANI / antibody onto the Microarray carbon electrodes was achieved electrochemically by sequentially cycling the working electrode potential from -200mV through to +800mV (vs. Ag/AgCl) and back to the starting potential at a scan rate of 50 mVs⁻¹. A linear sweep from -200 to +800mV (50mVs⁻¹) was performed at the end of cyclic voltammetry to leave the polyaniline in its protonated emeraldine salt form. A 0.2M aniline hydrochloride solution was prepared in a pH 4.0 acetate buffer (Myler, 2000). The solution pH fell to approximately 2.6 upon addition of monomer. Monoclonal antibody receptor was incorporated into the buffered monomer solution prior to polymerisation at a resultant concentration of 0.5mg ml⁻¹ within polyaniline ‘emeraldine’ mushroom-like protrusions. The pH of the monomer solution increases upon addition of antibody but was required to stay below pH 4.0 so that it was

the conductive 'emeraldine' form of polyaniline deposited at the working electrode. The conducting emeraldine mushrooms emanate from the micropores at the polymerised and sonicated carbon electrode offering hemi-spherical diffusion profiles and provide an enhanced electro-active interface. The conducting polymer increases in volume in three dimensions, building up as more charge was passed with each potential sweep.

The final monomer / antibody solution pH of ~3.5 and the polymerisation time (in terms of voltammetric sweeps applied) is a compromise between requiring the conductive form of aniline to be laid down efficiently without damaging the antibodies. The dimensions of the protrusion phenomena can be altered by varying the deposition cycle number, however, increasing polymerisation time will increase the time the receptor biocomponents are subjected to harsh environmental conditions.

3.5.3.2 Antibody immobilisation via electro-polymerisation and non-covalent affinity assembly at PANI, emeraldine, 'mushroom-like' protrusions.

The avidin-biotin system provides a powerful tool for research and analysis due to the specific and high affinity ($K_a = 10^{15} \text{ M}^{-1}$) interaction between avidin and biotin. Biotinylated antibodies, enzymes and other protein molecules are increasingly used as specific probes in immunological and immunocyto-chemical techniques (Wilchek & Bayer, 1988; Heggeness & Ash, 1977; Hnatowich *et al*, 1987; Suter & Butler, 1986; Gretch *et al*, 1987) and in medical applications (Hnatowich *et al*, 1987). Biotinylated derivatives of peptide hormones, growth factors and cytokines are also useful in various research applications involving affinity purification of receptors in combination with avidin affinity chromatography (Finn *et al*, 1984; Lee *et al*, 1989).

The primary application of the ImmunoProbe Biotinylation kit is the preparation of biotin labeled polyclonal and monoclonal antibodies which may be used in ELISA, immunoblotting, immunohistochemistry and other immunosensing techniques.

The kit may also be used for conjugation of biotin to other proteins, peptide hormones or cytokines.

Biotinylation is often performed with N-hydroxysuccinimide (NHS) esters of biotin or biotinamidohexanoic acid (biotinAC, BAC) (Wilchek & Bayer, 1990). The extended spacer arm from the hexanoic acid greatly improves the interaction between avidin and biotinylated macromolecules by overcoming steric hindrance present at the biotin binding sites of avidin (Green *et al*, 1971).

With the Sigma ImmunoProbe Kit, biotinylation is performed with BAC-SulfoNHS. This derivative is soluble in water and biotinylation proceeds at near neutral pH values. The reagent is particularly useful when mild reaction conditions are required for the biotinylation of sensitive biomolecules such as antibodies, enzymes and cell surface proteins (Suter & Butler, 1986; Gretch *et al*, 1987).

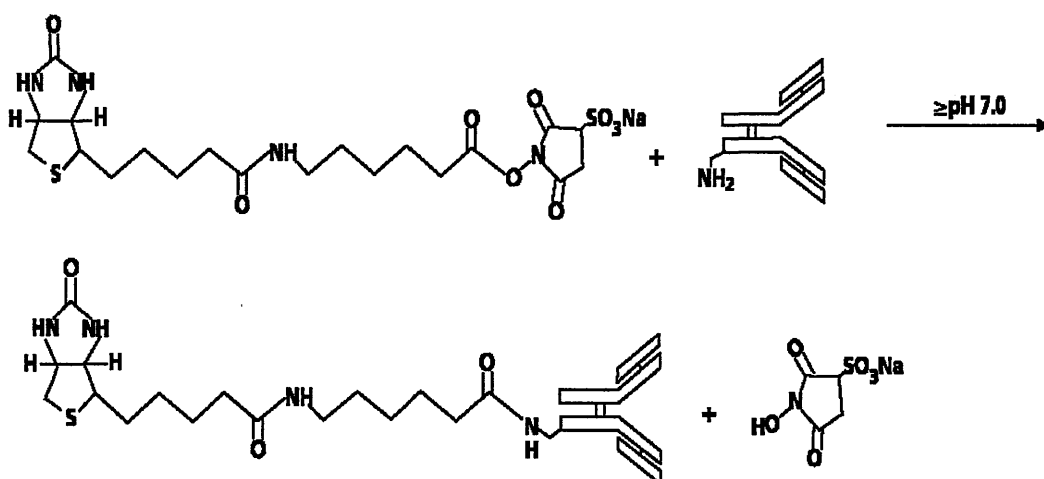


Fig 3.11 Schematic representation of the conjugation of BAC-SulfoNHS to an amino group of an IgG Molecule.

Following the labeling reaction, the biotinylated protein was separated from unreacted or hydrolysed reagent by a fast gel-filtration step. The extent of biotinylation and ratio of biotin to protein are determined by the avidin-HABA assay (Green, 1965).

The absorption of the avidin-HABA complex at 500nm (A_{500}) decreases proportionally with increased concentration of biotin as the HABA dye is displaced from avidin due to the higher affinity of avidin for biotin. Using a ratio of BAC-SulfoNHS / IgG of 5:1 or 10:1, the level of biotin incorporation is approximately 2-3 and 4-6 moles of biotin per mole of protein, respectively. The biotinylated antibodies were stored frozen in working aliquots at -20°C .

Following the biotinylation of antibody receptors, the construction of an orientation-specific scaffolding at the PANI-modified microelectrode array sensors resulted in their stable immobilisation as a monolayer at the electrode-solution interface and proceeds via the following 5 steps.

Step 1) The biotinylation of antibody bioreceptors.

Antibody biotinylation (Glazer, 1982) was performed prior to all other sensor fabrication steps and the biotinylated bioreceptors were stored frozen in working aliquots of approximately 1mg ml^{-1} at -20°C . To remove sodium azide preservative, the antibody was subject to gel-filtration using a Sephadex (G25) gel-filtration column (Millner, 1999). Sodium azide can have adverse effects on the biotinylation of antibodies.

Protein Labeling: The following procedure describes the preparation of biotin-labelled IgG. The molar ratio in the reaction mixture is 5:1 or 10:1 of biotinylation reagent (BAC-SulfoNHS, MW = 556.8) to IgG (MW = 150,000). These ratios result, in most cases, in 2-3 moles and 4-6 moles of biotin per mole of protein, respectively. This procedure can be modified if a protein of a different molecular weight or a different amount of IgG is used in the labeling reaction. The level of biotin labeling may vary from one protein to another, in part due to the different number of lysine residues available for modification.

A sodium phosphate buffer sachet was reconstituted with 5ml of deionised water to 0.1M.

The IgG (a-PSA, a-S100, a-NSE) for biotinylation was prepared at 1mg ml^{-1} in 0.1M sodium phosphate buffer. The A_{280} of an IgG solution at 1mg ml^{-1} is 1.4 (1.0cm path length).

30 μl of DMSO was used to dissolve 5mg of BAC-SulfoNHS. 0.1M sodium phosphate buffer was then added to a final volume of 0.5ml and the solution was vortexed well. The resulting concentration of BAC-SulfoNHS solution was 10mg ml^{-1} .

19 μl (ratio 5:1) or 38 μl (ratio 10:1) of the BAC-SulfoNHS was immediately added to 1ml of the IgG solution with gentle stirring. The reaction mixture solution was incubated with gentle stirring for a further 30 minutes at room temperature or for 2 hours at 2-8°C.

Isolation of labeled protein: 1 litre of 0.01M PBS was prepared for the fast gel-filtration step whereby the conjugate is separated from the reactants.

The gel filtration column was supported over a 100ml beaker.

The cap was removed from the top of the column. The lower tip of the column was cut open and the excess liquid was allowed to flow through.

The column was equilibrated with 30ml of PBS (6 x 5ml).

The reaction mixture (IgG with BAC-SulfoNHS) was applied to the column and the flow-through was collected as Fraction 1.

The column was then eluted with 9 x 1ml of PBS and the flow through was collected as Fractions 2-10. The gel-filtration column was then washed with a further 30 ml of PBS (6 x 5ml) and as such may then be regenerated up to five times.

The protein presence was monitored by measuring the absorbance at 280nm in the UV/visible spectrophotometer.

An elution profile was obtained and the fractions displaying a clear peak at 280nm (thus containing the protein) were pooled.

Working aliquots of 100µl were stored frozen at -20°C until they were required during the latter stages of sensor fabrication utilizing the biological affinity attachment bridges for their specifically orientated immobilisation.

Determination of the Biotin/Protein ratio: In order to determine the level of biotinylation in the proteins, it was necessary to measure the protein concentration (absorption at 280nm) and the biotin content (with the avidin-HABA assay).

0.1ml of the pooled biotinylated protein fraction was diluted to 1ml with PBS. The absorbance of this diluted sample was recorded at 280nm using the UV spectrophotometer. This sample was denoted as the 'Protein sample'.

Pronase was reconstituted in 1ml of de-ionised water.

10µl of pronase was added to another 0.1ml sample of the pooled biotinylated protein fraction and incubated overnight at room temperature. This sample was denoted as the 'Biotin sample'. The biotinylated IgG was digested with 0.1% pronase to prevent subsequent steric hindrance of biotin binding to avidin and nonspecific binding of the dye to the biotinylated protein when performing the avidin-HABA assay.

The avidin stock solution was reconstituted by dissolving the avidin in 19.4ml of deionised water.

A blank UV spectrophotometer trace was recorded at 500nm with PBS.

0.1ml of the 10mM HABA solution was added to 3.2ml of the avidin stock solution. The absorbance of the avidin-HABA solution at 500nm was 1.0 (1.0cm path length).

0.9ml of the avidin-HABA reagent was mixed with 0.1ml of PBS (HABA control).

0.9ml of the avidin-HABA reagent was mixed with 0.1ml of the pronase treated sample (Biotin sample).

The absorbance of the HABA control and Biotin sample were recorded at 500nm in 1.0cm path length cuvette allowing for the calculation of the ratio of biotin bound to protein. The pooled biotinylated antibody is then stored at -20°C in working aliquots of 100µl.

Step 2) The biotinylation of polyaniline.

Polyaniline is electrogenerated at microelectrode array sensor surfaces using optimum deposition conditions as no biomolecule is present in the monomer solution prior to deposition. A 0.2M aniline solution was prepared in a pH 1 chloride buffer to provide sufficient counter-ions for polymerisation. Cyclic voltammetry was performed at a scan rate of 50mVs⁻¹ from -200mV to +800mV and back to the starting potential (vs. Ag/AgCl). A linear sweep from -200mV to +800mV was performed at the end of cyclic voltammetry to leave the polyaniline in its conductive emeraldine salt form. The Biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt is prepared in de-ionised water at a concentration of 1mg ml⁻¹. The mushroom microelectrode array sensors were submerged in the biotinylating solution for 24 hours. The biotin couples to the primary amines at the end of the polyaniline chains and secondary amines throughout the polymer.

Step 3) Neutravidin.

Neutravidin biotin-binding agent was prepared in de-ionised water at 10µg ml⁻¹. The neutravidin was added to the working electrodes of the biotinylated sensors for a 1 hour period. The sensors were then thoroughly flushed with de-ionised water to remove any non-specifically weakly adsorbed matter.

Step 4) Addition of biotinylated antibody.

The biotinylated antibody was then defrosted and the sensor working electrodes are submerged in the antibody solution for another 1 hour fabrication stage. Again de-ionised water is used to thoroughly flush the sensors and remove any non-specifically bound antibody at the end of the exposure.

Avidin-biotin attachment bridges ‘sandwich self assembly’ takes advantage of the strong specific biological interactions between proteins and ligands.

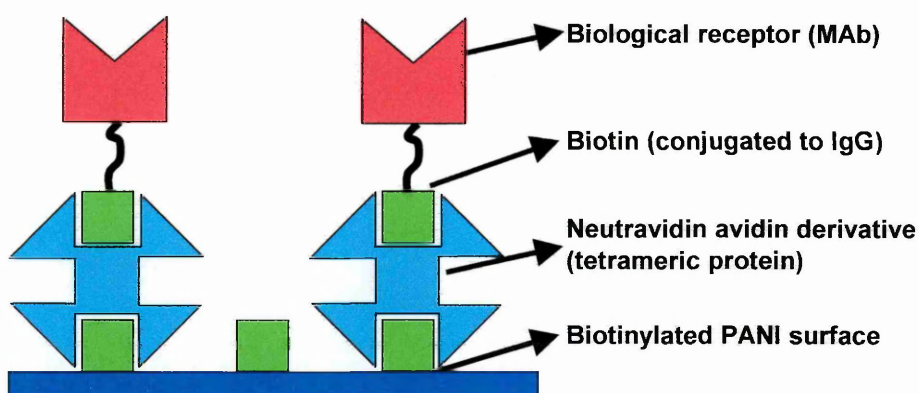


Fig 3.12: Schematic representation of the immobilisation via the sophisticated non-covalent affinity assembly at biotinylated PANI ‘mushroom’ microelectrode array sensors.

This approach for biomolecule immobilisation provides orientation specific attachment of a monolayer of receptors at the surface of the polyaniline protrusions. Optimum deposition criteria can be utilised for polyaniline electrogeneration and conditions for the antibody attachment are at neutral pH. The preservation of biological properties is far greater.

Step 5) HSA blocking.

Human serum albumin (HSA) was used to limit non-specific effects. HSA blocking solution was prepared at a concentration of 10^{-6} M in de-ionised water. In this study, the effect on the Impedimetric data of adding 10^{-6} M of HSA to the electrode surface during fabrication was examined. Albumin was used as an interfering agent in an attempt to reduce non-specific binding. HSA is quantitatively the most important protein in plasma and is commonly used in immunoassays as a blocking agent and for conjugating with haptens (Mariani, & Bianchi, 1982). The sensor assembly is exposed to a HSA solution for a 1-hour period.

3.5.4 Quartz Crystal Microbalance sensor fabrication validation.

QCM investigations were carried out following each of the fabrication steps to validate the experimental techniques employed on polyaniline-coated quartz crystals. This sophisticated approach of biotin-avidin scaffolding is monitored using a static cell experimental set-up. A thorough buffer flushing regime using distilled de-ionised water was used in between each fabrication step. In all cases sufficient time was allowed as a stabilisation period.

3.5.5 Electrochemical Impedance Spectroscopy.

Initial investigations were performed using the readily available BSA / ABSA system. Work was aimed towards the detection and quantification of further biomolecules, particularly those involved in the diagnosis of transient ischemic attack such as neuron specific enolase and S-100 protein. PSA and monoclonal antibody were also used extensively in the progression of experimental studies as readily available alternatives to S-100 & NSE systems as the similar physical and chemical natures of the proteins pairings permit an accurate model.

A protocol was established for the impedimetric evaluation of the immobilised antibody sensor platforms upon exposure to complementary antigen. This allows a relatively direct comparison between the varying immobilisation techniques employed and the electrode platforms. A series of control experiments have also been devised to validate and verify the specificity of any responses.

Identical experiments were performed for all antibody-immobilised carbon sensors. All experimental conditions are kept consistent. This permits an overall assessment of the two immobilisation strategies, in terms of immobilisation success and signal transduction quality, for use as impedimetric immunosensor platforms for the labelless detection of markers for stroke. The same experimental protocol is followed for all electrode assemblies, and is described as follows.

AC impedance measurements were performed with a Gill AC DSP Frequency Response Analyser performing both Faradaic and non-Faradaic electrochemical impedance spectroscopy (EIS). Impedance measurements were performed in the frequency range from 10,000Hz to 1Hz, 100 data points at 25 points per decade. A sinusoidal potential modulation (± 5 mV amplitude) was superimposed on the formal potential of the redox couple of $[\text{Fe}(\text{CN})_6]^{4-} / [\text{Fe}(\text{CN})_6]^{3-}$ (+0.22V vs. Ag/AgCl).

The impedance data was fitted to an electrical equivalent circuit using the ACM analysis software (version 4) as the data was extracted by the instrument. The equivalent circuit provides an electrical analogue of chemical/physical processes probed by ac EIS.

Prior to each experiment, the electrolyte test solutions were deoxygenated by bubbling with high-purity nitrogen for at least 20 minutes. All experiments were carried out at room temperature. When not in use all reagents were stored between 2-8°C.

Following the immobilisation of antibody receptor molecules, EIS was then performed to interrogate sensor electrodes. The following describes the EIS interrogation protocol.

Initially a baseline impedance trace was recorded in the relevant buffer test solution alone. This buffer solution contained 10mM $K_4[Fe(CN)_6]$ + 10mM $K_3[Fe(CN)_6]$ as a redox couple and no antigen. A 50 ml vessel of this solution was kept consistent and antigen-free for the recording of all spectral traces. A 1 litre stock solution was also prepared for the flushing and washing regime.

Antibody-loaded sensors were then exposed for 30 minute time periods to increasing concentrations of complementary antigen suspended in the relevant buffer solution. For consistency, the antigen solutions were identical to the test solution, aside from the presence of antigen. After 30 minutes exposure to a single concentration, the working electrode was removed from antigen and flushed with 50ml of the stock solution via a peristaltic pump to remove any non-specifically adsorbed or weakly-bound matter. The impedance trace was then recorded in the relevant buffer test solution between 10,000 Hz to 1Hz.

This process was then repeated for the full range of antigen concentrations, following exposure to the antibody-loaded sensors.

Complex plane impedance analyses allow differing redox states of the polymer, faradaic charge transfers and also capacitive transduction events to be related to the antibody/antigen binding event as a function of interactions occurring at the interface.

Results are plotted in representative Nyquist forms for analysis and interpretation of sensor responses. The traces obtained illustrate the changes in spectra obtained from the baseline trace for a range of clinically relevant concentrations of antigen; depending on the system under investigation.

Simultaneous experiments were also performed for each antigen under investigation using human polyclonal IgG serum antibody receptors instead of the specific monoclonal antibody. The IgG antibody is immobilised at the electrode in an identical manner to the specific sensor under investigation and subject to an identical experimental interrogation protocol. To determine the resultant specific response of the sensors, the IgG sensor response was subtracted from the MAb sensor response.

For BSA and PSA detection a pH 7.4 phosphate buffer (section 3.2) was used for preparation of test, stock and antigen solutions.

For S-100 detection a pH 7.8 phosphate buffer (section 3.2) was used for preparation of test, stock and antigen solutions.

For NSE detection a pH 6.8 tris buffer (section 3.2) was used for preparation of test, stock and antigen solutions.

For CPE-immobilised detection of BSA and PSA 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 & 300 ng ml⁻¹ antigen analyte solutions were prepared in the phosphate buffer.

For affinity-immobilised detection of PSA, S-100 and NSE 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 & 300 pg ml⁻¹ antigen analyte solutions were prepared in the appropriate buffer.

Chapter 4:

Fabrication of microelectrode array sensors.

4.0 Fabrication of microelectrode array sensors.

4.1 Base electrode modification: Electropolymerisation of poly(*o*-phenylenediamine).

4.1.1 Introduction.

Aniline, first known as aniline black (Fritzsche, 1840), and its derivatives including poly-(phenylenediamine) have been known to scientists for over a century (Prochazka, 1880), (Fieser, 1930). However it was not until 1954 that the electrode filming properties of poly(*o*-phenylenediamine), produced via electropolymerisation in acidic solution, were investigated using graphite electrodes (Lord & Rogers, 1954). In 1976, Yacynych and Mark investigated the nature of this film using infra-red, visible and ultra-violet spectroscopy, and speculated as to the nature of the conducting and non-conducting forms (Yacynych & Mark, 1976).

A key property of a conducting polymer is the presence of conjugated, i.e. alternating double and single bonds, along the backbone of the polymer. Each bond contains a localised *sigma* (σ) bond which results in a strong chemical bond. In addition, every double bond also contains a less localised *pi* (π) bond which is weaker in nature. However, conjugation alone is not enough to make a polymer conductive. In addition, charge carriers in the form of electrons or 'holes' have to be injected in to the material; a hole being a position where an electron is missing. When such a hole is filled by an electron jumping in from a neighbouring position, a new hole (or *vacancy*) is created, and so on, allowing charge to migrate a long distance through the polymer thereby, becoming conductive. Charge carriers, serving to either add or remove electrons from the polymer, are termed *dopants*, and generally take the form of an acid or an inorganic molecule. If an electron is removed from the top of the valence band of a semiconductive polymer, e.g. polyacetylene, the vacancy created in the carbon atom results in the formation of a radical cation.

The radical cation, or *polaron*, is partly localised because of the coulomb attraction to the counter ion with which it formed. The addition of a high concentration of dopant to a polymer allows the polaron to move in the field of closely spaced counter-ions, thereby permitting the flow of current. If a second electron is removed from a previously oxidised section of the polymer, a second independent polaron may be created, or, if it is the unpaired electron from the first polaron that is removed, a bipolaron is formed which allows two positive charges to move as a pair. Charge transfer through the bulk of the polymer material occurs by electron transfer of localised charged states between polymer chains (Winokur *et al*, 1987). Although less common, a second mechanism for current flow involves the formation of *solitons*. These are solitary wave defects forming stable free radicals, created for example by the thermal isomerisation of a *cis* polyacetylene chain to the *trans* structure (a *neutral* soliton). Bulk conductivity through the polymer is permitted by *intersoliton hopping* between chains. Measurements of the electrical conductivity of a number of polyaniline derivatives in acidic solution, including poly(*o*-phenylenediamine), referred to herein as PoPD, were carried out by Yano *et al* in 1985 (Yano *et al*, 1985). The conductivity of insulating PoPD polymerised in more neutral conditions was measured by Prasad *et al* to be approximately $3 \times 10^{-9} \text{ S.cm}^{-1}$ (Prasad *et al*, 1996), a million times lower than for PoPD polymerised under highly acidic conditions ($1.2 \times 10^{-3} \text{ S.cm}^{-1}$) (Yano, 1995).

Much research has been carried out into the possible uses of poly(*o*-phenylenediamine), that has included for example sensor modification for the permselectivity against blood interferents such as ascorbic acid (Garjonte & Malinaukas, 1999), and to H^+ ions for the application of pH sensing (Cheek *et al*, 1983), (Rubenstein, 1984). Other applications reported include the protection against metal corrosion (D'Elia *et al*, 2001), electrochromic properties (Oyama *et al*, 1988), (Yano & Higuchi, 1990), (Yano *et al*, 1993), humidity sensing (Ogura *et al*, 1996), biomimetic molecularly imprinted polymers (Malitesta *et al*, 1999), plus a wealth of biosensor related applications (Malitesta *et al*, 1990; Myler, 1997; Diekers *et al*, 2000; Ekinici *et al*, 2001; Barton *et al*, 2004; Pritchard *et al*, 2004; Davis & Higson, 2005).

The *o*-phenylenediamine monomer is an aromatic diamine (Figure 4.1) which can be synthesised by nitration and reduction processes involving benzene.

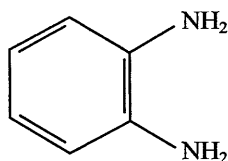


Fig 4.1: *o*-Phenylenediamine monomer (*o*PD).

Poly(*o*-phenylenediamine) can be deposited chemically (Bach, 1966), or electrochemically (Oyama *et al*, 1985) onto electrode surfaces. The benefits of electrochemical polymerisation noted earlier make it the method of choice for the modification of sensor surfaces by polymerisation. Despite the attention PoPD has received, there is relative uncertainty as to the precise structure of the polymer. Various analytical methods have been employed to elucidate its nature, including electrochemical techniques (Yano, 1995), quartz-crystal microbalance studies (Dai *et al*, 1998), Raman and UV-Vis spectroscopies (Wu *et al*, 1996; Losito *et al*, 2001), radiometry (Martinusz *et al*, 1995) and scanning tunnelling microscopy (Ogura *et al*, 1995).

Two principal structures of PoPD have been suggested (Mederos *et al*, 1999). Oyama and co-workers used phenazine as a model compound, comparing the IR absorption spectra of PoPD with that of phenazine in KBr pellets (Chiba *et al*, 1987). They found that the IR spectrum of PoPD was similar to that of phenazine, and that the absorption peak, indicated at 805 cm^{-1} , could be attributed to the skeletal vibrations of the phenazine rings. In contrast Yano found that electropolymerised PoPD films were soluble in common organic solvents and that a cast film of PoPD exhibited reversible redox behaviour, which was accompanied by electrochromism, just as it did before being dissolved. This indicated that the polymer structure remained more or less unchanged and allowed a representative elemental analysis to be carried out via H FT NMR spectra.

The polymeric backbone proposed by Yano was a 1,4-substituted benzoid-quinoid structure (Yano, 1995). Figures 4.2 and 4.3 illustrate these two suggested polymer configurations for polymerisation pH ranges 1-7 (Wu *et al*, 1996; Losito *et al*, 2001).

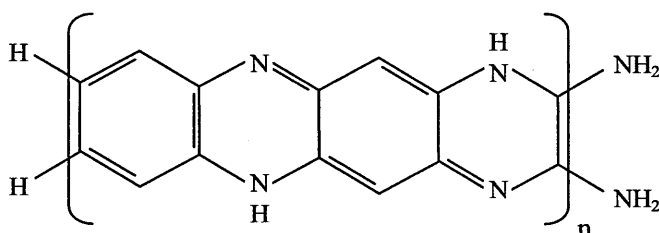


Fig 4.2: Phenazine type structure suggested by Oyama, 1985.

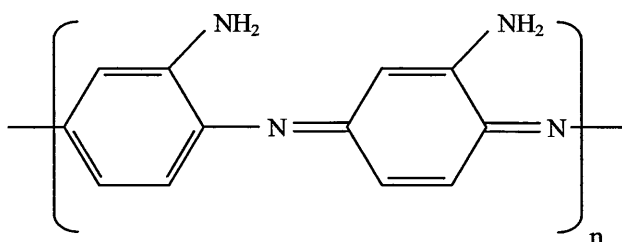


Fig 4.3: 1, 4-substituted benzoid-quinoid structure suggested by Yano, 1995.

The thickness of the film produced by the conducting form of PoPD has been reported to be as great as 850 nm (Yano, 1995). In contrast, the thickness of non-conducting PoPD assembled in more neutral conditions has been reported to be as little as 10 nm (Ohnuki *et al*, 1983; Malitesta *et al*, 1990).

A range of applications utilising electropolymerised poly(*o*-phenylenediamine) films have been investigated previously, including pH sensing applications (Cheek *et al*, 1983), synthesis of biomimetic molecularly imprinted polymers (Malitesta *et al*, 1999) and a wealth of biosensor related applications (Sasso *et al*, 1990; Almeida and Mulchandani, 1993; Castanón *et al*, 1997; Myler *et al*, 1997; Myler, 2000; Ekinici *et al*, 2001; Law, 2002; Barton *et al*, 2004; Gornall, 2004; Pritchard *et al*, 2004; Mills, 2005).

The electropolymerisation of poly(*o*-phenylenediamine) can be exploited to electrically insulate a sensor surface with an ultra-thin layer of polymer. As described in more detail later in this Chapter (section 4.2), this film can then be ultrasonically ablated to create numerous microscopic pores exposing the underlying conductive substrate. As the conductive pores are electrically connected, this results in the formation of a microelectrode array based sensor device.

Alternatively, it is possible to polymerise *o*-phenylenediamine via chemical polymerisation methods, employing a *chemical oxidant* as an initiator. These methods are often employed for industrial polymer syntheses, since the approach is highly appropriate for the bulk polymerisation of reaction mixtures. However, in the context of electrode surface modification, electrochemistry can provide a simple and efficient route to site specific polymerisations at electrode surfaces of any size or number (Imisides *et al*, 1991). The films produced can be easily controlled voltammetrically in terms of density and thickness (particularly for the case of self-limiting *insulating* polymers) and can provide complete coverage on a molecular level (Sasso *et al*, 1990; Castanón *et al*, 1997).

4.1.2 Electrochemical characterisation of planar carbon electrode sensor platform.

The planar carbon electrodes were screen-printed to the desired specifications required and were produced by Microarray Ltd. They were then subjected to electrochemical evaluation to confirm their electroactivity and hence, their successful fabrication.

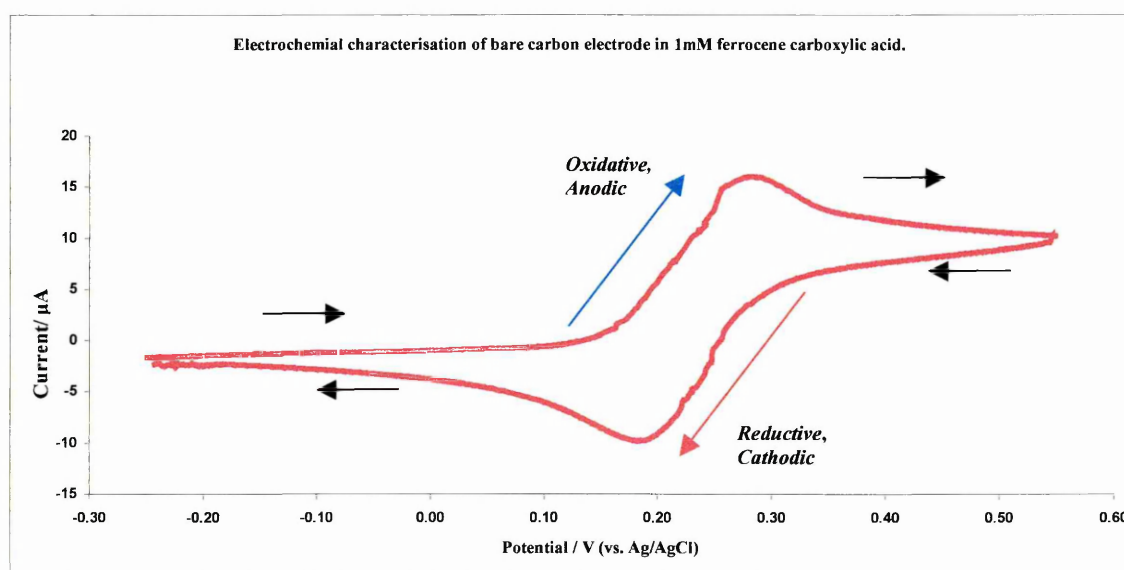


Fig 4.4: Typical cyclic voltammogram showing the electrochemical behaviour of a planar carbon electrode sensor.

Figure 4.4 shows a typical cyclic voltammogram obtained for a screen-printed carbon electrode supplied by Microarray Ltd. analysed potentiodynamically using the onboard reference, counter and working electrodes. The identical and consistent sensor dimensions and distances between the three electrodes yielded matching electrochemical responses when differing sensor were analysed in the chosen redox couple.

The planar electrodes display single clear peaks in both the forward, oxidative (anodic) and reverse, reductive (cathodic) directions. The working electrode potential is cyclically scanned from -250mV to $+550\text{mV}$ at a scan rate of 50mVs^{-1} (vs. Ag/AgCl) in 1mM ferrocene carboxylic acid redox couple. There is inherent irreversibility with carbon paste as the electrode material accounting for the peak-to-peak voltage separation of $\sim 90\text{mV}$ as opposed to 59mV expected for a fast single electron transfer process (Wang, 2000).

For a planar electrode convectional controlled mass transport is faster and the electrode is under diffusional mass transport control as the rate-determining step.

The planar screen-printed electrode is then subjected to several modification steps to form a microelectrode array and the results obtained in Figure 4.4 are used as a platform from which to assess the effects of the modifications on the electrochemical behaviour.

To further enhance the electro-active interface via improved diffusion characteristics, the working electrode was initially modified with a thin-film of insulating polymer. Discrete micron dimension pores of the underlying carbon electrode are subsequently exposed by a unique sonication process causing ablation of the polymeric surface via acoustic cavitation.

A novel sensor platform composed of an array of microelectrodes is thus fashioned that permits the resultant immobilisation of antibody receptor molecules by entrapment or affinity techniques within and/or at the surface of mushroom-like protrusion phenomena, emanating from the ablated pores (Chapter 5). Several techniques and visual assessments were utilised to optimise the fabrication procedure and initial comparative experimental investigations were performed on gold sputter-coated ground glass microscope slides.

Figure 4.4 will be used to assess the effects of the modifications on the base electrode platforms and will be discussed in Section 4.2.

4.1.3 Electropolymerisation of poly (*o*-phenylenediamine) onto gold substrates.

Initial investigations into the electrodeposition of insulating films of poly(*o*-phenylenediamine) utilised gold sputter-coated ground glass slide electrodes. A 5mM solution of *o*PD in pH 7.4 phosphate buffer was cycled 20 times between 0 and +800 mV at a scan rate 50 mVs⁻¹ (cyclic voltammogram illustrated in Figure 4.5). It is noted that unless otherwise stated, all potentials herein have been quoted with respect to Ag/AgCl.

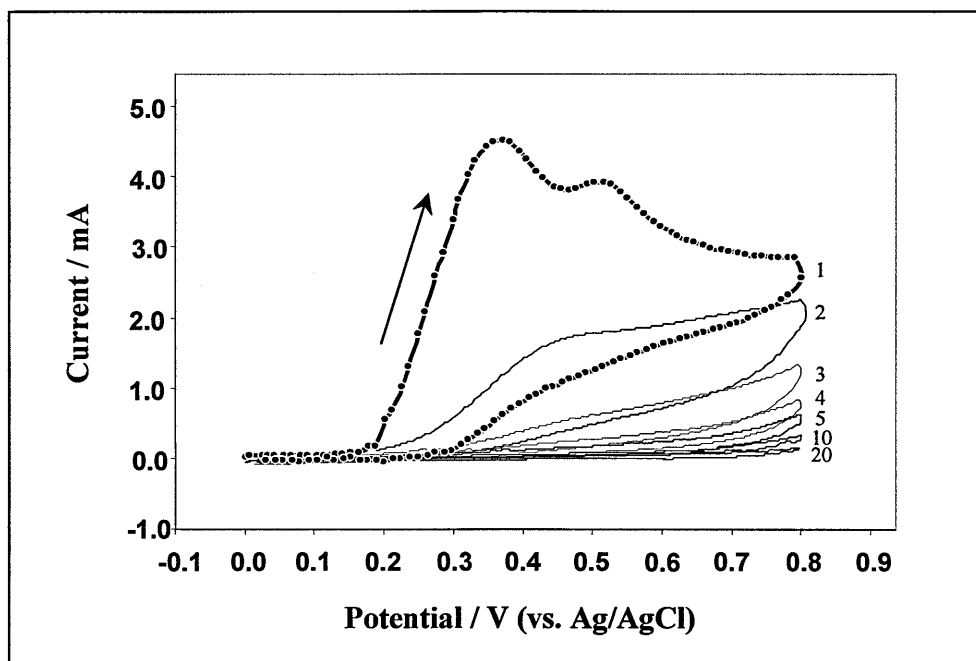


Fig 4.5: CV obtained during the polymerisation of *o*PD (at pH 7.4) on a gold slide electrode

A characteristic cyclic voltammogram for the electropolymerisation of *o*-phenylenediamine was obtained. Two anodic peaks are exhibited on the first sweep, at approximately +350 and +525 mV (vs. Ag/AgCl). Research by Jang *et al* investigated the nature of these peaks and showed that no complementary cathodic peaks can be found if the scan is reversed, indicating that the process is completely irreversible in nature (Jang *et al*, 1995). Using NMR spectroscopy, the first voltammetric peak was identified as due to the oxidation of the *o*PD monomer to form a radical monocation (Figure 4.6).

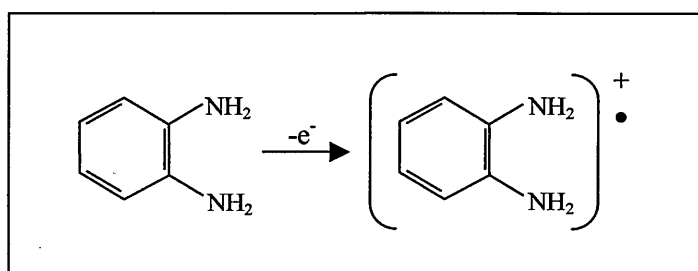


Fig 4.6: Initial oxidation of the *o*PD monomer to form a radical monocation.

The monocation species immediately combines via radical coupling to form one of three possible dimers (Figure 4.7).

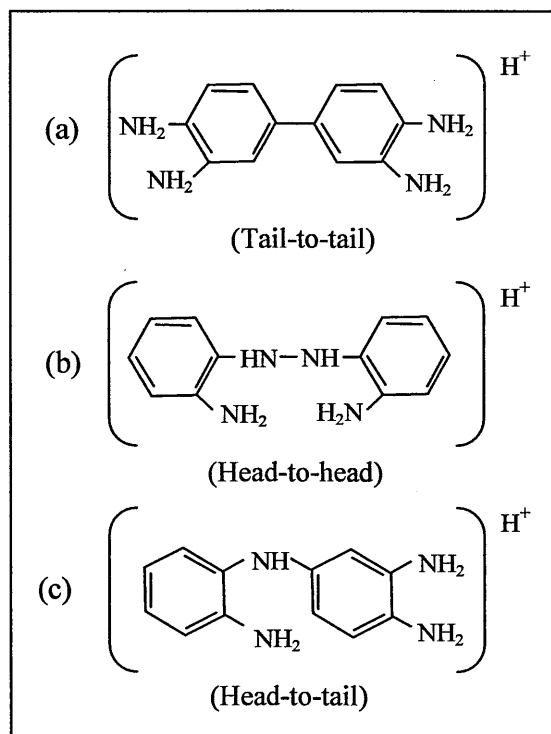


Fig 4.7: Dimerisation following oxidation of *o*PD.

As C–N coupling has been shown to be dominant for the anodic oxidation of aromatic amines in aqueous solution (Volkov *et al*, 1980; Deng & Van Berkel, 1999), the ‘head-to-tail’ dimer configuration is formed preferentially (Figure 4.7c). This structure is suitable for propagation, and the molecule can undergo further elongation to produce more (soluble) species (Figure 4.8).

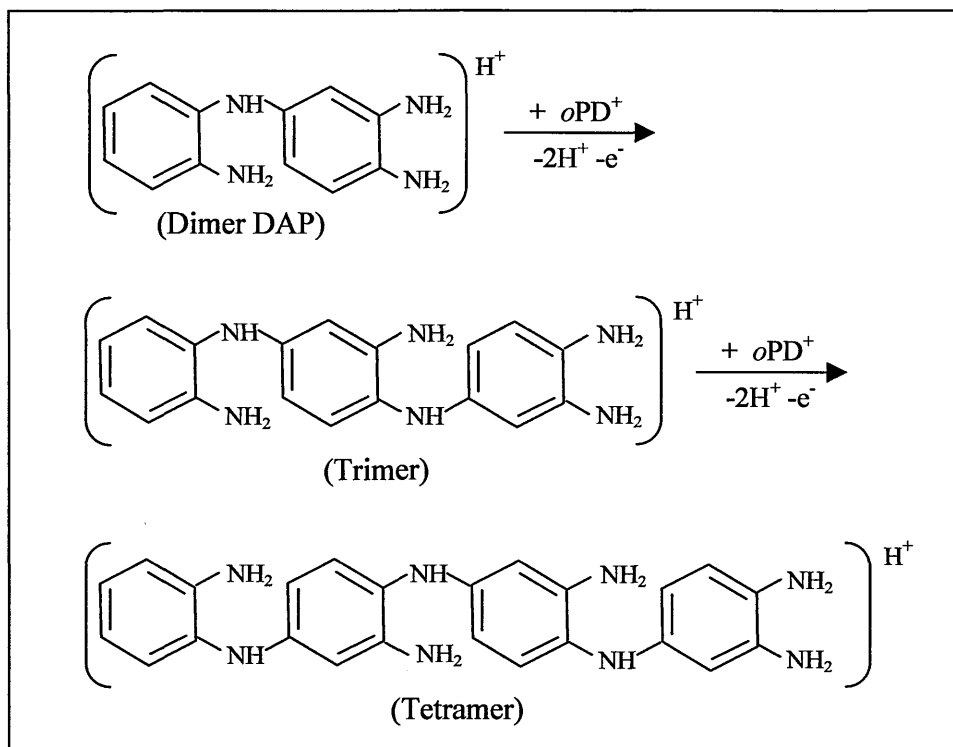


Fig 4.8: Chain propagation following dimerisation of *o*PD.

Detailed work by Losito *et al* recently showed that chain propagation beyond tetramer length occurs only when the applied potential is increased by around +200 mV beyond that required for initial monomer dimerisation, and this gives rise to the second anodic peak seen in Figure 4.2 (Losito *et al*, 2003). At this point, more complex, pH-sensitive reactions take place, where competition exists between oxidative coupling (leading to chain propagation), and intramolecular oxidations with cyclization, so leading to the incorporation of phenazine and 1,4 benzoquinonediimine into the polymeric structure. In acidic conditions, the oligomer formation favours phenazine-like subunits, whereas in more basic solutions, 1,4-benzoquinonediimine forming processes are preferred and this results in a less conjugated polymer structure (Figure 4.9).

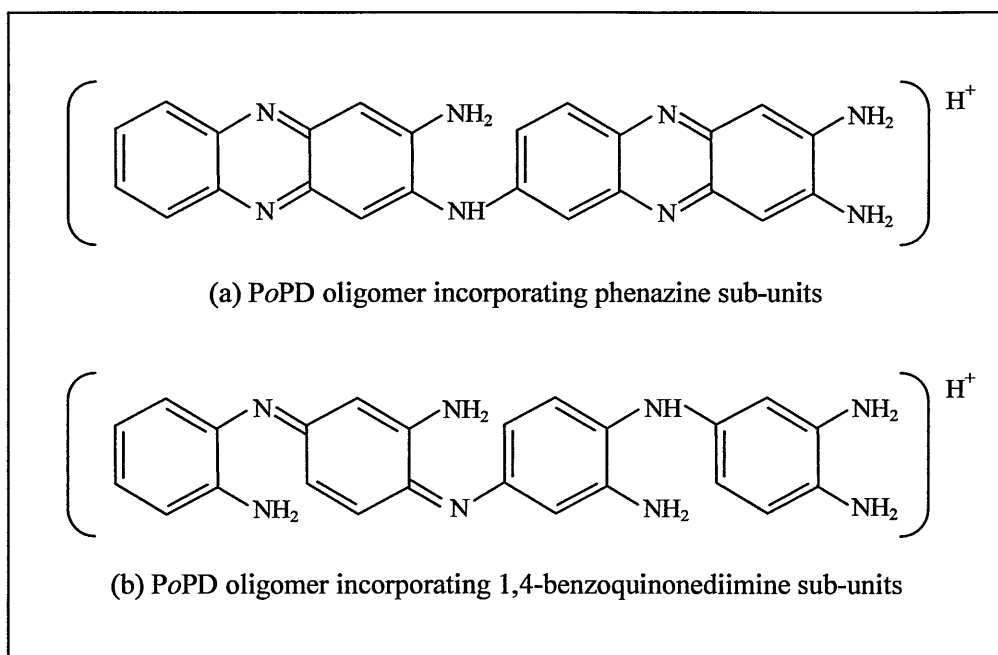


Fig 4.9: Schematic of PoPD oligomers formed in (a) acidic and (b) neutral conditions.

The degree of π -conjugation greatly influences the conductivity of the film, and it follows that the poly(*o*-phenylenediamine) polymerised in neutral conditions (pH 7.4), utilised in this investigation, is non-conducting. This has two important consequences for sensor modification: firstly the film is not electroactive, thereby passivating the electrode, and it follows that for any further reaction to take place the electroactive species must first diffuse through the film (Emr & Yacynych, 1995). Secondly, the insulating nature of the polymer provides a film of uniform and self-limiting thickness. Wu & Chang showed through direct measurement via atomic force microscopy (AFM) that for 5 mM *o*PD polymerised on gold slide electrodes at pH 7.4, that film thickness was approximately 20 nm in all areas of the coated electrode (Wu & Chang, 2004). They compared the results with other techniques for estimating film-thickness, illustrating that charge-transfer integration based methods (which first require the real surface area of the electrode to be established), do not account for the swelling of the hydrated polymer which increases film thickness by approximately three-fold.

It can be seen from figure 4.5 that the oxidation currents fall exponentially between subsequent scans and that after 20 cycles a current plateau has been reached.

This is in agreement with observations by Wu & Chang (Wu & Chang, 2004), who showed that for the same conditions, 96.5% of charge had passed after 7 scans. It is the deposition (adsorption) of the insoluble, higher oligomers onto the electrode surface which results in electrode-passivation with an ultra-thin film, readily suitable for ultrasonic ablation.

Figures 4.10 and 4.11 show a scanning electron-microscope (SEM) image of a gold sputter-coated slide electrode unmodified and electropolymerised with *o*PD respectively.

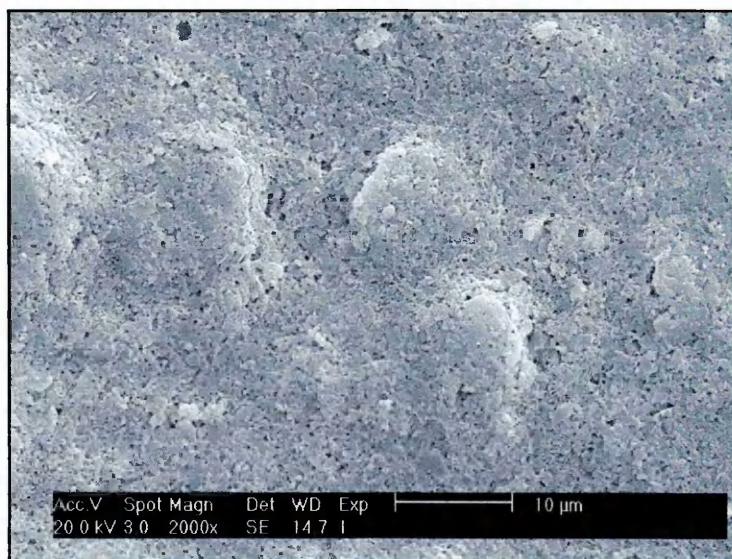


Fig 4.10: SEM image of the surface of an unmodified gold glass-slide electrode.

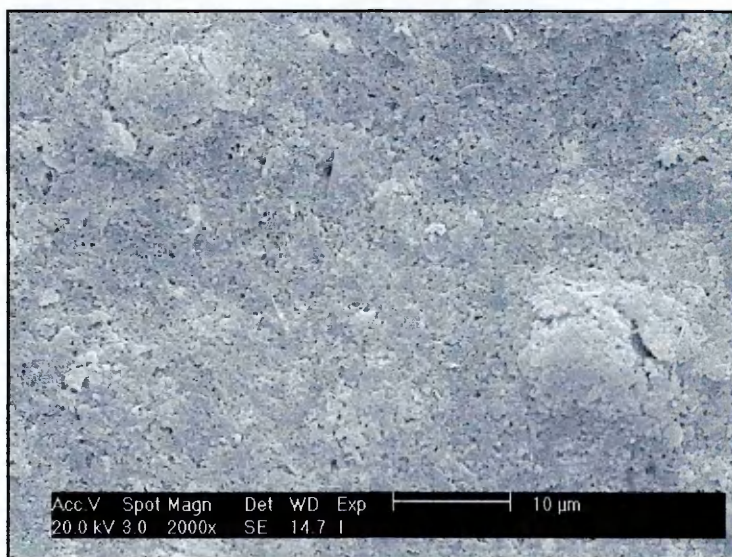


Fig 4.11: SEM image of a gold slide electropolymerised with *o*PD (pH 7.4, 20 scans, 50mVs⁻¹).



The surface roughness of the thin polymer film conforms to that of the gold sputter-coated ground glass slide, indicating that the polymer forms an ultrathin film, self-limiting in thickness. Given the electrically insulating nature of the film, it was necessary to coat the sample exhibited in Figure 4.11 with a second layer of gold across the surface to allow examination by SEM. Although sputter-coated gold deposition does not produce an atomically smooth finish, the size of the gold agglomerates do not exceed 100 nm and so maintain an accurate depiction of the surface beneath (Schug *et al*, 1999).

In order to confirm the presence and integrity of the poly(*o*-phenylenediamine) film, polymer coated gold slide electrodes were interrogated via cyclic voltammetry in 1 mM potassium ferricyanide solutions (pH 7.8). The experiment was repeated with uncoated bare gold electrodes to compare signal response and voltammetric profiles. Equation 4.1 indicates the reduction-oxidation (redox) couple of potassium ferricyanide:

Equation 4.1

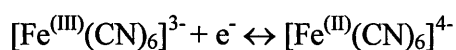


Figure 4.12 illustrates the cyclic voltammograms obtained; the initial sweep is in the cathodic direction in order to reduce Fe^{3+} to Fe^{2+} .

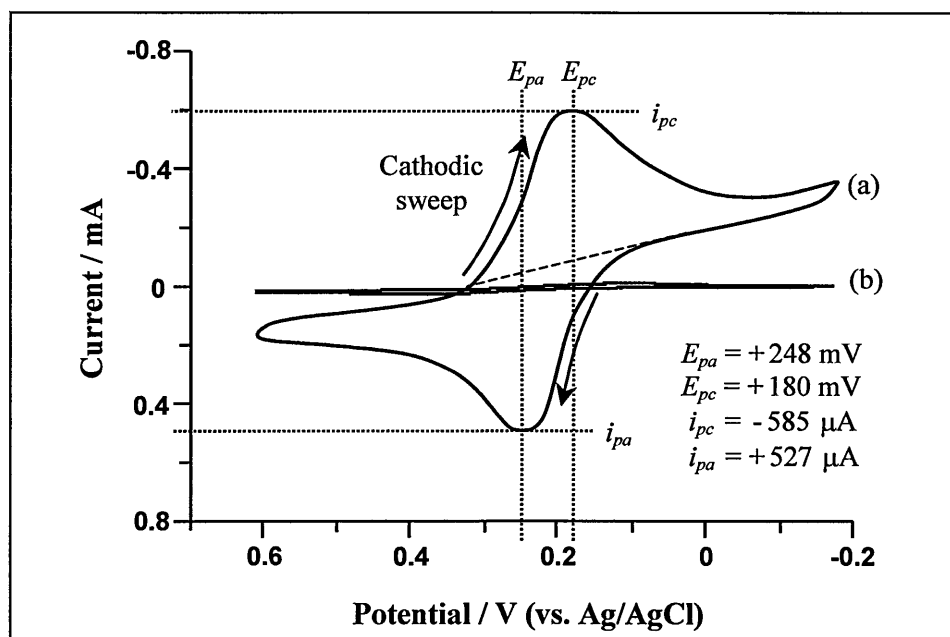


Fig 4.12: Cyclic voltammogram of (a) bare gold, and (b) poly(*o*-phenylenediamine) coated gold-slide electrode in a 1 mM potassium ferricyanide solution (15 mVs^{-1}).

The cyclic voltammogram yields a peak separation ($E_{pa}-E_{pc}$) of approximately 68 mV, which is close to the value of 59 mV expected for a reversible, diffusion-controlled single electron transfer reaction. It is likely that the source of this discrepancy can be attributed to solution resistance and surface topography leading to non-ideality. Furthermore, it has been shown that this redox couple is prone to causing the formation of a passivating film of Prussian blue on platinum electrodes (Pharr & Griffiths, 1997; Granger & Swain, 1999) - which also is possible in this instance. Even low levels of electrode passivation can cause a decrease in the rate of electron transfer, which in turn results in a decrease in peak potentials and an increase in peak separation.

A reversible reaction is also indicted by the ratio of i_{pc} and i_{pa} , which is approximately equal to one. Since the solution consisted mainly of ferricyanide, the reaction product from the cathodic sweep (ferrocyanide) experiences a diffusion gradient away from the electrode, causing it to move out of the diffusion layer and resulting in a charge bias in favour of reduction.

4.1.4 Electropolymerisation of poly (*o*-phenylenediamine) onto carbon substrates.

Single disposable carbon ink electrodes were subjected to the same electropolymerisation regime used for gold substrates. The carbon screen-printed electrodes lend themselves to mass production of microelectrode array sensors utilising the developed fabrication specifications (Mills, 2005). Figure 4.13 exhibits the cyclic voltammogram obtained for the electropolymerisation of 5 mM *o*PD (0 to +800 mV, 50 mVs⁻¹, pH 7.4) onto a 21.78 mm² carbon-paste electrode (Microarray Ltd).

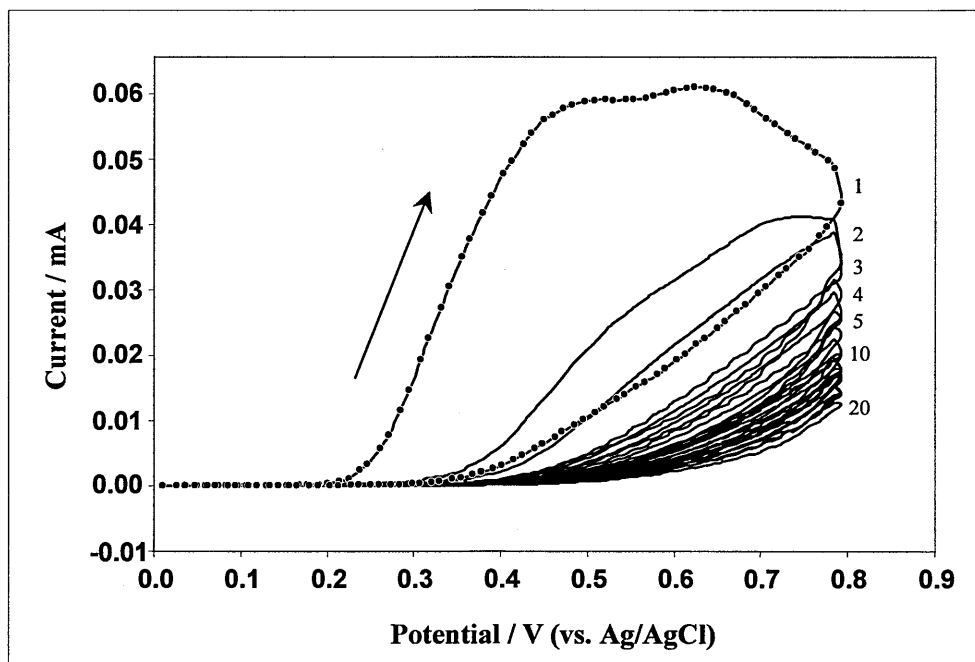


Fig 4.13: Electropolymerisation of 5 mM *o*PD at a screen printed carbon electrode (0 to +800 mV, 50 mVs⁻¹, pH 7.4 phosphate buffer).

The cyclic voltammogram obtained indicates two less pronounced anodic peaks at approximately +480 and +625 mV. When compared to the equivalent voltammogram obtained for gold electrodes (Figure 4.5), it can be seen that the peak potentials have been shifted anodically by approximately +150 mV, indicating that the screen-printed carbon surface is less electroactive when compared to gold. Therefore it can be said that the electron transfer associated with the oxidation of the *o*PD monomer and subsequent chain propagation is less kinetically favourable across the carbon / solution interface than that for gold.

Furthermore, in comparison to Figure 4.5, the second anodic peak appears to be less prominent and the rate of decrease of the anodic current appears to be diminished, suggesting the incomplete polymerisation of the oxidised monomer. This agrees with data provided by Losito *et al*, who showed that by limiting the anodic switching potential, or making the polymerisation stage less kinetically favourable by reducing the pH, that the

formation of soluble dimers, trimers and tetramers exceeded the rate formation of insoluble, passivating *o*PD *n*-mers (Losito *et al*, 2003). Consequently, for the case of electropolymerisation on carbon-paste electrodes, it was decided to extend the anodic potential sweep to +1000 mV in order to increase the thermodynamic favourability of chain propagation. Figure 4.14 indicates the cyclic voltammogram of *o*PD polymerised on the carbon-paste electrodes for this increased potential range.

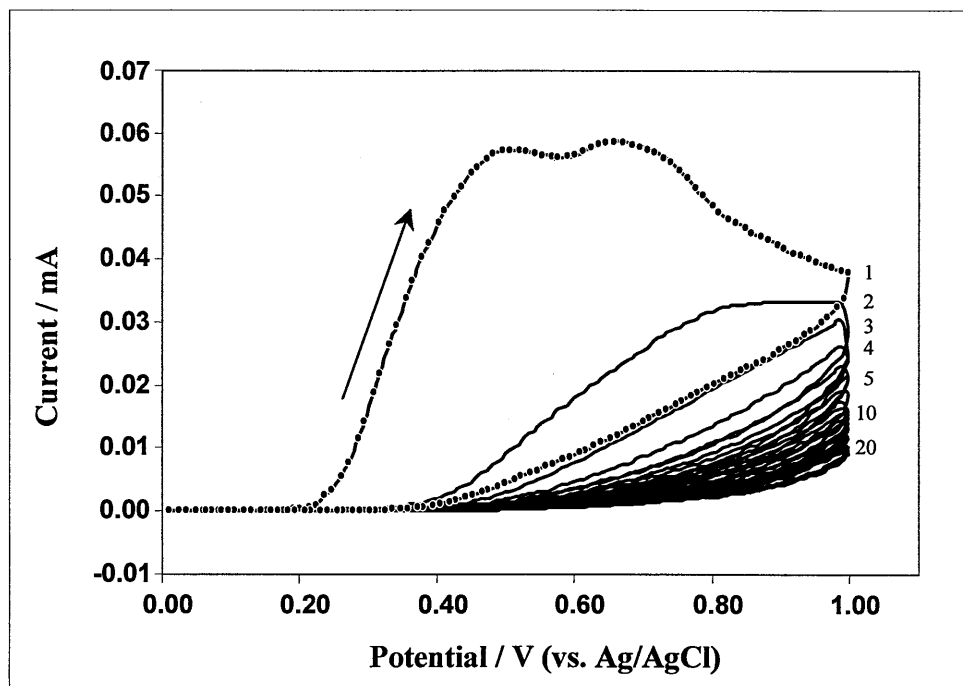


Fig 4.14: Electropolymerisation of *o*PD at a screen printed carbon electrode (0 to +1000 mV, 50 mVs⁻¹, pH 7.4 phosphate buffer).

It can be seen that there is a more rapid decrease in the current flow between each scan, indicating a more efficient chain propagation phase and resulting in the enhanced passivation of the electrode surface. Scanning electron micrographs were taken of bare carbon (Figures 4.15) and at an *o*PD electropolymerised carbon surface (Figure 4.16) respectively.

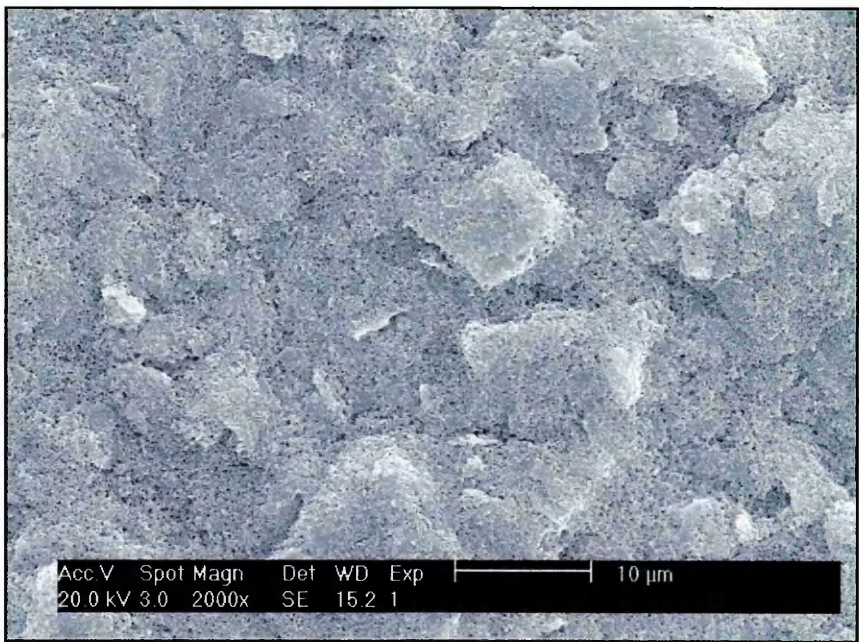


Fig 4.15: SEM image of the surface of a bare carbon-ink electrode.

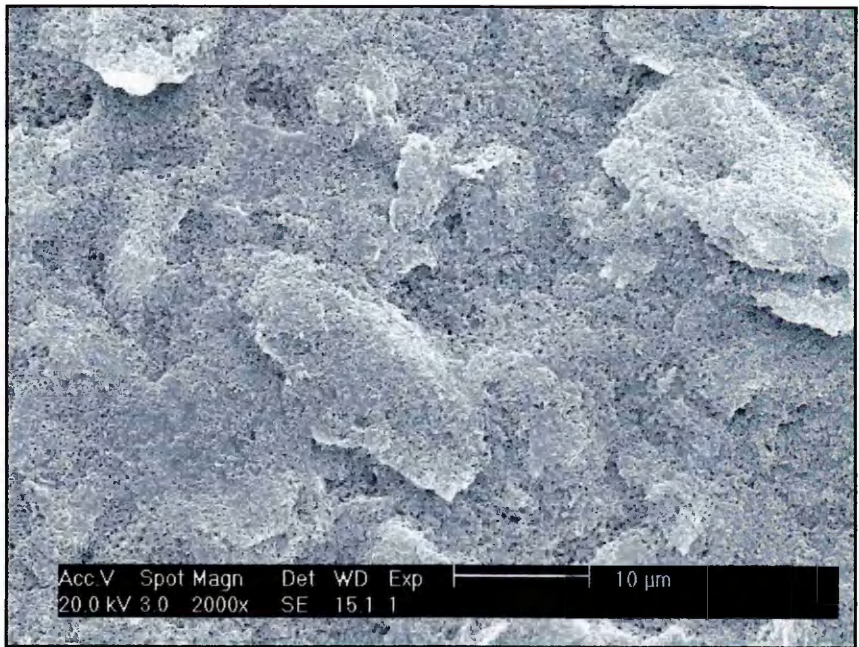


Fig 4.16: SEM image of an oPD polymerised carbon-ink electrode.

The micrographs indicate that the film formed by the electropolymerisation of oPD is again faithfully replicating the surface beneath it. The insulated surface requires to be coated with a conductive layer to allow examination via SEM (in this instance surfaces

were sputter-coated with gold). As a result, information concerning material conductivity by contrasting the level of brightness of the two images is lost. However, the insulating properties of the oPD film were indicated when an electropolymerised electrode without a conductive coating was examined, and was seen to cause rapid saturation of the micrograph image. It may be possible, however, to circumvent this problem by use of a microscope capable of employing a lower accelerating voltage.

Quasi-reversible and irreversible redox processes are commonly experienced with screen printed carbon electrodes (Dock & Ruzgas, 2003), whose electrochemical behaviour can be explained by their heterogeneous construction. The precise composition of the carbon-ink formulation used cannot be reported in this work because DuPont (UK) Limited (Bristol, UK) will not disclose the composition of their commercial inks. However, screen printed carbon-inks typically comprise a mixture of graphite powder, a polymeric binder (typically vinyl or epoxy based), and other chemical additives to aid dispersion and printing (Adams, 1958; Grennan *et al*, 2001; Svancara *et al*, 2001). The resultant paste is a composite of randomly oriented graphite particles, suspended in an insulating matrix of polymer. Electrical conductivity within the bulk system is believed to be provided by points of physical contact between graphite particles, providing a conductive path through the polymeric medium (Svancara *et al*, 1996). An alternative mechanism involving indirect electron tunnelling between particles in close proximity has also been suggested (Svancara & Vytras, 2000).

Graphite itself is a natural arrangement of flat layers of hexagonal rings of carbon, held closely together by strong, hybridised sp^2 covalent bonds. Consecutive layers of the ring structures are only held together via 'Van der Waals' forces, resulting in a weak and more distant association (Figure 4.17).

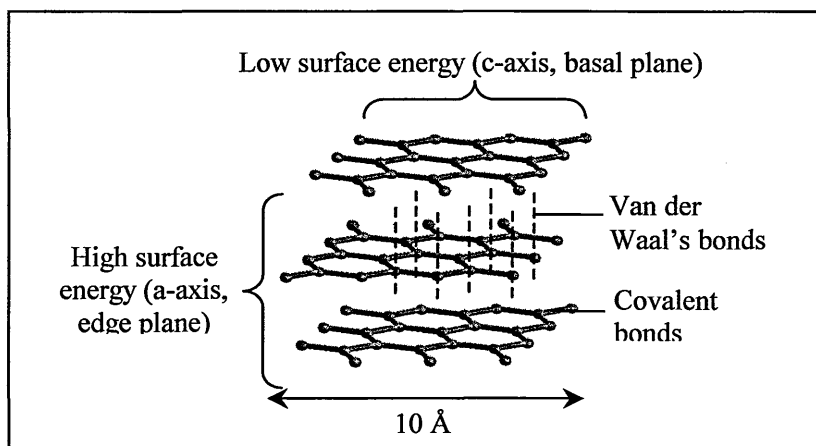


Fig 4.17: Structural arrangement of carbon graphite.

This structure leads to anisotropic physico-chemical properties. Firstly, the large difference in bond strength within the plane of the carbon rings (known as the *basal* plane) compared to the strength between the basal plane layers, results in a macro-molecular 'flake-like' structure, formed during processing. Secondly, the close atomic spacing, and electron mobility in the extended π system found within the carbon rings, leads to excellent conductivity parallel to the basal plane ($\sim 0.1 \Omega\text{cm}$). In contrast, a much more resistive path exists between the layers ($\sim 1 \times 10^{-4} \Omega\text{cm}$) (McCreery, 1991). Furthermore, the surface energies at the face of the basal plane are much lower than that of the edge plane, and offer varying wettability and electroactivity (Rice & McCreery, 1989; McCreery, 1991). The result is a complex heterogeneous surface with differing physical and chemical characteristics, in which the main electroactive component (graphite) is almost entirely coated, even at the surface, by a film of non-conducting pasting liquid whose presence is essential for printing processes (Kalcher, 1990). The polymer membrane causes hydrophobicity and reduces the electroactivity of the entire surface, including the areas containing exposed edge-planes of graphite. Consequently the electrochemical properties of a carbon-ink are sensitive to the ratio of graphite to pasting liquid. The edge plane of dry graphite will exhibit near Nernstian behaviour, even with a potassium ferricyanide redox couple, while in contrast the basal plane shows extremely low activity (McCreery, 1991). As the proportion of polymer binder increases

in the carbon-paste mixture, so the conductivity and electrode kinetics decrease accordingly (Rice *et al*, 1983; Hu *et al*, 1985).

Cui *et al* investigated a number of screen printed carbon-inks using techniques such as ac impedance and cyclic voltammetry (Cui *et al*, 2001). They found that, since the electrodes had all been produced in-house by the same method, variations in behaviour could only be attributed to differences in the proprietary polymeric binders and types of graphite employed. They also noted a poor response by carbon-inks to the potassium ferricyanide redox couple, and refer to a report by Deakin *et al*, who describe a negatively charged carbon-paste / solution interface, responsible for slow reaction kinetics with negatively charged redox couples (Deakin *et al*, 1985). In the same paper they identified the positively charged redox couple, hexaammineruthenium(III) chloride to show excellent reversibility on unmodified carbon-ink substrates. Consequently two redox couples which dissociate in water to form positively charged species were used to interrogate the screen printed carbon-ink substrates, namely hexaammineruthenium(III) chloride, $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$, and ferrocenemonocarboxylic acid, $\text{Fc}(\text{CO}_2\text{H})$ (illustrated with resultant voltammograms in Figures 4.18 and 4.19).

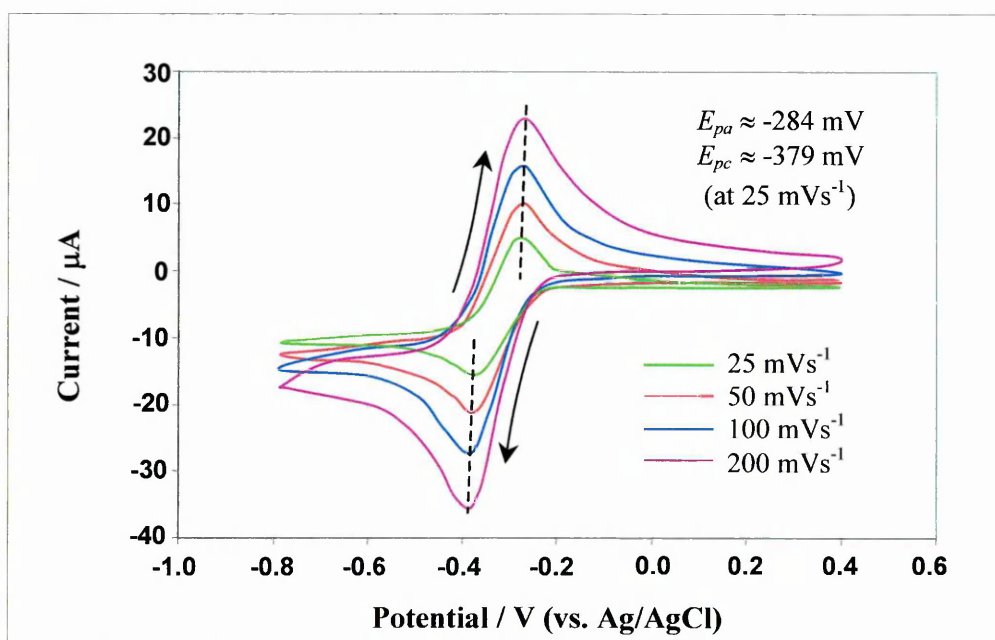


Fig 4.18: Cyclic voltammogram of 1 mM $[\text{Ru}(\text{NH}_3)_6]^{3+/2+}$ at a screen printed carbon ink electrode (25, 50, 100 and 200 mVs^{-1} sweep rate).

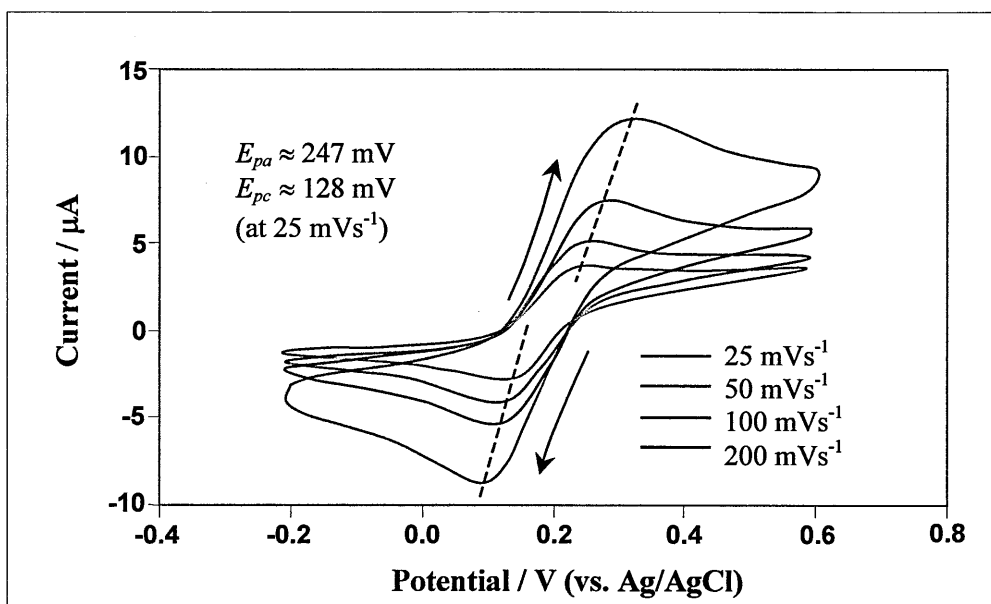


Fig 4.19: Cyclic voltammogram of 1 mM $[\text{Fe}(\text{CO}_2\text{H})]^{2+/3+}$ at a screen printed carbon paste electrode (25, 50, 100 and 200 mVs^{-1} scan rate)

The redox couples exhibit both oxidation and reduction peaks. However, even at the slowest scan rate of 25 mVs^{-1} , *quasi-reversible* behaviour is indicated by the non-Nernstian peak separations of 95 mV and 119 mV for the ruthenium and ferrocene complex respectively. The peak potentials of the ferrocene couples show significant dependence on the scan rate (Figure 4.19), whereas little change is observed for the ruthenium complex (Figure 4.18). When the shape of the voltammograms are also considered, it can be concluded that the hexaammineruthenium(III) chloride exhibits superior electron transfer kinetics with a carbon-ink electrode surface when compared to ferrocenemonocarboxylic acid or potassium ferricyanide.

4.2 Base electrode modification: Formation of microelectrode arrays via the ultrasonic ablation of poly(*o*-phenylenediamine) modified screen printed carbon ink electrodes.

4.2.1 Introduction.

The use of ultrasonic techniques in manufacturing processes extends through a variety of applications, including chemical synthesis, wastewater treatment (for the degradation of biomolecules and complex chemicals), textiles processing and biotechnology (primarily for biological cell disruption) (Mason & Lorimer, 2002). Ultrasonics are most widely used in the manufacturing industry for the purpose of cleaning, where the intense heat and pressures associated with cavitation bubble collapse greatly increase the efficiency of surface contamination removal (Mason *et al*, 1989). However, ultrasonics have now been utilised in a novel approach for the fabrication of microelectrodes by means of the ultrasonic ablation of thin polymer films (Higson, Filed 19th November 1996; publication date October 1996). Previous work shown here and within this research group (Myler *et al*, 1997) has shown that the electropolymerisation of poly(*o*-phenylenediamine) onto gold sputter-coated ground glass slide electrodes results in a largely featureless, insulating polymer layer (Figure 4.20). This process has been extended to a less expensive, screen printable carbon-ink substrate, which offers a commercially viable route suitable for disposable sensor manufacture (section 4.1.4).

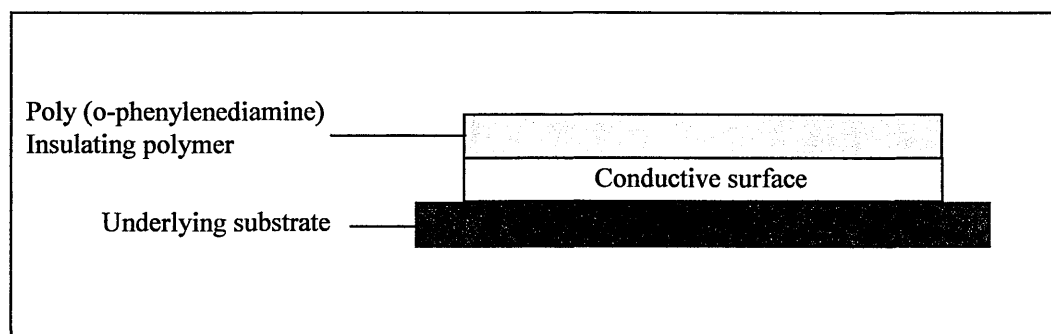


Figure 4.20: Schematic of a polymer insulated electrode.

Ultrasound (in the kHz range) passing through a solvent such as water induces cavitation, whereby localised hotspots of up to several thousand degrees Kelvin are created in the form of super-heated vapour bubbles (Suslick *et al*, 1986; Suslick, 1990; Taleyarkan *et al*, 2002). The surrounding medium, which is of ambient temperature, very rapidly cools the vapour bubbles, and causes them to collapse upon themselves (Mason, 2003(b)).

When these events occur close to a solid surface, the movement of water is hindered by the presence of the object and so the majority of the liquid enters the bubble from the opposite side. This results in an asymmetric bubble collapse, which produces a high velocity jet of liquid targeted towards the solid surface (Suslick *et al*, 1999). The velocity of these microjets (hundreds of metres per second) may cause the shattering of hard, brittle solids, and have been exploited for many years within medicine as a means of breaking up kidney stones (Brannen and Bush, 1984; Suslick, 1990). Softer, more ductile materials receive surface ablation from the impact of such jets. In the case of soft, ultrathin polymer films, such as poly (*o*-phenylenediamine), the extent of this surface ablation is sufficient to cause perforation through to the harder underlying conductive surface, as depicted in Figure 4.21.

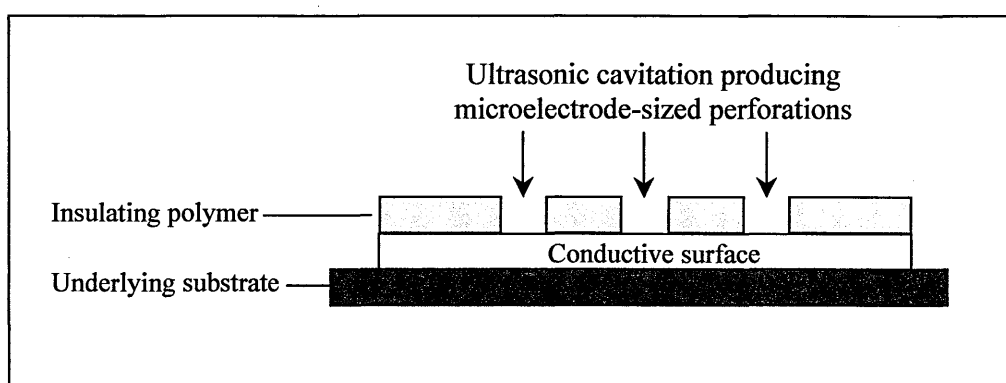


Figure 4.21: Ultrasonic ablation of a thin polymer layer revealing conductive substrate beneath.

The small size of the perforations formed ($<10\ \mu\text{m}$ diameter) and the minute thickness of the surrounding polymer ($<100\ \text{nm}$) permits a hemispherical diffusion profile to the conductive substrate beneath (Figure 4.22).

Furthermore, since the ablation process exposes a single, continuous piece of conductive material (such as gold or carbon), each perforation forms part of a microelectrode array construction, with an inherent stir independent electrochemical response characteristic.

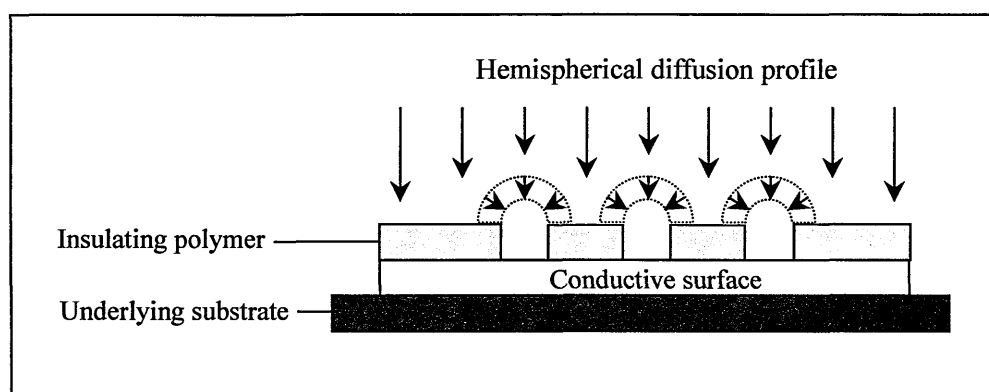


Figure 4.22: Hemispherical diffusion profile resulting from ultrasonic ablation of an ultra-thin polymer film.

The mass production of microelectrode array devices using existing microfabrication technologies, such as photolithography (Cunningham *et al*, 2001) or laser ablation (Fofonoff *et al*, 2004) have to date proved cost prohibitive for application to large-scale commercial manufacture of single-use sensors. However, the novel manufacturing approach described here lends itself well to rapid, large scale, and importantly low-cost fabrication of disposable microelectrode array sensors. Furthermore, the generic nature of this technology allows it to be applied to virtually any electroanalytical application.

Screen-printed carbon-ink sensors developed by Microarray Ltd. further described in Section 4.1.4 form precursors to this ultrasound-based research designed to create working sensor devices.

4.2.2 Initial investigations into the ultrasonic fabrication of microelectrode arrays on polymer insulated gold sputter-coated ground glass slide electrodes.

Previous work within this research group reported the production of microelectrode array sensors via the ultrasonic ablation of poly(*o*-phenylenediamine) passivated gold sputter-coated ground glass slide electrodes (Myler, 2000). Investigations were initially carried out into the fabrication of individual microelectrode array sensors based on this technique, using the base gold electrodes discussed in section 4.1.3, as exploratory phase ‘proof of concept’ studies.

As a precursor to developing the technique on carbon-ink based substrates, gold sputter-coated ground glass slide electrodes were insulated with 20 sweeps of poly(*o*-phenylenediamine) (5 mM PoPD, 0 to +800 mV vs. Ag/AgCl, 50 mVs⁻¹, pH 7.4 phosphate buffer, 5.1 M NaCl) prior to ultrasonic exposure. To prevent contamination by material present in the ultrasound bath, the passivated electrodes were suspended vertically in a flat-bottomed 50 ml glass beaker, containing de-gassed, distilled, deionised water (Mason *et al*, 1989). The beaker was then positioned in the centre of a 25 kHz Transsonic T460 bench top ultrasonic bath, with the water level of the beaker matching that of the surrounding medium to facilitate transfer of acoustic energy between the two liquid bodies. PoPD insulated electrodes were then exposed to ultrasonic energy emitted from a single, fixed position transducer attached to the base of the ultrasound bath (Figure 4.23).

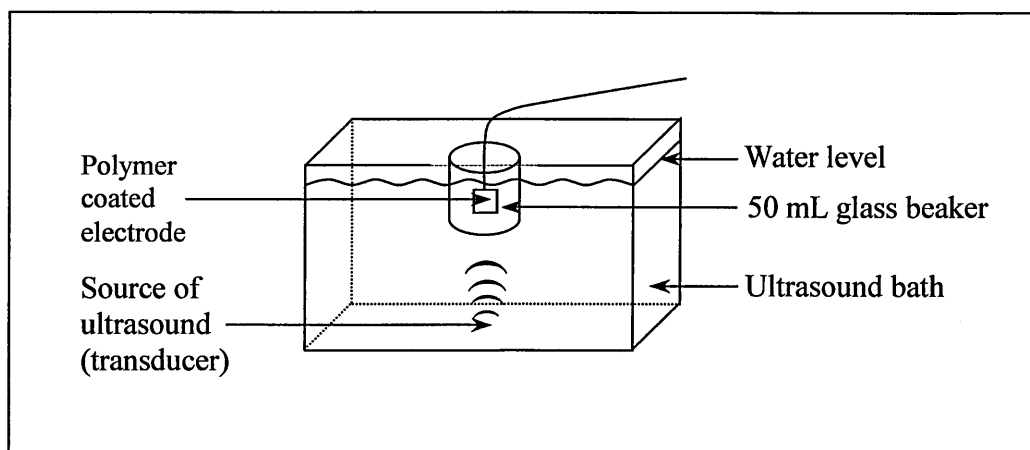


Figure 4.23: Laboratory-scale microelectrode array fabrication via ultrasonic ablation.

Figures 4.24 and 4.25 exhibit scanning electron micrographs obtained for a PoPD insulated gold sputter coated glass slide electrode exposed to 20 seconds sonication.

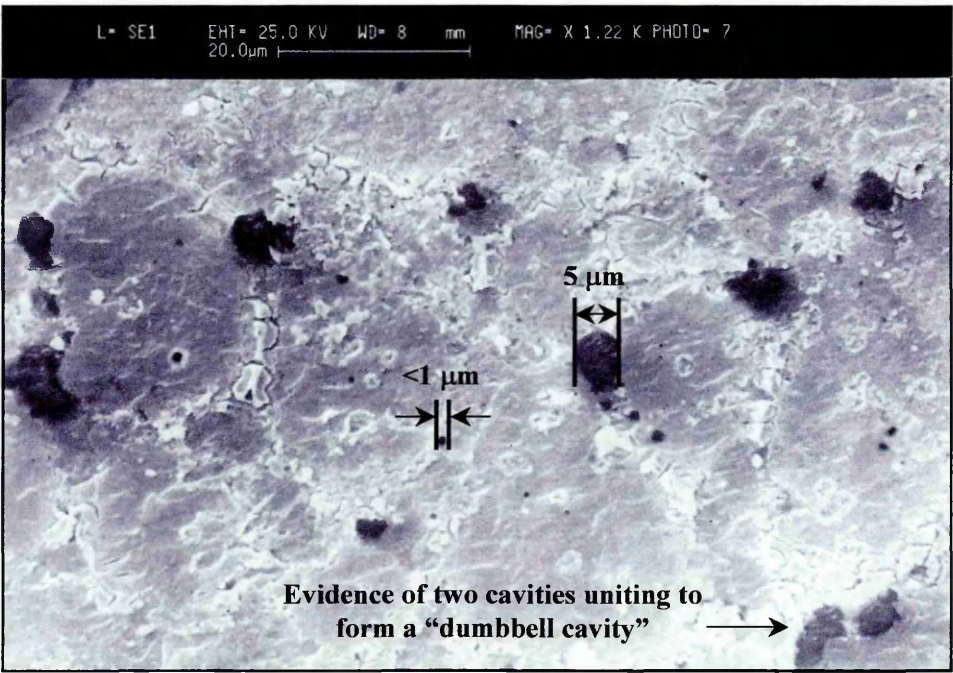


Figure 4.24: Electron micrograph of sonochemically fabricated microelectrode array.

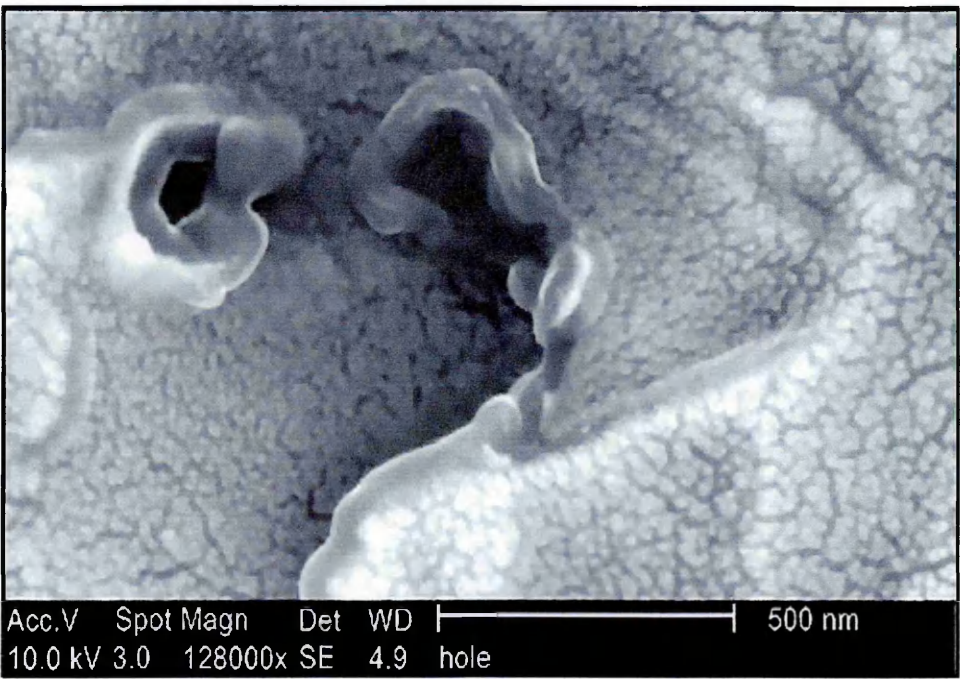


Figure 4.25: Magnified image of a sub-micron microelectrode showing pore structure.

There are a number of features of interest displayed within the micrographs. It can be seen that as a result of the chaotic nature of the cavitation process, the spatial distribution of pores over the polymer surface is random. In some instances this results in two pores forming in close proximity, eventually uniting to form a 'dumbbell' shaped cavity (Barton *et al*, 2004). It is also evident that almost all of the pores are bimodal in size, possessing either 4 μm ($\pm 1 \mu\text{m}$) or sub-micron diameters. The smaller cavities may result from the initial impact of the micro-jets of fluid, and it is thought that these pores may then act as nucleation sites for further bubble formation (Suslick, 1990), giving rise to a quantum enlargement in the diameter of the cavity. Since there is no evidence of pores with diameters above 5 μm , it is believed that this is the threshold size beyond which a pore ceases to function as a nucleation site. A higher magnification micrograph of an ultrasonically fabricated microelectrode is exhibited in Figure 4.25, and indicates that the initial impact of the cavitation micro-jet has caused the polymer to become thermoplastically distorted around the perimeter of the pore. Electrochemical characterisation of electrode assemblies was carried out via cyclic voltammetric interrogation of a 1 mM potassium ferricyanide (III) solution.

Voltammograms of unmodified (planar) electrodes were compared to both PoPD insulated, and microelectrode array sensors produced on polymerised and sonicated gold sputter coated glass slide electrodes (Figures 4.26 and 4.27 respectively). Microelectrode arrays were produced individually using a sonication period of 20 seconds, consistent with previous methodologies which determined the optimum conditions for obtaining stir-independent response characteristics (Myler, 2000).

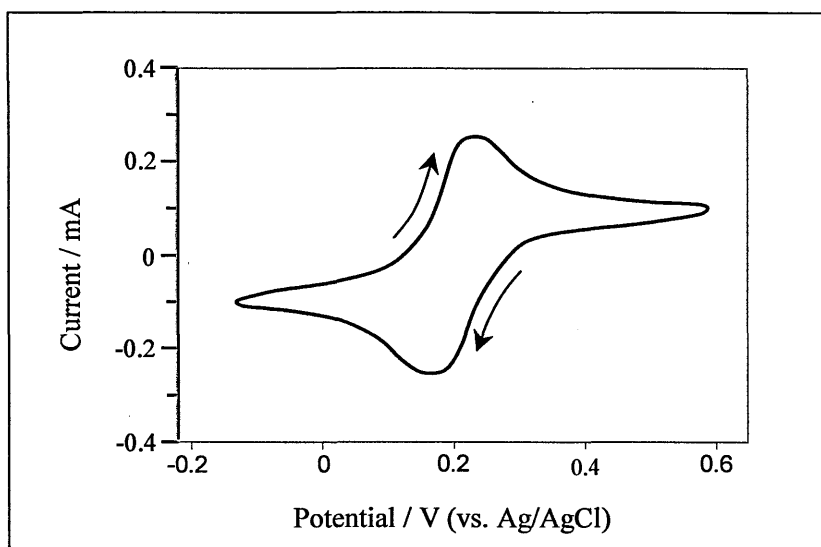


Figure 4.26: Cyclic voltammogram of ferri/ferrocyanide couple at a planar gold sputter-coated glass slide electrode (scan rate 50 mVs^{-1} , $1 \text{ mM Fe(CN)}_6^{3-}$, pH 7.8, 0.1 M KCl).

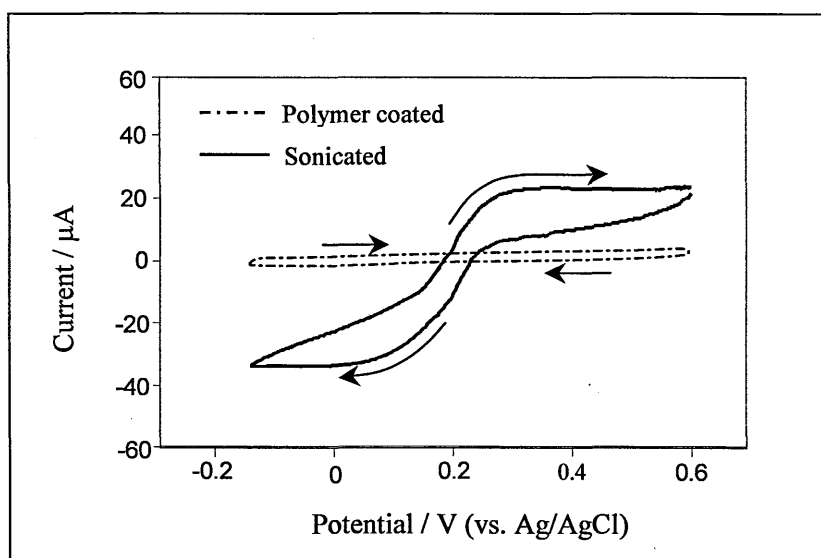


Figure 4.27: Cyclic voltammetry of ferri/ferrocyanide couple at an unsonicated PoPD coated gold slide electrode compared to that exposed to 20 s ultrasonic ablation (scan rate 50 mVs^{-1} , $1 \text{ mM Fe(CN)}_6^{3-}$, 0.1 M KCl).

The voltammograms corresponding to the planar (uncoated) electrodes (Figure 4.26) indicate a Nernstian peak separation of approximately 59 mV, as would be expected for a reversible, diffusion controlled single electron transfer process. In contrast, very little faradaic response is observed for the unsonicated, PoPD coated electrode, confirming the presence of a pristine, insulating polymeric layer (Figure 4.27).

Interrogation of the ultrasonically ablated microelectrode array sensors revealed a sigmoidal response, suggesting a non-linear (hemispherical) diffusion profile typically associated with microelectrode constructions (Greef *et al*, 1985). Theory predicts that for an ideal microelectrode, the oxidative and reductive sweeps will overlay perfectly, however it can be seen that in Figure 4.27 there is some separation of the forward and reverse sweeps. This is a consequence of the chaotic nature of the cavitation process, whereby microelectrode pores are positioned randomly across a sensor surface. Inevitably, some of the microelectrodes formed will be in such close proximity (for example, the dumbbell cavity displayed in Figure 4.24) that there is an overlap of the diffuse parts of their double layers resulting in a charging capacitance. The overall stir dependence of the sensors was examined by means of chronoamperometric interrogation in unstirred (quiescent) and artificially convected solutions whereby very little change in amperometric response was observed when convection was introduced, <6%, (Mills, 2005). This is a consequence of the hemispherical diffusion profiles associated with microelectrodes, whose characteristic dimension is smaller than the diffusion layer thickness under the conditions applied (Stulik *et al*, 2000).

4.2.3 Investigations into the ultrasonic fabrication of microelectrode arrays on insulated carbon-ink screen printed electrodes.

Once the planar electrodes were insulated with non-conducting polymer (section 4.1.4), an array of micron scale pores are created in the insulating layer, exposing the underlying conductive carbon working electrode surface, through a cavitation process via ablation of the polymer with controlled exposure to ultra-sound.

Microarray Ltd. provide the expertise and process details for sonication of insulated electrode assemblies. Sonication times, frequency, power, water temperature, organics removal and de-ionisation of sonication tank have all been previously optimised in house by Microarray (Myler, 2000; Barton *et al*, 2004; Law, 2004; Mills, 2005; Gornall, 2005).

Profiling of the tank and the introduction of a mechanical movement system / jig have enabled much greater reproducibility within a batch - and batch to batch variations for microelectrode production yield RSD values <5% (Mills, 2005).

4.2.3.1 Electrochemical characterisation of modified screen printed carbon-ink electrodes.

Superior sigmoidal (microelectrode) behaviour was exhibited in both the anodic & cathodic regions when electrochemical characterisation of a planar carbon sensor was repeated following the insulation and sonication modification steps and these results (Figure 4.28) serve to validate the fabrication techniques employed.

Figure 4.28 shows the electrochemical behaviour of a screen-printed carbon electrode throughout the differing stages of the fabrication process applying the experimental procedure outlined in (section 3.5.2.1). For a planar electrode convectional controlled mass transport is faster and the electrode is under diffusional mass transport control as the rate-determining step. Clear peaks are observed in both the forward and reverse current transients.

The insulation step can also be confirmed by the lack of any electroactivity, whereby the forward and reverse peaks are both seen to diminish, after 20 cycles to deposit the self-limiting film of poly (*o*-phenylenediamine). This can be seen as the green line (planar) in Figure 4.28 is compared to blue line (insulated & diminished electroactivity).

The difference in electrochemical behaviour between the planar electrode and an electrode exposed to controlled ultrasound is also clear. This can be seen in Figure 4.28 when the green line (planar) is compared to the red line (microelectrode array).

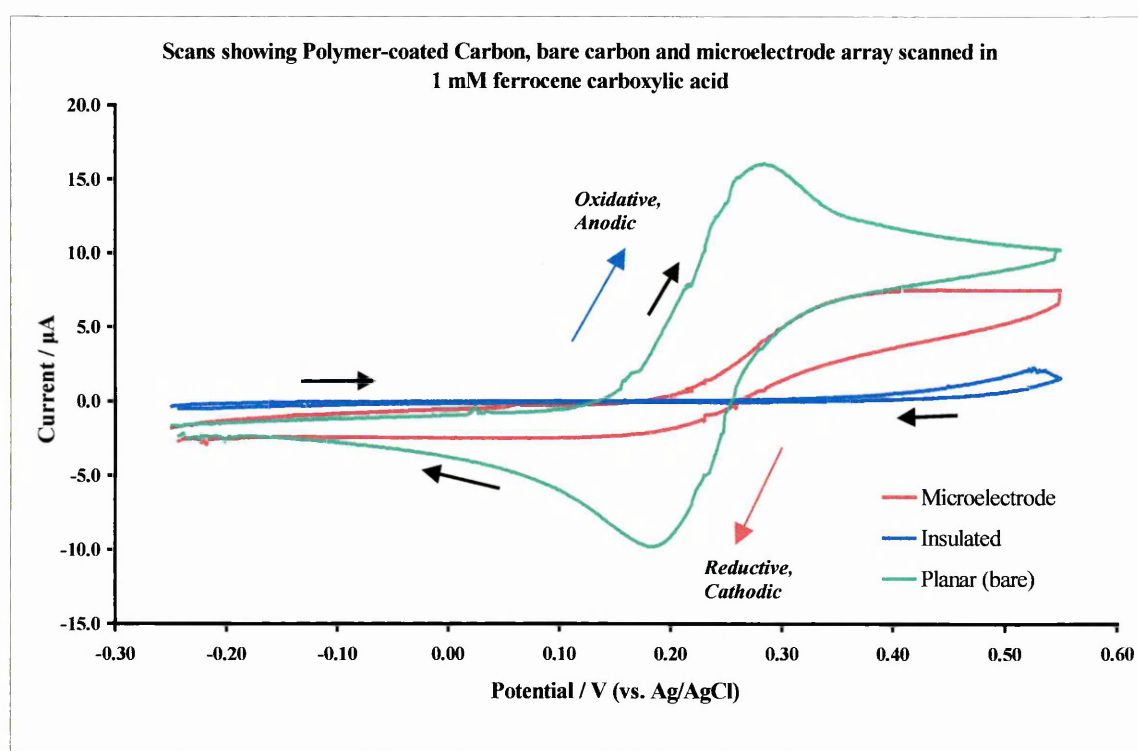


Fig 4.28: Cyclic voltammogram showing the electrochemical behaviour of the carbon electrode sensor array at differing stages of the fabrication process.

During sonication, micropores are created when water molecules are heated to extreme temperatures in very short time periods. Micro-jets are created within intimate proximity of the insulated carbon electrode penetrating the film thereby exposing discrete micron dimension areas of the underlying conductive carbon substrate. This array of microelectrodes produces the electrochemical behaviour observed above.

Voltammetric microelectrode behaviour is exhibited in ferrocene carboxylic acid couple, Figure 4.28 (red line); displaying good sigmoidal behaviour in both anodic and cathodic directions. In both the forward, oxidative, and reverse, reductive, current transients there is a characteristic plateau in which diffusion no longer acts as the rate limiting step but instead the electrode is now under kinetic control.

For a planar electrode convectional controlled mass transport is faster and the electrode is under diffusional controlled mass transport as the rate-determining step (Higson, 2003).

Electrochemical evaluation of microelectrode assemblies was performed to allow for their possible use within sensors or biosensors. Microelectrodes characteristically exhibit sigmoidal shaped cyclic voltammograms for reversible solution bound redox couples such as potassium ferricyanide $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, in contrast to the characteristic diffusion limited reversible voltammograms experienced at planar electrode surfaces. (Figure 4.28 green line).

The voltammogram corresponding to the planar carbon electrode reveals an approximate 59mV (~90mV) peak separation as would be expected for a $1e^-$ diffusional controlled reversible reduction/oxidation process. This inherent irreversibility associated with carbon, as opposed to gold for example, is due to the reduction in the electrical activity of the surface in part due to the lower quality of the surface topography of carbon. A cyclic voltammogram for the polymer-insulated electrode is also shown. Very little voltammetric response is observed at the polydiaminobenzene-coated electrode, confirming that it has been insulated electrochemically.

The microelectrode array electrode yields a sigmoidal shaped voltammetric profile confirming that all of the microelectrode arrays formed in this way were indeed exhibiting true microelectrode-like behavior and this was indeed the case for all microelectrode array electrodes tested.

As mentioned previously individual microelectrodes would be expected to see forward and reverse current transients that would fall almost on top of each other with little evidence of double-layer charging capacitance. Separate forward and reverse peaks displaying evidence of double-layer charging are however observed in this case due to the very large pore density (2×10^5 microelectrodes cm^{-2}) that will give rise to a significant cumulative double-layer charging current as illustrated in Figure 4.29.

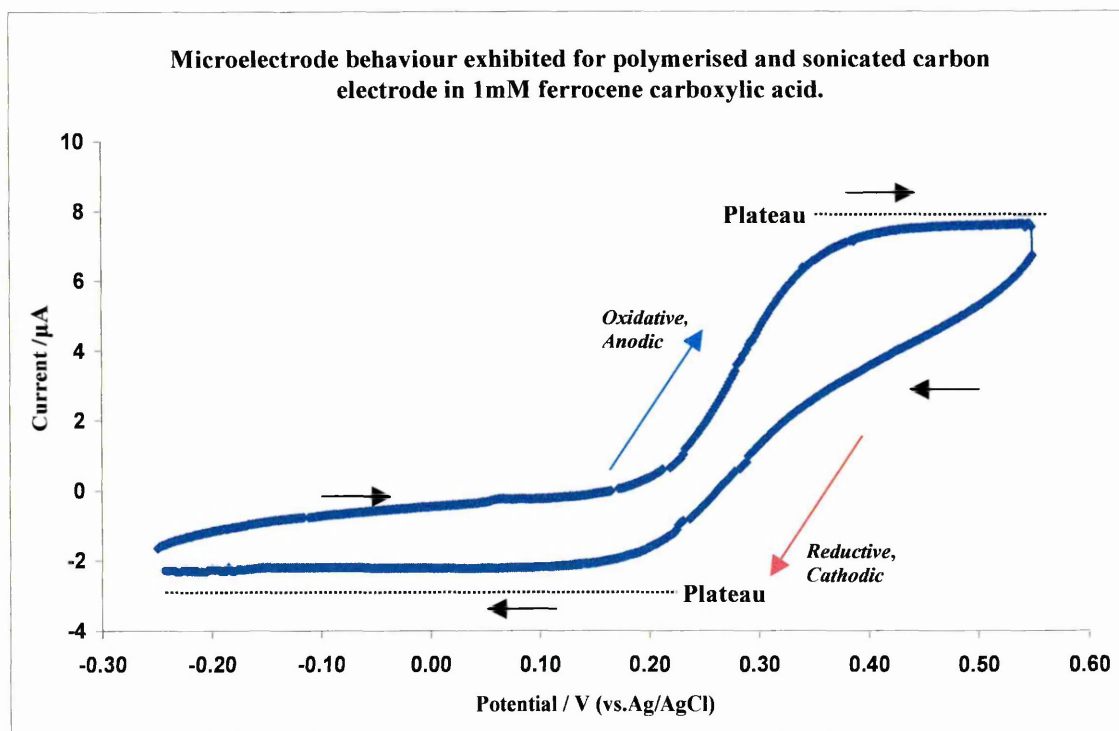


Fig 4.29: Cyclic Voltammogram obtained for a fabricated carbon microelectrode array sensor displaying characteristic microelectrode dual plateau behaviour.

The diffusion of reactants to and products from the electrode surface is now no longer controlled by diffusion but by the kinetics at the electrode interface. Stir-independence is offered by the microelectrode behaviour lending itself to biosensor applications where measurements may be taken from flow cell devices.

To assess the reproducibility of the fabrication process 10 Carbon electrodes were selected and electrochemically evaluated for their voltammetric response from differing positions on the sheet of 100 polymerised and sonicated electrodes.

The working electrode potential was scanned from -200mV to +550mV in 1mM ferrocene carboxylic acid redox couple and the sigmoidal behaviour in both the anodic and cathodic directions for all 10 sensors are shown in Figure 4.30.

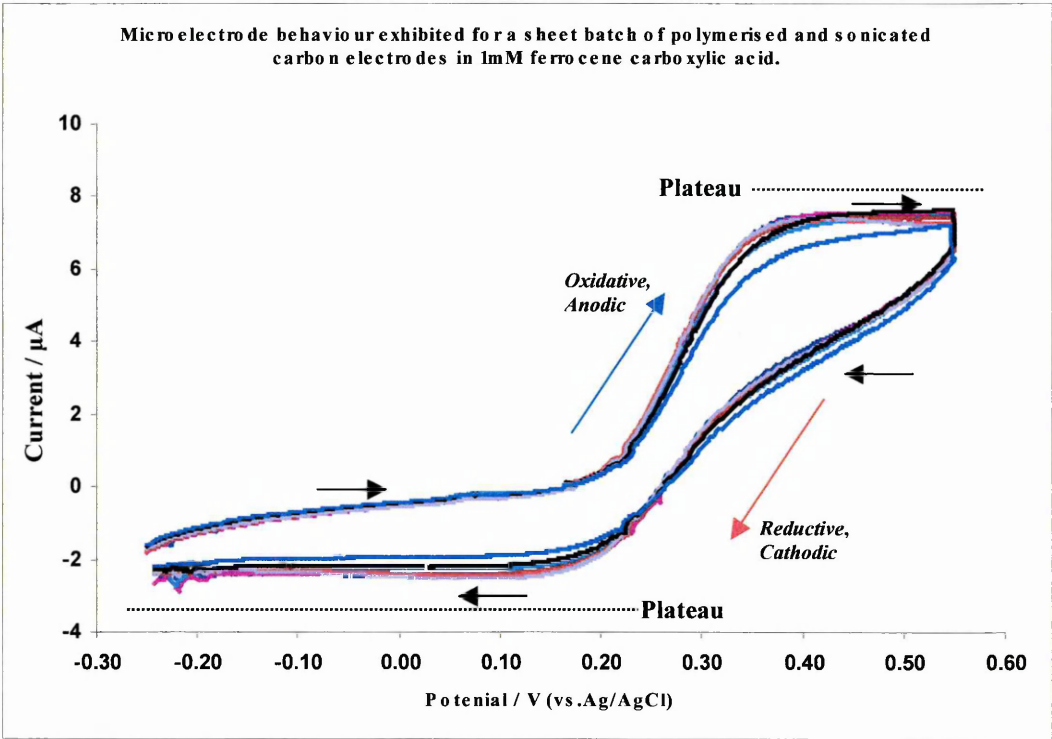


Fig 4.30: Cyclic Voltammograms obtained for sensors from a selection from a sheet batch of 100 *o*-phenylene diamine insulated carbon electrodes subjected to 10 seconds sonochemical ablation.

The 10 selected carbon electrodes from the fabricated batch of 100 show high reproducibility upon electrochemical analysis in 1mM ferrocene carboxylic acid and variation within this batch is low as displayed by near perfect reproducibility (in terms of current-transients in both directions) between sensor voltammograms.

It should be acknowledged that sigmoidal behaviour such as this and indeed microelectrode hemispherical diffusional behaviour are effects that may be modulated by i) the rate of reduction or oxidation of an electro-active solute at the surface of a microelectrode or ii) the rate of convectonal mass transport of the solutes to and from the electrode surface.

As well as scanning electron microscopy of the finalised sensors, several other imaging techniques have been employed to assist the optimisation of microelectrode array production assessing various influencing factors throughout the stages of the fabrication process to further increase the reproducibility from sensor to sensor within a batch and from batch to batch.

4.2.3.2 Visual assessment via sonoluminescence of cavitation site distribution.

The fluorescent energy map within the sonication tank under differing baffling regimes can be assessed with the human eye and recorded using conventional photographic techniques. The images obtained reflect the ultrasound profile within the tank and this aids in the refinement of the distribution of potential cavitation sites to increase the homogeneity within the tank. The use of an aqueous probe to reveal the natural distribution of cavitation intensity throughout the tank is clearly beneficial.

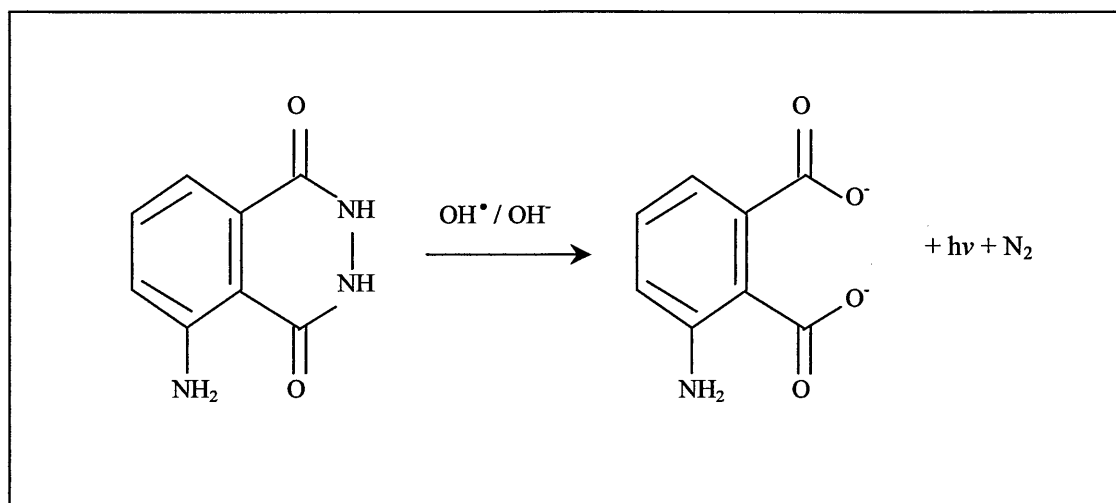


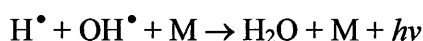
Figure 4.31: Decomposition of Luminol in aqueous alkaline conditions (Renaudin *et al*, 1994)

The enormous energies associated with transient cavitation bubble collapse have for many years been known to causing the emission of light (termed *sonoluminescence*). (Frenzel and Schultes, 1934).

Sonoluminescence is a third-order process, stemming from the second order effect of high frequency sound in water, namely cavitation (Suslick *et al*, 1990).

Thermal hydrolysis of water vapour contained within collapsing cavitation bubbles is believed to give rise to high energy free radicals, which in turn react with surrounding bodies, releasing light as a by-product (Equation 4.2) (Sehgal *et al*, 1980; Riesz *et al*, 1985).

Equation 4.2



where M represents a third body (such as a gas or water molecule) that assists the above process by providing a stabilised intermediate complex, h is Plank's constant ($6.26 \times 10^{-34} \text{Js}^{-1}$) and ν is the speed of light ($2.998 \times 10^8 \text{ms}^{-1}$).

Unfortunately, light emission experienced during *multi-bubble* sonoluminescence (which exists in ultrasonic tanks) is too weak to provide useful data on the spatial distribution of ultrasonic cavitation (Verrall & Sehgal, 1987). In order to enhance the intensity of light emitted from transient bubble collapse, so permitting the visualisation of cavitation intensity, a handful of researchers have added the fluorescent chemical *Luminol* (3-aminophthalhydrazine) to the cavitating liquid medium (Renaudin *et al*, 1994; Yanagida *et al*, 2000; Birkin *et al*, 2003). Luminol, ordinarily employed for detection in molecular biology or for the forensic detection of blood at a crime scene, is a di-cyclic oxygenated amine which can decompose to release blue light of a characteristic wavelength of 430 nm. Hydroxyl radicals generated during transient cavitation bubble collapse (Equation 4.2) are able to react with Luminol under alkaline conditions, producing light of an intensity many orders of magnitude greater than that experienced in ordinary multibubble sonoluminescence (Figure 4.32).

Finely ground luminol was added to deionised water in the tank containing $1.5 \times 10^{-2} \text{M Na}_2\text{CO}_3$, stirring to dissolve and the pH was adjusted to pH 10.7 according to

the method outlined in section 3.5.2.2. The final concentration of luminol in the tank was 5×10^{-5} M. The light emitted by the mixture upon exposure to ultrasound was barely visible to the naked eye experimental imaging was performed under 'black-out' conditions.

A range of exposure times, aperture settings and photographic film speeds were employed to attain a reasonable image.

Figure 4.32 shows the pattern of light emission obtained and the distribution of potential sites for cavitation when no baffling regime is incorporated into the sonication tank at a film exposure time of 30 minutes. Figure 4.32 clearly shows the heterogeneous nature of the ultrasound in the liquid medium.

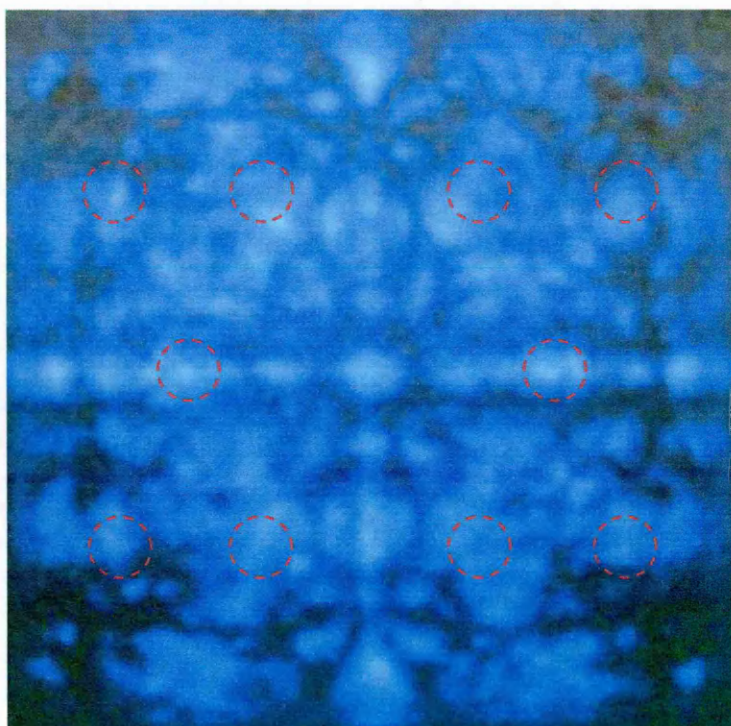


Fig 4.32: Sonoluminescence tank profiling with no baffling regime.

Bright areas represent regions of high intensity and dark areas indicate low cavitation activity. The positions of the transducers attached to the base of the tank are indicated. The deflection in the tank floor partially directs the ultrasound towards the tank centre.

It is possible to associate some of the brighter regions on the picture with transducer location, however, it can also be seen that regions of the tank that are not directly above a transducer also experience intense cavitation, suggesting multiple reflections and spread of acoustic energy with the medium.

With the intention of establishing a homogeneous cavitation distribution throughout the tank, an internal ultrasonic baffling system was developed.

The baffling is a stainless steel cage of porous sheets, which is inserted into the tank on rubber feet to separate it from the tank floor by a distance of 5 cm. This served to prevent the cage from hindering the motion of the tank floor or disrupting the formation of the acoustic wave at the emitting surface. The base and four walls of the tank are thus lined with these sheets and this helps to disrupt the standing wave effects created when the sonication tank is in operation.

Initial investigations into the disruption of the sound field based on the small-scale microelectrode manufacture indicated that the steel with the smallest and most closely packed perforations available provided the best disruption of the ultrasonic waveform while maintaining cavitation intensity. Consequently, a full-sized ultrasound baffle was constructed using 1 mm thickness grade 307 stainless steel, perforated with 3 mm diameter and 5 mm pitch round holes. The distribution of cavitation within the tank was once again assessed using sonochemical luminescence, displayed in Figure 4.33.

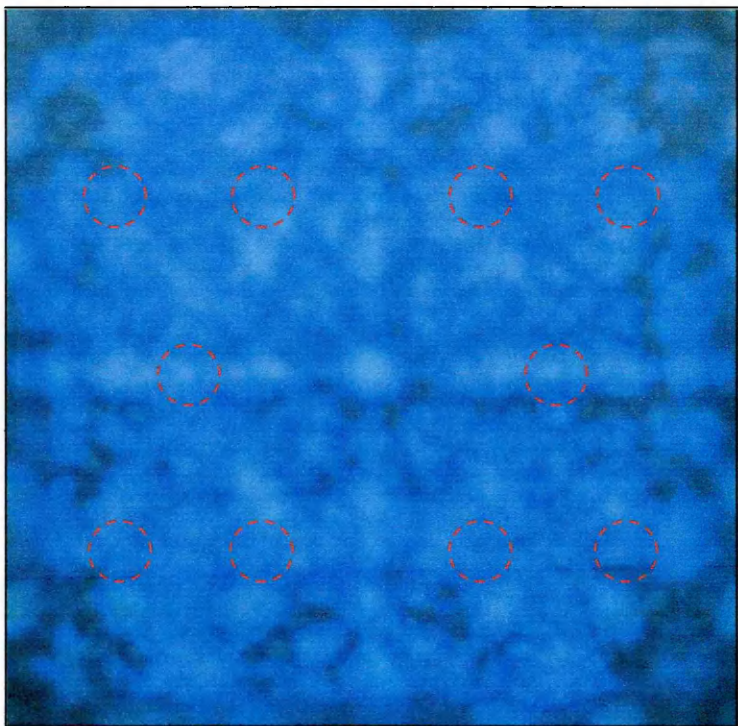


Fig 4.33: Sonoluminescence with the addition of baffling in the tank to disrupt standing waves i.e) nodes and antinodes.

Although ultrasound is only applied for 10 seconds during fabrication and the image is a cumulative light exposure image over thirty minutes it is still clear that the homogeneity of the ultrasound energy distribution and therefore the cavitation sites is greatly increased by the development of the baffling as shown by comparing Figure 4.33 to Figure 4.32.

It can be seen that the presence of the baffling significantly improved the uniformity of cavitation intensity throughout the tank since the numerous, regularly spaced perforations act as point sources of acoustic emission. Importantly, since the diameter of the perforations in the steel baffling are much smaller than the range of incident wavelengths, the sound wave is diffracted as it passes through the gap, resulting in an increased homogeneity of node and antinodal positions (Figure 4.34). In addition, the baffling diminishes the effect of the tank floor deflection as a flattened base is created and this in turn permits a more even distribution of acoustic energy when compared to emissions from the unmodified tank floor.

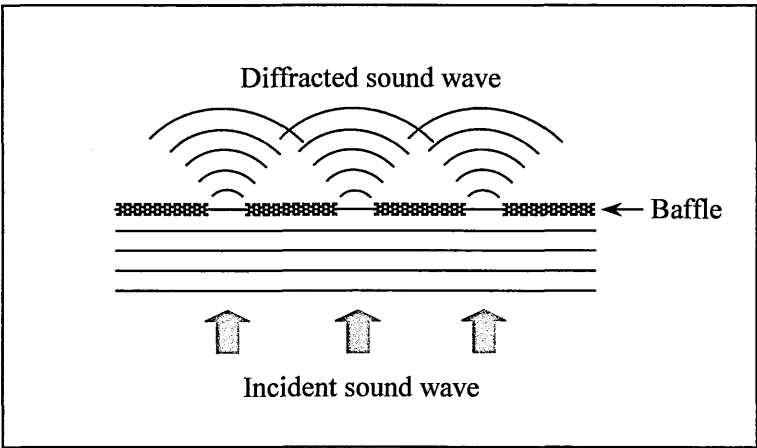


Figure 4.34: Schematic representation a sound wave passing through a narrow gap.

The baffling has been designed to disrupt standing waves (nodes and antinodes). This improves ultrasound homogeneity and in turn has increased the reproducibility of microelectrode response both within a batch and batch to batch. The experimental techniques employed clearly demonstrate the benefits and principles of the baffling system. Unfortunately, the true detail of the fluorescent phenomenon that occur is only encountered when making a visual assessment under blackout conditions and is a far better alternative for the optimisation of the baffling design.

4.2.3.3 Visual assessment of pore density and distribution.

A quick and simple visual assessment of the number and distribution of the pores can be obtained using gold cyanide labelling. The resultant image can be visualised under a light microscope. This permits density calculations to confirm that the dimensions of the array of microelectrodes are within the specifications for microelectrode behaviour and is a good assessment of the fabrication process thus far. Experiments are performed using the procedure described in section 3.5.2.3.

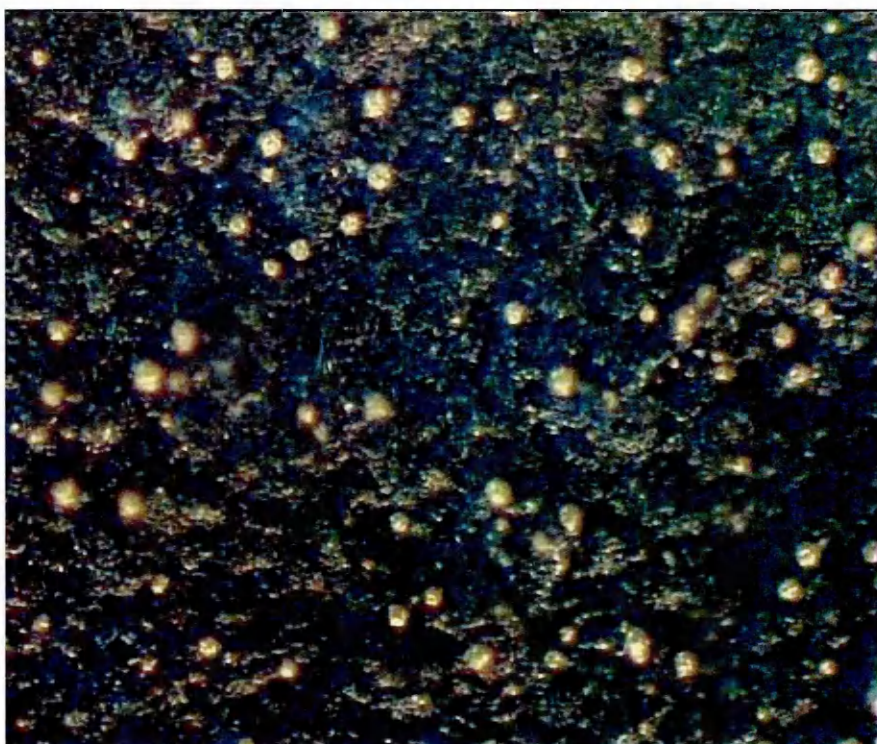


Fig 4.35: Sensor Dimension Specifications via Gold Cyanide Labelling.

This technique does not offer a quantitative result but does offer a qualitative assessment and the concentration of gold spots determines ‘exactly’ how many pores there are on a sensors working electrode.

It can be seen from Figure 4.35 that the gold cyanide occupies each cavitation site following electro-deposition.

The diameter of the gold spots on the image is greater than that of the actual pores created via sonication as the gold ‘wells’ out of the cavities and hence imaging can be obtained using a light microscope.

Electrodes are fabricated with the greatest population densities achievable whilst still maintaining microelectrode behaviour. This is apparent as long as no hemispherical diffusion profiles overlap which diminishes the stir-independent microelectrode responses.

4.3 Chapter 4 Conclusions.

The electropolymerisation of poly(*o*-phenylenediamine) onto gold sputter-coated ground glass slide electrodes was characterised in neutral conditions to show a two electron transfer mechanism for the formation of a non-conducting, ultrathin film. This was then applied to screen printed carbon-ink electrode surfaces, which offer a less expensive route to the mass production of disposable sensors. As a consequence of the decreased electron transfer kinetics associated with carbon-ink host electrodes, the electropolymerisation process was refined by extending the polymerisation potential window from 0 to +800 mV to 0 to +1000 mV (vs. Ag/AgCl), allowing the more anodic reactions to take place and so a more efficient passivation of the electrode surface to occur. A pre-polymerisation sonocation step (three 0.5 s pulses of ultrasonic energy) was identified as being required to remove trapped air from the electrode surface, thereby permitting intimate contact with the monomer solution and preventing disruption of the polymer layer.

The area under each peak produced can be related to the quantity of charge passed due to the faradaic reaction and can therefore be linked to the thickness of the formed film. The irreversible peak observed is seen to diminish in size with each successive cycle, as the working electrode becomes coated with more polymer, and therefore insulated.

The electropolymerisation of *o*-phenylenediamine is a self-regulating process since poly(*o*-phenylenediamine) is an insulating polymer and thus the working electrodes become fully insulated after 20 potential sweeps, indicated by the reduction in area under the curve with each cycle (i.e. charge passed), and this limits the deposition of further polymer to regulate the final thickness of the film.

Confirmation of insulation was confirmed by the lack of any electroactivity in a redox couple if cyclic voltammetry is performed following the electrode modification step.

One of the most attractive characteristics associated with using electropolymerisable non-conducting polymers, as functional separational films, such as polydiaminobenzene, is their ability to produce self-insulating coatings, which cover the entire electrode surface irrespective of electrode topography. Poly *o*-phenylenediamine also offers a biocompatible coating (Cosnier, 1999).

Microelectrode array assemblies were produced initially via the ultrasonic ablation of poly(*o*-phenylenediamine) modified gold sputter-coated ground glass slide electrodes. Sensors were interrogated using cyclic voltammetry in a ferri/ferrocyanide redox couple to show sigmoidal behaviour and offer good microelectrode array performance.

Novel microelectrode array sensors were successfully fabricated and evaluated via several techniques to confirm microelectrode-type electrochemical behaviour and to validate the fabrication steps on poly(*o*-phenylenediamine) modified screen printed carbon-ink electrodes. Reproducibility was high and the reduction in sensor variation has been supported by several imaging techniques and the development and optimisation of key experimental factors.

An industrial-sized ultrasonic tank was procured and a modulated frequency sweep introduced to enhance the uniformity of the ultrasonic field. A procedure for analysing the distribution of cavitation activity via the sonochemical luminescence of Luminol was developed and used to successfully expose regions of high and low cavitation activity within the ultrasound tank, these relating to energy focused by the positioning of the ultrasonic transducers and the asymmetry in the profile of the tank base.

The uniformity of the distribution of acoustic energy was then further enhanced through the development of an acoustic baffling system.

The bio recognition entity (antibody receptor) is subsequently immobilised at the novel modified microelectrode array transducer within and at the surface of conductive PANI 'emeraldine' mushroom-like protrusions or alternatively attached to the PANI protrusion

surface via non covalent affinity techniques. These immobilisation strategies are discussed and reported in Chapter 5. The sensors are finally interrogated by electrochemical impedance spectroscopy for the application of immunosensors for markers of TIA such as NSE & S-100 β protein; the results for both immobilisation approaches are discussed in the final chapters.

The results in this chapter have been published in *Biosensors and Bioelectronics* as part of a series focussing on the widespread applicability of microelectrode array sensors in the field of biosensor technologies (Barton *et al*, 2004).

Chapter 5:

Site-specific immobilisation techniques.

5.0 Site-specific immobilisation techniques on screen printed carbon-ink microelectrode array sensor platforms.

5.1 Introduction.

This chapter deals with the concept and understanding of electrochemical procedures of biomolecule immobilisation based on electrochemically polymerised species. The biomolecule entrapment in a conventional electrogenerated polymer, PANI, was compared to the attachment of biomolecules to biotinylated PANI through affinity interactions based on avidin-biotin bridge construction. Both approaches have been reported in the literature to offer viable immobilisation procedures within intimate proximity of the working electrode. During the past few years immunosensors (Karube & Suzuki, 1986; Gizeli & Lowe, 1996; Lippa *et al*, 2001; Bakker & Telting-Diaz, 2002) have been the subject of increasing interest because of their potential application as an immunoassay tool in several fields such as clinical diagnostics (Aizawa, 1994) and environmental control (Marco *et al*, 1995). Owing to their low cost, small size, possible use in vivo (where applicable) and also their short response time, these devices can compete seriously with classical immunoassays. However, the immobilisation of the molecular receptor on the transducer is of key importance for the control and improvement of the performance of such a sensor. Conversely, over the last 40 years, considerable attention has been focussed on conducting polymers because of their unique electronic properties and their great potential for biomolecule immobilisation. Several immobilisation procedures were used to ensure the coupling of receptor species onto conducting polymers; these range from the entrapment within the polymer matrix (Foulds & Lowe, 1986; Umana & Waller, 1986; Bartlett & Whittaker, 1987; Yabuki *et al*, 1990; Deshpande & Hall, 1990; Battaglini *et al*, 1991; John *et al*, 1991; Shaolin *et al*, 1991; Bartlett & Cooper, 1993; Marchesiello & Genies, 1993; Shaolin, 1994; Gros and Bergel, 1995; Trojanowicz *et al*, 1996; Cosnier, 1997; Uchiyama *et al*, 1997; Yang & Shaolin, 1997; Gooding *et al*, 1998; Cosnier, 1999; Muguruma & Karube, 1999; Sargent *et al*, 1999; Sargent & Sadik, 1999 Wallace *et al*, 1999; Cosnier, 2005) to the electrostatic binding through non-covalent affinity interactions (Snejdarkova *et al*, 1993; Hoshi *et al*,

1995; Katz *et al*, 1998; Cosnier *et al*, 1998; Cosnier, 1999; Cosnier *et al*, 1999; Cosnier & Gondran, 1999; Cosnier & Lepellec, 1999; Turkova, 1999; Wallace *et al*, 1999; Amounas *et al*, 2000; Luppá *et al*, 2001; Ouerghi *et al*, 2002; Corry *et al*, 2003; Cui *et al*, 2003; Darain *et al*, 2003; Limoges *et al*, 2003; Da Silva *et al*, 2004; Ionescu *et al*, 2004; Song & Vo-Dinh, 2004; Cosnier, 2005; Hleli *et al*, 2005).

Polyanilines have been known for more than a century, since the synthesis of the so-called “aniline blacks” that enjoyed an early use as cotton dyes in the 1860’s. Systematic studies (Green & Woodhead, 1910; Willstater & Cramer, 1910; Green & Woodhead, 1912) of their properties prior to World War I led to the proposal by Green and Woodhead of an aniline octamer structure and the existence of several oxidation states. Only sporadic studies of polyanilines were undertaken over the following 70 years, until investigations by MacDiarmid *et al* in the mid 1980s (MacDiarmid *et al*, 1985; Huang *et al*, 1986), and, in particular, the discovery of electrical conductivity for its emeraldine salt form, led to an explosion of interest in this fascinating polymer.

5.2 Electrochemical entrapment of biomolecules in organic polymers throughout their electrogeneration.

Figure 5.1 shows a typical cyclic voltammogram obtained for the electrogeneration of PANI via the potentiodynamic electrodeposition of a solution of monomer (0.2M Aniline Hydrochloride in pH 4.0 phosphate buffer) and biomolecule (0.5 mg ml⁻¹ antibody).

The working electrode was cyclically scanned from -200mV to +800mV and back to the starting potential at a scan rate of 50mV s⁻¹ (vs. Ag/AgCl). A linear sweep from -200mV to +800mV was performed to leave the PANI deposited in its conductive ‘emeraldine’ form.

Figure 5.1 shows a voltammogram for 50 cyclic depositions.

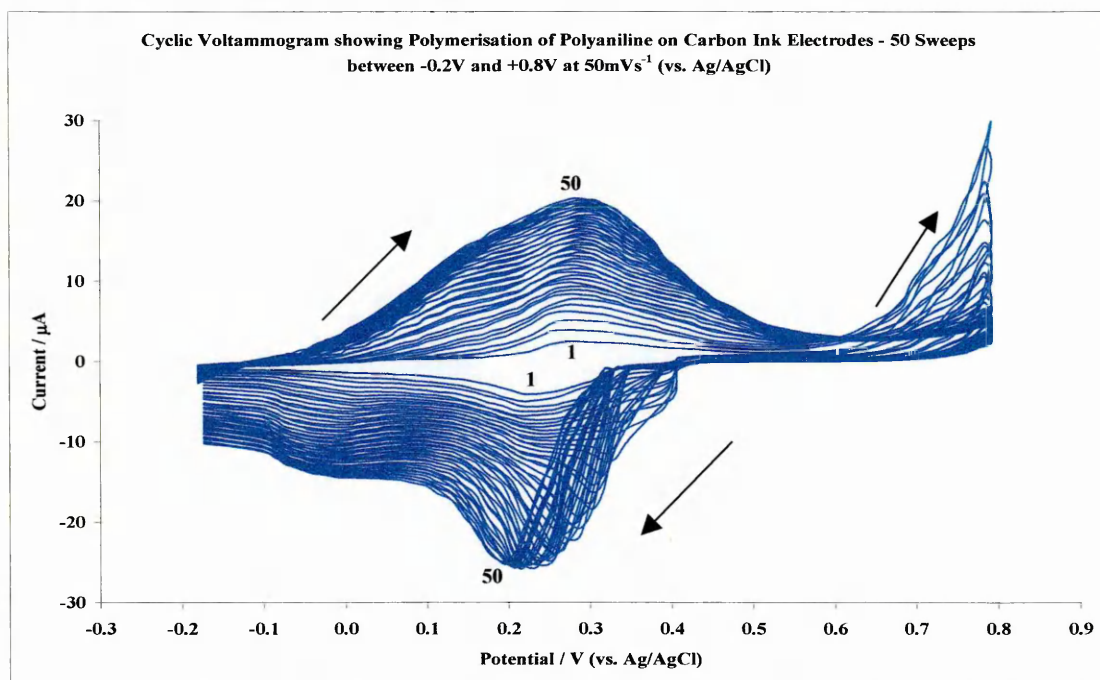


Fig 5.1: Typical Cyclic voltammogram for electrodeposition of PANI on host carbon microelectrode array sensors.

Within last two decades an elegant strategy of biosensor construction based on the entrapment of biomolecules in polymers during their electrogeneration on electrode surfaces has appeared. In various pioneering works (Foulds & Lowe, 1986; Umana & Waller, 1986; Bartlett & Whitaker, 1987) authors described the entrapment of glucose oxidase within the growing network of conducting polypyrroles.

Here the immobilization of antibody at the immunosensor surface is reported. Antibody was added to a monomer solution and the conductive form of PANI, emeraldine, was subsequently electropolymerised at the modified microelectrode array sensor assemblies. As the antibody is added the monomer solution pH increases yet is still below pH 4 (the pH of the monomer solution settles at a pH of approximately 2.6) as shown in Figure 5.1 whereby the peak currents increase with each potential sweep in both the forward and reverse current transients, thus showing the polymer is deposited in its conductive form.

The electrochemical formation of polymer layers of controlled thickness constitutes a reproducible and 'non-manual' procedure of biosensor modification / fabrication.

The electrochemical method involves the entrapment of biomolecules in organic polymers during their electrogeneration on an electrode surface. The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers and biomolecules.

Biomolecules in the immediate vicinity of the electrode surface are thus incorporated in the growing polymer (Shinohara *et al*, 1988; Bartlett & Caruana, 1992; Schuhmann, 1995). In addition, the entrapment of biomolecule occurs without chemical reaction that could affect their reactivity (Cosnier, 1999).

An advantage of electrochemical polymerisation is that films can be prepared easily in a rapid one-step procedure. Furthermore, this method enables exact control of the thickness of the polymer layer based upon the measurement of the electrical charge passed during the electrochemical polymerisation.

Another major advantage of electrochemical deposition procedures over more conventional methods is the possibility to precisely electrogenerate a polymer over small electrode surfaces of complex geometry.

Most of the electrochemically deposited polymer films used for the biomolecule immobilisation are conducting polymers, for example, polyacetylene, polythiophene, PANI, polyindole and polypyrrole (Pandey, 1988; Shinohara *et al*, 1988; Bartlett & Cooper, 1993).

PANI exists in three well-defined oxidation states: leucoemeraldine, emeraldine and pernigraniline, as shown in Figure 5.2.

Leucoemeraldine and pernigraniline represent the fully reduced and fully oxidised forms, respectively. In emeraldine the ratio of amine to imine nitrogen atoms is ~ 0.5 . Electrically conducting emeraldine can be obtained by standard chemical or electrochemical oxidation.

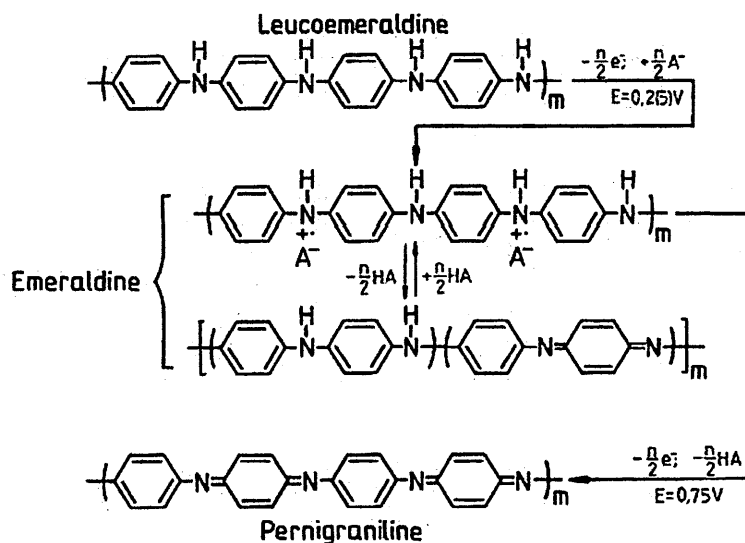


Fig 5.2: Generalised Scheme of the oxidative and non-oxidative (protonic acid) doping of PANI:

n , number of aniline units, $m = 4n$. (Wallace *et al*, 2003).

During the electrochemical polymerisation at pH 3.5, aniline is polymerised thus forming the electrically conductive emeraldine form. The generally accepted mechanism of oxidative polymerisation of aniline (Wei *et al*, 1989; Wei *et al*, 1990(a); Wei *et al*, 1990(b); Wei *et al*, 1994), to produce the conducting emeraldine PANI has been discussed (section 2.7.1), yet is still a matter of speculation (Genies *et al*, 1985(a); Genies *et al*, 1988; Syed & Maravattick, 1991; Gospondinova *et al*, 1994; Chiou *et al*, 1997; Mu *et al*, 1997; Gospodinova & Terlemezyan, 1998; Innis & Wallace, 2002; Wallace *et al*, 2003(a)). The polymerization has been assigned to the oxidative category since it is concomitant with the oxidation of aniline by standard methods of chemical or electrochemical oxidation.

Owing to their conductivity, the thickness and volume of the polymer can be easily varied and is not restricted to very thin, low volume depositions, in comparison to those formed using non-conducting, self-limiting polymers.

The polymers that are termed conducting are those that permit some electrical conductivity in at least one redox state. The polymer may be oxidised or reduced, so the conductivity of the polymer depends on its redox state. As shown in Figure 5.2 PANI has three redox states, which permit the polymer to exhibit either *reasonable* conductivity or insulating behaviour via its polarization (Higson, 2003).

Most polymeric materials are poor conductors of electricity because of the unavailability of large numbers of free electrons to participate in the conduction process. The mechanism of electrical conduction in these materials is not well understood, but it is understood that conduction in polymers of high purity is electronic (Callister, 1997).

Within the past several years, polymeric materials have been synthesised that have electrical conductivities on par with metallic conductors; they are appropriately termed *conducting polymers*. Conductivities as high as $1.5 \times 10^{-7} (\Omega\text{m})^{-1}$ have been achieved in these materials (Callister, 1997).

This phenomenon is observed in a dozen or so polymers, including polyacetylene, polyparaphenylene, polypyrrole and PANI that have been doped with appropriate impurities.

As is the case with semiconductors, these polymers may be made either *n*-type (i.e., free radical dominant) or *p*-type (i.e., hole dominant) depending on the dopant. However, unlike semiconductors, the dopant atoms or molecules do not substitute for or replace any of the polymer atoms. High purity polymers have electron band structures characteristic of electrical insulators. The mechanism by which large numbers of free electrons and holes are generated in these conducting polymers is complex and not well understood (Focke & Wnek, 1988; Stejskal & Gilbert, 2002; Wallace *et al*, 2003(b)).

In very simple terms, it appears that the dopant atoms lead to the formation of new energy bands that overlap the valence and conduction bands of the intrinsic polymer,

giving rise to a partially filled conduction or valence band and thus the production, at room temperature, of a high concentration of free electrons or holes.

In this instance PANI is easily electropolymerised from an aqueous solution of aniline monomer.

Further molecules, such as enzymes, may be immobilised within the polymer, with the polymer acting as a matrix. In this way, a number of redox state dependent conductimetric properties may be monitored - and, in this way, a number of 'chemical transistors' or 'enzyme transistors' have been developed.

Other biomolecules may be immobilised within the PANI matrix and for the purposes of this research a range of antibodies have been immobilised within PANI at microelectrode array sensors. Although only minute quantities of the biological component (antibody) are required, the purer it is, the more reliable it will be for applications within biosensors (Turner *et al*, 1987).

The buffer species, cycle number (up to 50 cycles) and hence polymerisation time have all been thoroughly investigated to achieve the most efficient immobilisation of antibody receptor molecules within the matrix of emeraldine PANI.

Once the PANI was formed the electrode was thoroughly washed with de-ionised water and left to air-dry over 24 hours. It is beneficial to keep the polymerisation time to a minimum to reduce the time spent at a low pH to maintain the biological activity and physical integrity of the antibodies, thus preventing denaturation, as well as allowing for a quick fabrication step. 15 cyclic sweeps from -200mV to +800mV and one linear sweep to +800mV (vs. Ag/AgCl), 50mVs⁻¹, ensures immobilisation of antibody within 12 minutes and permits adequate polymer formation.

The phenomenon associated with conducting polymer growth at microelectrode array sensor surfaces is that as the PANI 'emeraldine' is electrogenerated, mushroom-like

protrusions are observed. The polymer protrusions co-entrap antibody receptor molecules during the polymerisation process and hence throughout the polymer matrix and as a randomly orientated monolayer at the outer surface.

The three dimensional structures confer a hemi-spherical diffusion profile enhancing the electroactive interface of the modified electrodes. The conducting polymer emanates from the micropores at the polymerised and sonicated carbon electrode surface (Chapter 4).

Optimisation of pore dimensions and distribution and therefore mushroom distribution have been undertaken in conjunction with Microarray Ltd. Cycle number and polymerisation time investigations have been undertaken. Studies have shown that the antibody receptor bio-molecules maintain their biological activity and are not de-natured since they are not immersed for prolonged periods in the low pH environment and are thoroughly washed after entrapment. 15 cycles along with 1 linear sweep to +800mV (vs. Ag/AgCl) was sufficient for immobilisation within the resulting emeraldine protrusions to be formed. Sensors were then left to air-dry over 24 hours.

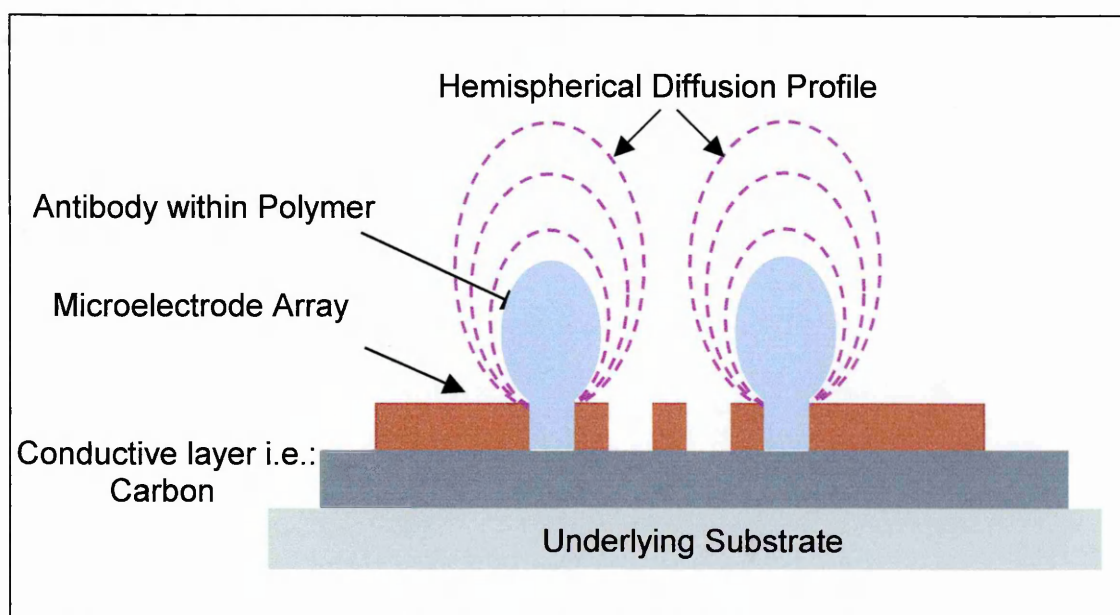


Fig 5.3: Schematic representation of diffusional profile for PANI microelectrode array.

Figure 5.3 depicts a schematic representation of the diffusional characteristics of the PANI mushroom microelectrode array.

Due to the difference in conductivity between the conductive ‘emeraldine’ protrusions and the insulated carbon-working electrode, scanning electron microscopy can be used to analyse the growth and distribution of the phenomena throughout the surface. Samples are prepared as outlined in Section 3.4.5.

Figures 5.4 - 5.6 show the images obtained via scanning electron microscopy of these arrays, following the electrodeposition of PANI, confirming that emeraldine protrusions were indeed formed via growth of the polymer (PANI) from the sonochemically fabricated pores. The projections form in the shape of mushroom-like growths or protrusions as the conducting polymer fills and spreads out on top of each microelectrode cavity.

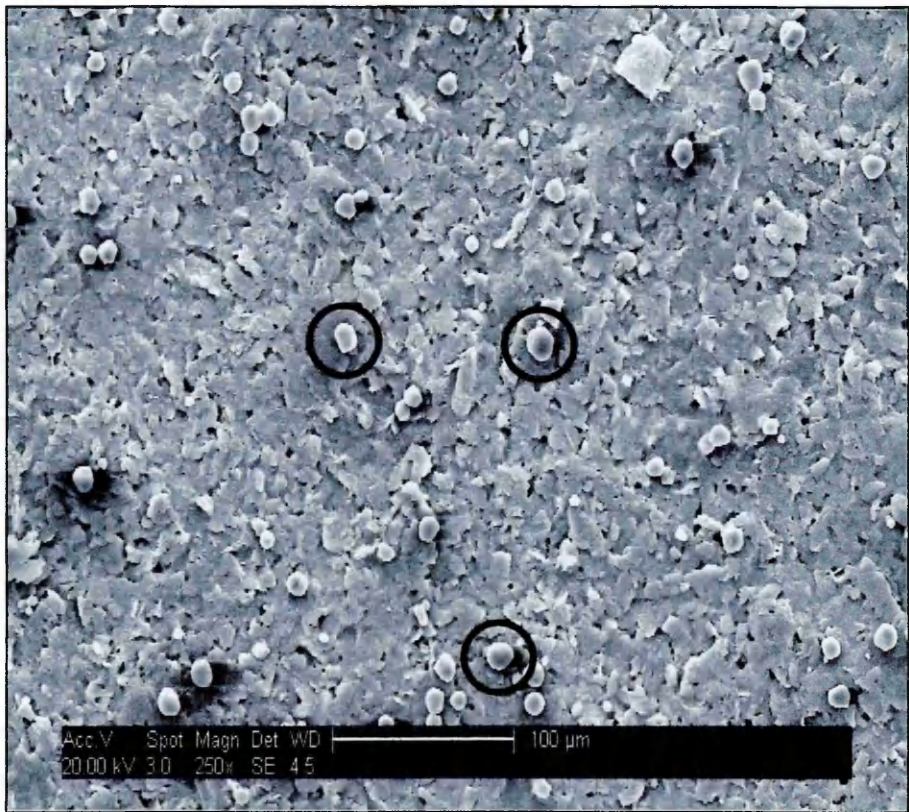


Fig 5.4: SEM images of ‘emeraldine’ protrusion phenomena at M = 250x.

Several interesting points should be noted from the electron micrograph of Figure 5.4. Firstly it can be seen that all of the microelectrode cavitations appear to be filled with PANI. This shows all ablative cavitations of the insulating polymer film lead to exposure of the underlying conductive surface, since aniline could not be electropolymerised without access to the underlying conductive surface of the host carbon electrode.

Higher magnification on a particular area of the working electrode, Figure 5.5, reveals in better detail the diameter sizes of the observed protrusion phenomena, typically in the region of 8-15 μm .

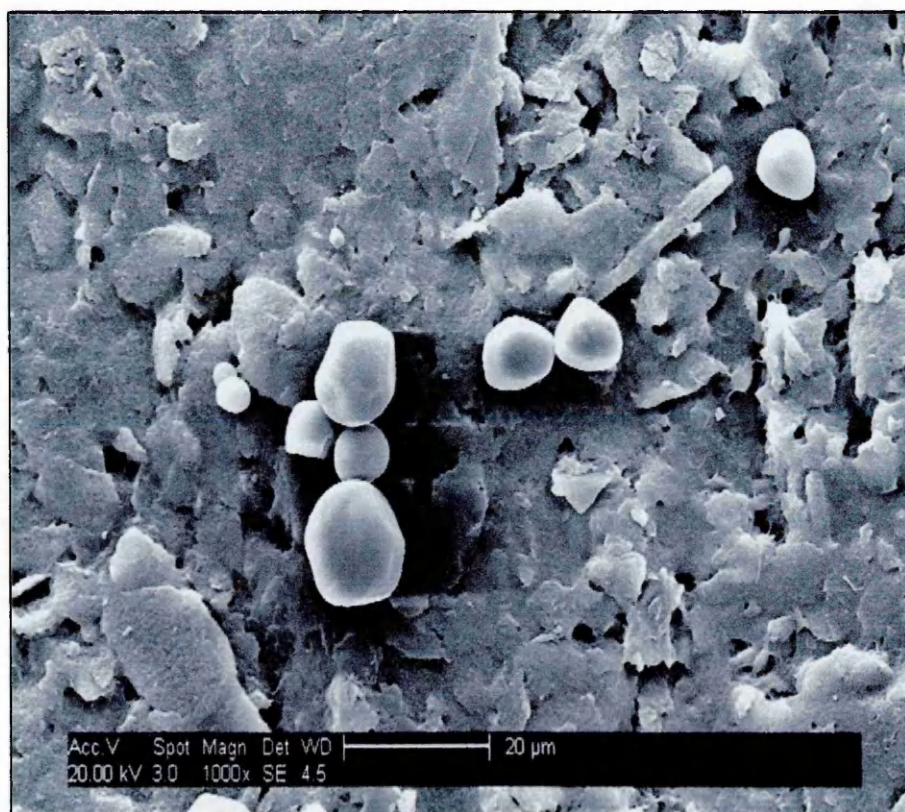


Fig 5.5: SEM images of ‘emeraldine’ protrusion phenomena at M = 1000x.

To reveal more about the overall dimensions of the protrusions and to obtain a better visual representation of the surface topography, the scanning electron microscope was used to evaluate the surface of the electrode at an angle of 55°.

This gives a clearer idea of the size and dimensions of individual protrusions as demonstrated in Figure 5.6 showing increasing magnifications.

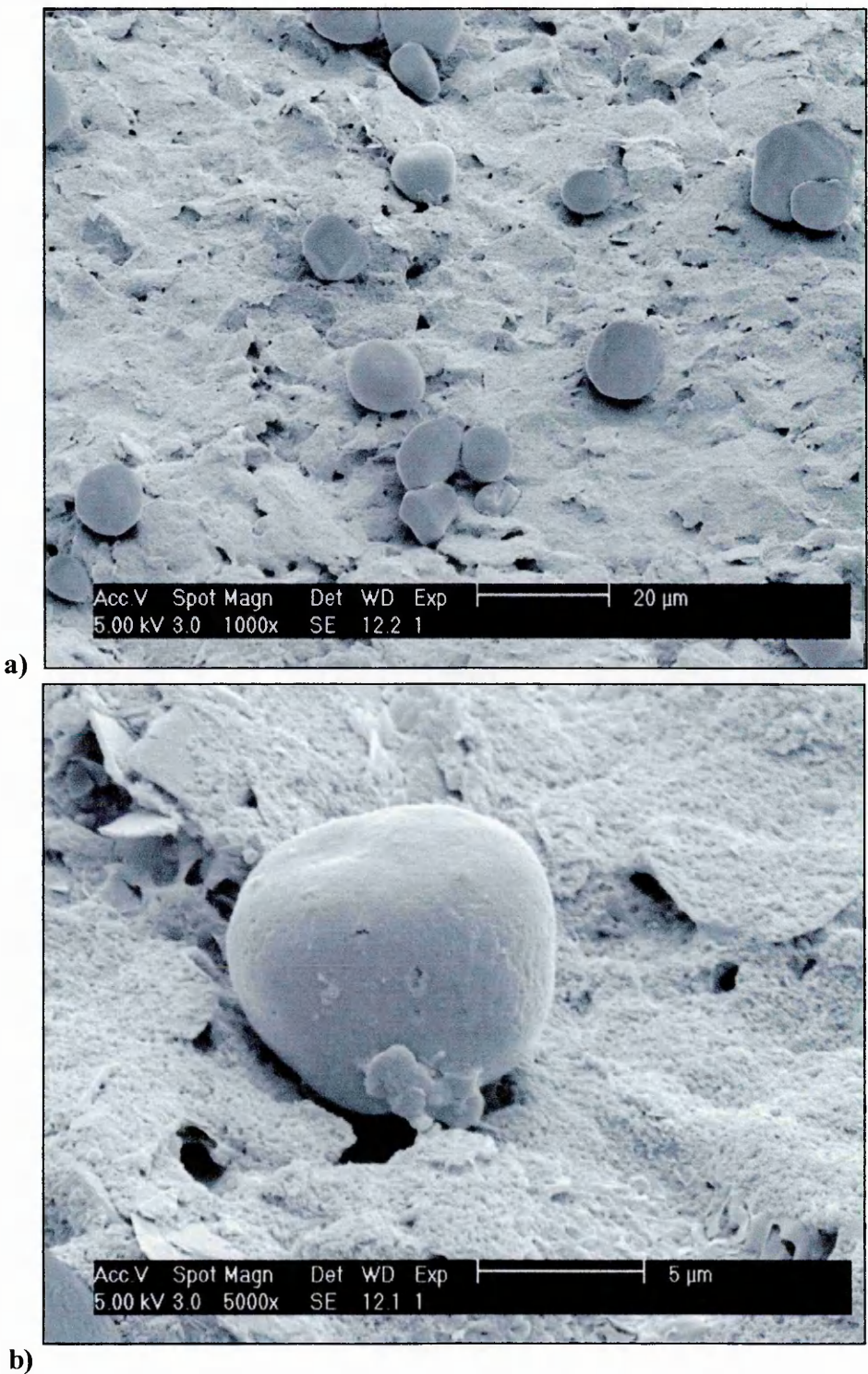


Fig 5.6: SEM imaging performed at 55° to the electrode at a) M = 1000x & b) M = 5000x.

The mushroom dimensions are typically 8 to 15 μm and generally spherical. Growth is controlled by interfacial and surface energies, along with surface tension, surface chemistry and the polymer volume conductively building up with each cycle. The estimated population density of protrusion phenomena is $2 \times 10^5 \text{ cm}^{-1}$ (Higson, 1996; Myler, 2000; Barton *et al*, 2004; Gornall, 2005; Mills, 2005).

Immobilisation of antibody occurs during the polymerisation step within and at the surface of the conductive 'emeraldine' protrusions. The number of active sites available to the analyte antigen due to the orientation of the co-entrapped antibody is of paramount importance for the application of labelless immunosensor.

The microelectrode array sensors were subsequently interrogated as to their potential for application as impedance immunosensors via electrochemical impedance spectroscopy techniques and the results are reported in Chapter 6.

5.3 Non-covalent affinity attachment of biomolecules following PANI protrusion growth.

The possibility of developing an electrodeposited biotinylated PANI immobilisation matrix for the fabrication of impedimetric immunosensors was investigated as an alternative to the conventional entrapment approach. It was proposed that the retention of biomolecule from the aniline monomer solution prior to electropolymerisation would allow for optimum deposition conditions of the emeraldine PANI. Additionally, the low pH required for electrodeposition would no longer be able to adversely affect the biological properties of the antibody receptors. Validation of this immobilisation procedure may also be obtained throughout the stepwise scaffolding construction utilising quartz crystal microbalance techniques for real-time monitoring.

The stable immobilisation of macromolecular biomolecules on conductive microsurfaces with complete retention of their biological recognition properties is a crucial problem for the commercial development of miniaturised biosensors (Turner *et al*, 1987; Cosnier,

1999). Effectively most of the conventional procedures of biomolecule immobilisation such as cross-linking, covalent binding and entrapment in gels or membranes suffer from low reproducibility and a poor spatially controlled deposition. Apart from these conventional methods, the immobilisation of biomolecules in electropolymerised films has gained importance over recent years.

The electrochemical method employed in Section 5.2 involves the entrapment of biomolecules in organic polymers during their electrogeneration on a modified microelectrode array sensor surface. The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers and biomolecules. However, the immobilisation of immunoagent in this manner may prevent the formation of antigen-antibody complexes.

The orientation of immobilised antibody is random and the availability of antigen binding sites and hence subsequent stable complex formation is hindered. Steric hindrance may also prevent affinity interactions from occurring between antigen analyte and antibody immobilised within the conducting polymer matrix. The low pH required for deposition of emeraldine PANI might also affect the biological properties of the antibodies to some degree.

To increase the specific response between antibody and antigen in test solution advances in the procedures employed for the site-specific immobilisation of receptors has been thoroughly investigated.

Within the literature, attempts to overcome some of these outstanding issues have been to initially electrogenerate functionalised conducting polymers. Then the attachment of biomolecules to the polymer surface can be obtained by covalent binding to adequate functional groups such as amino, (Schuhmann *et al*, 1990; Schuhmann *et al*, 1993; Rockel *et al*, 1994) carboxylic (Willner *et al*, 1992), *N*-hydroxysuccinimide and *N*-hydroxyphthalimide ester groups (Hiller *et al*, 1996; Korri-Yousoufi, 1997).

The main advantage of electropolymerisation and covalent binding lies in the possibility of using optimal conditions for both. However, the covalent binding could induce chemical denaturation of the protein or influence protein flexibility. These effects may affect the catalytic activity or recognition properties of the biomolecules (Cosnier *et al*, 1999).

Another approach is to electropolymerise functionalised conducting polymers. Then the attachment of biomolecules to the surface can be obtained by affinity of the biomolecule at the functional group. This approach enables optimum conditions to be utilised throughout the fabrication.

In comparison to the physical entrapment of biomolecules within polymers such as polypyrrole, polythiophene, polyacetylene or PANI, this approach facilitates macromolecular interactions. However, the amount of immobilised biomolecules is restricted to a monolayer at the interface polymer-solution.

As well as facilitating antibody-antigen interactions, an affinity immobilisation system would also preserve the complete biological properties of the biomolecules unlike immobilisation via electropolymerised entrapment - or via covalent binding following electropolymerisation.

The immobilisation of proteins on electrogenerated polymer films has been attempted by the use of an affinity system (Yang *et al*, 1998; Leipert *et al*, 1998; Cosnier *et al*, 1998; Torres-Rodriguez *et al*, 1998; Cosnier *et al*, 1999; Cosnier & Lepellec, 1999).

Within this work, the use of an affinity-based immobilisation system is reported to specifically orientate a monolayer of monoclonal antibody receptors at novel PANI mushroom protrusions formed at microelectrode array sensors.

Due to the high affinity constant of the avidin-biotin interaction (association constant $K_a = 10^{15} \text{ M}^{-1}$) (Wilchek & Bayer, 1998), this coupling system has been used for protein

immobilisation on biotinylated polymer films. The attachment of proteins is achieved via the simple formation of an avidin-biotin bridge with biotinylated proteins or avidin-conjugated proteins. The avidin-biotin interaction is the strongest known biochemical (non-covalent) bond (Green, 1963(a); Vermette *et al*, 2003).

The high affinity interaction of avidin-biotin leads to an affinity-driven immobilisation protocol of biomolecules solely involving a single attachment point that fully retain their biological activity (Gitlin *et al*, 1988(a); Gitlin *et al*, 1988(b)). More interestingly, binding of a protein monolayer displaying excellent accessibility to each immobilised protein has been recently achieved by using avidin-biotin bridges between electropolymerised biotinylated polypyrrole films and biotinylated antibodies (Ouerghi *et al*, 2002).

Among conventional methods utilised for the detection of antigen-antibody binding events, the electrochemical transduction offers attractive advantages such as its ease of use in turbid samples, portability, low cost and compatibility with bulk manufacturing procedures.

Besides the direct reagentless immunosensing based on frequency impedance measurements, (Kalab & Skladal, 1995; Sargent *et al*, 1999; Katz & Willner, 2003) the detection of an immunoreaction is commonly performed either by optical (Marks *et al*, 1997; Konry *et al*, 2003) or by amperometric transduction following the classical enzyme-linked immunosorbent assay strategy (Rishpon, 1996; Ghidilis *et al*, 1998; Santandreu *et al*, 1999; Liu *et al*, 2001; Zayats *et al*, 2002; Dai *et al*, 2003). As a consequence, the diffusion of the electroactive probes indicates the immunoreaction through the underlying biotinylated polymer film and hence the sensitivity of the amperometric immunosensor is strongly related to the permeation properties of the biotinylated polymer used for the immobilisation of antibody or antigen.

However, the biotinylated polymers described until now exhibited poor permeabilities since their formation passivated the electrodes (Yang *et al*, 1998; Kuramitz *et al*, 2003).

Most of them were thus employed for the modification of affinity sensors based on gravimetric measurements (Dupont-Filliard *et al*, 2001; Torrez-Rodriguez, 2002).

Avidin (pI of 10.5, ~67 kDa, 5.6 x 5 x 4 nm) is a basic glycoprotein, found in the egg white and tissues of birds, reptiles and amphibians (Green, 1962; Green, 1963 (a); Green, 1963(b); Green, 1963(c); Melamed & Green, 1963; Green, 1964; Green, 1965; Green, 1966; Green *et al*, 1971; Green, 1975; Green, 1990; Rosano *et al*, 1999).

It consists of 10% carbohydrate (Melamed & Green, 1963; Gitlin *et al*, 1987; Hiller *et al*, 1987) and it is composed of 4 x 128 amino acid residue chains (DeLange, 1969; DeLange, 1970; DeLange & Huang, 1971; Green *et al*, 1971; Huang & DeLange, 1971). Avidin can also bind up to four molecules of vitamin H, D-biotin, in a non-covalent affinity interaction (Gitlin *et al*, 1988(a); Gitlin *et al*, 1988(b)).

For model recognition systems, streptavidin is more commonly used than avidin. Streptavidin (pI of ~5-6, ~60kDa, 4.5 x 4.5 x 5.8 nm) is also a tetrameric protein, obtained from *Streptomyces avidinii*. It is composed of four identical chains, each 159 amino acid residues long (Chalet & Wolf, 1964; Tausig & Wolf, 1964; Green, 1990).

In bioengineering terms, the properties of streptavidin are improved when compared to those of avidin, in that most of the basic amino acids of avidin have been converted to neutral or acidic residues and the molecule is not glycosylated (Green, 1990; Wilchek & Bayer, 1999). The biotin-binding properties of non-glycosylated avidin have been reported to be equivalent to those obtained for the native (glycosylated) avidin molecule indicating that the oligosaccharide moiety is not essential for the binding activity (Hiller *et al*, 1987). Like avidin streptavidin binds biotin at four binding sites, two binding sites on each of two opposing faces of the molecule, with an extremely high binding constant ($K_D \sim 10^{-13}$ M) (Wilchek & Bayer, 1999; Sano & Cantor, 1995). The four binding sites together with the high affinity of avidin-like proteins for biotin serve as an aid in amplifying the sensitivity of immunoassays, which have become widespread as a general tool in all fields of biology (Wilchek & Bayer, 1999).

The avidins (including streptavidin and neutravidin) all have weights in the region of $M_r \sim 60\text{kDa}$ and are tetrameric, with one biotin binding site per monomer (Green, 1964). Avidin is prepared from egg white and has an acidic pI due to any attached carbohydrate leading often to high non-specific binding. Streptavidin is much more basic and suffers from much less non-specific binding; however, it is much more expensive. A good compromise is Neutravidin which is recombinant avidin with its glycosylation site engineered out.

Neutravidin biotin-binding protein is a superior replacement for avidin and streptavidin when non-specific binding must be minimized. Neutravidin is a modified avidin derivative that combines several key features to provide a biotin-binding protein with exceptionally low non-specific binding properties (Wooley & Longworth, 1942). The neutravidin protein does not contain carbohydrate, thus eliminating the potential for binding to lectins. The deglycosylation of neutravidin does not compromise or impact the stability or biotin-binding activity of the protein (Hiller *et al*, 1987).

The relatively neutral isoelectric point (6.3 ± 0.3) of neutravidin is achieved through modification of charged groups on the surface of the protein. This modification ensures that sufficient lysines remain available on the surface of neutravidin, so that protein conjugates can still be successfully prepared through traditional amine-reactive chemistries and as mentioned the neutral pI minimises nonspecific adsorption. The specific activity for biotin-binding is approximately $14\mu\text{g/mg}$ of protein, which is near the theoretical maximum activity (Brett *et al*, 1987; Unson *et al*, 1999; Wojciechoski, 1999; Glover & McHenry, 2001; Guo *et al*, 2001; Claypool *et al*, 2002).

The simplest way of using the avidin-biotin reagents is to use a biotinylated surface, add on the tetrameric avidin, and then add on the biotinylated antibody / enzyme. There are many biotinylation reagents available with a range of functional groups for attachment, and various spacer arms. The biotinylating agent used herein was Biotin 3-sulfo-N-hydroxysuccinimide ester. Essentially, the antibody/enzyme is reacted at a slightly alkaline pH in a non-amine buffer; the reaction is quenched by adding excess

amine (e.g. excess glycine at pH 7.0) and then the unincorporated biotin is removed from the biotinylated protein by gel filtration on Sephadex G-25 superfine or similar.

Biotinylation of antibody was performed in accordance with section 3.5.3.2 and the biotinylated capture bio components were stored frozen at -20°C until required for further use. Sensor construction was performed in accordance with 3.5.3.2 steps 1-5. UV spectrophotometry and QCM techniques were employed to validate specific fabrication stages.

5.3.1 Antibody biotinylation – UV spectrum profile.

Following the biotinylation of Polyclonal Human IgG & monoclonal APSA, AS-100 and ANSE antibodies according to section 3.5.3.2-step-1 UV spectroscopy is employed to obtain an elution profile. A clear peak at 280nm indicates the presence of protein in the fraction. In this and most cases fractions 4, 5 & 6 exhibited peaks and were therefore pooled and subjected to the avidin-HABA assay to determine the extent of biotinylation. Figure 5.7 shows a representative elution profile obtained for monoclonal anti S-100 (S53) antibodies following biotinylation at 1mg ml^{-1} .

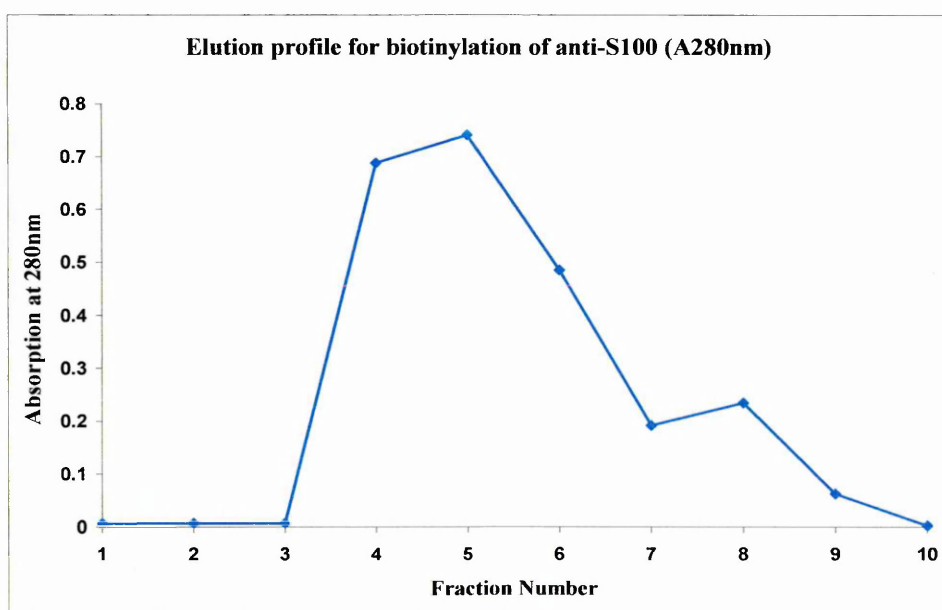


Fig 5.7: Elution profiles obtained following biotinylation (S-100 MAb).

The second peak (fraction 8) is due to unincorporated high molecular weight protein. Approximately 6 moles of biotin were bound to each mole of protein (Ab) following the Avidin-HABA assay. Biotinylated antibodies were attached to free biotin groups on the electrogenerated PANI using neutravidin as a coupling reagent.

5.3.2. Quartz Crystal Microbalance immobilisation validation.

The immobilisation method permits the production of a highly reproducible and stable device. Optimum deposition conditions of PANI 'emeraldine' were utilised as no biomolecule was present during electrogeneration. Figure 5.8 shows the deposition of PANI on the carbon microelectrode array sensors using this methodology. A monomer solution was prepared in pH1 chloride buffer, 0.2M Aniline, 0.3M HCl, 0.5M KCl. 15 cyclic sweeps from -200mV to +800mV (vs. Ag/AgCl) and 1 linear sweep to +800mV.

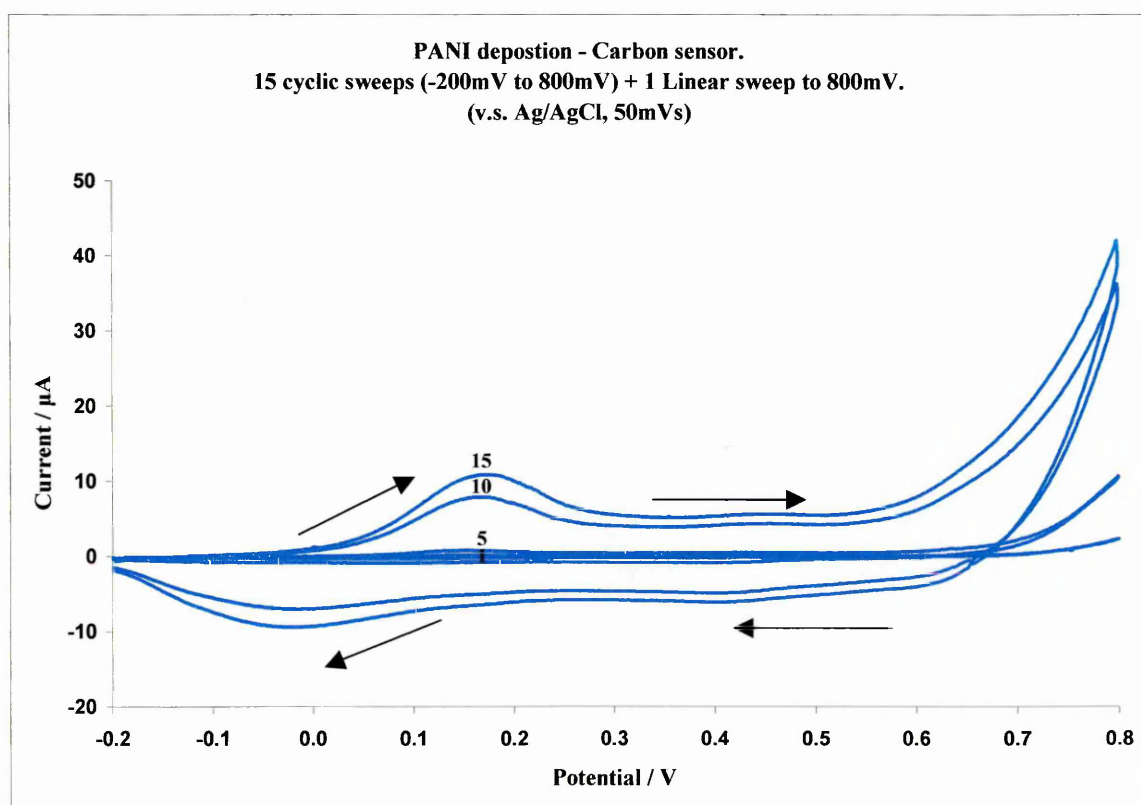


Fig 5.8: Cyclic voltammogram showing optimum electrodeposition of PANI from a pH1 chloride buffer on carbon microelectrode array sensor.

The voltammogram obtained is comparable to that observed in Figure 5.1 since both forward and reverse current transient peaks are again seen to increase with each potential sweep. It is the conductive 'emeraldine' form of PANI that is laid down on the microelectrode array sensors.

Voltammograms are highly reproducible from sensor to sensor and share near perfect reproducibility when plotted on the same axes. This suggests a repeatable fabrication protocol that generates protrusions of similar dimensions and quantity at the sensor surface within the electrochemical cell.

Figure 5.9 shows a cyclic voltammogram of PANI electrogeneration under the same deposition conditions and parameters at a QCM chip - a gold-coated quartz crystal. The QCM was utilised to validate the surface attachment chemistry techniques. Again the deposition can be precisely controlled from chip to chip.

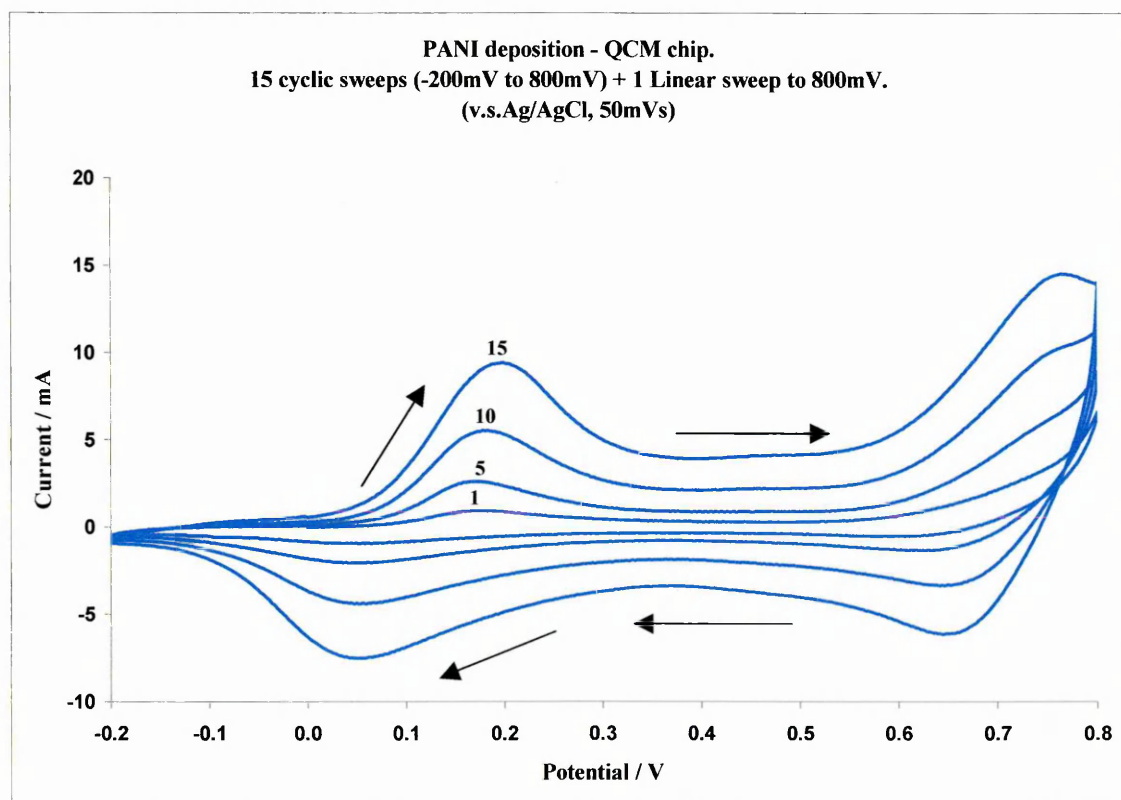


Fig 5.9: Cyclic voltammogram showing optimum electrodeposition of PANI from a pH1 chloride buffer on a QCM chip.

The Curie brothers showed in 1880 that crystals of Rochelle salt could produce a flow of electrons when pressure was applied in certain crystallographic directions (Curie & Curie, 1880(a); Curie & Curie, 1880(b)). Later they also showed the converse effect, i.e. production of strain by application of electricity. These findings were the discovery of the piezoelectric effect. Piezoelectricity did not receive a lot of interest originally and a more detailed study of piezoelectricity was not started until 1917 when it was reported that quartz crystals could be used as transducers and receivers of ultrasound in water.

In 1919 several devices of everyday interest based on the piezoelectricity of Rochelle salt were described; i.e. loudspeakers, microphones and sound pick-ups. In 1921 the first quartz crystal controlled oscillator was described (Farrand, 1921; Shepherd, 1986; Lewis, 1990). These first quartz crystal controlled oscillators were based on X-cut crystals, which have the drawback of being very temperature sensitive. Therefore, the X-cut crystals are nowadays used in applications where the large temperature coefficient is of little importance, such as for use as transducers in space sensors. The cut angle determines the piezoelectric response.

A typical “thick” crystal plate is a cylindrical disk of a diameter 10mm and thickness about 0.7 to 0.1mm for resonant operation in the 2 to 15 MHz frequency range. Most commonly plate oscillators are cut to exhibit longitudinal (thickness expander) modes or thickness shear modes. For quartz, cuts across the X-axis, Y-axis or so called rotated Y-cuts are used. Various designers of cuts appear in the literature: X-cut (thickness expander), AC, AT (thickness shear), DT (plate shear), BT, ST (horizontal shear), SC (stress compensated), etc. Symbol T specifies a cut with minimal temperature dependence of the resonant frequency. Within these general cuts, there are subgroups (e.g., planar, plano-convex and others). These cuts should be specified, as their properties are very important for the piezoelectric sensor performance. Electronic surface films and electrical contacts are usually accomplished by vacuum deposition, sputtering or electroplating of metal films on both crystal faces (Bruckenstein & Shay, 1985; Buttry & Ward, 1992; Brucker, 2005).

The dominance of the quartz crystal for all kind of frequency control applications started in 1934 when the AT-cut quartz crystal was introduced. The majority of crystals that are used in everyday applications operate in the frequency range of 1MHz up to 200MHz. The crystals in this category are cut along particular angular orientations with respect to the major axes of the original quartz stone and are identified as AT-cut resonators. The advantage with the AT-cut quartz crystal is that it has nearly zero frequency drift with temperature around room temperature, that is, they possess thermal characteristics that are suitable for both wide temperature and controlled temperature applications depending on the angle of cut selected which is the reason for their wide use. This crystal wafer is prepared by cutting the quartz at approximately 35.17° from its Z-axis. If ac voltage is applied across the crystal, by means of two excitation electrodes deposited on its opposite faces, then shear vibration is generated in the X-axis parallel to the electric field, i.e., one face displacing parallel to the other without thickness change (Mason, 1934; Froehlich *et al*, 1993)

From the very beginning the use of quartz crystal resonators as frequency control elements it was common to increase the frequency of the resonator by drawing pencil marks on the electrodes, or decreasing the frequency by rubbing off some electrode material with an eraser. The understanding of this mass induced frequency shift was only known on a qualitative basis.

However, in 1959 Sauerbrey published a paper that showed that the frequency shift of a quartz crystal resonator is directly proportional to the added mass. Sauerbrey's work is generally taken as the breakthrough and the first step towards a new quantitative tool to measure very small masses; i.e. the quartz crystal microbalance (Sauerbrey, 1959).

Hence, one can describe the QCM to be an ultra-sensitive mass sensor. The heart of the QCM is the piezoelectric AT-cut quartz crystal sandwiched between a pair of electrodes. When the electrodes are connected to an oscillator and an AC voltage is applied over the electrodes the quartz crystal starts to oscillate at its resonance frequency due to the piezoelectric effect.

This oscillation is generally very stable due to the high quality of the oscillation (high Q factor). If a rigid layer is evenly deposited on one or both of the electrodes, then the resonant frequency will decrease proportionally to the mass of the adsorbed layer according to the Sauerbrey equation:

Equation 5.1

$$\Delta f = - [2 \times f_0 \times \Delta m] / [A \times (\rho_q \mu_q)^{1/2}], \text{ where}$$

Δf = measured frequency shift,

f_0 = resonant frequency of the fundamental mode of the crystal,

Δm = mass change per unit area (g/cm^2),

A = piezo-electrically active area,

ρ_q = density of quartz, $2.648 \text{ g}/\text{cm}^3$,

μ_q = shear modulus of quartz, $2.947 \times 10^{11} \text{ g}/\text{cm} \times \text{s}^2$.

There are situations where the Sauerbrey equation does not hold, for example, when the added mass is a) not rigidly deposited on the electrode surface(s), b) slips on the surface or c) not deposited evenly on the electrode(s). Therefore, the Sauerbrey equation is only strictly applicable to uniform, thin-film deposits. Due to this the QCM was for many years just regarded as a gas-phase mass detector. It was not until the beginning of the 1980s that scientists realised that a quartz crystal can be excited to a stable oscillation when it was completely immersed in a liquid. Much of the pioneering work in liquid phase QCM measurements have been done by Kanazawa and co workers, who showed that the change in resonant frequency of a QCM taken from air into a liquid is proportional to the square root of the liquid's density-viscosity product (Kanazawa & Gordon, 1985):

Equation 5.2

$$\Delta f = - f_u^{3/2} [(\rho_L \eta_L) / (\pi \times \rho_q \mu_q)]^{1/2}, \text{ where}$$

Δf = measured frequency shift,

f_u = resonant frequency of the unloaded crystal,

ρ_L = density of liquid in contact with the crystal,

η_L = viscosity of liquid in contact with the crystal,

ρ_q = density of quartz, 2.648 g/cm^3 ,

μ_q = shear modulus of quartz, $2.947 \times 10^{11} \text{ g/cm} \times \text{s}^2$.

After it was found out that an excessive viscous loading would not prohibit use of the QCM in liquids and that the response of the QCM is still extremely sensitive to mass changes at the solid-liquid interface, QCMs have been used in direct contact with liquids and/or visco-elastic films to assess changes in mass and visco-elastic properties. Even in air or vacuum, where the damping of layers has been considered to be negligible or small the QCM has been used to probe dissipative processes on the quartz crystal. This is especially true for soft condensed matters such as thick polymer layers deposited on the quartz surface.

The QCM is basically a mass sensing device with the ability to measure very small mass changes on a quartz crystal resonator in real-time. The sensitivity of the QCM is approximately 100 times higher than an electronic fine balance with a sensitivity of $0.1 \mu\text{g}$. This means that QCMs are capable of measuring mass changes as small as a fraction of a monolayer or single layer of atoms. The high sensitivity and the real-time monitoring of mass changes on the sensor crystal make QCM a very attractive technique for a large range of applications. Particular application is the development of QCM systems for use in fluids or with visco-elastic deposits and this has dramatically increased the interest towards the QCM technique. Major advantages of the QCM technique used for liquid systems are that it allows for a label-free detection of molecules.

The following stepwise modifications to the PANI 'mushroom' sensor platform are performed via exposure of the sensor to the relevant reagent-containing solution. A piezoelectric 'proof of concept' study was performed in order to investigate the techniques employed.

An electrochemical quartz crystal microbalance was used with the gold-coated quartz crystal chip detecting changes in the oscillating frequency that are related to mass changes at the surface by the Sauerbrey equation.

Simultaneous control experiments were performed with both channels monitored in identical static cell set-ups to provide 'real-time' monitoring of mass changes. A thorough washing and flushing regime was employed in between steps and a stable frequency response was obtained before analysis.

The aim was to validate the surface attachment chemistry techniques and results are as follows.

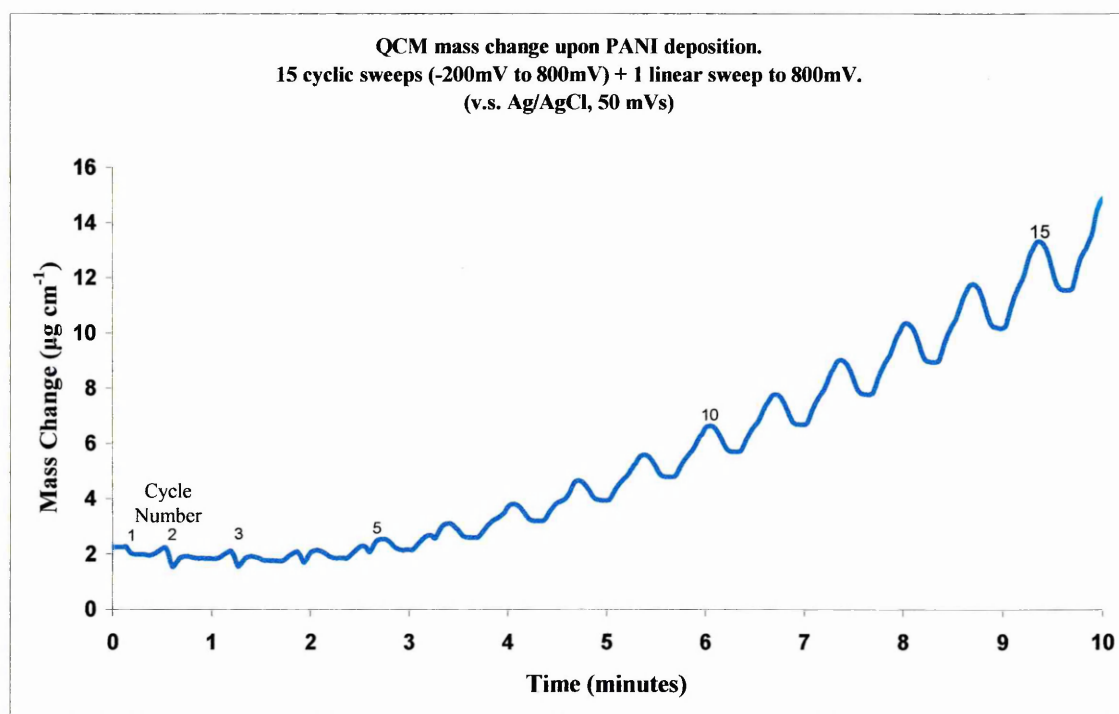


Fig 5.10: QCM data showing optimum electrodeposition of PANI from a pH1 chloride buffer on a QCM chip.

Figure 5.10 illustrates the cyclic mass change for the electrogeneration of a PANI film on a QCM chip after 15 cyclic depositions and 1 linear sweep to +800mV.

The electrochromic phenomenon of PANI was also observed throughout the electrodeposition as PANI transitions from colourless leucoemeraldine (-200mV) to its protonated green emeraldine salt (+800mV). PANI can also be overoxidised to pernigraniline (lilac/purple, +1000mV). The deprotonated form of emeraldine salt, emeraldine base is blue.

The frequency shift related to mass increase at the surface by the Sauerbrey equation equates to approximately $15\mu\text{g cm}^{-1}$ of PANI growth.

With the QCM it is possible to observe the reproducible potentiodynamic growth of PANI and control the mass deposited at the QCM chip.

Both channels of the QCM had an identical mass of PANI deposited at their surface via 15 cyclic sweeps and 1 linear sweep to +800mV (vs. Ag/AgCl). The static electrochemical cells were thoroughly flushed with de-ionised water.

PANI formation by both the chemical and electrochemical processes have been extensively studied to establish structure-property relationships. PANI can exist in three different forms (leucoemeraldine, emeraldine and pernigraniline as either salt or base in each form). In addition, the protonated forms of PANI also have counter-anions intimately associated with the positively charged PANI chains. The evidence comes mainly from electronic transport studied in which the temperature dependence of conductivity fits a charge hopping model (Zuo *et al*, 1989). Finally, it has also been observed that PANIs may contain considerable amounts of solvent molecules. PANI formed by either the electrochemical or chemical process give essentially linear chains with predominantly para head-to-tail couplings.

There is considerable evidence and widespread acceptance that PANI forms a heterogeneous structure in its emeraldine salt form. The evidence comes mainly from electronic transport studied in which the temperature dependence of conductivity fits a charge hopping model (Zuo *et al*, 1989).

It is proposed that small domains of highly ordered polymer are surrounded by less ordered material and that electron transport is dominated by the slow conduction through the latter. In the ordered regions, it is suggested that the polymer chains lie flat, allowing a longer conjugation length as the π orbitals can overlap. In the amorphous regions, the chains are more twisted, causing less overlap of π orbitals and shorter conjugation lengths. The exact nature of the structure of the ordered phase in PANI is still subject to ongoing research. The elucidation of such structure is complicated by the various means of producing emeraldine salt (ES) films or coatings, direct electropolymerisation - or through acid doping (protonation) of the emeraldine base (EB).

The latter technique has the advantage of the EB's being highly soluble - and good quality films and coatings can be cast from the solution. Subsequent treatment of the EB films gives the emeraldine salt. It has been known for some time that certain dopants render the PANI emeraldine salt soluble. Thus, it is also possible to form films and coatings by direct casting from ES solutions. However, the nature of the solvent used for the ES can profoundly affect the conformation of the PANI in solution; these conformational differences are also carried over to the cast films.

Pouget *et al* gave an exhaustive study to PANI crystallinity in 1991 (Pouget *et al*, 1991) and this work remains the main reference point for more recent studies. Pouget and co-workers report two forms of crystallinity in the emeraldine salt form of PANI, and that these two structures are produced by different processing methods.

Following the identical deposition of PANI on two QCM chips the static measurement cell was flushed with de-ionised water. One of the QCM chips was biotinylated overnight for 24 hours with a 1mg ml^{-1} solution of Biotin 3-sulfo-N-hydroxysuccinimide ester dissolved in de-ionised water. The other control chip was left overnight in de-ionised water alone.

At the end of the 24 hours, de-ionised water was used to rinse both electrochemical cells and a stable frequency was reached before the success of biotinylation was assessed indirectly via the addition of Neutravidin to both surfaces (PANI-biotin vs. PANI). Results for QCM measurements are shown in Figure 5.11.

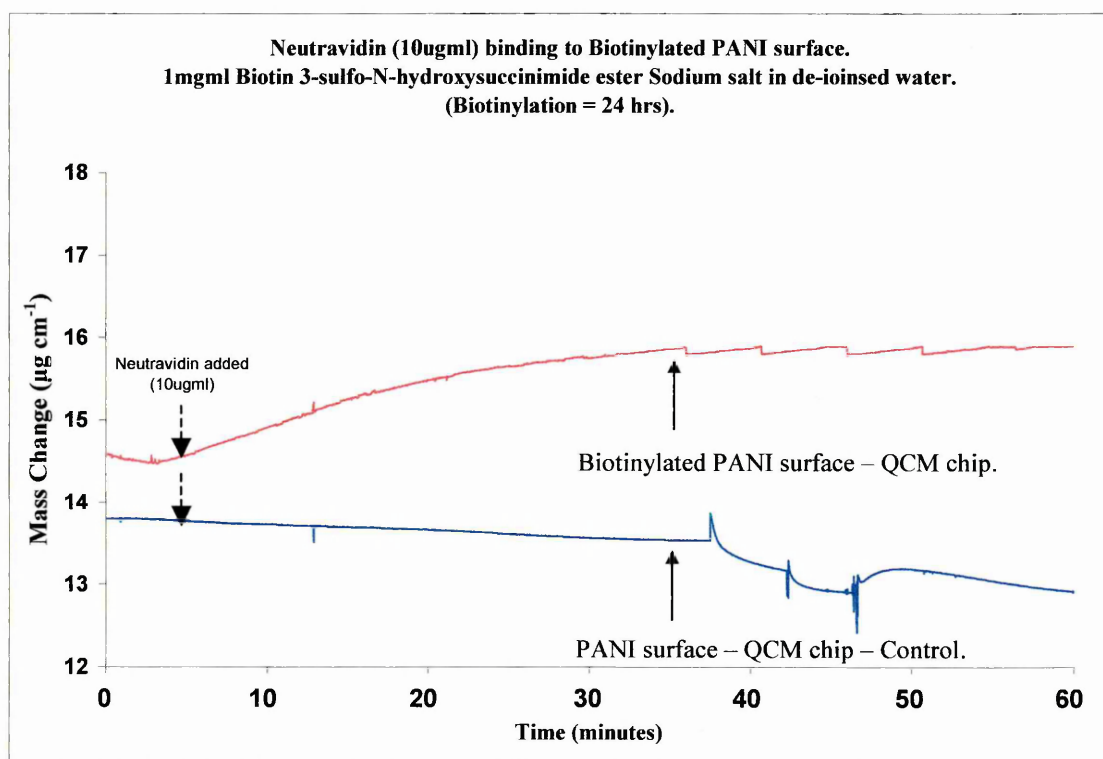


Fig 5.11: QCM data obtained for a biotinylated PANI film exposed to a solution of neutravidin (biotin-binding protein) at $10\mu\text{g ml}^{-1}$.

Figure 5.11 clearly shows a significant mass change from the stabilised QCM cell for the biotinylated PANI surface (red plot) and a negligible change for the experimental control (blue plot) indicating that neutravidin is binding to the biotinylated PANI surface and that no interaction is occurring in the control.

It is postulated here that the neutravidin is binding to the biotinylated surface with at least one of the two available sites on one side of the neutravidin molecule. This would leave two further binding sites on the opposing side for further attachment of biotin species.

Again the QCM chips were thoroughly flushed with de-ionised water and a stabilisation period was allowed to achieve a consistent frequency before further experiments were performed.

Monoclonal antibody (APSA) was exposed to both the Biotinylated PANI, neutravidin-coated QCM chip and the control (PANI-coated) chip at a concentration of 1 mg ml^{-1} for one hour.

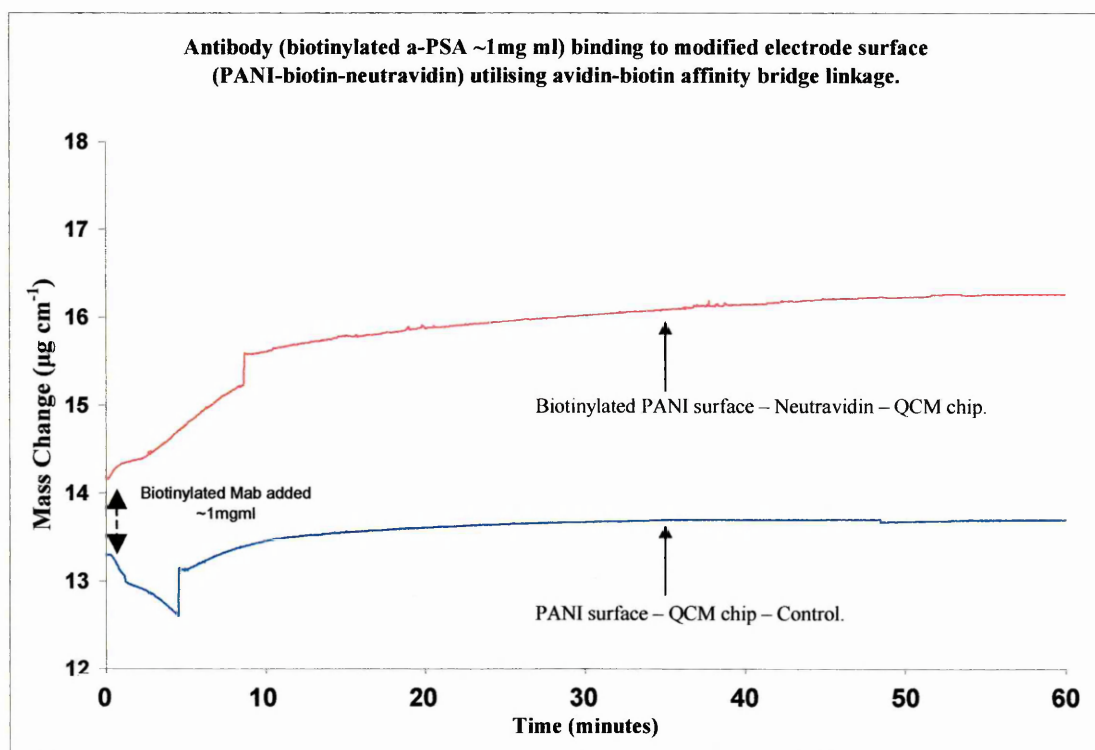


Fig 5.12: QCM data obtained for a biotinylated PANI / neutravidin-coated film exposed to a solution of biotinylated MAb at 1 mg ml^{-1} .

Again Figure 5.12 clearly illustrates a significant mass change for the addition of biotinylated antibody receptors to the modified QCM chip (red plot) and negligible mass change for the control experiment (blue plot) suggesting that the technique employed here offers a viable approach towards antibody immobilisation.

There is a significant mass change on the fully modified QCM chip suggesting that biotin labelled antibody is binding to neutravidin via at least two of its binding sites on the opposing side to which neutravidin is bound to PANI.

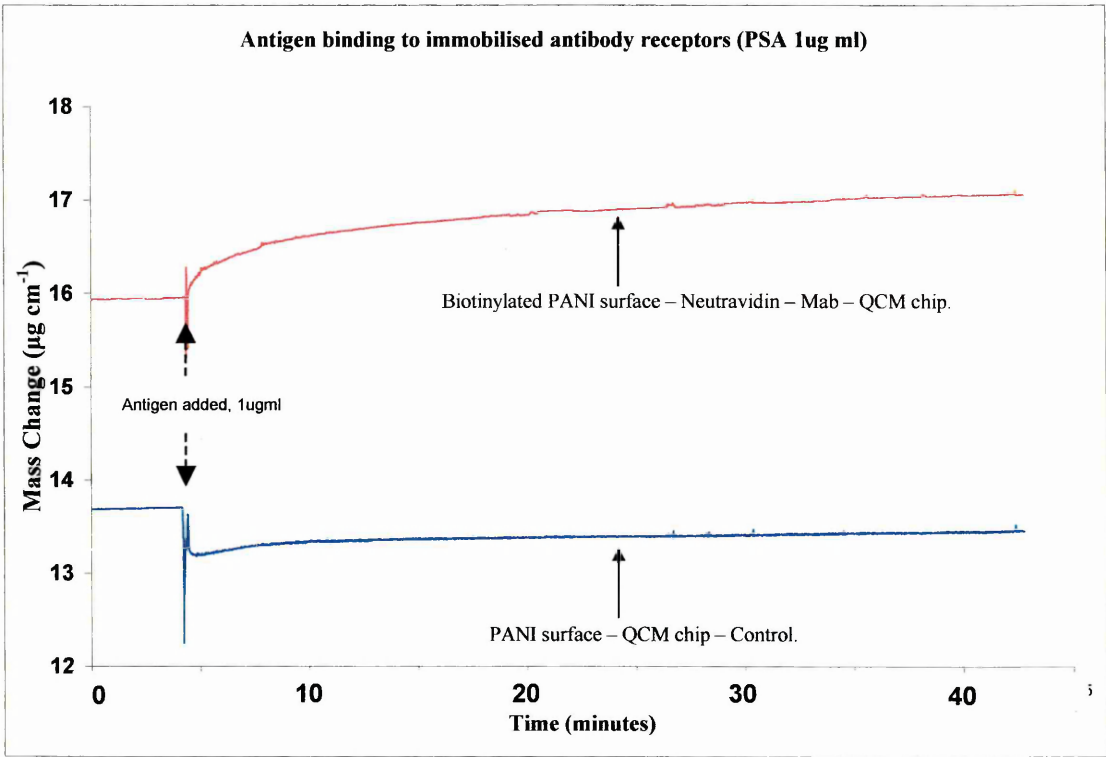


Fig 5.13: QCM data obtained for a biotinylated PANI / neutravidin-coated / antibody immobilised film exposed to a solution of antigen at $1\mu\text{g ml}^{-1}$

As an indirect assessment of antibody immobilisation and for further validation of the experimental protocol, complementary antigen (PSA) was exposed to the modified antibody-immobilised chip at a concentration of $1\mu\text{g ml}^{-1}$. Figure 5.13 clearly illustrates the recognition of antigen by the APSA chips, immobilised through an elegant non-covalent affinity assembly (red plot), as opposed to a QCM chip coated in PANI only (blue plot) that shows insignificant change from the stabilised baseline frequency. The experiments performed validate the fabrication protocol employed for the immobilisation of antibody receptors at novel microelectrode array sensors.

Non-covalent affinity techniques create a monolayer of specifically-orientated receptors immobilized at the PANI protrusions with greater availability of active sites for antigen binding and with the full preservation of their biological properties such as recognition capability, stability & flexibility all in turn expected to increase sensitivity.

Potentiometric immunoassays have been of great interest since 1975 (Turner *et al*, 1987). These are based on the premise that proteins in solution are polyelectrolytes and hence, as an antibody is a protein, its electrical charge will be affected upon binding with an antigen. The potential difference between an electrode, to which an antibody has been immobilised, and a reference electrode will depend on the concentration of the free antigen, assuming that the antibody binding site is free to participate in the interaction with the antigen.

More recently, impedance spectroscopy has become a rapidly developing electrochemical technique for the characterisation of biomaterial-functionalised electrodes and biocatalytic transformations at electrode surfaces, and specifically for the transduction of biosensing events at electrodes (Sargent & Sadik, 1999; Bardea *et al*, 2000; Berggren *et al*, 2000; Kharitonov *et al*, 2000; Alfonta *et al*, 2001; Katz *et al*, 2001; Kharitonov *et al*, 2000; Ouerghi *et al*, 2002; Katz & Willner, 2003; Guan *et al*, 2004; Tang *et al*, 2004; Wang *et al*, 2004; Wu *et al*, 2005). The immobilisation of biomaterials, in this case antibody, alters the capacitance and interfacial electron transfer resistance of the conductive or semiconductive surface. Impedance spectroscopy allows analysis of interfacial changes originating from biorecognition events at electrode surfaces. Kinetics and mechanisms of electron transfer processes corresponding to biocatalytic reactions can also be derived by Faradaic impedance spectroscopy. The recognition complex formed between the sensing biomaterial and the analyte alters the capacitance and resistance at the modified surface-electrolyte interface. These two components can be measured as separate entities using impedance spectroscopy. In summary, impedance spectroscopy is a very powerful tool for the analysis of interfacial properties changes of modified electrodes upon biorecognition events occurring at the modified surfaces.

5.4 Chapter 5 Conclusions.

Biomolecules, in this case antibody receptors, have been successfully immobilised within intimate proximity of the transducer surface of novel microelectrode array sensors.

Polymeric protrusions emanate from the micron pore ablations at the microelectrode array surface conferring a hemispherical diffusion profile across the modified working electrode.

A simple and rapid one-step immobilisation protocol involving co-entrapment of biomolecules throughout electrogeneration of PANI within the matrix and at the surface was exploited for the creation of immunosensors for impedimetric investigations into the quantification of analyte specific to TIA diagnosis.

A more sophisticated and elegant strategy involving non-covalent affinity attachment was also employed whereby orientation of receptor can be controlled to a greater extent to facilitate antibody-antigen interactions. QCM techniques were employed and confirm that this immobilisation approach was successful.

The suitability of both immobilisation approaches for utilization within impedimetric immunosensors for stroke (TIA) was subsequently evaluated and results are reported in the following chapters.

Immobilisation techniques discussed in section 5.2 and employed according to section 3.5.3.1 can subsequently be interrogated as impedimetric immunosensors; results are reported in chapter 6.

Immobilisation techniques discussed in section 5.3 and employed according to section 3.5.3.2 can subsequently be interrogated as impedimetric immunosensors; results are reported in chapter 7.

**Chapter 6: Electrochemical Impedance studies
utilising conductive polymer entrapment (*CPE*)
as the site-specific immobilisation
technique employed.**

6.0 Electrochemical Impedance studies utilising conductive polymer entrapment (*CPE*) as the site-specific immobilisation technique employed.

6.1 Introduction:

The antibody – antigen (Ab-Ag) interactions at novel microelectrode array sensor platforms, modified with conducting PANI, have been investigated utilising electrochemical impedance spectroscopy (EIS) techniques. Generally faradaic impedance spectroscopy (as opposed to non-faradaic capacitance measurements) have been employed. The specific aim of the investigation is to develop an analytical test for the labelless detection of biochemical markers for transient ischemic attack such as neuron specific enolase (NSE) and S-100 protein (Aurell *et al*, 1991; Hatfield & McKernan, 1992; Barone *et al*, 1993; Schaarschmidt *et al*, 1994; Butterworth *et al*, 1996; Cunningham *et al*, 1996; Kim *et al*, 1996; Abrahams *et al*, 1997; Buttner *et al*, 1997; Ingebrigsten *et al*, 1997; Missler *et al*, 1997; Martens *et al*, 1998; Missler & Wiesmann, 1998; Hermann *et al*, 1999; Raabe *et al*, 1999; Schoerhuber *et al*, 1999; Wunderlich *et al*, 1999; Hermann *et al*, 2000).

The immobilisation strategy involved the electrogeneration of emeraldine polyaniline (PANI) protrusions and the simultaneous co-entrapment of specific biomolecule receptors at the microelectrode array surface. Initial experimental investigations are performed on the readily available anti-bovine serum albumin (ABSA) and bovine serum albumin (BSA) Ab-Ag couple.

The experimental protocol developed is then applied for the determination of further bio-analytes, particularly targeted towards the detection of biochemical markers currently perceived to be critical in the diagnosis of transient ischemic attack.

Prostate specific antigen (PSA) and anti-prostate specific antigen monoclonal antibody (APSA) have also been used to provide a model of the behaviour of such protein marker immunosensors and they have greatly aided the development of sensors of this type.

Following the immobilisation of antibody receptor molecules, within and at the surface of conducting emeraldine PANI mushroom-like protrusions, the microelectrode array sensor assemblies were exposed to increasing concentrations of complementary antigen in buffered test solution - and subjected to electrochemical impedance spectroscopy. For every specific sensor assembly analysed a matched control sensor was also interrogated to allow for a corrected non-specific response. In this case the antibody immobilised was non-specific to the target analyte.

Complex plane impedance measurement analyses allow differing redox states of the polymer, faradaic charge transfers and also capacitive transduction events to be studied. The experimental technique is evaluated for the development of a labelless detection analytical protocol for determining biochemical markers for transient ischemic attack.

At the onset of a stroke event a range of biochemical components can be released into the blood supply for the brain and the spinal cord depending on the stroke type and the severity of the brain attack. Several authors have identified key markers for the diagnosis and differentiation of stroke. The determination of markers such as these is required at pg ml^{-1} concentration ranges and there is an emphasis on rapid quantification to permit the intervention of the appropriate therapy.

For transient ischemic attack there is a three-hour treatment window whereby tissue plasminogen activator (t-PA) thrombolytic therapy could permit a much higher degree of recovery for the patient with no adverse effects from the attack (National Institute of Neurological Disorders and Stroke, 1995; Kwiatkowski *et al*, 1999; Marler *et al*, 1999; Marler *et al*, 2000).

In previous investigations by several authors classical thermodynamic concepts were applied extensively to the characterisation of Ab-Ag interaction at organic conducting polymer interfaces under equilibrium conditions (Eddowes, 1987; Karlsson *et al*, 1991). These did not, however, adequately explain the non-equilibrium kinetics of the Ab-Ag interaction at organic conducting polymer interfaces. To obtain the kinetic and mechanistic contributions to the response of conducting polymer electrode surfaces, an in-situ, non-destructive, electrochemical technique would be desirable.

This study has chosen the use of EIS since it allows the resolution of both the kinetic and/or the transport processes occurring within the system at various applied potentials. This involves the use of minimal ac perturbation to determine the ionic and/or the electronic transport phenomena.

Sensor characterisation using EIS over a wide range of frequencies and applied potentials can provide a variety of information on the non-faradaic processes. These include the double-layer capacitance, C_{dl} , solution resistance, R_s , and the diffusion behaviour. faradaic processes can be probed by extracting the electron charge-transfer resistance R_{et} , under various applied potentials and conditions.

6.2 Impedimetric sensing of the fabrication of immunosensors via *CPE*.

Figure 6.1 shows one popular format for evaluating electrochemical impedance data, the Nyquist plot. This format is also known as a Cole-Cole plot or complex impedance plane plot. The imaginary impedance component ($-Z''_{(im)}$) is plotted against the real impedance component ($Z'_{(re)}$) at each excitation frequency for the fabrication of immunosensor assemblies.

Plot 6.1 a) represents the trace obtained for a planar carbon electrode in pH 7.4 phosphate buffered test solution containing $KFe(CN)_6]^{3-/4-}$. Plot 6.1 b) is obtained in the test solution following modification steps to create a microelectrode array sensor.

Plots 6.1 c), 6.1 d) and 6.1 e) represent completed immunosensor traces obtained in test solution after antibody receptor components have been immobilised within and at the surface of PANI via the electrogeneration of PANI protrusion phenomena utilising *CPE*.

From Figure 6.1 a) to b) it is apparent that the modification of the planar electrode to form the microelectrode array sensor causes significant change in the impedimetric spectral trace.

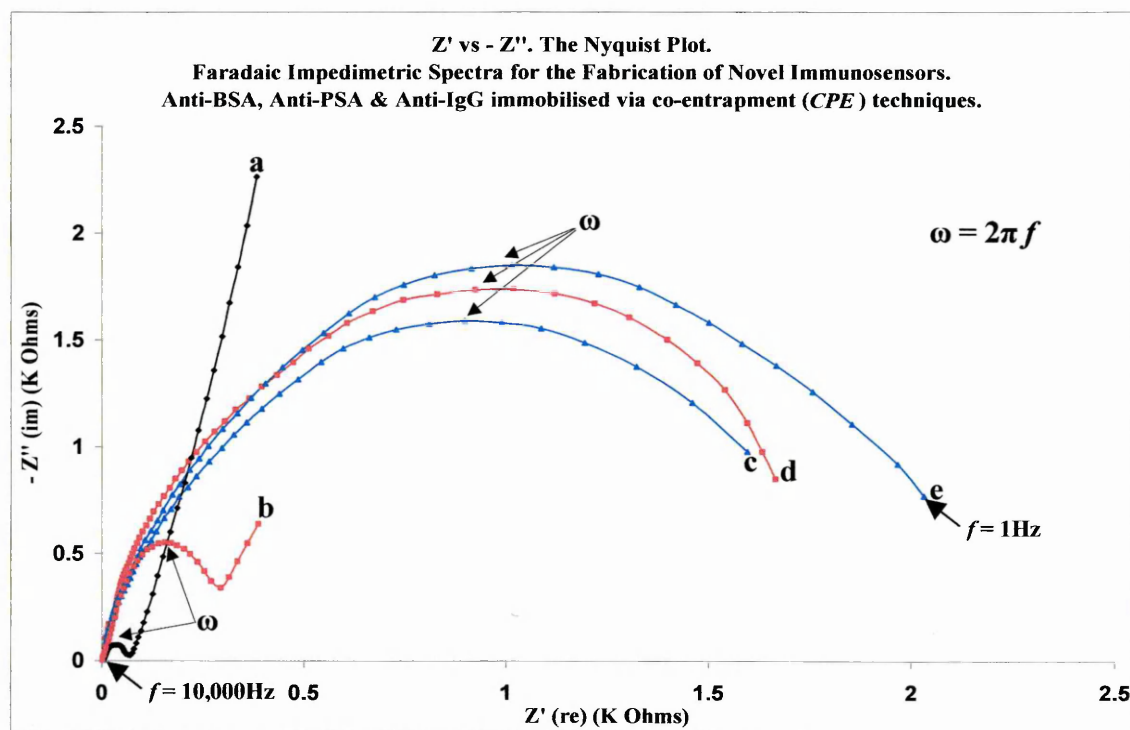


Fig 6.1: Faradaic Impedimetric sensing of the Fabrication of *CPE* Immunosensors: a) Planar Carbon electrode, b) Polymerised & Sonicated Carbon Microelectrode Array, c) anti-PSA (monoclonal), d) anti-BSA (monoclonal) and e) anti-IgG (polyclonal) antibody-immobilised PANI-modified sensors.

Plot 6.1 a) represents very fast electron transfer processes and hence only the linear part of the trace is observed as would be expected for a planar electrode. The impedance is mainly controlled by diffusion of the redox probe. Both the $Z'_{\text{(re)}}$ and $-Z''_{\text{(im)}}$ components are seen to increase with decreasing frequency over the whole range of applied experimental frequencies. (10,000Hz to 1Hz, +/- 5mV amplitude perturbation).

For plot 6.1 b) the Z' (re) component is also seen to increase with decreasing frequency over the whole range of frequencies from 10,000Hz to 1Hz. The $-Z''_{(im)}$ component, however, is seen to increase from 10,000Hz to the characteristic frequency, ω , of the impedance trace, indicated at the point where the $-Z''_{(im)}$ value is at its maximum. Also, the Warburg impedance contribution is seen to dramatically reduce and shift to lower frequencies from Figure 6.1 a) to 6.1 b). Plot 6.1 b) shows a modified electrode where the impedance is controlled by diffusion of the redox probe at the low frequencies and by interfacial electron transfer at high frequencies.

Likewise, there is significant difference between plot 6.1 b) and plot 6.1 c), 6.1 d) and 6.1 e).

The immobilisation of antibody bio-receptors within and at the surface of PANI protrusions causes the appearance of a large semicircle region in the trace. Significant insulation at the electrode surface has occurred confirming the successful electrodeposition of PANI.

At this modification step the contribution from the Warburg impedance is seen to completely diminish so that there is no linear portion and the trace is indicative of a slow electron transfer step over the whole range of frequencies. A large semicircle region that is not accompanied by a straight line trace relates to a sensor impedance that is controlled by interfacial electron transfer within the entire range of applied frequencies and has no diffusion limited behaviour. Traces c), d) and e) are very similar in form and suggest a reproducible fabrication process. When antibody of the same species is immobilised the spectra show near identical traces.

The frequency corresponding to the top of the semicircle, $\omega_{(\theta = \max)}$, can be used to calculate the capacitance if R_{et} is known.

Equation 6.1

$$\omega_{(\max Z'')} = 1 / C R_{et} \quad \text{or} \quad \omega_{(\theta = \max)} = (C R_{et})^{-1}$$

The semicircle diameter equals to the electron transfer resistance, R_{et} , thus the maximum value of the imaginary impedance occurs at the radius of the semicircle. The corresponding $Z'_{(re)}$ value at this frequency is equivalent to $0.5R_{et}$ and is achieved at this characteristic frequency, ω_0 . In this way R_{et} can be easily extrapolated from the experimental data obtained or directly calculated by the instrument software via fitting to a simple electronic equivalent circuit.

The Capacitance can also be calculated directly from the $-Z''_{(im)}$ value at the characteristic frequency whereby $\omega = 2\pi f$, the frequency in radians (Wu *et al*, 2005).

Equation 6.2

$$C = -1/(2\pi f Z'')$$

Figure 6.2 shows the $-Z''_{(im)}$ component variation (translated as capacitance changes) for the stepwise fabrication of immunosensors calculated at the characteristic frequency of each trace where the phase angle is closest to -90° indicating that the system exhibits near ideal capacitor behaviour (Berney, 2000). a) is the planar carbon electrode, b) is the carbon microelectrode array sensor platform following polymerisation and sonication, c), d) & e) are the PANI modified microelectrode array sensors whereby anti-PSA, anti-BSA & anti-IgG are immobilised respectively via *CPE*.

Following each modification there is a decrease in capacitance. Substitution of the electrolyte solution with polymer and protein molecules at the electrode surface and further increase of the thickness and density of the organic layer to immobilise antibody receptors results in a significant change of the capacitance, C , which is derived from the imaginary part of the impedance spectra.

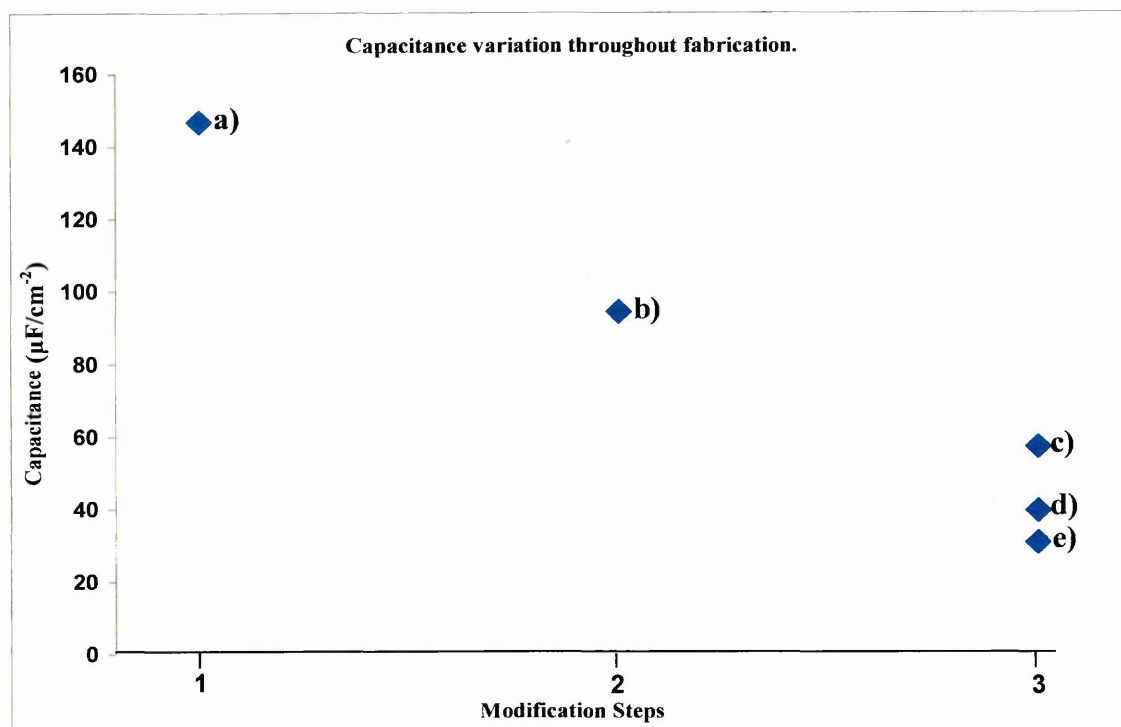


Fig 6.2: Non-Faradaic Impedimetric sensing of the Fabrication of *CPE* Immunosensors:
a) Planar Carbon electrode, b) Polymerised & Sonicated Carbon Microelectrode Array,
c) anti-PSA (monoclonal), d) anti-BSA (monoclonal) and e) anti-IgG (polyclonal)
antibody-immobilised PANI-modified sensors.

The decrease of the effective dielectric constant of the medium at the interface yields the respective decrease of the capacitance. Nonetheless the changes in the real part of the impedance are expected to be substantially higher. Consequently faradaic EIS was utilised to monitor the detection of antigen. Faradaic impedance spectroscopy is usually considered to be more sensitive to the insulation of the electrode surface upon the binding of antigen to antibody functionalised electrode surfaces, (Katz & Willner, 2003).

6.3 Faradaic impedimetric interrogation of PANI ‘mushroom’ microelectrode array sensors: ABSA immunosensor studies.

Following immobilisation of antibody, ABSA, according to section 5.2, impedance analyses were performed from 1Hz to 10,000Hz (5mV a.c. amplitude perturbation) in pH 7.4 phosphate buffer, i.e. containing no antigen, as a baseline trace. This buffer solution did, however, contain a 50:50 mixture of $[\text{KFe}(\text{CN})_6]^{3-/4-}$, at a concentration of

10mM as redox mediator. The potential of the electrochemical cell is offset to the formal potential of the redox probe (+0.22V vs. Ag/AgCl). Subsequently, ABSA doped sensors were exposed to a range of concentrations from 1 to 300ng ml⁻¹ bovine serum albumin (BSA). BSA antigen was suspended in pH 7.4 phosphate buffers containing [KFe(CN)₆]^{3-/4-} at pre-prepared analyte concentrations of 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 and 300 ng ml⁻¹. The pH of 7.4 was utilised to coincide with human physiological pH.

Following the recording of a baseline spectral trace (0 ng ml⁻¹), the same sensor is used for all concentrations and exposed for 30 minute time periods to the increasing concentrations of BSA antigen in phosphate buffered test solution.

After each 30 minutes exposure to a known concentration, the sensor is thoroughly flushed with 50ml of pH 7.4 phosphate buffer (containing [KFe(CN)₆]^{3-/4-} & no antigen) to remove any non-specifically adsorbed matter before ac impedance spectra were recorded in the phosphate buffered test solution (containing [KFe(CN)₆]^{3-/4-} & no antigen). This sensor was then exposed to an increasing antigen concentration and the process repeated for the full range of applied antigen concentrations under investigation. Traces recorded may then be compared to the baseline trace. Section 3.5.5 thoroughly details the impedimetric interrogation procedure.

6.3.1 ABSA immunosensor: Specific recognition of BSA antigen.

Electrochemical Impedance Spectra obtained for an ABSA-doped PANI mushroom microelectrode array sensor exposed to increasing BSA concentrations.

Figure 6.3 & Figure 6.4 depict the experimental data obtained, and presented in the form of Nyquist plots showing the faradaic ($Z'_{(re)}$) and capacitive ($-Z''_{(im)}$) component variation throughout the recording of an ac impedance spectrum from 1 to 10,000 Hz for an ABSA-doped PANI sensor following exposure to 1 to 300 ng ml⁻¹ BSA antigen.

For clarity, Figure 6.3 represents the experimental data obtained at the lower concentrations of 1, 2, 3, 4, 5 and 10 ng ml⁻¹ BSA to allow resolution of the spectra.

Figure 6.4 represents the experimental data obtained for the whole analytical concentration range.

From Figure 6.3 it is apparent that the $Z'_{(re)}$ component and the $-Z''_{(im)}$ component of impedance both increase with decreasing frequency for the baseline trace.

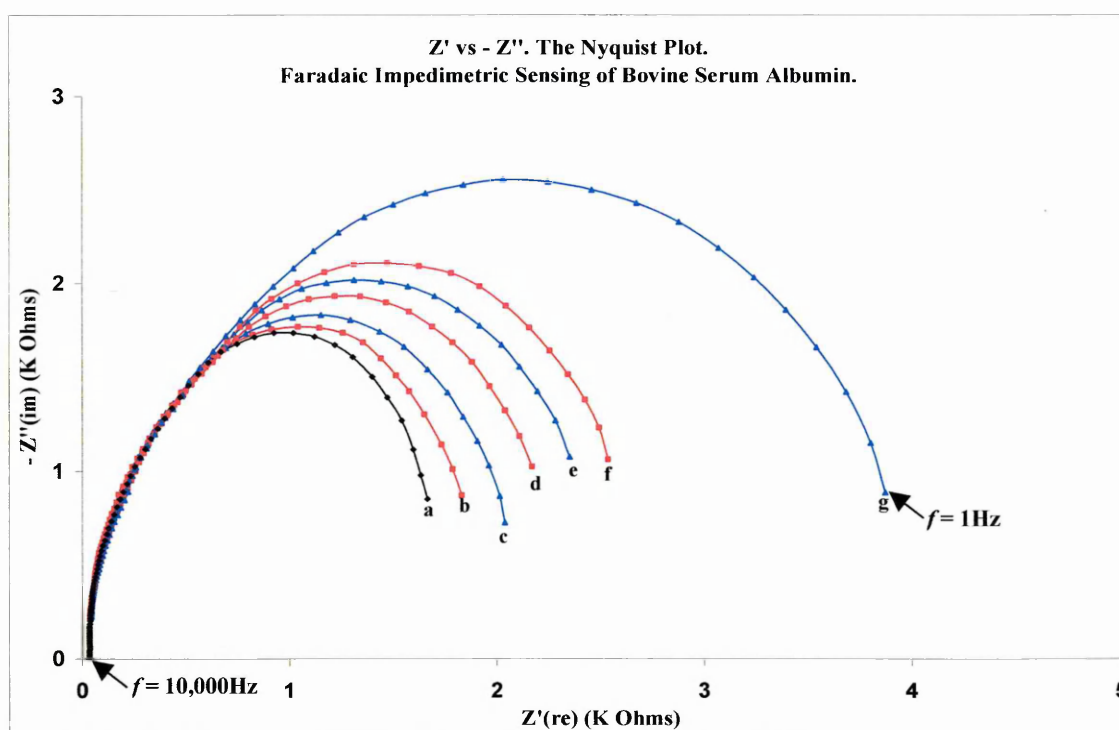


Fig 6.3: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel ABSA-doped PANI immunosensor following exposure to various concentrations of BSA. The concentration of antigen (ng ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 ng ml⁻¹ BSA.

The $Z'_{(re)}$ component is seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the final applied frequency (1Hz).

The impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow (Katz & Willner, 2003). All subsequent traces follow this general form.

A typical shape of a faradaic impedance spectrum (presented in the form of a complex plane diagram – a Nyquist plot) includes a semicircle region lying on the x-axis followed by a straight line.

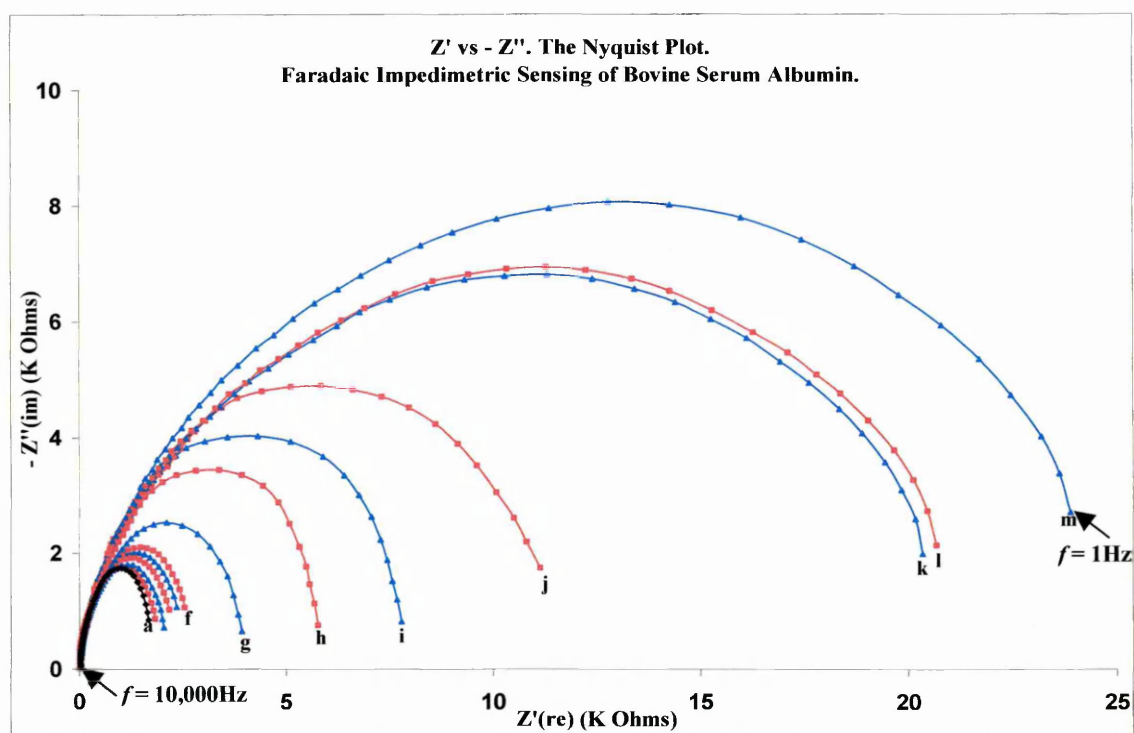


Fig 6.4: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel ABSA-doped PANI immunosensor following exposure to various concentrations of BSA. The concentration of antigen (ng ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 ng ml^{-1} BSA.

From Figure 6.4 it is apparent that the forms of the Nyquist plots remain similar to those encountered in Figure 6.3 and again all impedance spectrum are indicative of a surface-modified electrode system where the electron transfer is slow.

A slow electron-transfer step results in a large semicircle region that is not accompanied by a straight line. The semicircle portion corresponds to the electron-transfer-limited

process, whereas the linear part represents the diffusionally limited electrochemical process. In the case of very fast electron transfer processes, the impedance spectrum could include only the linear part as observed in Figure 6.1, trace a) for a planar carbon electrode. The electron transfer kinetics and diffusional characteristics can be extracted from the spectra. The semicircle diameter (extrapolated) is equal to the electron transfer resistance, R_{et} . The intercept of the semicircle with the Z' axis at high frequencies ($\omega \rightarrow \infty$) is equal to the solution resistance; R_s . Extrapolation of the circle to lower frequencies yields an intercept corresponding to $R_s + R_{et}$.

The characteristic frequency, ω_0 , given by Equation 6.1, has the meaning of the reciprocal of the time constant of the equivalent circuit. The maximum value of the imaginary impedance in the semicircle part is achieved at the characteristic frequency. This value occurs at the radius of the semicircle. The corresponding $Z'_{(re)}$ component value at this frequency equates to $0.5 R_{et}$.

One of the most important parameters governing the technique of faradaic impedance spectroscopy is the applied frequency. At low frequencies ($f < 1$ MHz) the impedance value is basically determined by the DC-conductivity of the electrolyte solution. At very high frequencies ($f > 100$ kHz) inductance of the electrochemical cell and conducting wires can contribute to the impedance spectra. Thus, the analytically meaningful impedance spectra are usually recorded at frequencies where they are mainly controlled by the interfacial properties of the modified electrodes ($10 \text{ MHz} < f < 100 \text{ kHz}$).

The ACM auto DSP frequency response analyser completes an impedance spectrum over a frequency range of 10 kHz to 1Hz in 210 seconds.

This frequency range gives details on the interfacial properties of the modified electrode. From the shape of the Nyquist curve of the baseline trace and lack of linear portion it is apparent that within this entire range of frequencies the impedance is controlled by the interfacial electron transfer and electron-transfer limited processes at the interface.

The electron transfer resistance, R_{et} , controls the electron transfer kinetics of the redox probe at the electrode interface. Thus, an insulating modifier on the electrode is expected to retard the interfacial electron transfer kinetics and to increase the electron transfer resistance. The electron transfer resistance at the electrode is given by Equation 6.3 where R_c and R_{mod} are the constant electron transfer resistance of the unmodified carbon electrode and the variable electron transfer resistance introduced by the modifier (fabrication process or antigen analyte), in the presence of the solubilised redox probe, respectively.

Equation 6.3

$$R_{et} = R_c + R_{mod}$$

The experimental results can be analysed graphically (Bard & Faulkner, 1980; Stoyanov *et al*, 1991) and in the case of faradaic impedance spectra usually by the use of the Nyquist coordinates: $(-Z''_{(im)} \text{ vs } Z'_{(re)})$ in the frame of the theoretical model (Randles, 1947 & Ershler, 1947). A computer fitting of the experimental data to a theoretical model represented by an equivalent circuit is usually performed; in this case the software provided with the frequency response analyser is automated to perform this analysis as data is extracted by the instrument.

All electronic characteristics of the equivalent circuit and the corresponding physical parameters of the real electrochemical system can be extracted from such an analysis. Since the variable parameters of the system represent the functions of the modifying layer and its composition, they can be used to quantitatively characterise the layer. Analysis of the $Z'_{(re)}$ and $-Z''_{(im)}$ values observed at different frequencies allows the calculation of the following important parameters: a) the double-layer capacitance, C_{dl} and its variable component C_{mod} ; b) the electron transfer resistance, R_{et} , and its variable component R_{mod} and c) the electron transfer rate constant, k_{et} , for the applied redox probe derived from the electron transfer resistance, R_{et} . Thus, impedance spectroscopy represents not only a suitable transduction technique to follow the interfacial interactions of biomolecules, but

it also provides a very powerful method for the characterisation of the structural features of the sensing interface and for explaining mechanisms of chemical processes occurring at the electrode/solution interface (Janata, 2002).

It can be seen from Figure 6.3 & Figure 6.4 that there was a measurable increase in signal response for the Faradic component ($Z'_{(re)}$) and the capacitive component ($-Z''_{(im)}$) of the Nyquist Plot for increasing concentrations of BSA exposed to ABSA-doped (MAB), PANI modified, carbon sensors.

The $Z'_{(re)}$ component change can be translated into an electron transfer resistance change and the values obtained were used to create a calibration profile as they provide the most convenient and sensitive means of response analysis (Figure 6.5).

In order to measure the full electrical impedance at the interface, including its real part, $Z'_{(re)}$, a redox probe ($[Fe(CN)_6]^{3-/4-}$ ions were used in these investigations) has to be included in the electrolyte solution.

A baseline impedance trace was recorded in this electrolyte solution and then following exposure to antigen for 30 minutes and a thorough washing of the sensor surface the impedance trace was recorded again in the redox-containing electrolyte solution. The redox probe provides a faradaic current at the electrode surface as the electrode is biased at the standard potential of the redox probe (+0.22V vs. Ag/AgCl). It is the changes in the real component of the impedance spectrum observed upon the bioaffinity complex formation at the electrode surface that are of paramount interest in these investigations as they are usually higher than the respective capacitance changes. Faradaic impedance spectroscopy is generally regarded to be more sensitive to the insulation of the electrode surface upon the binding of antigen to the antibody-functionalised electrode surfaces.

However, it should be noted that the changes in the faradaic impedance spectrum upon the antigen-antibody complex formation are not always large. If the sensing layer is not well organised and contains many pinholes between the biomolecules, and the redox

probe is sufficiently small to freely penetrate through these pinholes the changes observed in the faradaic impedance spectra could be minor. From Figure 6.3 and Figure 6.4 it is apparent that the fabrication process produces a sensor assembly that gives significant change following exposure to antigen. From these results we can say that the experimental protocol and interrogation techniques employed may offer a viable approach towards labelless impedimetric detection of markers of TIA.

The $Z''_{(im)}$ component is also seen to increase with increasing antigen concentrations (up to the characteristic frequency) and similarly the capacitance of the sensor could be calculated to provide the basis of the calibration profile. An increase in the $Z''_{(im)}$ component corresponds to a decrease in capacitance but again these changes are generally less sensitive to the binding of antigen to antibody-functionalised electrodes. It is worth noting that there is greater evidence of $Z'_{(re)}$ signal differentiation observed at the lower frequencies ($\sim 1\text{Hz}$).

The formation of a recognition complex between the sensing biomaterial and the analyte can cause insulation at the modified electrode surface. This alters the capacitance and resistance at the modified surface-electrolyte interface. These two components are measured as separate entities using impedance spectroscopy.

For a more thorough evaluation of the data obtained in Figures 6.3 & 6.4, the changes in the Nyquist curves were translated into electron transfer resistance changes. The main reasons for presenting the data in this manner are that the differing antibody-antigen systems under investigation can be compared in a standard format and the clarity of the responses obtained increases. If a prototype device was developed this would also provide a simple test variable for software development. From Figures 6.3 & 6.4 the electron transfer resistance values were calculated as follows:

The maximum value of the imaginary component, $-Z''_{(im)}$, in the semicircle region of a Nyquist plot corresponds to a value on the real component axis, $Z'_{(re)}$, equivalent to the radius of the semicircle and is achieved at the characteristic frequency of the trace. Thus

the $Z'_{(re)}$ component value obtained at this frequency is doubled to obtain a low frequency intercept on the $Z'_{(re)}$ axis. This value corresponds to the electron transfer resistance (diameter of semicircle) + solution resistance. The solution resistance is constant ($30\Omega + - 5\Omega$) for all of the traces recorded and is obtained at the high frequency starting point on the $Z'_{(re)}$ axis. For each trace recorded the solution resistance is subtracted to obtain the diameter of the semicircle and the diameter of the semicircle is the electron transfer resistance. Figure 2.12 should be analysed to clearly illustrate these facts.

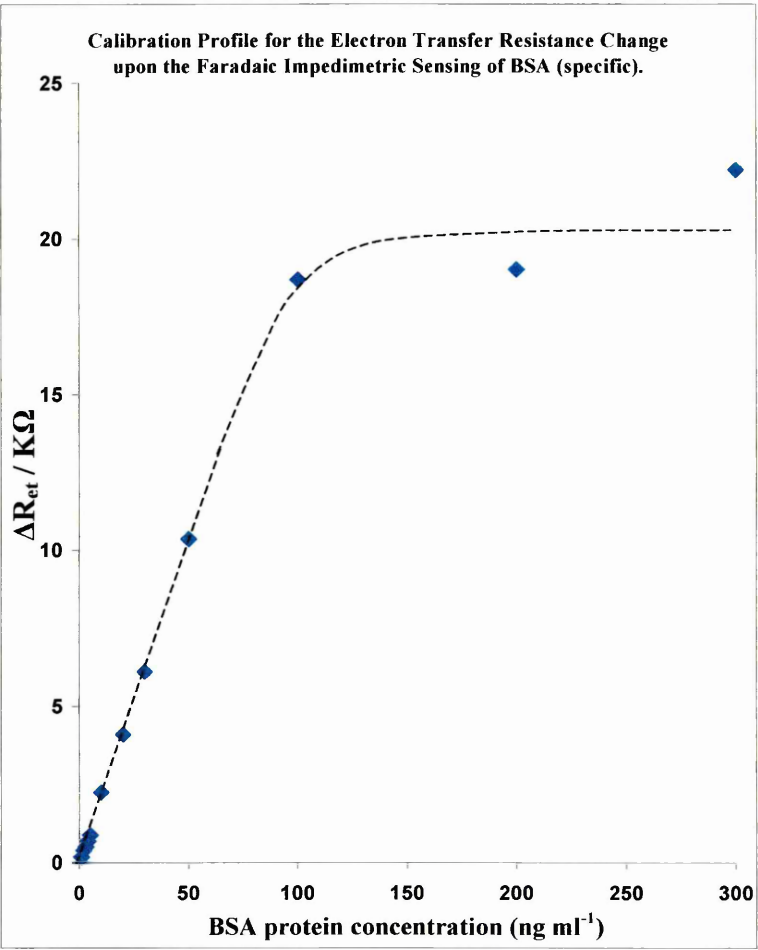


Fig 6.5: Calibration curve for the change in electron transfer resistance from the baseline response for a range of BSA antigen concentrations (specific).

The ACM software provides an electron transfer resistance value with each trace recorded via computer fitting of the experimental data to a theoretical model represented by a simple equivalent circuit. The R_{et} values obtained from the ACM software correlate

exactly to two decimal places with those calculated using the independently simulated method outlined above.

The electron transfer resistance increases with increasing BSA concentration and Figure 6.5 illustrates this fact clearly. The results depict the change in the electron transfer resistance at each concentration calculated with respect to the baseline trace (0 ng ml^{-1} BSA) for the ABSA-modified sensor following exposure to complementary BSA as the target analyte. Equation 6.4 details the calculation of this respective change.

The interaction of BSA with the electrode modified with ABSA results in significant insulation of the electrode reflected by the increase in the electron transfer resistance. It is postulated that as antigen concentration is increased the number of complexes formed also increases and this is reflected by the observed electron transfer resistance increase.

Equation 6.4

$$\Delta R_{\text{et}} = R_{\text{et}} ([\text{Ag}]) - R_{\text{et}} (\text{Baseline})$$

The insulation of the modified electrode upon formation of stable ABSA-BSA complexes hinders the electron transfer kinetics of the redox probe resulting in the increase of electron transfer resistance. The electron transfer resistance increases with increasing antigen concentration in a linear-type response from 1 to 100 ng ml^{-1} . The lower limit of detection of the specific BSA immunosensor is 1 ng ml^{-1} . There is negligible change from 100 ng ml^{-1} to 300 ng ml^{-1} suggesting that the immunosensor approaches saturation and stable antibody-antigen complex formation is no longer occurring at these concentrations.

6.3.2 AIGG immunosensor: BSA non-specific control investigations.

Electrochemical Impedance Spectra obtained for an AIGG-doped PANI mushroom microelectrode array sensor exposed to increasing BSA concentrations.

In order to validate the specificity of the observed response for the ABSA-modified sensor assembly, control experiments were performed. In this experiment IgG (human polyclonal) antibody was immobilised as the capture biocomponent and the sensor was exposed to BSA over the same concentration range. In every other sense the ABSA sensor and AIGG sensor were identical. The results obtained permit an overall assessment of the contribution of non-specific effects to the changes encountered in Figures 6.3 and 6.4. A correction can therefore be made to the electron transfer resistance changes from the baseline response at each concentration (Figure 6.5) to acquire the true specific ABSA immunosensor system response.

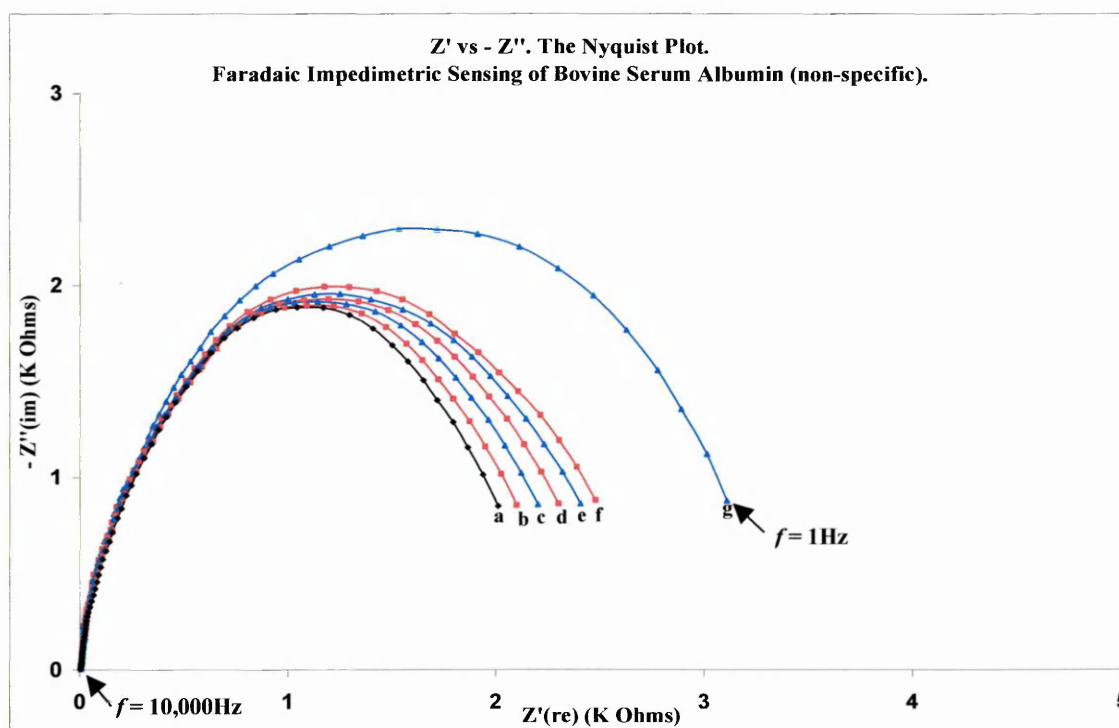


Fig 6.6: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel AIGG-doped PANI immunosensor following exposure to various concentrations of BSA. The concentration of antigen (ng ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 ng ml^{-1} BSA.

The data shown in Figure 6.6 and Figure 6.7 are plotted on the same axes scales as Figures 6.3 and 6.4 respectively. This enables a direct comparison between the specific sensor and non-specific sensor responses.

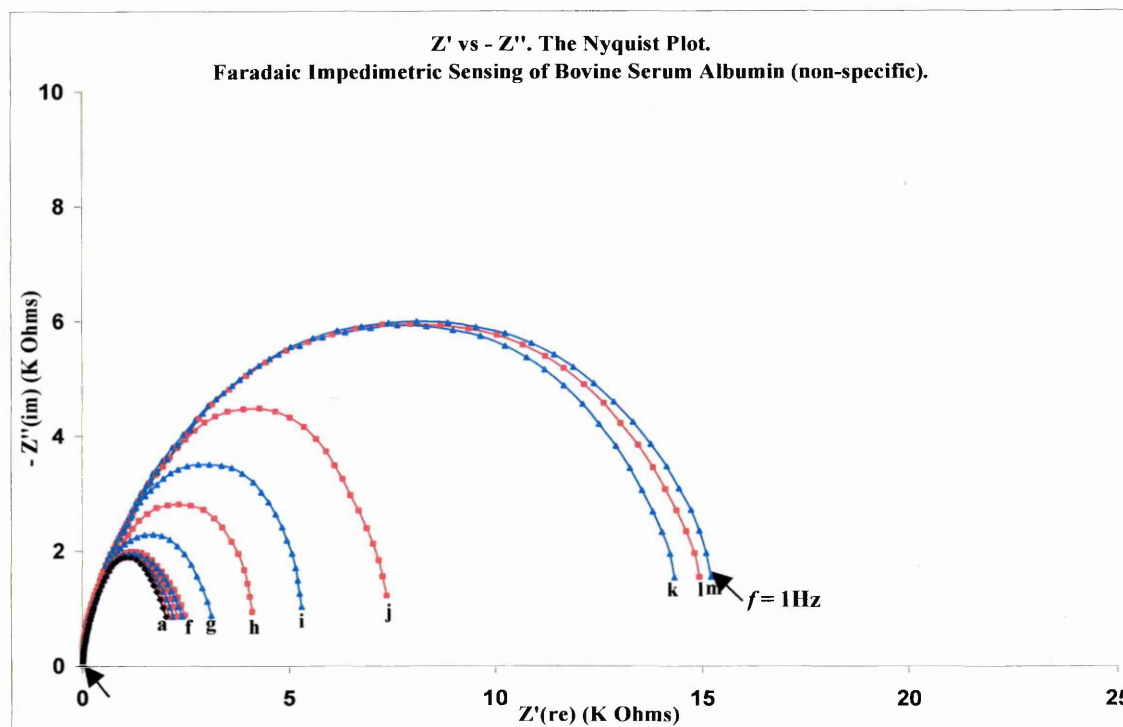


Fig 6.7: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel AlgG-doped PANI immunosensor following exposure to various concentrations of BSA. The concentration of antigen (ng ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 ng ml^{-1} BSA.

All impedimetric spectral traces obtained follow a similar general form to those obtained in section 6.3.1. The $Z'_{(re)}$ component is again seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the final applied frequency (1Hz). The impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow.

It is apparent from Figures 6.6 and 6.7 that a similar response is obtained to the specific sensor even though the receptor biocomponent is non-specific to the target analyte BSA.

That is, the electron transfer resistance (diameter of semicircle region) in the electrochemical impedance spectrum for an AIGG-immobilised sensor is seen to increase with increasing antigen concentration in a linear-type response until a point at which the concentration of antigen no longer causes an increase in diameter of the Nyquist diagram.

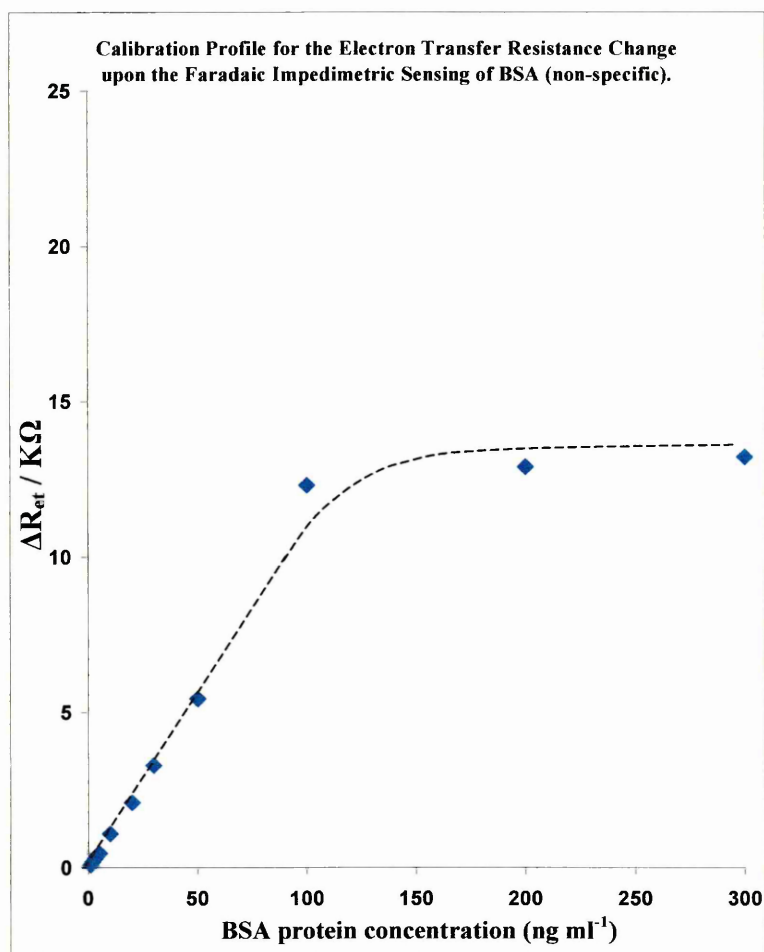


Fig 6.8: Calibration curve for the change in electron transfer resistance from the baseline response for a range of BSA antigen concentrations (non-specific).

For clarity, Figure 6.8 shows the non-specific experimental data presented as the relative change in electron transfer resistance from the baseline trace (0 ng ml⁻¹ BSA) for each concentration analysed. Figure 6.8 has the same y-axis scale for ease of comparison to Figure 6.5 and the values are extracted from the instrument and independently calculated identical manner. A linear-type non-specific response is observed from 1 to 100 ng ml⁻¹ BSA.

Upon comparison of Figure 6.8 with Figure 6.5 it is apparent that approximately 50 - 60 % of the ABSA-immobilised sensor response encountered is, in fact, non-specific.

In this case the antibody immobilised via *CPE* is non-specific to the target analyte BSA. The non-specific response observed in Figures 6.6 and 6.7 could arise from factors such as non-specifically adsorbed matter not being removed via the flushing regime and thus remaining at the sensor surface and / or weakly bound matter, polymer swelling, proton doping and transitional changes of the polyaniline at the neutral test pH, i.e. becoming less conductive. All non-specific contributions cease to affect the shape of the Nyquist diagrams significantly above 100 ng ml^{-1} BSA and the calibration curve begins to plateau suggesting that an optimum electron transfer resistance change from the baseline response has been reached and non-specific occurrences are at their maximum.

6.3.3 'Corrected' specific response: ABSA immunosensor.

To obtain a calibration profile, in terms of 'corrected' electron transfer resistance change for a BSA immunosensor, accounting for any non-specific responses, the changes obtained in Figure 6.8 (non-specific) are subtracted from those obtained in Figure 6.5 (specific) across the entire analytical concentration range. In total 10 ABSA-immobilised sensors and 10 corresponding AIGG-immobilised sensors were interrogated over the concentration range, according to section 6.3.1 and section 6.3.2 respectively, to assess the reproducibility of the observed responses. Identical experimental procedures were employed throughout investigations and all parameters were kept consistent.

Figure 6.9 represents the 'corrected' profile for 10 replicate pairs of matched sensors. The mean electron transfer resistance response at each experimental analyte concentration is plotted. The resultant response is due entirely to the interaction of ABSA with BSA analyte and arises from the effect on the electron transfer resistance that the formation of stable antibody-antigen complexes at the sensor surface generates.

From Figure 6.9 a linear-type response is observed from 1 to 50 ng ml⁻¹ BSA. At concentrations above 50 ng ml⁻¹ the electron transfer resistance change begins to plateau as the sensor response approaches saturation and stable antibody-antigen complex formation no longer correlates with the increase in analyte concentration.

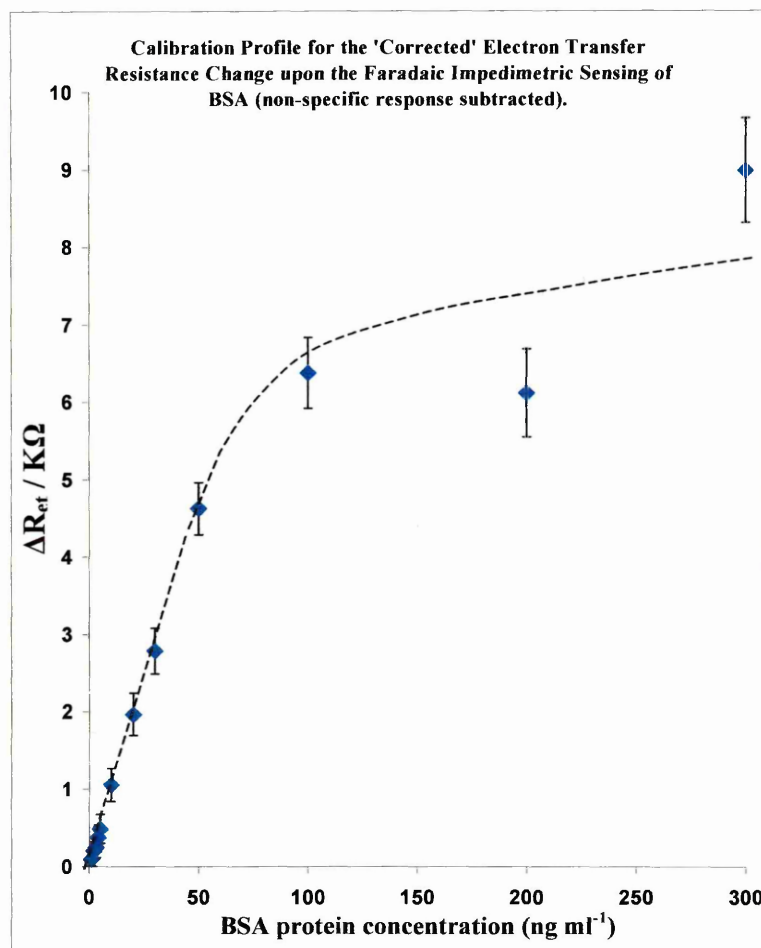


Fig 6.9: Calibration curve for the 'corrected' change in electron transfer resistance from the baseline response for a range of BSA antigen concentrations (non-specific response subtracted).
n = 10, error bars = standard deviation.

The mean baseline electron transfer resistance value is plotted and the mean change in electron transfer resistance value at each experimental concentration is also plotted. The most satisfactory and most widely used measure of dispersion is the *standard deviation* and is always measured from the MEAN and never from the median or mode. The error bars indicate the standard deviation obtained for the 10 matched sensor pairs from the mean 'corrected' values. Figure 6.10 is the same data shown only for the lower end of analytical concentrations.

From Figure 6.10 we can say that from 1 to 10 ng ml⁻¹ BSA there is a linear-type response for the 10 ‘corrected’ matched sensor pairings. From the standard deviations obtained we can say that discrimination of BSA antigen analyte is possible at 5 ng ml⁻¹. The limit of detection of the ABSA immunosensor is 1.5 ng ml⁻¹ BSA and is calculated from 3 x the standard deviation of the baseline response and extrapolated from the line of best-fit according to IUPAC guidelines (Analytical methods committee, 1987).

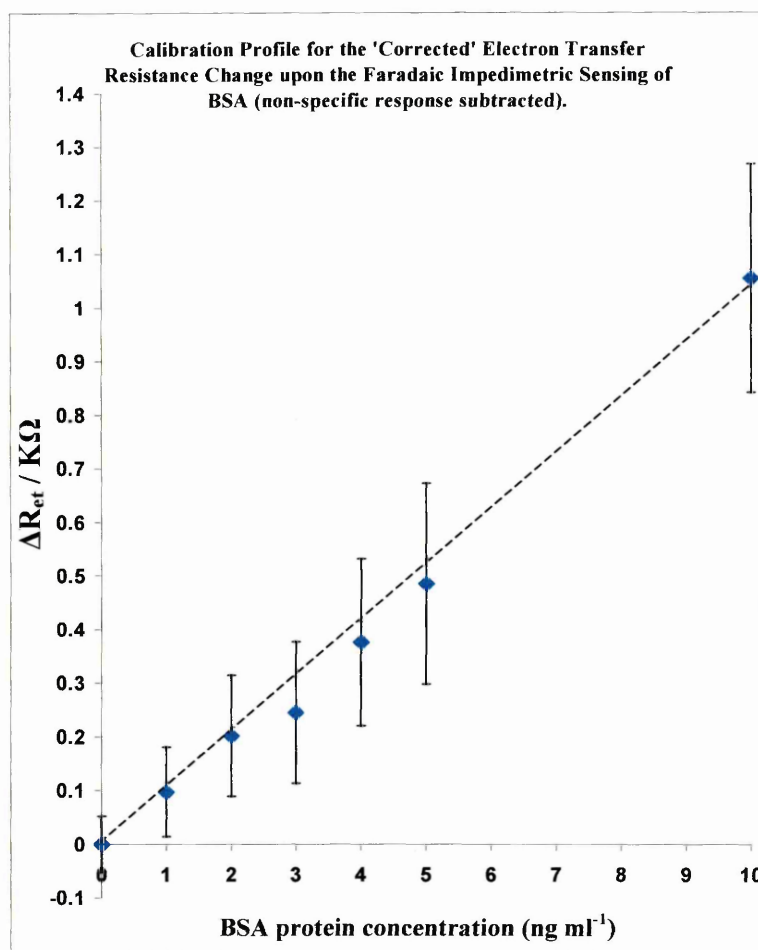


Fig 6.10: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of BSA antigen concentrations (non-specific response subtracted).
n = 10, error bars = standard deviation.

6.3.4 ABSA immunosensor: Reversibility studies.

The poor reversibility of immunological reactions presents a crucial problem to overcome in the development of commercially successful bio / immunosensors. The binding between an antigen and an antibody is usually very strong.

By applying acidic media or high concentration of salts or urea, the proteins associated with the antigen-antibody complex are unfolded, leading to separation of antigen and antibody.

These severe conditions affect the protein that acts as the receptor component of the sensor and indeed the reactivation of the sensing interface after separation of the antigen-antibody complex is often impossible. Even if the receptor component is not a protein and has high chemical stability, the structure of the monolayers could be perturbed and the thiol anchor groups could be dissociated from the surface upon the conditions required for the affinity complex dissociation. The regeneration of the binding sites of the antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilised antibodies and insidious drift problems.

Several experimental protocol approaches were investigated, a range of concentrations of differing buffer species (pH 1 – 3) and differing washing regimes were employed. Unfortunately in all cases the response recovery for the ABSA-BSA immunosensor system could never be regenerated. An identical baseline response could be achieved which suggests that the sensor surface integrity remains yet the antigen recognition and binding capabilities of the receptor antibodies are severely damaged by the treatments.

6.3.5 ABSA immunosensor: Stability studies.

Although a long-term sensor stability test was not performed a batch of 18 sensor pairs was assessed from storage in dry state, at 4°C, for a period of 12 weeks. Again an identical experimental protocol was employed for electrochemical impedance interrogation and the ‘corrected’ electron transfer change was the analysis method for response recovery.

Every 2 weeks a set of 3 matched sensor pairings was removed from storage and interrogated over the full BSA antigen analyte concentration range from 1 to 300 ng ml⁻¹. Figure 6.11 shows the data obtained at each time interval and the saturation concentration of 50 ng ml⁻¹ BSA was chosen as a means of final evaluation of sensor stability and the mean change in 'corrected' electron transfer resistance response is shown plotted against storage time. The error bars indicate the standard deviation for the three sensor pairs.

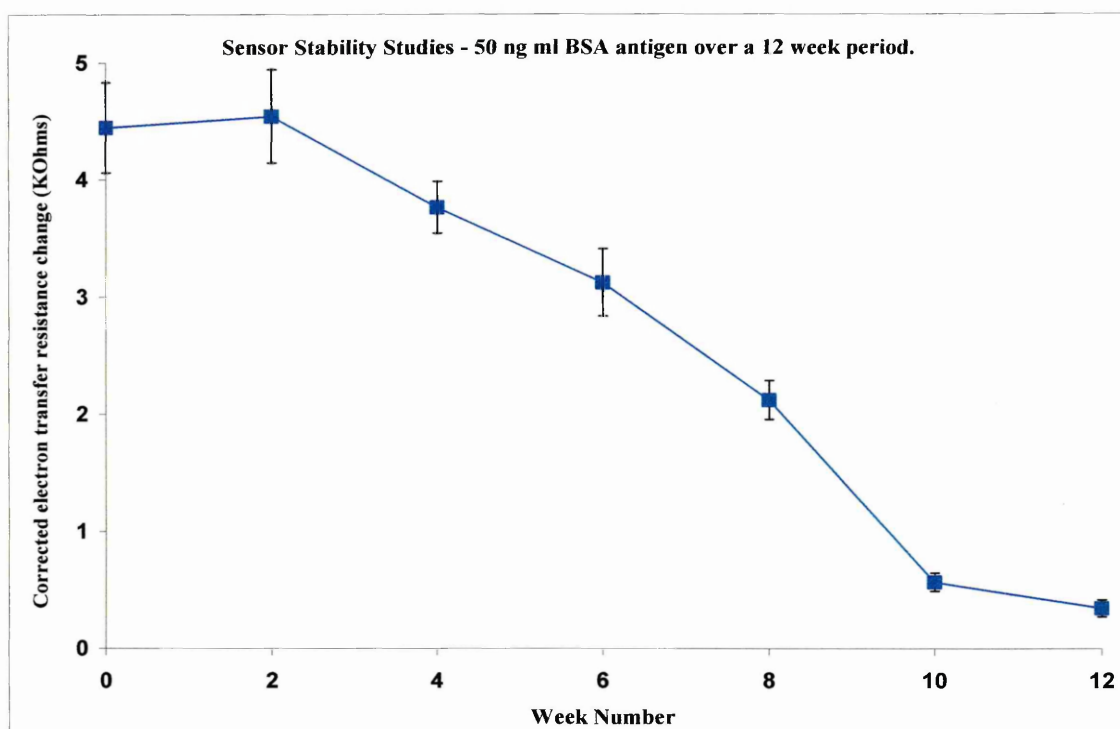


Fig 6.11: Corrected electron transfer resistance change at 50 ng ml⁻¹ BSA.
Stability study – Variation over 12 week period. ($n = 3$, error bars = SD).

Figure 6.11 shows that the sensor response is fully recovered after two weeks in storage with negligible difference from the initial response. Following this encouraging result there is a general decrease in sensitivity up to 10 weeks over which time the sensor response is virtually entirely lost. The activity of the antibody receptor biocomponent diminishes over time. The results do not lend themselves well to packaging applications and suggest an unsatisfactory shelf-life.

6.3.6 ABSA immunosensor: Conclusions.

The results acquired are promising in that the experimental protocol developed and the experimental techniques employed have offered a viable approach towards the labelless quantification of complementary antigen in test solution. Further development is required to overcome the difficulties encountered. A large non-specific response was observed, a complete lack of reversibility was encountered and stability studies also yielded disappointing results.

The ABSA immunosensor gave a 'corrected' linear-type response from 1 to 50 ng ml⁻¹. The higher the BSA antigen concentration in the test solution the greater the number of antibody-antigen complexes formed at the sensor surface. The complex formation introduces a barrier to electron transfer and hence the electron transfer resistance extracted from the spectra obtained in turn increases. A concentration of analyte above 50 ng ml⁻¹ in the test solution causes the sensor surface to approach saturation. The limit of detection of the system was found to be 1.5 ng ml⁻¹ and there was an ability to discriminate at 5 ng ml⁻¹ BSA analyte. However, only relatively minor electron transfer resistance changes were observed at these lower concentrations and hence the ABSA immunosensor does not allow for operation at lower experimental analyte concentration ranges of further clinical significance.

6.4 Faradaic impedimetric interrogation of PANI ‘mushroom’ microelectrode array sensors: APSA immunosensor studies.

Following the development of ABSA immunosensors, prostate specific antigen (PSA) and its monoclonal antibody (APSA) were selected as the next system for development and exploratory phase research. The system represents a better model than BSA in terms of more comparable physical properties and chemical nature to the BMBD (stroke) such as NSE and S-100 that are to be ultimately quantified. PSA is the most reliable tumor marker (Fernandez-Sanchez, 2004; Lin & Ju, 2005) for the early diagnosis of prostate cancer and monitoring of disease recurrence after treatment (Polascik, 1999).

Following immobilisation of antibody, APSA, according to section 5.2 impedance analyses were performed from 1Hz to 10,000Hz ($\pm$ 5mV amplitude perturbation) in pH 7.4 phosphate buffer, i.e. containing no antigen, as a baseline trace. This buffer solution did, however, contain a 50:50 mixture of $[\text{KFe}(\text{CN})_6]^{3-/4-}$, at a concentration of 10mM as redox mediator so as to perform faradaic impedance spectroscopy. The potential of the electrochemical cell is offset to the formal potential of the redox probe (+0.22V vs. Ag/AgCl). Subsequently, APSA doped sensors are exposed to a range of concentrations from 1 to 300 ng ml⁻¹ PSA. PSA antigen was suspended in pH 7.4 phosphate buffers containing potassium ferri / ferro cyanide at pre-prepared analyte concentrations of 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 and 300 ng ml⁻¹. The pH of 7.4 was utilised to coincide with human physiological pH. The experimental protocol developed and described in section 3.5.5 is applied for the electrochemical impedimetric interrogation of APSA immunosensors. A consistent sensor is used for all analytical concentrations and changes encountered are compared to the initial baseline trace (0 ng ml⁻¹ PSA).

Results are presented in the form of Nyquist plots depicting the faradaic ($Z'_{(re)}$) and capacitive ($-Z''_{(im)}$) components of impedance and their variation throughout the recording of the ac impedance spectra. Lower non-specific interactions are expected and the possibilities of lower analytical concentrations are anticipated.

6.4.1 APSA immunosensor: Specific recognition of PSA antigen.

Electrochemical Impedance Spectra obtained for an APSA-doped PANI mushroom microelectrode array sensor exposed to increasing PSA concentrations.

Figure 6.12 shows the specific response to PSA for a novel APSA-immobilised, PANI modified, carbon microelectrode array sensor assembly. The concentration of PSA analyte represented is 1 to 10 ng ml⁻¹. For the baseline trace, the $Z'_{(re)}$ component is seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the final applied frequency (1Hz).

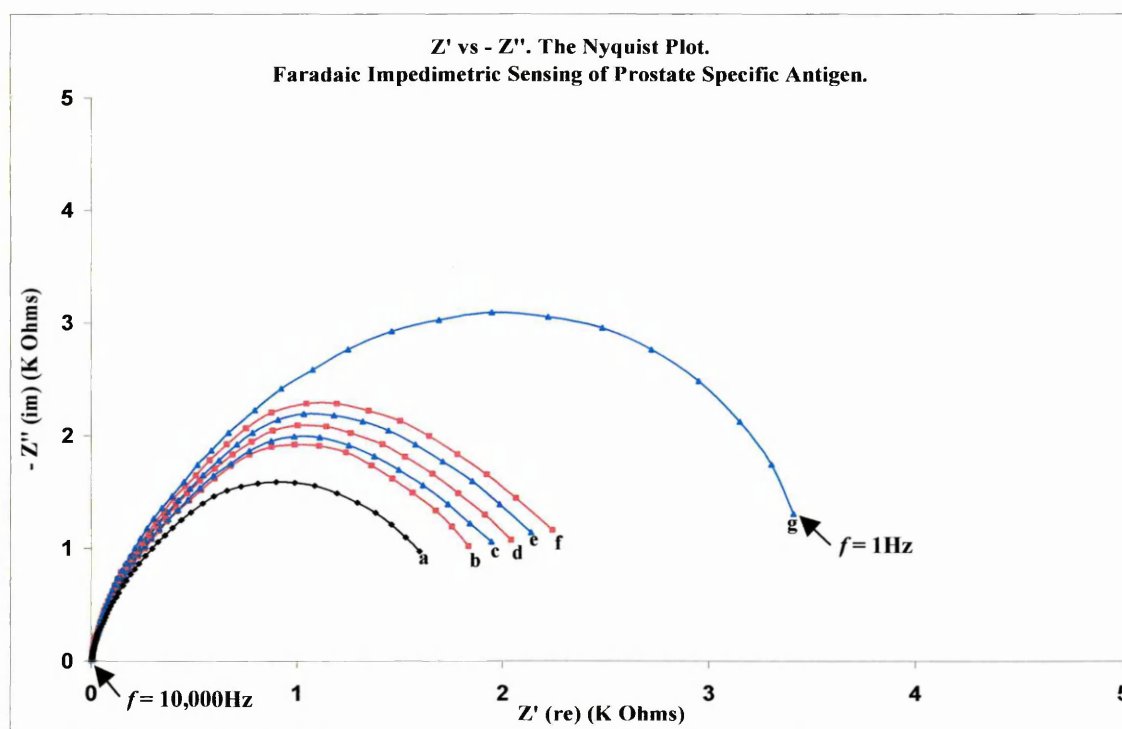


Fig 6.12: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel APSA-doped PANI Immunosensor following exposure to various concentrations of PSA. The concentration of antigen (ng ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 ng ml⁻¹ PSA.

The impedance spectrum is again indicative of a surface-modified electrode system where the electron transfer is slow (Katz & Willner, 2003). All subsequent traces following antigen exposure also follow this general form.

The response is not too dissimilar to the response encountered for the specific ABSA immunosensor described in section in 6.3.1.

Figure 6.13 represents the specific response to PSA analyte over the entire experimental concentration range up to 300 ng ml^{-1} PSA.

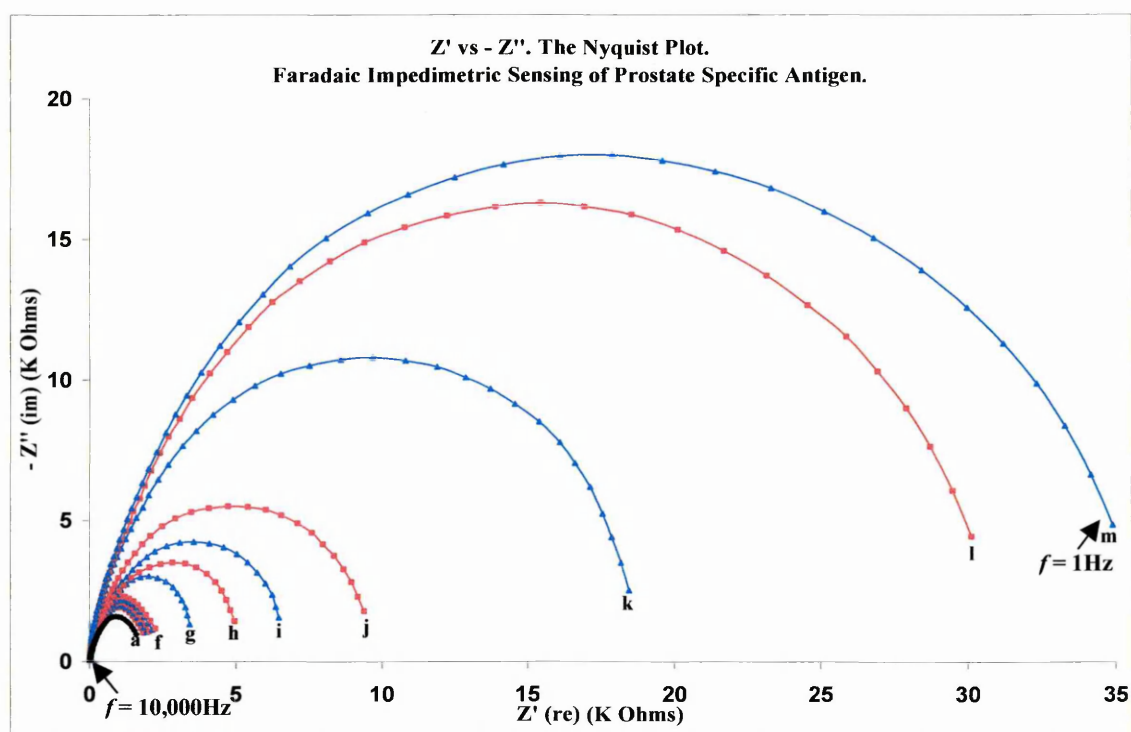


Fig 6.13: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel APSA-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (ng ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 ng ml^{-1} PSA.

Again the Nyquist curves follow the same general format as those in Figure 6.12 and those previously encountered in section 6.3.1 for the specific ABSA immunosensor. A computer fitting of the experimental data to a theoretical model, represented by a simple equivalent circuit, is performed by the software provided with the frequency response

analyser as data is extracted by the instrument. For a more thorough evaluation of the data obtained in Figure 6.12 & Figure 6.13 the changes in the Nyquist curves are translated into electron transfer resistance changes to provide a clear and consistent format. Experimentally determined and simulated values of electron transfer resistance were compared, showing an exact correlation to two decimal places, and are plotted in Figure 6.14.

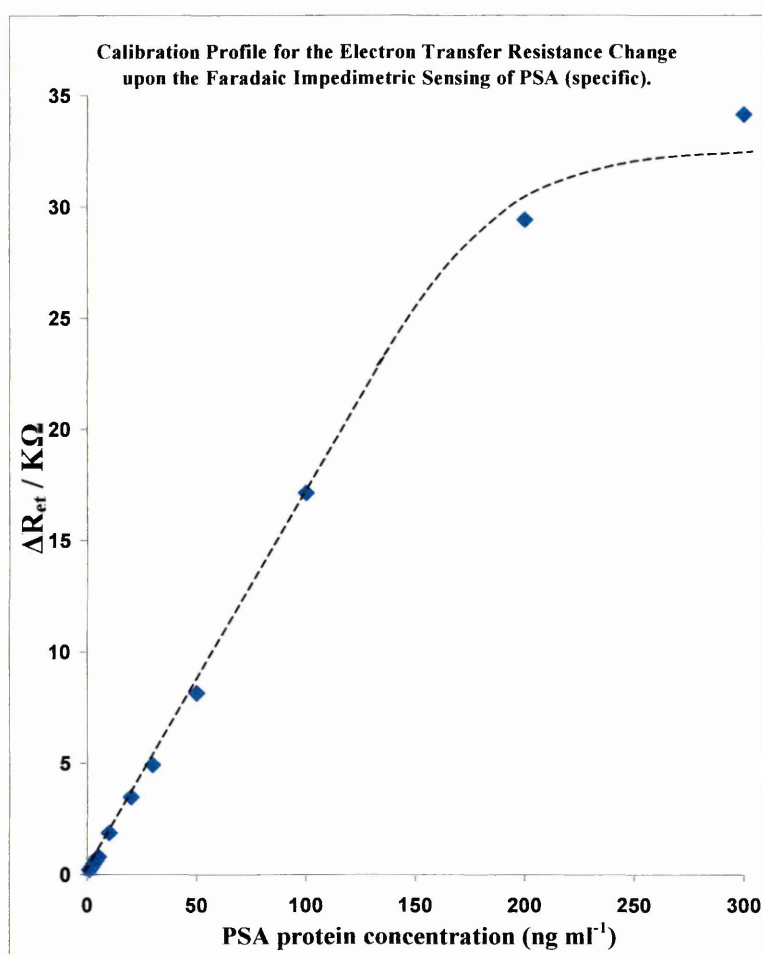


Fig 6.14: Calibration curve for the change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (specific).

The electron transfer resistance increases with increasing PSA concentration and Figure 6.14 illustrates this clearly. The results depict the change in the electron transfer resistance at each concentration calculated with respect to the baseline trace

(0 ng ml⁻¹ PSA) for the APSA-modified sensor following exposure to complementary PSA as the target analyte. Equation 6.4 details the calculation of the respective changes.

The interaction of PSA with the electrode modified with APSA results in significant insulation of the electrode reflected by the increase in the electron transfer resistance. It is postulated that as antigen concentration is increased, the number of complexes formed also increases and this is reflected by the observed electron transfer resistance increase.

The insulation of the modified electrode upon formation of stable APSA-PSA complexes hinders the electron transfer kinetics of the redox probe resulting in the increase of electron transfer resistance. The electron transfer resistance increases with increasing antigen concentration in a linear-type response from 1 to approximately 200 ng ml⁻¹. The sensitivity of the specific APSA immunosensor is 1 ng ml⁻¹. There is negligible change from 200 ng ml⁻¹ to 300 ng ml⁻¹ suggesting that the immunosensor approaches saturation and stable antibody-antigen complex formation is no longer occurring at these concentrations.

6.4.2 AIG immunosensor: PSA non-specific control investigations:

Electrochemical Impedance Spectra obtained for an AIG-doped PANI mushroom microelectrode array sensor exposed to increasing PSA concentrations.

Experiments were repeated using a matched AIG-immobilised sensor in which the antibody is non-specific to the target analyte PSA. Otherwise, identical experimental procedures are employed and all parameters are kept consistent. The experiments serve to validate the observed specific response of section 6.4.1 and account for all non-specific contributions to the sensing of PSA.

Figures 6.15 and 6.16 represent the AIG sensor response to PSA antigen from 1 to 300 ng ml⁻¹ and use the same axes as Figures 6.12 and 6.13 respectively to enable a direct comparison with the specific sensor response.

Figure 6.15 depicts the response to the lower PSA concentrations of 1, 2, 3, 4, 5 and 10 ng ml⁻¹ PSA antigen.

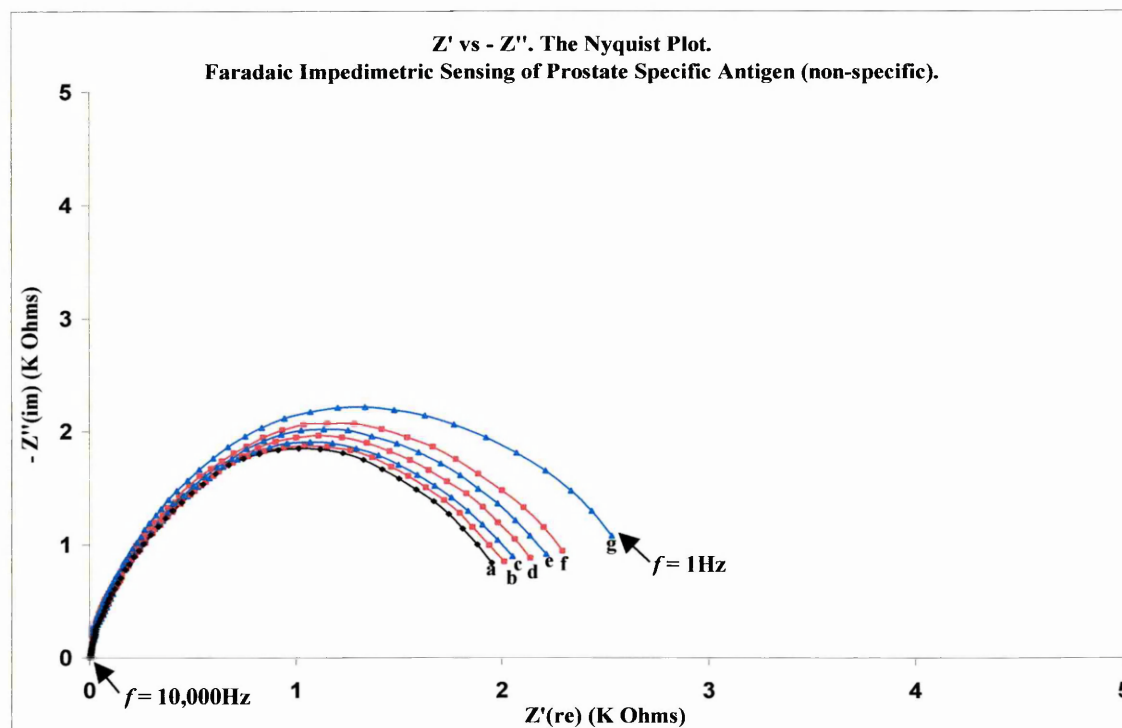


Fig 6.15: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AIGG-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (ng ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 ng ml⁻¹ PSA. Refer to Fig 6.12 for direct comparison on same axes scale.

All impedimetric spectral traces obtained follow the same general form and similar shaped Nyquist curves are obtained, indicative of a surface-modified electrode system where the electron transfer is slow.

Figure 6.16 represents the experimental data obtained for the whole analytical concentration range up to 300 ng ml⁻¹ PSA.

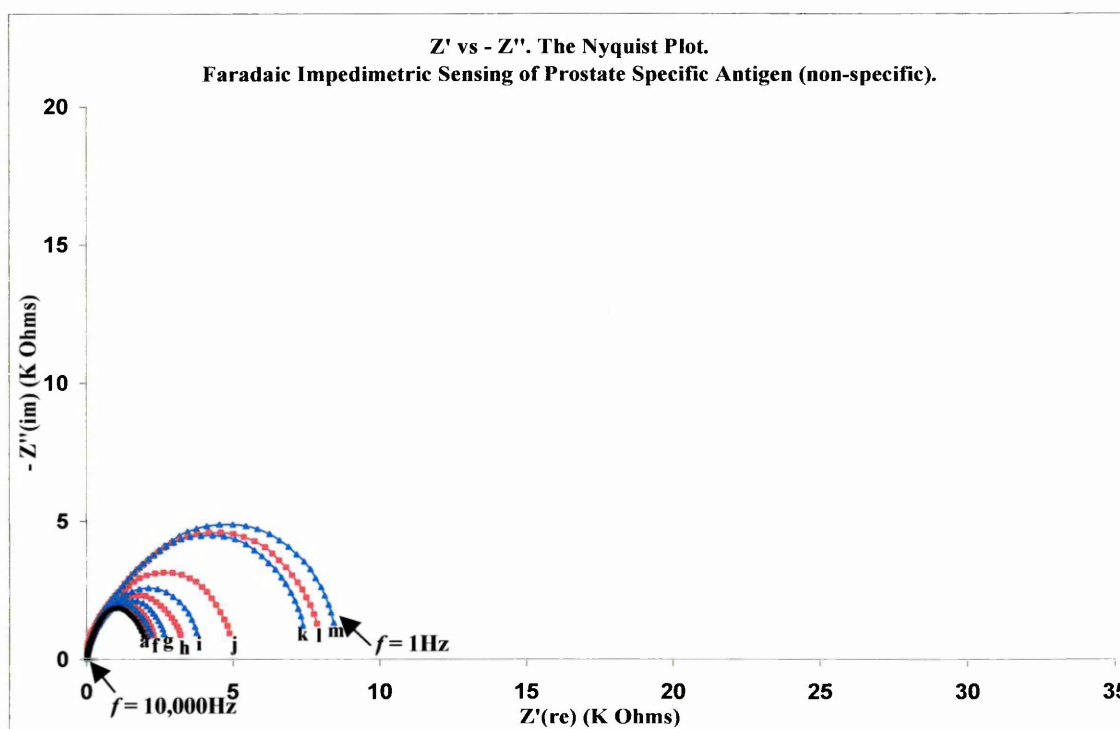


Fig 6.16: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AlG-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (ng ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 ng ml^{-1} PSA. Refer to Fig 6.13 for direct comparison on same axes scale.

For clarity, Figure 6.17 shows the non-specific experimental data presented as the relative change in electron transfer resistance from the baseline trace (0 ng ml^{-1} PSA) for each concentration analysed.

Figure 6.17 uses the same y-axis scale as Figure 6.14.

The electron transfer resistance values are extracted from the instrument and also independently calculated showing an identical correlation to two decimal places. The change in electron transfer resistance is calculated according to equation 6.4 with respect to the baseline trace at each experimental concentration.

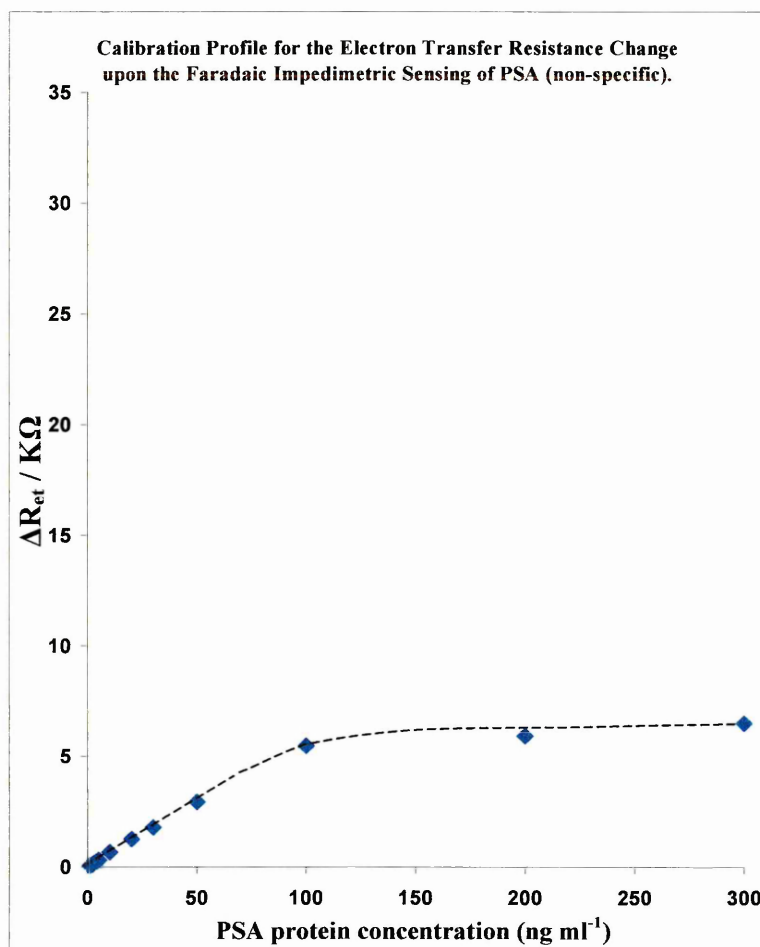


Fig 6.17: Calibration curve for the change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific).

A linear-type non-specific response is observed from 1 to 100 ng ml⁻¹ PSA. Upon comparison of Figure 6.17 with Figure 6.14 it is apparent that approximately 30 - 40% of the APSA-immobilised sensor response encountered is, in fact, non-specific. The calibration curve plateaus at approximately 100 ng ml⁻¹ PSA suggesting that an optimum electron transfer resistance change from the baseline response has been reached and non-specific occurrences are at their maximum.

6.4.3 'Corrected' specific response: APSA immunosensor.

To obtain a calibration profile, in terms of 'corrected' electron transfer resistance change for an APSA immunosensor, accounting for any non-specific responses - the changes obtained in Figure 6.17 (non-specific) were subtracted from those obtained in Figure 6.14 (specific) over the entire analytical concentration range. This matched sensor approach enables a true understanding of the APSA-PSA immunosensor system. In total 10 APSA-immobilised sensors and 10 corresponding AIGG-immobilised sensors were interrogated over the concentration range, according to section 6.4.1 and section 6.4.2 respectively, to allow assessment of the reproducibility of the observed responses. Figure 6.18 represents the 'corrected' profile for 10 replicate pairs of matched sensors.

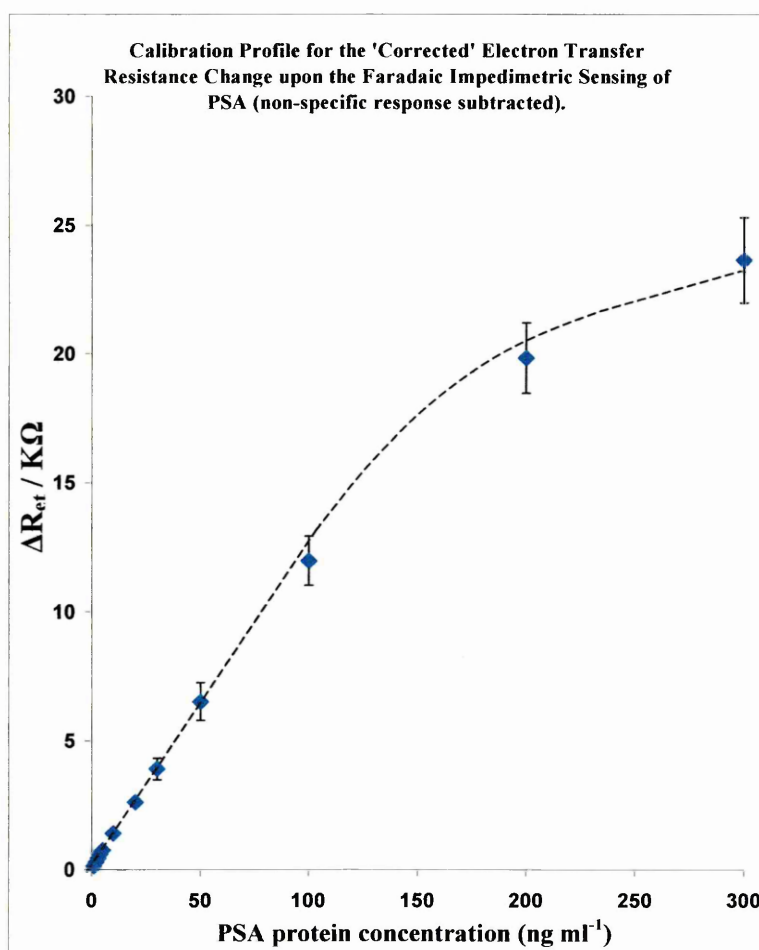
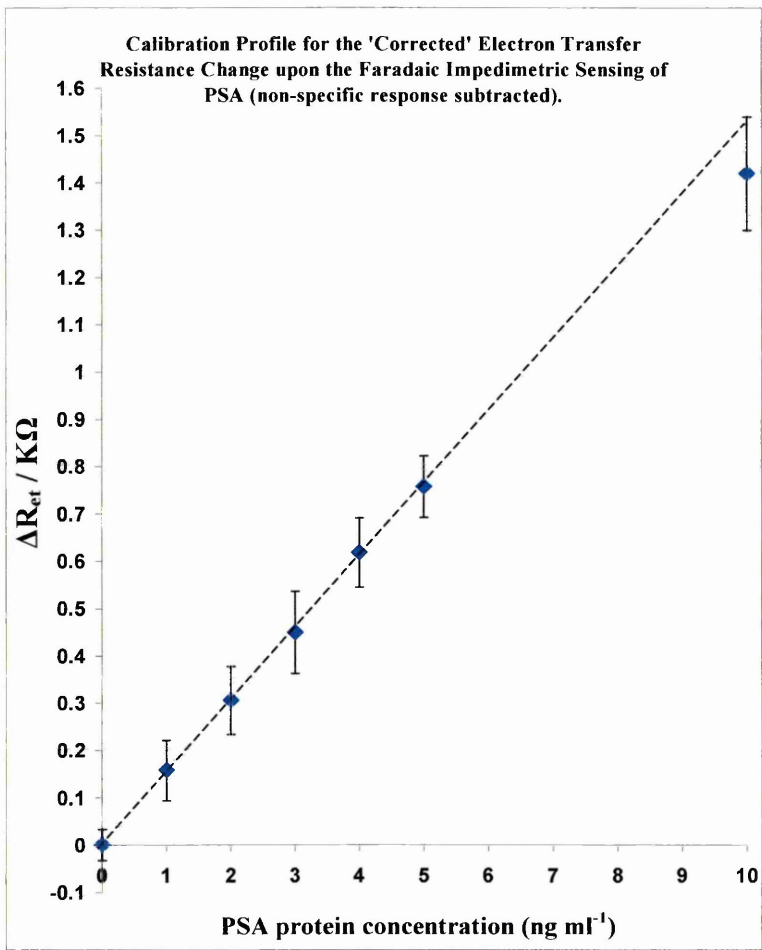


Fig 6.18: Calibration curve for the 'corrected' change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific response subtracted).
 $n = 10$, error bars = standard deviation.

Identical experimental procedures were employed throughout investigations and all parameters were kept consistent. The mean electron transfer resistance response at each experimental analyte concentration is plotted. The error bars indicate the standard deviation obtained for the 10 matched sensor pairs from the mean ‘corrected’ values. The resultant response is due entirely to the interaction of APSA with PSA analyte and arises from the effect on the electron transfer resistance that the formation of stable antibody-antigen complexes at the sensor surface generates.

For clarity, Figure 6.19 represents the calibration curve from 1 to 10 ng ml⁻¹ PSA. At these concentrations the response, in terms of electron transfer resistance change from the baseline trace (0 ng ml⁻¹ PSA) is fairly linear.



**Fig 6.19: Calibration curve for the change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific).
n = 10, error bars = standard deviation.**

From Figure 6.18 and Figure 6.19 a linear-type response is observed from 1 to approximately 100 ng ml^{-1} PSA. At concentrations above 100 ng ml^{-1} the electron transfer resistance change is no longer linear with increasing antigen concentration and the curve begins to tend towards a plateau. The sensor response approaches saturation and stable antibody-antigen complex formation no longer correlates with the increase in analyte concentration.

The reproducibility of the 'corrected' response obtained from the standard deviation for 10 matched sensor pairings enables discrimination to be possible at 1 ng ml^{-1} PSA antigen analyte. The limit of detection of the APSA immunosensor is 0.7 ng ml^{-1} PSA.

6.4.4 APSA immunosensor: Reversibility studies.

Several experimental protocol approaches were investigated. A range of concentrations of differing buffer species (pH 1 – 3) and differing washing regimes were employed. Unfortunately in all cases the response recovery for the APSA-PSA immunosensor system could never be regenerated following an initial response to PSA. An identical baseline response could be achieved which suggests that the sensor surface integrity remains yet the antigen recognition and binding capabilities of the receptor antibodies are severely damaged by the treatments.

6.4.5 APSA immunosensor: Stability studies.

The stability of the APSA-PSA immunosensor system was assessed from storage in dry state, at 4°C , for a period of 12 weeks. An identical experimental protocol is employed for electrochemical impedance interrogation and the 'corrected' electron transfer change was the analysis method for response recovery. Initially 18 matched sensor pairings of APSA (specific) and AIGG (non-specific) *CPE* immunosensors are refrigerated at 4°C in sealed, sterilised vessels.

Every 2 weeks a set of 3 matched sensor pairings was removed from storage and interrogated over the full PSA antigen analyte concentration range from 1 to 300 ng ml⁻¹. Figure 6.20 shows the data obtained at each time interval and the saturation concentration of 100 ng ml⁻¹ PSA was chosen for the final evaluation of sensor stability. The mean change in 'corrected' electron transfer resistance response is shown plotted against storage time. The error bars indicate the standard deviation for the three sensor pairs.

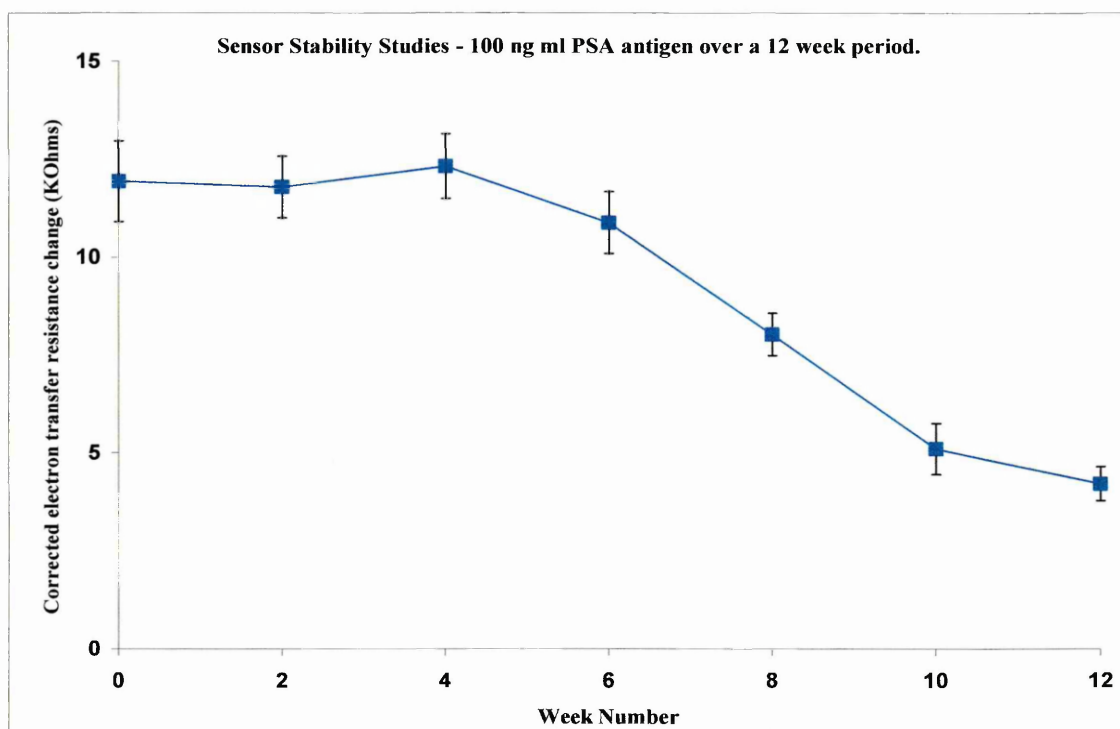


Fig 6.20: Corrected electron transfer resistance change at 100 ng ml⁻¹ PSA.
Stability study – Variation over 12 week period. ($n = 3$, error bars = SD).

Figure 6.20 shows that the sensor response is fully recovered after two weeks in storage with negligible difference from the initial response to PSA, at the saturation concentration of 100 ng ml⁻¹ PSA. At 4 and 6 weeks the response is fully recovered and there is negligible difference from the initial response and the standard deviations are comparable. In the following six weeks, however, there is a general decrease in sensitivity to PSA until the final test period. At 12 weeks the sensor response is approximately one third of the initial response to PSA. Further prolonged storage would cause the sensor response to be entirely lost.

The baseline trace remains unaffected by the storage regime suggesting that the PANI modified microelectrode array sensor matrix remains intact and that the activity of the antibody receptor biocomponent must diminish over time to account for the loss of response. A reduced number of stable antibody-antigen complexes are formed. The results do not lend themselves well to packaging applications and suggest an unsatisfactory shelf-life.

6.4.6 APSA immunosensor: Conclusions:

The results obtained verify the fabrication techniques and experimental interrogation protocol as a viable approach towards the labelless sensing of markers for TIA. The APSA-PSA immunosensor system has a reduced non-specific response associated with the faradaic sensing of PSA compared with the ABSA-BSA immunosensor system upon the faradaic sensing of BSA analyte. The APSA immunosensor was not, however, reversible and all regeneration attempts led to a detrimental effect on the performance of the immobilised antibody as a receptor biocomponent. The stability of the sensor assemblies was also disappointing with only a third of the sensitivity remaining after storage for twelve weeks at 4°C in dry state.

The APSA immunosensor gave a 'corrected' linear-type response from 1 to 100 ng ml⁻¹. The higher the PSA concentration in the test solution the greater the number of stable antibody-antigen complexes are formed at the sensor surface. The complex formation introduces a barrier to the electron transfer of the redox couple within the test solution at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration. At concentrations of analyte above 100 ng ml⁻¹, the response begins to plateau as the sensor saturates, with the recognition entities becoming occupied with analyte and active sites for further antigen binding being no longer available. The limit of detection of the APSA-PSA immunosensor system was found to be 0.7 ng ml⁻¹. Insufficient changes from the baseline trace are encountered for 1 ng ml⁻¹ and investigations did not proceed at lower quantification levels and the limits of detection of PSA had been reached with the

APSA-immobilised *CPE* sensor assemblies. The APSA immunosensors described display sensitivity that is of clinical significance for the diagnosis of prostate cancer via the quantification of total PSA in serum, the so called diagnostic grey zone of 4-10 ng ml⁻¹ (µg L⁻¹) (Fernandez-Sanchez, 2005).

6.5 Chapter 6 Conclusions:

In summary, the ABSA-BSA system gave rise to a linear-type response from 1 to 50 ng ml⁻¹ BSA analyte with a limit of detection of 1.5 ng ml⁻¹ and the ability to discriminate at 5 ng ml⁻¹ following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. This is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was not reversible and the stability of the sensors was unsatisfactory.

50% of the initial electron transfer resistance changes were found to be non-specific. Attempts to lower the antigen concentration quantification lead to only minor changes from the baseline response and insufficient sensitivity to proceed to pg ml⁻¹ detection levels. BSA is a notoriously difficult analyte species and investigations into the detection of PSA were pursued.

A linear-type response was observed for APSA immunosensors from 1 to 100 ng ml⁻¹ PSA with a detection sensitivity of 0.7 ng ml⁻¹ and the ability to discriminate at singular ng ml⁻¹ concentration levels. This is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. 10 matched sensor pairings were analysed over the entire concentration range to gauge the reproducibility. Approximately 30% non-specific response was encountered during the control experiments yielding the 'corrected' response which is significant and of clinical relevance for PSA quantification.

The APSA-PSA immunosensor system was not reversible and the stability of the sensors was unsatisfactory.

Attempts to reduce the analytical concentration range below 1 ng ml^{-1} led to only very minor electron transfer resistance changes.

In general, for the linear responses, the higher the analyte concentration in the test solution the greater the number of stable antibody-antigen complexes formed at the sensor surface. The stable antibody-antigen complex formation introduces a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration. At specific concentrations of analyte the number of antibody-antigen couples formed begins to plateau as the sensor saturates, the recognition entities become occupied with analyte and active sites for further antigen binding are no longer available. For the ABSA immunosensor the sensor surface approaches saturation at 50 ng ml^{-1} BSA and for the APSA immunosensor the surface approaches saturation at 100 ng ml^{-1} PSA.

Although not ideal the results have demonstrated the potential for EIS interrogation as a viable approach towards the labelless detection and quantification of biochemical markers of stroke (TIA) providing some significant developments can be made.

It is postulated that a lot of the sensitivity of the immunosensor systems is swamped by non-specific events, thus, the organisation of the antibody recognition layer, including the receptors orientation and the availability of active sites, with the minimisation of non-specific events is of paramount importance for the lower limits of detection available and to allow sensors to be used over concentration ranges of clinical relevance for markers of stroke. A greater control of the antibody immobilisation is required.

It is the aim to achieve detection limits and discrimination levels at the pgml^{-1} level and thus for results to be significant and of clinical relevance for markers of stroke such as S-100 and NSE.

Although it is a rapid one-step procedure, simple entrapment antibody immobilisation techniques within and at the surface of the PANI mushroom protrusions do not allow for the successful elaboration of *CPE* immunosensors via electrochemical impedance spectroscopy. The antibody orientation is random and the spatial immobilisation is not well controlled. A poorly organised recognition layer can cause a severe lack of sensitivity and this is thought to be the primary factor for the disappointing responses obtained.

The immunosensors investigated within this chapter do not suffice for low quantification of target analyte at the required concentration levels of stroke markers. Clinically significant data was obtained for potential use as an immunosensor for prostate cancer. However, the large non-specific response encountered will cause problems when analysis is performed in serum test samples as opposed to buffered test solutions that contain no interfering species.

A greater degree of stability and reversible behaviour are also required and would provide highly desirable assets for the immunosensors intended application purpose.

The main factors responsible for the insufficient sensitivity are the organisation of the recognition layer of the sensor, as in no way does it serve to encourage stable antibody-antigen complex formation and the high non-specific response swamping the signal response.

Chapter 7: Electrochemical Impedance studies utilising non-covalent affinity immobilisation techniques.

7.0 Electrochemical Impedance studies utilising non-covalent affinity as the site-specific immobilisation technique employed.

7.1 Introduction:

Immunosensors interrogated in this chapter were successfully fabricated via a step-wise protocol to ensure orientation specific immobilisation of antibodies utilising avidin-biotin non-covalent affinity technologies as detailed in section 5.3. This approach allows polyaniline to be formed as protrusions emanating at the microelectrode array surface, using optimum conditions and helps preserve the physical properties of the antibody receptors since they are no longer present in the monomer solution. This protocol, however, is slightly more time-consuming than *CPE*. Sensors were also HSA blocked to reduce the non-specific response. SEM imaging, QCM and voltammetric techniques were used to monitor each fabrication step at the microelectrode array sensors and validate, where applicable, the successful sensor assembly as discussed in Chapter 5.

The formation of a bioaffinity complex on the electrode surface is expected to introduce a barrier for the electron transfer at the electrode interface. This results in an enhanced electron transfer resistance at the electrode surface. However, it should be noted that the changes in the Faradaic impedance spectrum upon the antibody-antigen complex formation are not always large. Faradaic impedance spectroscopy was employed to assess the specific response to the relevant target analyte in test solution.

If the sensing layer is not well organised and contains pinholes between the biomolecules, and the redox probe is sufficiently small to freely penetrate through these pinholes, the changes observed in the Faradaic impedance spectra could be minor. The organisation of the recognition layer is thought to be the greatest influencing factor determining the sensitivity of the immunosensors interrogated thus far. Other important considerations in sensor development are regeneration of the sensor surface through disruption of Ab-Ag binding and release of antigen, long term stability of the sensor and insensitivity of the sensing surface to sample matrix species.

Attempts have already been made to minimise the non-specific response during fabrication and also control experiments with matched sensors of Human IgG (polyclonal) receptors allow a 'correction' to be made to the response curve showing the true specific response only. Reversibility and stability studies were also performed following the successful creation of viable labelless impedimetric immunosensors for the detection of key markers of stroke (TIA).

7.2 Impedimetric sensing of the fabrication of immunosensors via non-covalent affinity techniques.

Figure 7.1 shows the electrochemical impedance data obtained, following several modification steps, throughout the fabrication of immunosensors interrogated in this chapter. Figure 7.1 is a plot of the data represented in the Nyquist format.

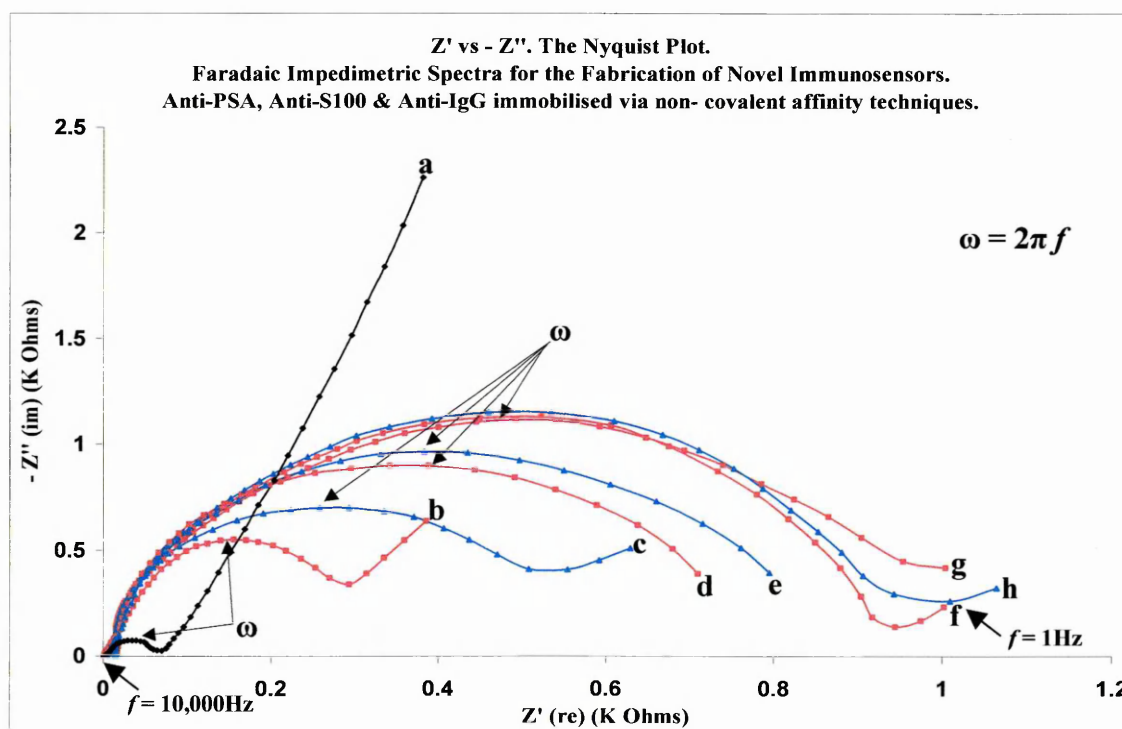


Fig 7.1: Faradaic Impedimetric sensing of the Fabrication of CPE Immunosensors:
 a) Planar Carbon electrode, b) Polymerised & Sonicated Carbon Microelectrode Array,
 c) PANI modified, d) Biotinylated PANI, e) Neutravidin f) anti-PSA (monoclonal),
 g) anti-S100 (monoclonal) and h) anti-IgG (polyclonal) immobilised.

The imaginary impedance component ($-Z''_{(im)}$) was plotted against the real impedance component ($Z'_{(re)}$) at each excitation frequency for the fabrication of immunosensor assemblies.

Plot 7.1 a) represents the trace obtained for a planar carbon electrode in pH 7.4 phosphate buffered test solution containing potassium ferri/ferro cyanide. Plot 7.1 b) is obtained in the test solution following modification steps to create a microelectrode array sensor. Plot 7.1 c) is obtained following modification of the microelectrode array sensor surface with emeraldine PANI utilising optimum electrodeposition conditions as no biomolecule is present in the monomer solution. The PANI is electrogenerated as protrusions from the microelectrode array. Plot 7.1 d) is obtained after the PANI-modified sensor is biotinylated and plot 7.1 e) is obtained after neutravidin is bound to the biotinylated PANI surface. Finally, plots 7.1 f), 7.1 g) and 7.1 h) represent completed immunosensor traces obtained in test solution after biotinylated antibody receptor components have been immobilised, binding to the free groups on the neutravidin molecule via non-covalent affinity interactions.

From Figure 7.1 a) to 7.1 b) it is apparent that the modification of the planar electrode to form the microelectrode array sensor causes significant change in the impedimetric spectral trace. Plot 7.1 a) represents very fast electron transfer processes, hence only the linear part of the trace is observed and this is as would be expected for a planar electrode. The impedance is mainly controlled by diffusion of the redox probe. Both the $Z'_{(re)}$ and $-Z''_{(im)}$ components are seen to increase with decreasing frequency over the whole range of applied experimental frequencies (10,000Hz to 1Hz, +/- 5mV amplitude perturbation).

For plot 7.1 b) the $Z'_{(re)}$ component is also seen to increase with decreasing frequency over the whole range of frequencies from 10,000Hz to 1Hz. The $-Z''_{(im)}$ component, however, is seen to increase from 10,000Hz to the characteristic frequency, ω , of the impedance trace, indicated at the point where the $-Z''_{(im)}$ value is at its maximum. Also, the Warburg impedance contribution is seen to dramatically reduce and shift to lower frequencies from Figure 7.1 a) to 7.1 b).

Plot 7.1 b) shows a modified electrode where the impedance is controlled by diffusion of the redox probe at the low frequencies and by interfacial electron transfer at high frequencies.

From plot 7.1 b) to plot 7.1 c) it is apparent that the PANI modification has introduced a further barrier to electron transfer, reflected by the increase in diameter of the semicircle portion of the trace. The Warburg contribution is again seen to decrease and shift to even lower frequencies. The trace is indicative of a surface-modified electrode where impedance is controlled mainly by interfacial electron transfer within the range of applied experimental frequencies. However, there is some diffusion controlled behaviour at the lowest applied frequencies.

Plots 7.1 d) and 7.1 e) show negligible differences from one another. The difference in diameter of the semicircle portion of the traces compared to plot c) indicates that further matter has been successfully incorporated at the sensor surface. The larger the semicircle diameter, the greater the barrier introduced for electron transfer. These traces are indicative of a surface-modified electrode where impedance is controlled mainly by interfacial electron transfer within the range of applied experimental frequencies. There is no contribution from the Warburg impedance and hence no diffusion-controlled behaviour.

The immobilisation of antibody bio-receptors at the sensor surface via non-covalent affinity attachment to the biotinylated, neutravidin modified-PANI protrusion phenomena causes a further increase in the semicircle portion of the traces. Insulation at the electrode surface has occurred introducing another increase in the barrier to electron transfer. This confirms the successful creation of a recognition entity immobilised within intimate proximity of the transducer at the polymerised and sonicated microelectrode array surface. At this modification step a small contribution from the Warburg impedance is seen to re-emerge. Plots 7.1 f), 7.1 g) and 7.1 h) depict insignificant differences and suggest a reproducible immunosensor fabrication process.

When antibody of the same species is immobilised the spectra show near identical traces. The differences between traces 7.1 f), 7.1 g) and 7.1 h) are due to the biomolecular species that are immobilised.

Prior to the subsequent Faradaic sensing of complementary target analyte (section 7.3.1) the sensor surface is blocked with HSA to minimise non-specific interactions throughout the interrogation of the novel immunosensors.

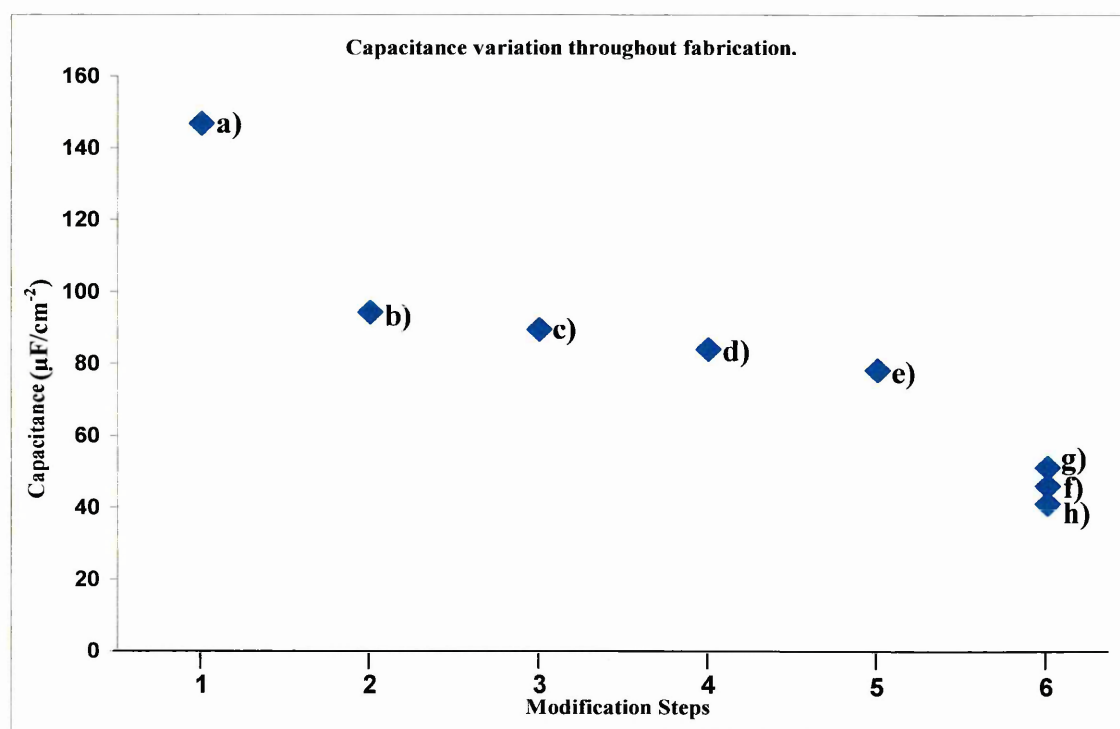


Fig 7.2: Non-Faradaic Impedimetric sensing of the Fabrication of CPE Immunosensors:
a) Planar Carbon electrode, b) Polymerised & Sonicated Carbon Microelectrode Array,
c) PANI modified, d) Biotinylated PANI, e) Neutravidin f) anti-PSA (monoclonal),
g) anti-S100 (monoclonal) and h) anti-IgG (polyclonal) antibody.

Figure 7.2 shows the $-Z''_{(im)}$ component variation (translated as capacitance changes) for the stepwise fabrication of immunosensors calculated at the characteristic frequency of each trace where the phase angle is closest to -90° indicating that the system is exhibiting near ideal capacitor behaviour (Berney, 2000). Capacitance values are calculated according to Equation 6.2. Again, 7.2 a) is the planar carbon electrode, 7.2 b) is the carbon microelectrode array sensor platform following polymerisation and sonication,

7.2 c) is the PANI modified microelectrode array sensor, 7.2 d) is obtained following biotinylation of PANI, 7.2 e) follows neutravidin modification and 7.2 f), 7.2 g) and 7.2 h) represent the fabricated immunosensors with antibody immobilised via non-covalent affinity.

Following each modification there is a decrease in capacitance. Substitution of the electrolyte solution with polymer and / or protein molecules at the electrode surface and further increase of the thickness and density of the organic layer to immobilise antibody receptors results in a significant change of the capacitance, C , which is derived from the imaginary part of the impedance spectra. The decrease of the effective dielectric constant of the medium at the interface yields the respective decrease of the capacitance.

The changes in the real part of the impedance are expected to be substantially higher. Consequently Faradaic EIS was utilised to monitor the detection of antigen. Faradaic impedance spectroscopy is usually considered to be more sensitive to the insulation of the electrode surface upon the binding of antigen to antibody functionalised electrode surfaces, (Katz & Willner, 2003).

7.3 Faradaic impedimetric interrogation of PANI 'mushroom' microelectrode array sensors: APSA immunosensor studies.

Screen-printed carbon electrodes were supplied by Microarray, Ltd. An innovative technique transforms the planar electrode into an array of micro-electrodes via the application of controlled ultrasound following insulation of the planar electrode surface with poly-*o*-phenylenediamine. The sonication technique causes ablation of the polymer surface and up to 2×10^5 sub-micron pores are created via exposure of the underlying carbon working electrode. Each of the pores is essentially a microelectrode and all microelectrode are connected together cumulatively acting as an array.

In order to provide a platform for biomolecule immobilisation further modification of the microelectrode array sensor is performed.

Polyaniline is electrogenerated in its' conductive form, emeraldine, as protrusion phenomena that grow at each of the ablated sub-micron pores as three-dimensional spherical structures. The polyaniline is biotinylated for attachment of the monoclonal antibody bio-recognition entities utilising avidin-biotin non-covalent techniques.

Novel monoclonal antibodies, developed and supplied by Canag Diagnostics, Ltd, were biotinylated and subsequently immobilised at novel polyaniline-modified microelectrode array sensors via a sophisticated avidin-biotin scaffolding construction. The monoclonal antibodies are specific to the chosen antigen analyte. The chemistry controls the orientation of antibodies ensuring greater availability of active sites for analyte detection and encourages stable recognition-complex formation. The spherical polyaniline protrusion phenomena, to which the antibodies are attached, confer a hemispherical diffusion profile to the novel sensor platform.

When engineering an immunosensor it is critical that the immobilisation method allows retention of the native conformation and binding characteristics of the antibody. The evolution of site-specific immobilisation techniques from simple entrapment to the method discussed above and described in detail in chapter 5 has ensured that these requirements are met.

Non-specific adsorption was minimised by HSA blocking as the final fabrication step and during sensor interrogation a washing regime was employed following antigen exposure. Matched sensors, differing only in the fact that the antibody immobilised is non-specific to the target analyte, were interrogated simultaneously to further gauge non-specific events that may still be occurring. The matched sensors were analysed identically over the full concentration range permitting a 'corrected' calibration profile by simply subtracting the non-specific sensor response at each concentration from the specific sensor response. 10 specific and 10 non-specific sensors were interrogated for each antigen system under investigation. A consistent sensor was analysed over the full concentration range. It is anticipated that the fabrication developments incorporated will allow for greater immunosensor sensitivity.

Following immobilisation of antibody, APSA, according to section 5.3 impedance analyses were performed from 1Hz to 10,000Hz (+/- 5mV amplitude perturbation) in pH 7.4 phosphate buffer, i.e. containing no antigen, as a baseline trace. This buffer solution did, however, contain a 50:50 mixture of $[\text{KFe}(\text{CN})_6]^{3-/4-}$, at a concentration of 10mM as redox mediator so as to perform faradaic impedance spectroscopy. The potential of the electrochemical cell is offset to the formal potential of the redox probe (+0.22V vs. Ag/AgCl). Subsequently, APSA doped sensors were exposed to a range of concentrations from 1 to 300 pg ml^{-1} PSA. PSA antigen was suspended in pH 7.4 phosphate buffers containing $[\text{KFe}(\text{CN})_6]^{3-/4-}$ at pre-prepared analyte concentrations of 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 and 300 pg ml^{-1} . The pH of 7.4 was utilised to coincide with human physiological pH. The experimental protocol developed and described in section 3.5.5 is applied for the electrochemical impedimetric interrogation of APSA immunosensors. A consistent sensor is used for all analytical concentrations and changes encountered are compared to the initial baseline trace (0 pg ml^{-1} PSA).

Initial exploratory investigations with these novel APSA immunosensors showed significant change from the baseline trace for 1 ng ml^{-1} PSA. An approximate increase in electron transfer resistance from ~1 K ohm to ~20 K Ohm was observed. However, EIS analysis across the entire analyte concentration range up to 300 ng ml^{-1} PSA in the test solution caused negligible response. The sensor assembly behaviour was indicative of a saturated surface. It was concluded that analyte concentration in the test solution was too high and thus a pg ml^{-1} concentration range was employed for response assessment.

Results are presented in the form of Nyquist plots depicting the faradaic ($Z'_{\text{(re)}}$) and capacitive ($-Z''_{\text{(im)}}$) components of impedance and their variation throughout the recording of the ac impedance spectra.

7.3.1 APSA immunosensor: Specific recognition of PSA antigen.

Electrochemical Impedance Spectra obtained for an APSA-doped PANI mushroom microelectrode array sensor exposed to increasing PSA concentrations.

Figure 7.3 shows the specific response to PSA for a novel APSA-immobilised, PANI modified, carbon microelectrode array sensor assembly. The concentration of PSA analyte represented is 1 to 10 pg ml⁻¹. For the baseline trace, the $Z'_{(re)}$ component is seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the lower applied frequencies (~1Hz). In general, the impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow and the impedance is controlled by the interfacial electron transfer (Katz & Willner, 2003).

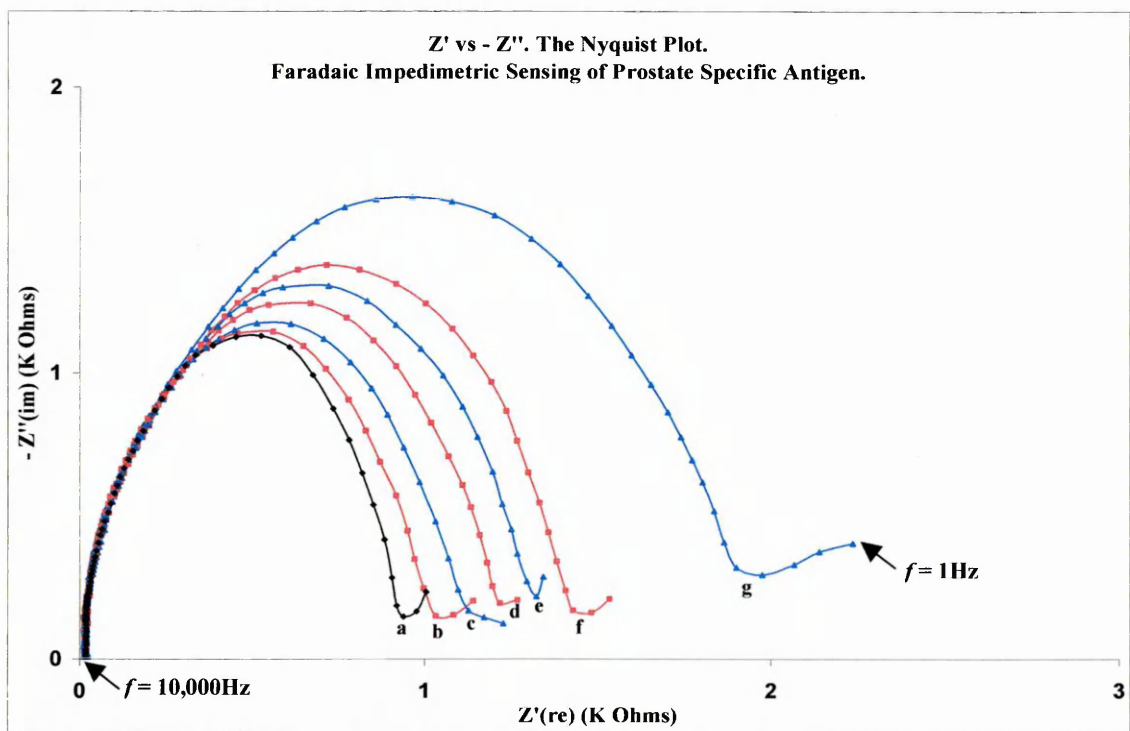


Fig 7.3: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel APSA-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (pg ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 pg ml⁻¹ PSA.

All subsequent traces following antigen exposure also follow this general form.

Figure 7.4 represents the specific response to PSA analyte at the higher concentrations of the experimental range up to 300 pg ml⁻¹ PSA. Again the Nyquist curves follow the same general format as those in Figure 7.3.

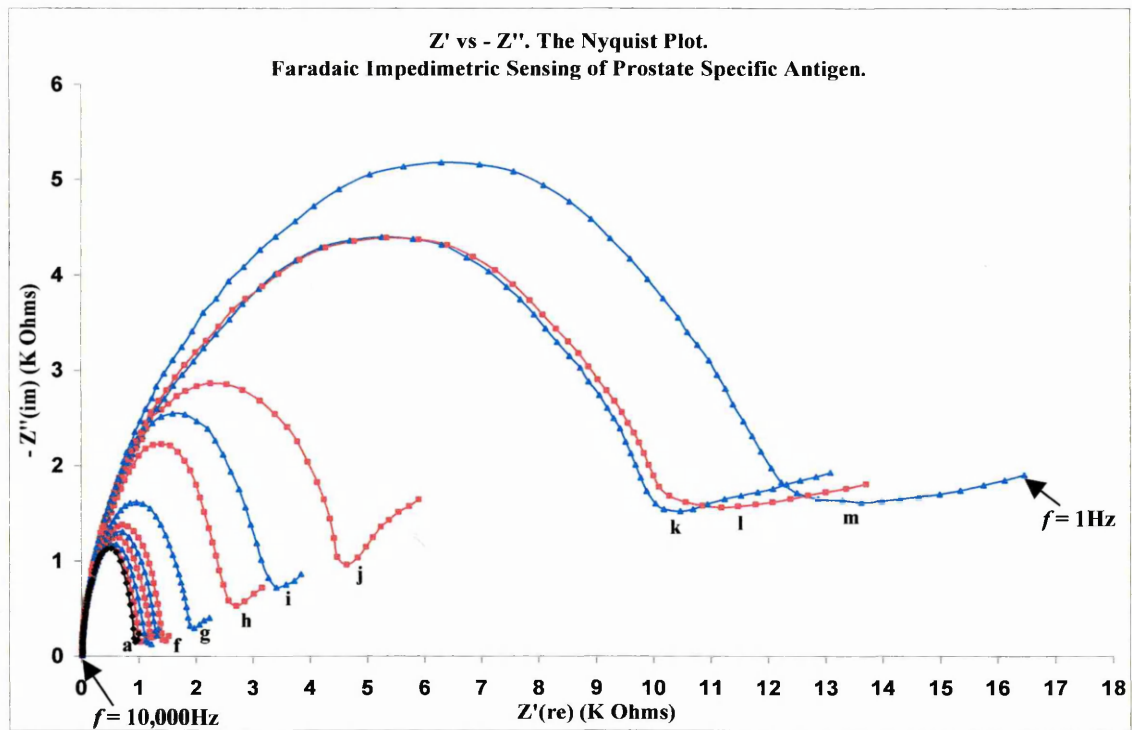


Fig 7.4: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel ABSA-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (pg ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml⁻¹ PSA.

A computer fitting of the experimental data to a theoretical model, represented by a simple equivalent circuit, is performed by the software provided with the frequency response analyser as data is extracted by the instrument. For a more thorough evaluation of the data obtained in Figures 7.3 and 7.4 the changes in the Nyquist curves are translated into electron transfer resistance changes to provide a clear and consistent format. Experimentally determined and simulated values of electron transfer resistance were compared, showing an exact correlation to two decimal places, and are plotted in Figure 7.5.

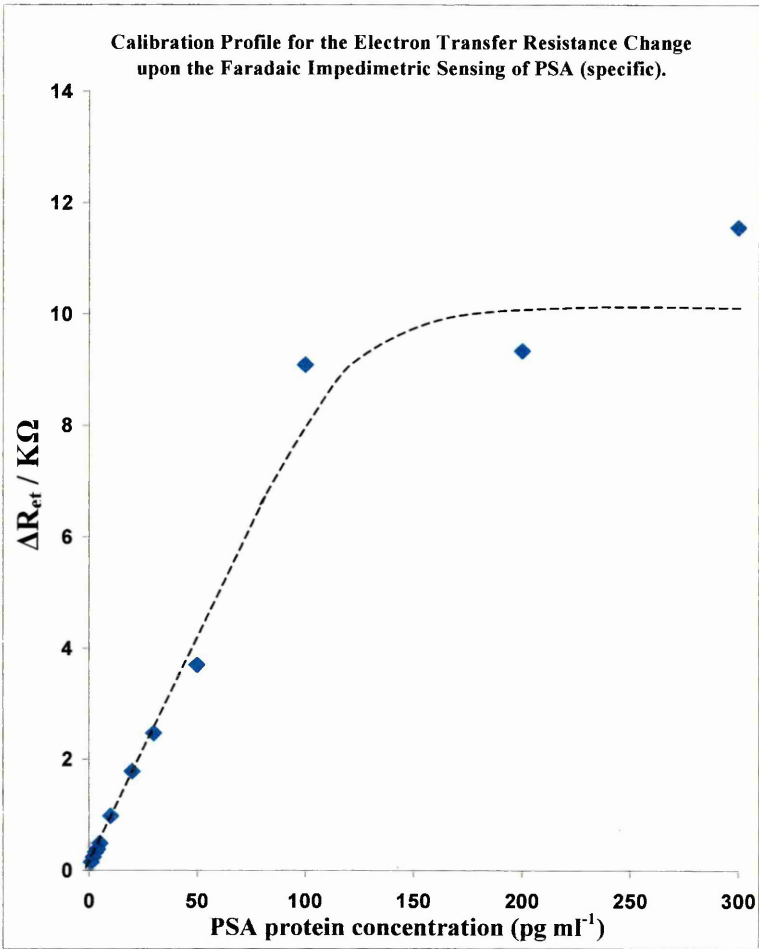


Fig 7.5: Calibration curve for the change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (specific).

Figure 7.5 depicts the electron transfer resistance change calculated with respect to the baseline trace (0 pg ml⁻¹). Equation 6.4 details the calculation of the electron transfer resistance change.

Figure 7.5 shows that the APSA immunosensor has a generally linear response to PSA analyte from 1 pg ml⁻¹ to approximately 100 pg ml⁻¹ PSA. The electron transfer resistance increases with increasing antigen analyte concentration up to approximately 100 pg ml⁻¹.

The interaction of PSA with the electrode modified with APSA results in significant insulation of the electrode reflected by the increase in the electron transfer resistance.

It is postulated that as antigen concentration is increased the number of complexes formed also increases and this is reflected by the observed electron transfer resistance increase.

The insulation of the modified electrode upon formation of stable APSA-PSA complexes hinders the electron transfer kinetics of the redox probe resulting in the increase of electron transfer resistance. The electron transfer resistance increases with increasing antigen concentration in a linear-type response from 1 to approximately 100 pg ml^{-1} . The sensitivity of the specific APSA immunosensor is 1 pg ml^{-1} . There is negligible change from 100 pg ml^{-1} to 300 pg ml^{-1} suggesting that the immunosensor approaches saturation and stable antibody-antigen complex formation no longer relates to the analyte concentration.

7.3.2 AIG immunosensor: PSA non-specific control investigations.

Electrochemical Impedance Spectra obtained for an AIG-doped PANI mushroom microelectrode array sensor exposed to increasing PSA concentrations.

Experiments were repeated using a matched AIG-immobilised sensor in which the antibody is non-specific to the target analyte PSA. Otherwise, identical experimental procedures were employed and all parameters are kept consistent. Figures 7.6 and 7.7 represent the AIG sensor response to PSA antigen from 1 to 300 pg ml^{-1} and use the same axes scale as Figures 7.3 and 7.4 respectively to enable a direct comparison with the specific sensor response.

Figure 7.6 represents the non-specific sensor response from 1 to 10 pg ml^{-1} PSA. The experiments serve to validate the observed specific response of section 7.3.1 and account for all non-specific contributions to the sensing of PSA.

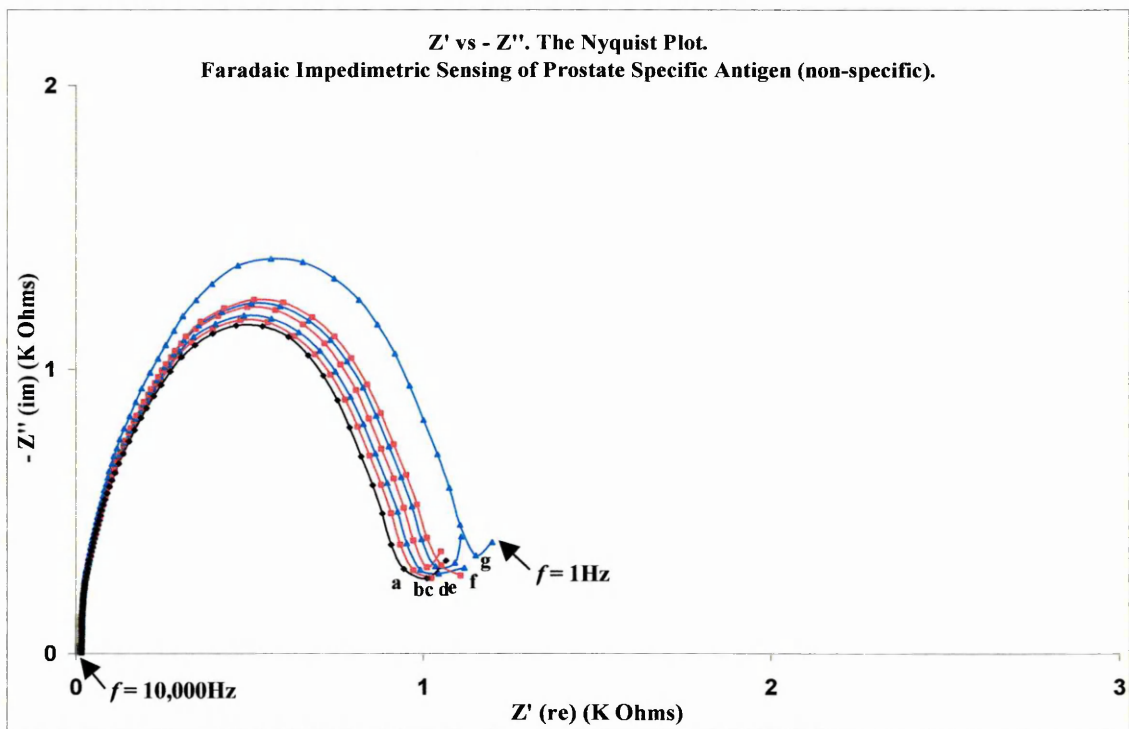


Fig 7.6: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel AlgG-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 pg ml^{-1} PSA. Refer to Fig 7.3 for direct comparison on same axes scale.

All impedimetric spectral traces obtained follow the same general form and similar shaped Nyquist curves are obtained, indicative of a surface-modified electrode system where the electron transfer is slow and controlled by interfacial electron transfer.

For each trace, the $Z'_{(re)}$ component was seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component was seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the lower applied frequencies ($\sim 1\text{Hz}$).

Figure 7.7 represents the non-specific sensor response from 1 to 300 pg ml^{-1} PSA.

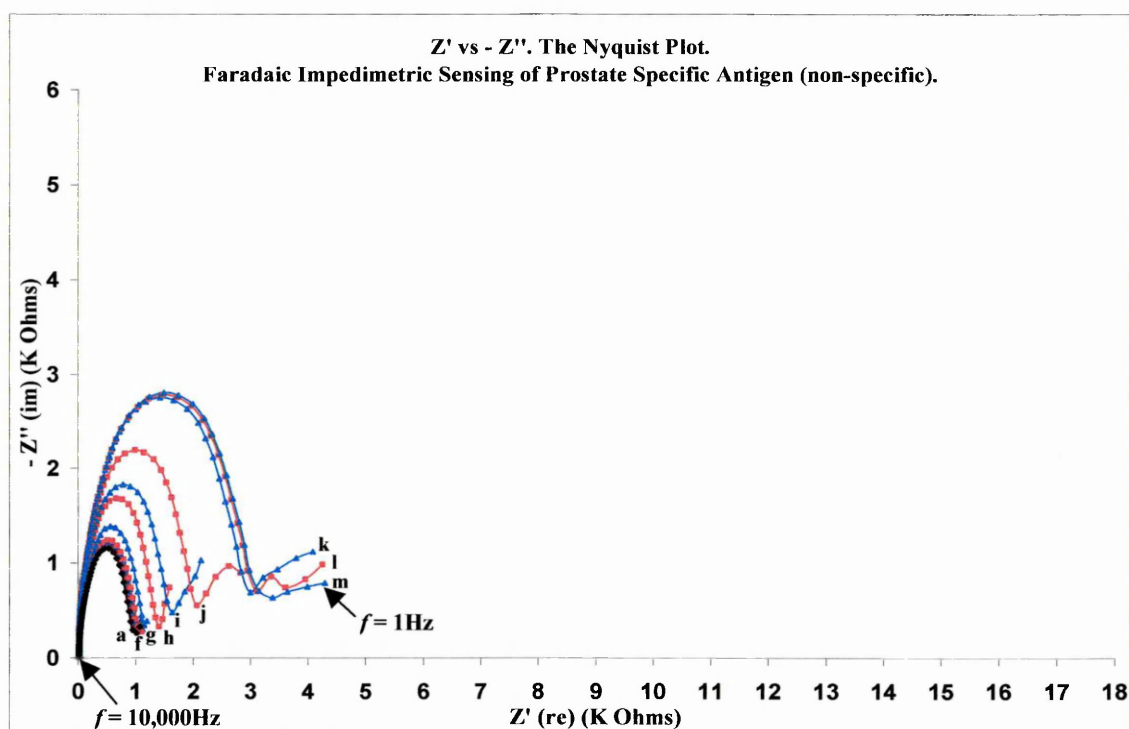


Fig 7.7: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs. capacitive component ($-Z''_{(im)}$) for a novel AlG-doped PANI immunosensor following exposure to various concentrations of PSA. The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml^{-1} PSA. Refer to Fig 7.4 for direct comparison on same axes scale.

For clarity, Figure 7.8 shows the non-specific experimental data presented as the relative change in electron transfer resistance from the baseline trace (0 ng ml^{-1} PSA) for each concentration analysed. Figure 7.8 uses the same y-axis scale as Figure 7.5.

The electron transfer resistance values were extracted from the instrument and also independently calculated showing an exact correlation between the two methods to two decimal places. The change in electron transfer resistance is calculated according to equation 6.4 with respect to the baseline trace at each experimental concentration.

A linear-type non-specific response is observed from 1 to 100 pg ml^{-1} PSA.

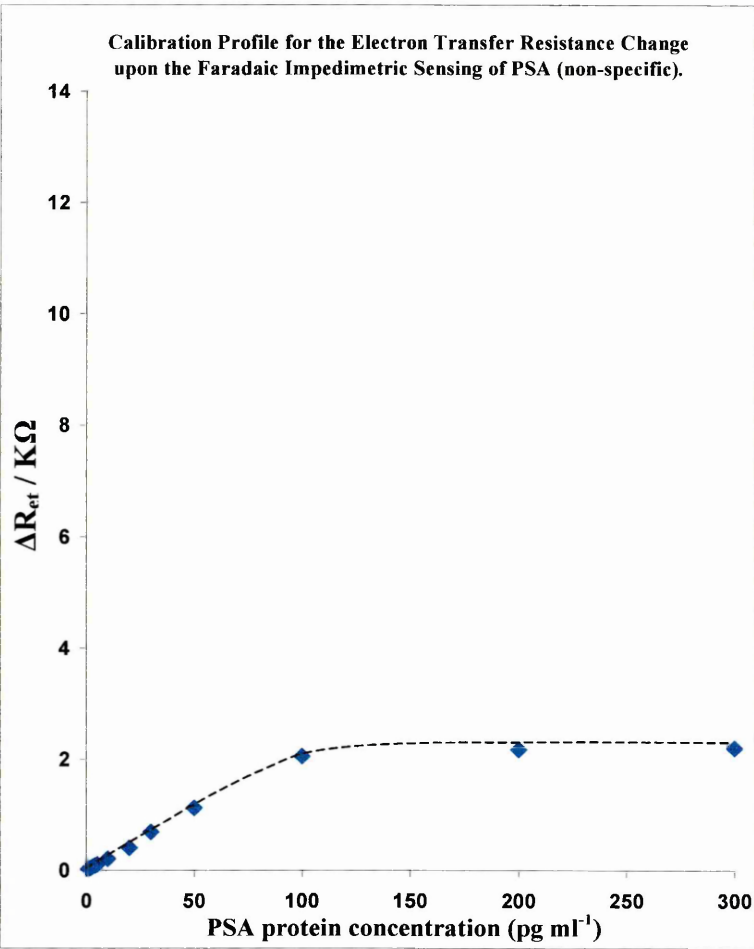


Fig 7.8: Calibration curve for the change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific). Refer to Fig 7.5 for direct comparison on same axes scale.

Upon comparison of Figure 7.8 with Figure 7.5 it is apparent that approximately 20% of the APSA-immobilised sensor response encountered was, in fact, non-specific. The calibration curve plateaus at approximately 100 pg ml⁻¹ PSA suggesting that an optimum electron transfer resistance change from the baseline response has been reached and non-specific occurrences are at their maximum.

7.3.3 'Corrected' specific response: APSA immunosensor.

To obtain a calibration profile, in terms of 'corrected' electron transfer resistance change for an APSA immunosensor, accounting for any non-specific responses the changes obtained in Figure 7.8 (non-specific) are subtracted from those obtained in Figure 7.5 (specific) for the entire analytical concentration range to yield Figure 7.9. This matched sensor approach enables a true understanding of the APSA-PSA immunosensor system. In total 10 APSA-immobilised sensors and 10 corresponding AlgG-immobilised sensors were interrogated over the concentration range, according to section 7.3.1 and section 7.3.2 respectively, to gauge the reproducibility of the observed responses.

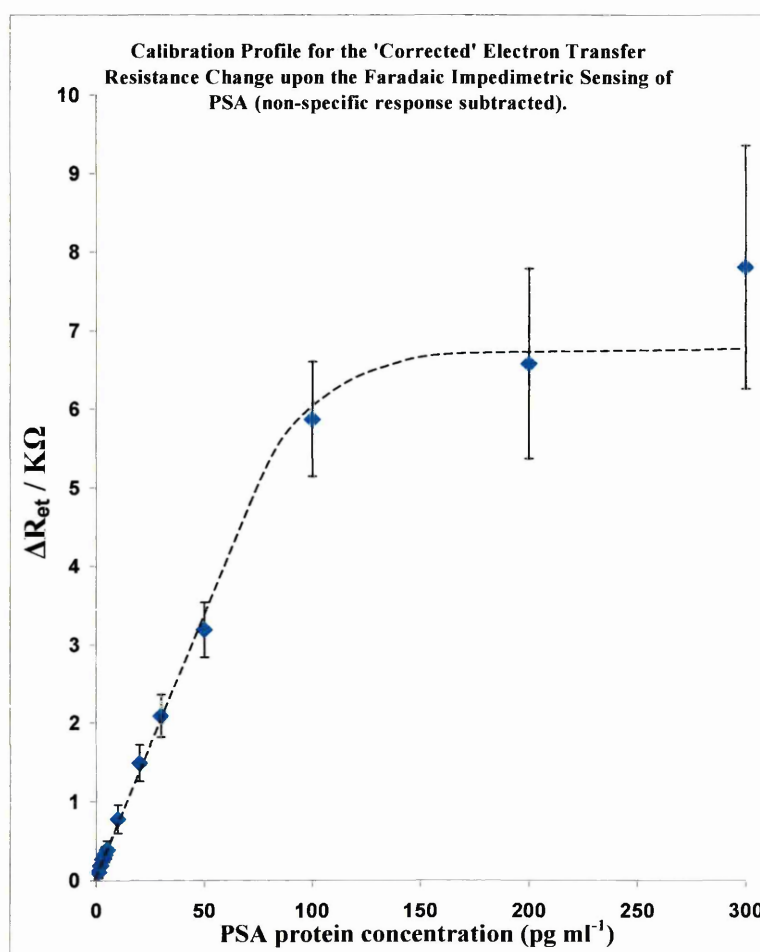


Fig 7.9: Calibration curve for the 'corrected' change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific response subtracted).
n = 10, error bars = standard deviation.

Figure 7.9 represents the ‘corrected’ profile for 10 replicate pairs of matched sensors. Identical experimental procedures were employed throughout investigations and all parameters were kept consistent. The mean electron transfer resistance response at each experimental analyte concentration was plotted. The error bars indicate the standard deviation obtained for the 10 matched sensor pairs from the mean ‘corrected’ values. The resultant response is due entirely to the interaction of APSA with PSA analyte and arises from the effect on the electron transfer resistance that the formation of stable antibody-antigen complexes at the sensor surface generates.

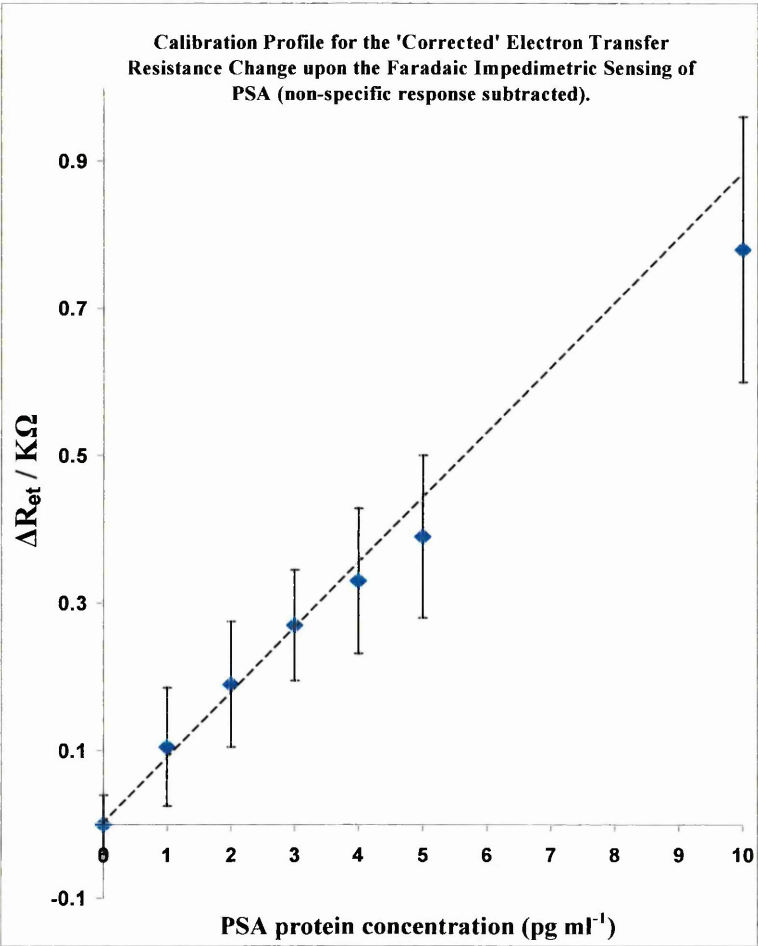


Fig 7.10: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of PSA antigen concentrations (non-specific response subtracted). n = 10, error bars = standard deviation.

For clarity Figure 7.10 represents the calibration curve from 1 to 10 pg ml^{-1} PSA. At these concentrations the response, in terms of electron transfer resistance change from the baseline trace (0ng ml^{-1} PSA) was fairly linear.

From Figure 7.9 a linear-type response was also observed from 1 to approximately 100 pg ml^{-1} PSA. At concentrations above 100 pg ml^{-1} the electron transfer resistance change no longer relates to antigen concentration and the curve begins to tend towards a plateau. The sensor response approaches saturation and stable antibody-antigen complex formation was absolute.

The reproducibility of the 'corrected' response obtained from the standard deviation for 10 matched sensor pairings enables discrimination to be possible at 5 pg ml^{-1} PSA antigen analyte. The limit of detection of the APSA immunosensor is 1.4 pg ml^{-1} PSA.

7.3.4 APSA immunosensor: Reversibility studies.

The regeneration of the binding sites of the antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilised antibodies and insidious signal drift problems. Several approaches were investigated with varying degrees of success. A range of concentrations of differing buffer species (pH 1 – 3) and differing washing regimes were employed and the most effective procedure is reported.

5 APSA-immobilised sensors and 5 matched non-specific AIGG-immobilised sensors were exposed to the full interrogation procedure prior to reversibility investigations. That is, the sensors were exposed to the full range of PSA antigen concentrations for 30 minutes at each concentration and in between concentrations a washing / flushing of weakly-bound or non-specifically adsorbed matter from the sensor surface was performed before the impedance trace is recorded in redox-containing buffer.

Once the trace was recorded for both APSA & IgG-immobilised sensors at a particular concentration, the experimental protocol was continued up to a concentration of 300 pg ml^{-1} .

The 5 sensor pairs under investigation formed part of the 10 sensor pairings analysed to obtain the data in Figures 7.9 and 7.10. A mean 'corrected' electron transfer change for each concentration was obtained with a standard deviation for the 5 pairs. After the regeneration treatment the same experimental procedure was followed again to assess the recovery of the initial response in terms of 'corrected' electron transfer change over the concentration range. If the Faradaic response can be restored the sensor has potential for reusability. 5 regenerations were attempted and the saturation concentration of 100 pg ml^{-1} PSA was selected for detailed analysis.

In order to split the antibody-antigen complex and to regenerate the sensors, 0.1M HCl acidic buffer (pH 2.3) is applied for 3 separate 1 minute time periods. In between each acidic buffer exposure pH 7.4 PBS was used to rinse the sensor surface. Antigens are released from the sensor surface and antibodies are still bound when the APSA antibody / PSA antigen immunocomplexes are disrupted. Finally the sensor was rinsed with 50ml PBS to produce the PSA-free sensors and the experimental EIS analysis could be repeated.

The results in Figure 7.11 indicate that negligible changes in 'corrected' electron transfer response at the saturation concentration of PSA were encountered after the first regeneration for the 5 sensor pairings. Both specific and non-specific sensors are showing reproducible behaviour. For 100 pg ml^{-1} PSA the mean 'corrected' electron transfer change and the standard deviation were comparable to the initial response obtained. For one sensor regeneration the response was reversible.

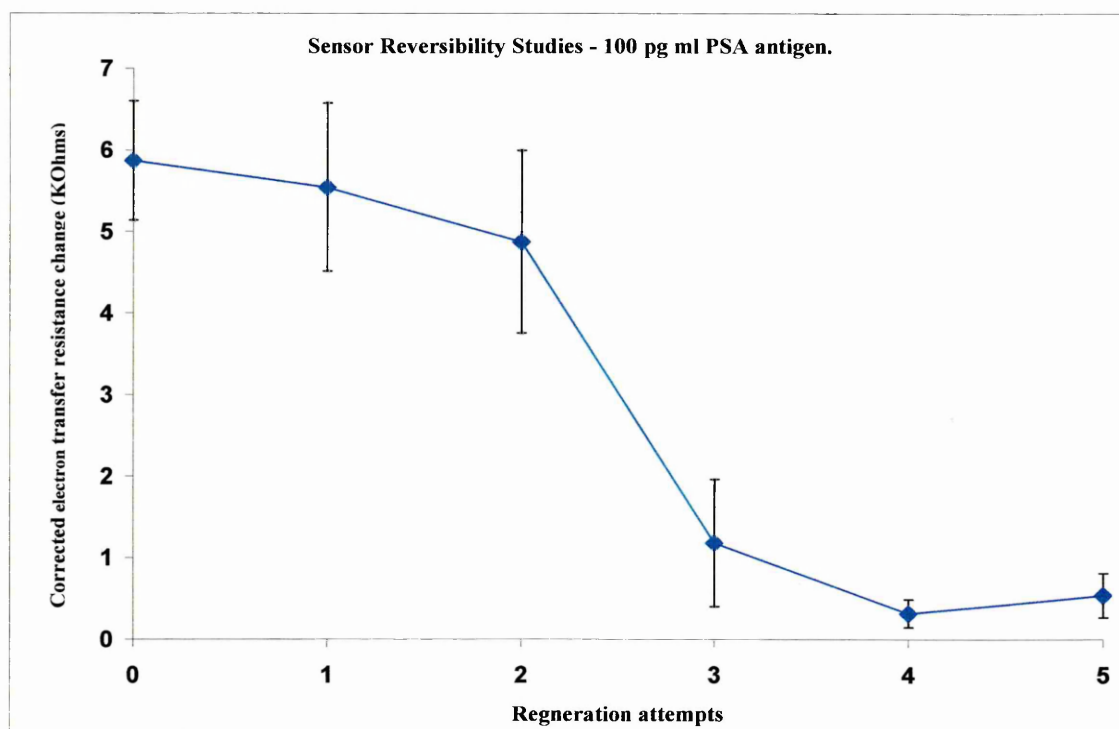


Fig 7.11: Recovery of sensor response following a number of regeneration attempts.

After the second regeneration it is apparent that the ‘corrected’ electron transfer change has been slightly reduced with a response approximately 85% that of the initial response. The standard deviation is comparable. However, following the second regeneration and recovery of response there is a dramatic reduction in sensitivity. All subsequent regeneration attempts for the 5 sensor pairings show little variation from the baseline response. The fact that the baseline response is near identical to the initial baseline response suggests that the PANI-modified microelectrode array sensor surface remains intact; however, all antibody activity has been lost.

The reversible phenomenon observed after two sensor regeneration attempts can be attributed to the restoration of the availability of the binding sites of the antibody after a short-time acidic washing. Unfortunately, repeated washing of the sensing layer reduced the activity of the antibody, which can explain the decrease of the sensitivity of the sensors after the second regeneration.

Subsequent attempts to regenerate the surface show that further exposure to the acidic conditions removes all analyte recognition properties and the antibody no longer acts as the receptor component of the immunosensor.

The results are still significant in the fact that they demonstrate the potential for the development of the regeneration process. Further work should yield an optimum protocol for further, or perhaps continual, true reversibility. Greater initial response recovery over a greater number of regenerations would be highly beneficial. The phenomenon of reversible regeneration at immunosensor surfaces is a property that is rarely encountered and is a highly desirable asset of these APSA immunosensors.

7.3.5 APSA immunosensor: Stability studies.

The stability of the APSA-PSA immunosensor system was assessed from storage in dry state, at 4°C, for a period of 12 weeks. An identical experimental protocol was employed for electrochemical impedance interrogation (section 3.5.5) and the 'corrected' electron transfer change was the analysis method for response recovery. Initially 18 matched sensor pairings of APSA (specific) and AIGG (non-specific) immunosensors are refrigerated at 4°C in sealed, sterilised vessels. Every 2 weeks a set of 3 matched sensor pairings was removed from storage and interrogated over the full PSA antigen analyte concentration range from 1 to 300 pg ml⁻¹.

Figure 7.12 shows the data obtained at each time interval and the saturation concentration of 100 pg ml⁻¹ PSA was chosen as a means of final evaluation of sensor stability and the mean change in 'corrected' electron transfer resistance response is shown plotted against storage time. The error bars indicate the standard deviation for the three sensor pairs.

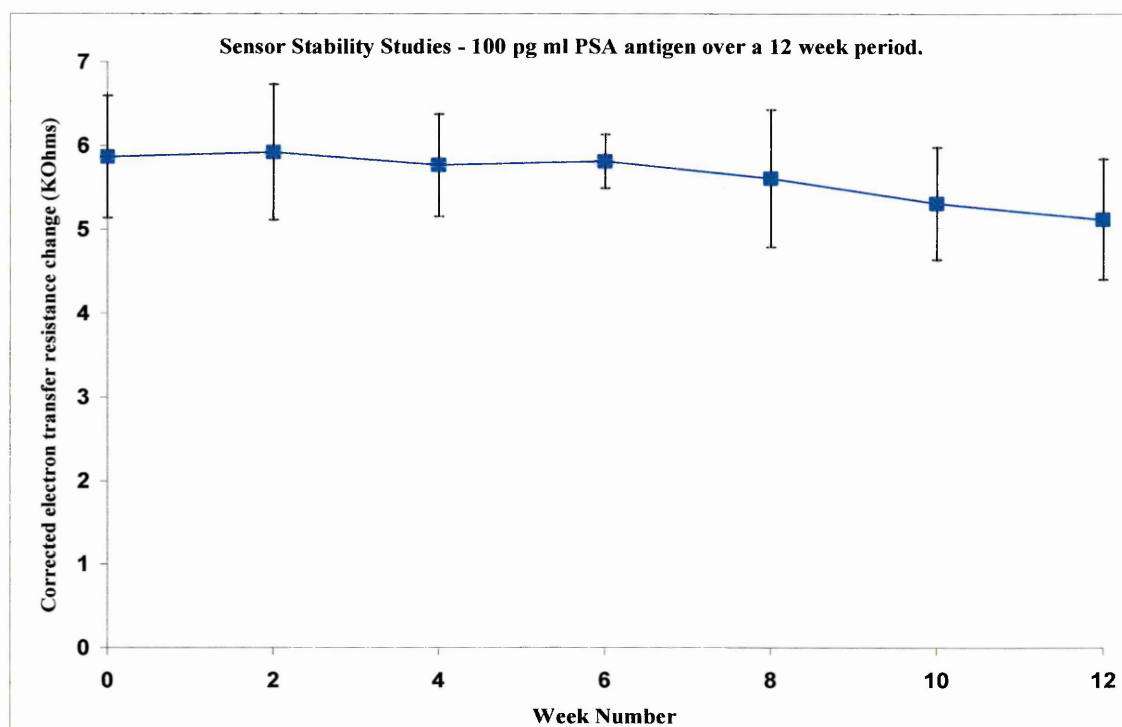


Fig 7.12: Corrected electron transfer resistance change at 100 pg ml⁻¹ PSA.
Stability study – Variation over 12 week period. ($n = 3$, error bars = SD).

Figure 7.12 shows that the sensor response is fully recovered after 12 weeks in storage with negligible difference from the initial response at $t = 0$. The activity of the antibody receptor biocomponent is unaffected by storage over time. The results lend themselves well to possible packaging and suggest a satisfactory shelf-life.

7.3.6 APSA immunosensor: Further non-specific studies.

Impedimetric immunosensors very often suffer from non-specific adsorption of biomolecules (e.g., proteins) accompanying the analyte. Special attempts are made to minimise non-specific adsorption phenomenon and its influence on impedance measurements. The matched sensors are essentially dual-experimental cells operating as two detection channels: with and without receptors specific to the target analyte. HSA is also co-immobilised in the immunosensing layer to decrease non-specific binding of proteins, acting as an interferent.

The sensor responses obtained throughout investigations were obtained in buffered test solutions containing only target analyte and no other interfering proteins.

Immunosensor should demonstrate sensitivity and specificity in the presence of foreign biological materials that could be adsorbed on the electrode surface non-specifically.

Five differing protein analytes were added to the test solution and 10 sensor pairings were analysed over the full concentration ranges for the antibody / antigen system using the protocol described in section 3.5.5. Aside from the foreign species present in test solutions the experimental protocol is repeated in an identical manner. 10 ng ml⁻¹ BSA, HSA, CA125 (tumor marker), NSE & S-100 are added for PSA sensing.

In this case results did not differ significantly from the original calibration figures (Figure 7.9 and Figure 7.10). The mean 'corrected' electron transfer changes showed a variation of less than 5% for all concentrations and standard deviations that were comparable to the original.

These results demonstrate that the modified electrodes keep biosensing abilities after their exposure to target analyte in a test solution containing multiple foreign species. Thus, adsorption of BSA / HSA / CA125 / NSE and S-100 and any non-specific interactions at the sensor surface do not proceed or do not affect significantly the biorecognition events (stable immunocomplex formation) and their impedance response. The matched AIgG sensor (non-specific) response does not alter so it is postulated that the HSA blocking during fabrication minimises non-specific effects almost totally. The AIgG sensor response is thus not due to the presence of the target analyte or the presence of foreign proteins. This control sensor must be responding to any other effects at the sensor surface that may be occurring such as changes in the polymer or effects from the solution itself on the sensor. In any case, the effects are always minor compared to the signal obtained when a sensor immobilised with a specific receptor antibody is exposed to its target analyte. Again these results are promising for prototype development since they suggest that sensing of target in 'real' samples is a plausible proposal.

7.3.7 APSA immunosensor: Conclusions.

The results obtained verify the fabrication techniques and experimental interrogation protocol as a viable approach towards the labelless sensing of markers for TIA. The APSA-PSA immunosensor system gave a low non-specific response associated with the faradaic sensing of PSA. The APSA immunosensor was reversible for up to two regeneration attempts before the acid treatment had a detrimental effect on the performance of the immobilised antibody as a receptor biocomponent. The stability of the sensor assemblies was encouraging with only a slight loss of sensitivity after storage for twelve weeks at 4°C in dry state.

The APSA immunosensor gave a 'corrected' linear-type response from 1 to 100 pg ml⁻¹. The higher the PSA concentration in the test solution the greater the number of stable antibody-antigen complexes are being formed at the sensor surface. The complex formation introduces a barrier to the electron transfer of the redox couple, present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration.

At concentrations of analyte above 100 pg ml⁻¹ the number of complexes formed begins to plateau as the sensor approaches saturation. The recognition entities become occupied with analyte and active sites for further antigen binding are no longer available. The limit of detection of the APSA-PSA immunosensor system was found to be 1.4 pg ml⁻¹ and there was an ability to discriminate at 5 pg ml⁻¹ PSA analyte which is of clear significance for the clinical diagnosis of prostate cancer (Fernandez-Sanchez, 2005).

The low quantification levels and limits of detection for PSA has illustrated the potential use of these immunosensor assemblies for the labelless detection of markers for TIA. Several other highly beneficial properties are also associated with the immunosensors and it is hoped that the techniques and technology employed are generic in nature when applied to further biochemical systems.

7.4 Faradaic impedimetric interrogation of PANI ‘mushroom’ microelectrode array sensors: AS-100 immunosensor studies.

S-100 is a recognised biochemical marker of brain disease and as such is an early indicator of transient ischemic attacks. Rapid detection and quantification of S-100 can help diagnose and differentiate stroke type and severity and can prevent further attacks of greater severity via the introduction of the appropriate thrombolytic therapy.

Following immobilisation of antibody, AS-100, according to section 5.3 impedance analyses were performed from 1Hz to 10,000Hz ($\pm$ 5mV amplitude perturbation) in pH 7.8 phosphate buffer, i.e. containing no antigen, as a baseline trace. This buffer solution did, however, contain a 50:50 mixture of $[\text{KFe}(\text{CN})_6]^{3-/4-}$, at a concentration of 10mM as redox mediator so as to perform faradaic impedance spectroscopy. The potential of the electrochemical cell is offset to the formal potential of the redox probe (+0.22V vs. Ag/AgCl). Subsequently, AS-100 doped sensors are exposed to a range of concentrations from 1 to 300 pg ml^{-1} S-100. S-100 antigen was suspended in pH 7.8 phosphate buffers containing $[\text{KFe}(\text{CN})_6]^{3-/4-}$ at pre-prepared analyte concentrations of 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 and 300 pg ml^{-1} . The pH 7.8 buffers drop to pH 7.4 upon suspension of S-100 and this resultant pH was utilised to coincide with human physiological pH. The experimental protocol developed and described in section 3.5.5 is applied for the electrochemical impedimetric interrogation of AS-100 immunosensors. A consistent sensor is used for all analytical concentrations and changes encountered are compared to the initial baseline trace (0 pg ml^{-1} S-100).

Results are presented in the form of Nyquist plots depicting the faradaic ($Z'_{(\text{re})}$) and capacitive ($-Z''_{(\text{im})}$) components of impedance and their variation throughout the recording of the ac impedance spectra.

7.4.1 AS-100 immunosensor: Specific recognition of S-100 antigen.

Electrochemical Impedance Spectra obtained for an AS-100-doped PANI mushroom microelectrode array sensor exposed to increasing S-100 concentrations.

Figure 7.13 shows the specific response to S-100 for a novel AS-100-immobilised, PANI modified, carbon microelectrode array sensor assembly. The concentration of S-100 analyte represented is 1 to 10 pg ml^{-1} .

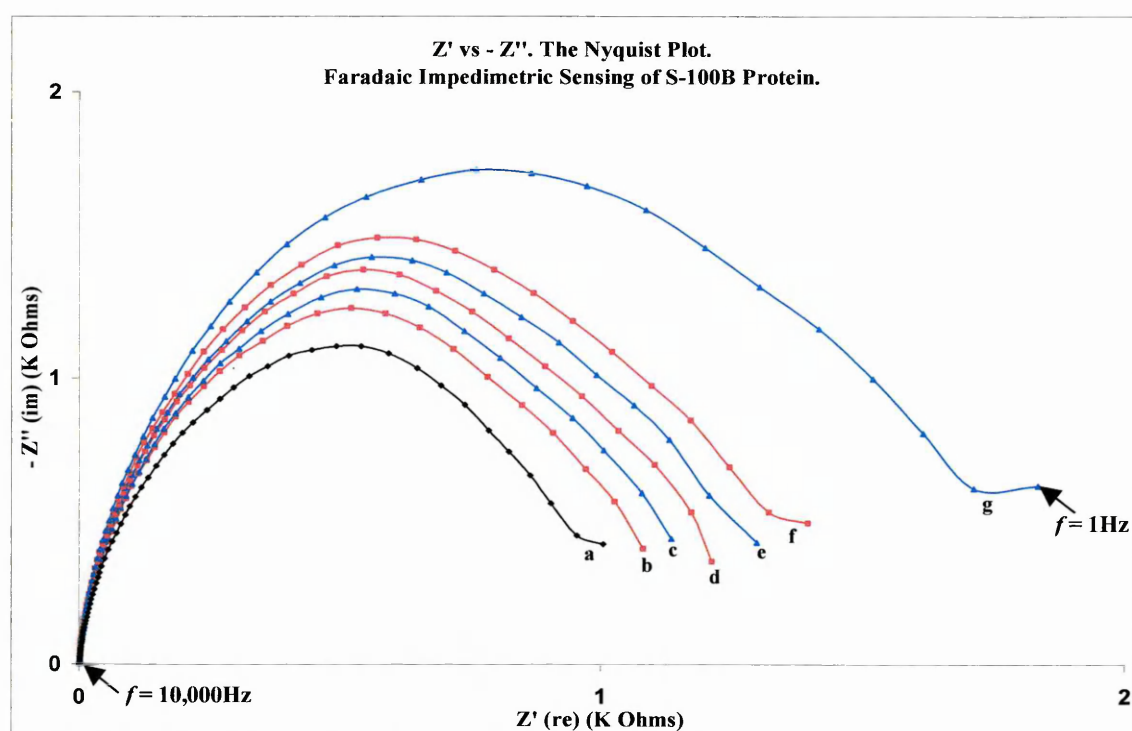


Fig 7.13: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AS-100-doped PANI immunosensor following exposure to various concentrations of S100. The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 pg ml^{-1} S100.

For the baseline trace, the $Z'_{(re)}$ component is seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the lower applied frequencies ($\sim 1\text{Hz}$).

In general, the impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow and the impedance is controlled by the interfacial electron transfer (Katz & Willner, 2003).

Figure 7.14 represents the specific response to S-100 analyte at the higher concentrations of the experimental range up to 300 pg ml^{-1} S-100 antigen.

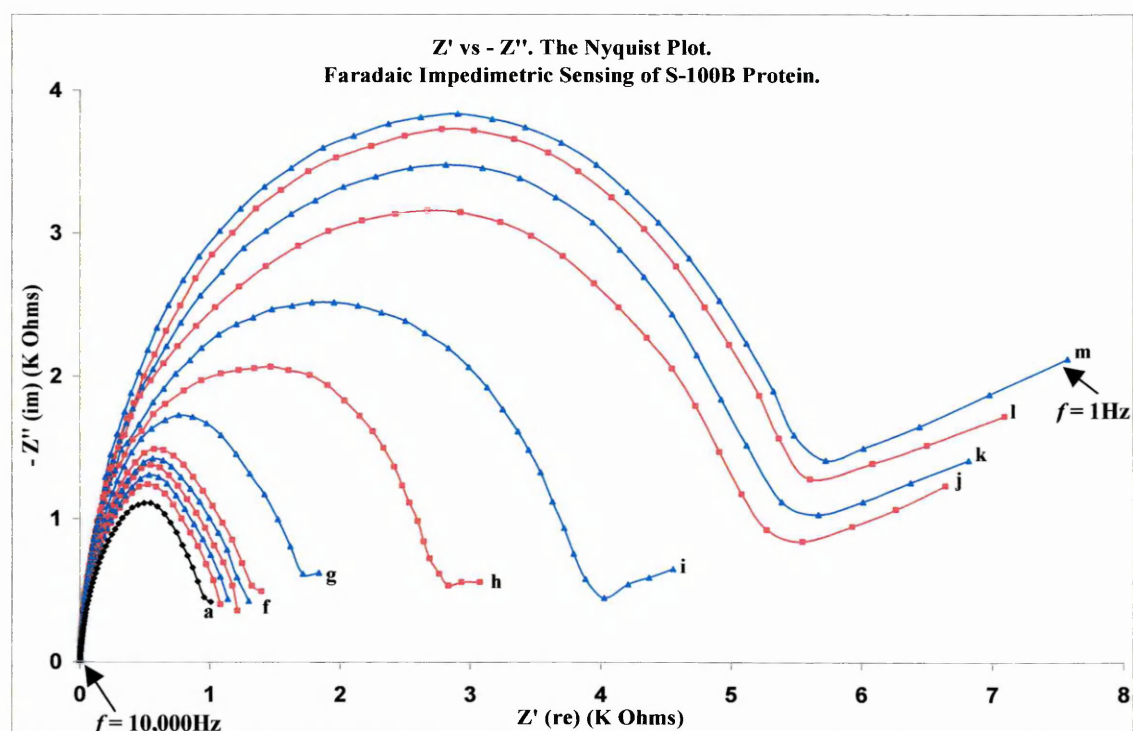


Fig 7.14: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AS100-doped PANI immunosensor following exposure to various concentrations of S100. The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml^{-1} S100.

Again the Nyquist curves follow the same general format as those in Figure 7.13. A computer fitting of the experimental data to a theoretical model, represented by a simple equivalent circuit, is performed by the software provided with the frequency response analyser as data is extracted by the instrument. For a more thorough evaluation of the data obtained in Figures 7.13 and 7.14, the changes in the Nyquist curves are translated into electron transfer resistance changes to provide a clear and consistent format.

Experimentally determined and simulated values of electron transfer resistance were compared, showing a 100% correlation to two decimal places, and are plotted in Figure 7.15.

Figure 7.15 depicts the electron transfer resistance change calculated with respect to the baseline trace (0 pg ml⁻¹). Equation 6.4 details the calculation of the electron transfer resistance change.

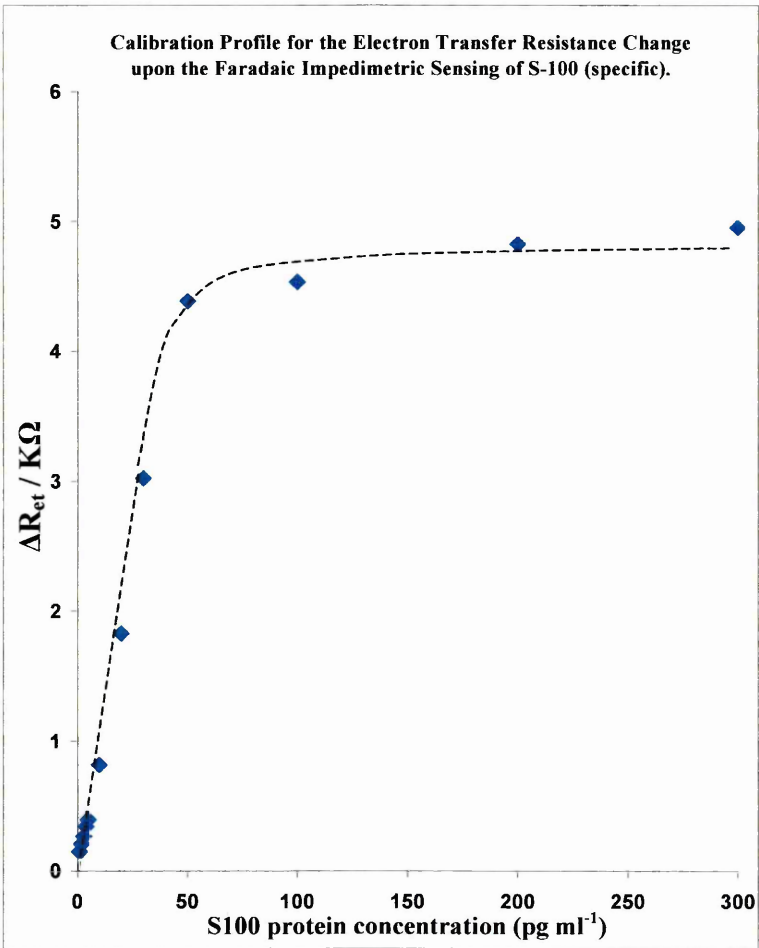


Fig 7.15: Calibration curve for the change in electron transfer resistance from the baseline response for a range of S100 antigen concentrations (specific).

Figure 7.15 shows that the AS-100 immunosensor gave a generally linear response to S-100 analyte from 1 $pg\ ml^{-1}$ to approximately 50 $pg\ ml^{-1}$ S-100.

The electron transfer resistance increased with increasing antigen analyte concentration up to approximately 50 pg ml^{-1} .

The interaction of S-100 with the electrode modified with AS-100 resulted in significant insulation of the electrode reflected by the increase in the electron transfer resistance.

It is postulated that as antigen concentration is increased the number of complexes formed also increases and this is reflected by the observed electron transfer resistance increase.

The insulation of the modified electrode upon formation of stable AS-100-S-100 immunocomplexes hinders the electron transfer kinetics of the redox probe resulting in the increase of electron transfer resistance. The electron transfer resistance increases with increasing antigen concentration in a linear-type response from 1 to approximately 50 pg ml^{-1} . The sensitivity of the specific AS-100 immunosensor was 1 pg ml^{-1} . There is negligible change from 50 pg ml^{-1} to 300 pg ml^{-1} suggesting that the immunosensor approaches saturation and stable antibody-antigen complex formation no longer relates to the analyte concentration.

7.4.2 AIG immunosensor: S-100 non-specific control investigations.

Electrochemical Impedance Spectra obtained for an AIG-doped PANI mushroom microelectrode array sensor exposed to increasing S-100 concentrations.

Experiments were repeated using a matched AIG-immobilised sensor in which the antibody is non-specific to the target analyte S-100. Otherwise, identical experimental procedures were employed and all parameters were kept consistent. Figures 7.16 and 7.17 represent the AIG sensor response to S-100 antigen from 1 to 300 pg ml⁻¹ and are presented using the same axes scale as Figures 7.13 and 7.14 respectively to enable a direct comparison with the specific sensor response. Figure 7.16 represents the non-specific sensor response from 1 to 10 pg ml⁻¹ S-100. The experiments serve to validate the observed specific response of section 7.4.1 and account for all non-specific contributions to the sensing of S-100.

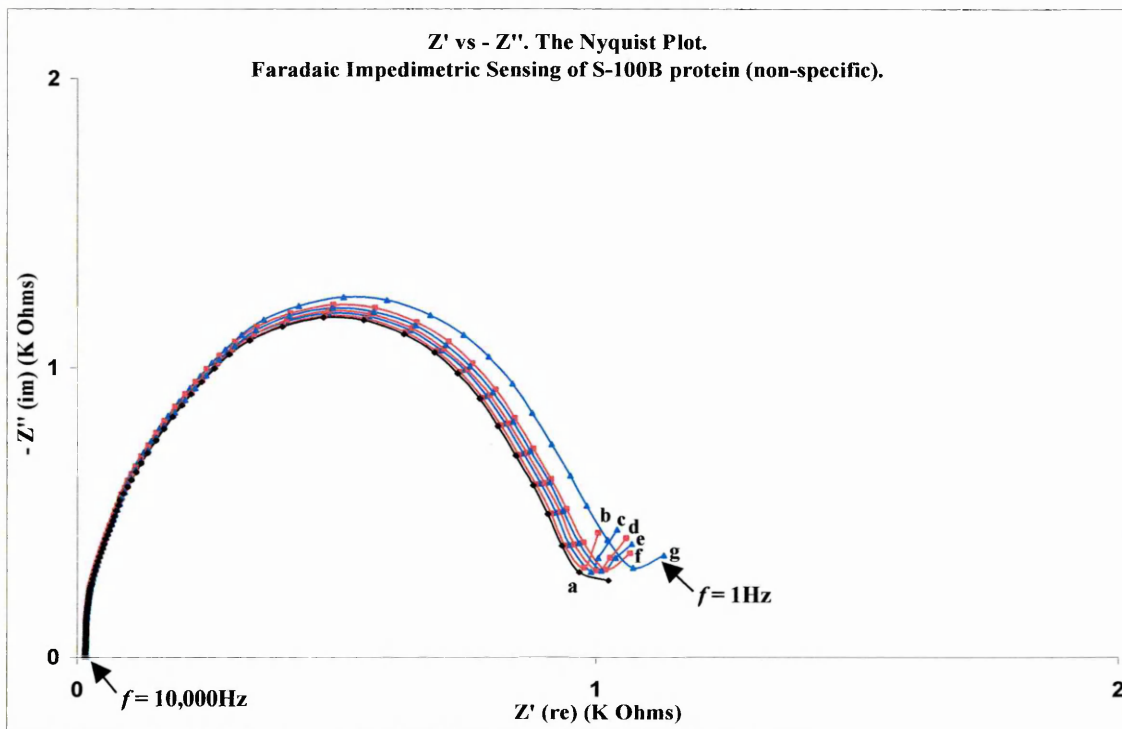


Fig 7.16: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AIG-doped PANI immunosensor following exposure to various concentrations of S100. The concentration of antigen (pg ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 pg ml⁻¹ S100. Refer to Fig 7.13 for direct comparison on same axes scale.

Figure 7.17 represents the non-specific sensor response from 1 to 300 pg ml⁻¹ S-100.

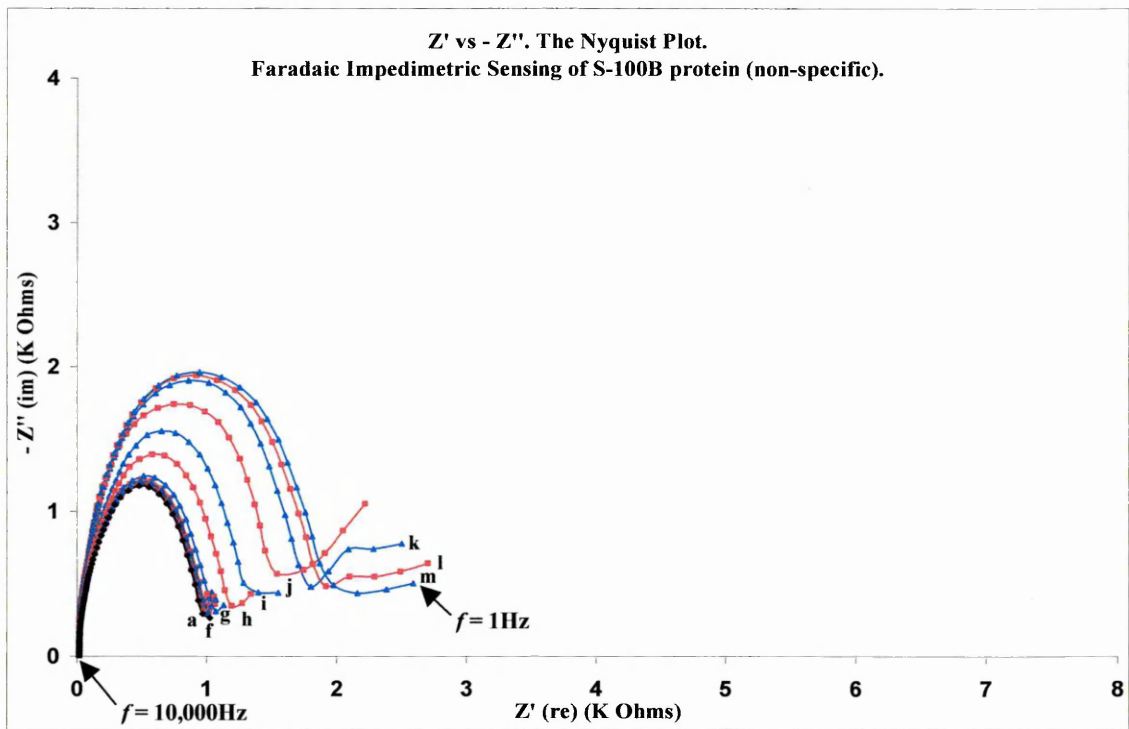


Fig 7.17: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel AlG-doped PANI immunosensor following exposure to various concentrations of S100. The concentration of antigen (pg ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml⁻¹ S100. Refer to Fig 7.14 for direct comparison on same axes scale.

All impedimetric spectral traces obtained follow the same general form and similar shaped Nyquist curves are obtained, indicative of a surface-modified electrode system where the electron transfer is slow and controlled by interfacial electron transfer.

For clarity, Figure 7.18 shows the non-specific experimental data presented as the relative change in electron transfer resistance from the baseline trace (0 ng ml⁻¹ S-100) for each concentration analysed. Figure 7.18 has the same y-axis scale as Figure 7.15.

The electron transfer resistance values are extracted from the instrument and also independently calculated showing a 100% correlation to two decimal places. The change in electron transfer resistance is calculated according to equation 6.4 with respect to the baseline trace at each experimental concentration.

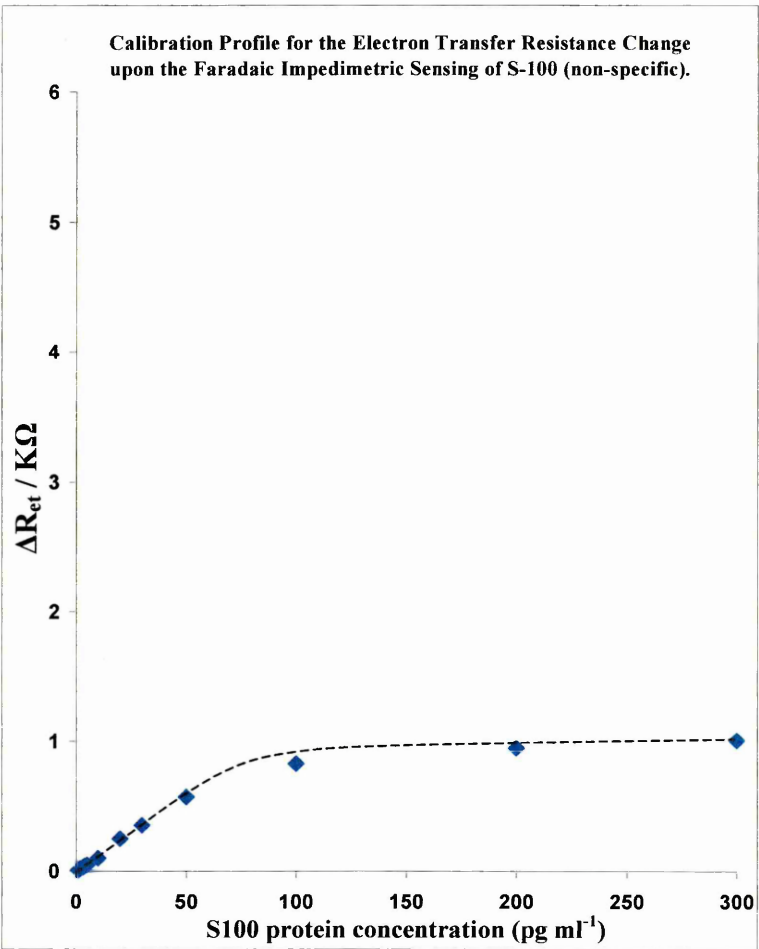


Fig 7.18: Calibration curve for the change in electron transfer resistance from the baseline response for a range of S100 antigen concentrations (non-specific). Refer to Fig 7.15 for direct comparison on same axes scale.

A linear-type non-specific response was observed from 1 to 100 pg ml⁻¹ S-100. Upon comparison of Figure 7.18 with Figure 7.15 it was apparent that approximately 15% of the APSA-immobilised sensor response encountered was, in fact, non-specific. The calibration curve plateaus at approximately 100 pg ml⁻¹ S-100 suggesting that an optimum non-specific electron transfer resistance change from the baseline response has been reached.

7.4.3 ‘Corrected’ specific response: AS-100 immunosensor.

To obtain a calibration profile, in terms of ‘corrected’ electron transfer resistance change for an AS-100 immunosensor, accounting for any non-specific responses the changes obtained in Figure 7.18 (non-specific) are subtracted from those obtained in Figure 7.15 (specific) over the entire analytical concentration range. This matched sensor approach enables a true understanding of the AS-100-S-100 immunosensor system.

Figure 7.19 represents the ‘corrected’ profile for 10 replicate pairs of matched sensors.

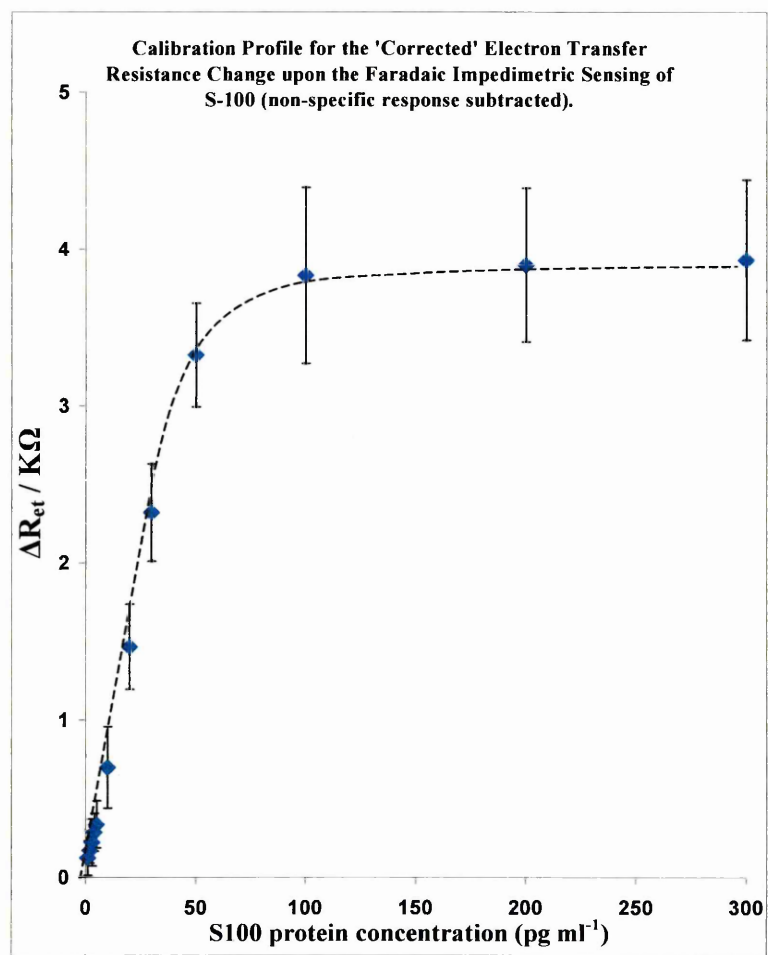


Fig 7.19: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of S100 antigen concentrations (non-specific response subtracted). n = 10, error bars = standard deviation.

In total 10 AS-100-immobilised sensors and 10 corresponding AlgG-immobilised sensors were interrogated over the concentration range, according to section 7.4.1 and section 7.4.2 respectively, to permit assessment of the reproducibility of the observed responses.

Identical experimental procedures were employed throughout investigations and all parameters were kept consistent. The mean electron transfer resistance response at each experimental analyte concentration was plotted. The error bars indicate the standard deviation for the 10 matched sensor pairs from the mean ‘corrected’ values obtained at each concentration. The resultant response is due entirely to the interaction of AS-100 with S-100 analyte and arises from the effect on the electron transfer resistance that the stable antibody-antigen complexes at the sensor surface generate.

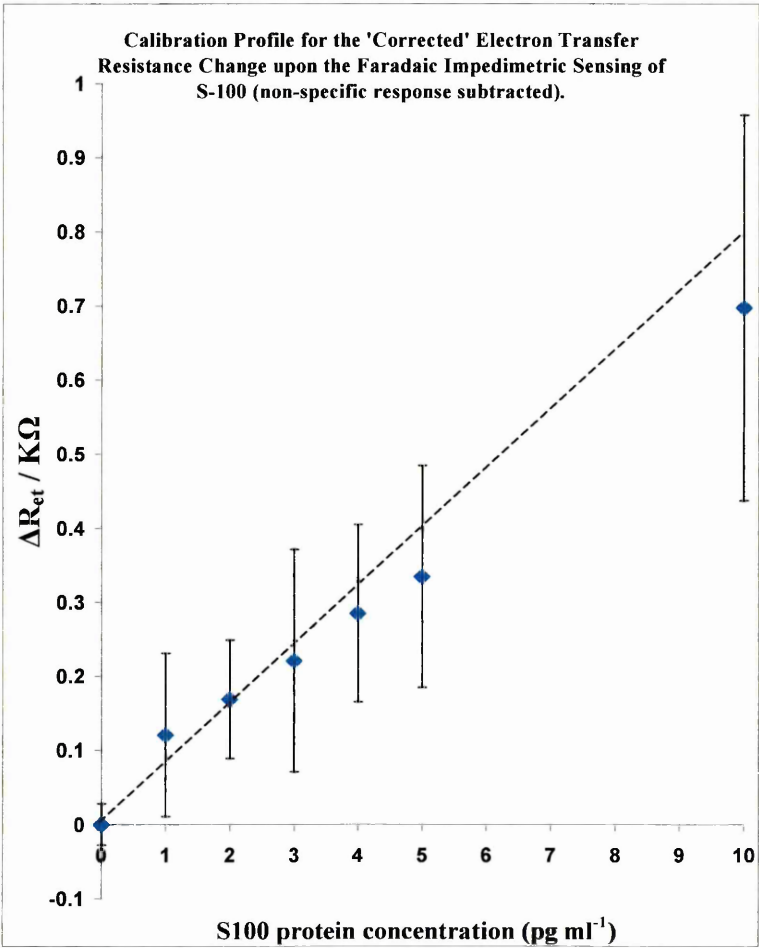


Fig 7.20: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of S100 antigen concentrations (non-specific response subtracted). n = 10, error bars = standard deviation.

For clarity Figure 7.20 represents the calibration curve from 1 to 10 pg ml^{-1} S-100. At these concentrations the response, in terms of electron transfer resistance change from the baseline trace (0 ng ml^{-1} S-100) was fairly linear. From Figure 7.19 a linear-type response was also observed from 1 to approximately 50 pg ml^{-1} S-100. At concentrations above 50 pg ml^{-1} the electron transfer resistance change no longer relates to antigen concentration and the curve begins to tend towards a plateau. The sensor response approaches saturation and stable antibody-antigen complex formation was absolute.

The reproducibility of the 'corrected' response obtained from the standard deviation for 10 matched sensor pairings enables discrimination to be possible at 10 pg ml^{-1} S-100 antigen analyte. The limit of detection of the AS-100 immunosensor was 1 pg ml^{-1} S-100.

7.4.4 AS-100 immunosensor: Reversibility studies.

5 AS-100-immobilised sensors and 5 matched non-specific AIgG-immobilised sensors were exposed to the full interrogation procedure prior to reversibility investigations. That is, the sensors were exposed to the full range of S-100 antigen concentrations for 30 minutes at each concentration and in between exposure to differing concentrations a washing / flushing of weakly-bound or non-specifically adsorbed matter from the sensor surface was performed before impedance traces were recorded in redox-containing buffer. Once the trace was recorded for both AS-100 & IgG-immobilised sensors at a particular concentration, the experimental protocol was continued up to a concentration of 300 pg ml^{-1} .

The 5 sensor pairs under investigation formed part of the 10 sensor pairings analysed to obtain the data in Figures 7.19 and 7.20. A mean 'corrected' electron transfer change for each concentration was obtained with a standard deviation for the 5 pairs. Following the regeneration treatment, the same experimental procedure is followed again to assess the recovery of the initial response in terms of 'corrected' electron transfer change over the concentration range.

If the faradaic response can be restored the sensor has potential for reusability. 5 regenerations were attempted and the saturation concentration of 50 pg ml⁻¹ S-100 was selected for detailed analysis.

In order to split the antibody-antigen complex and to regenerate the sensors, 0.1M HCl acidic buffer (pH 2.3) is applied for 3 separate 1 minute time periods. In between each acidic buffer exposure pH 7.4 PBS was used to rinse the sensor surface. Antigens are released from the sensor surface and antibodies are still bound when the AS-100 antibody / S-100 antigen immunocomplexes are disrupted. Finally the sensor was rinsed with 50ml PBS to produce the S-100-free sensors and the experimental EIS interrogation could be repeated.

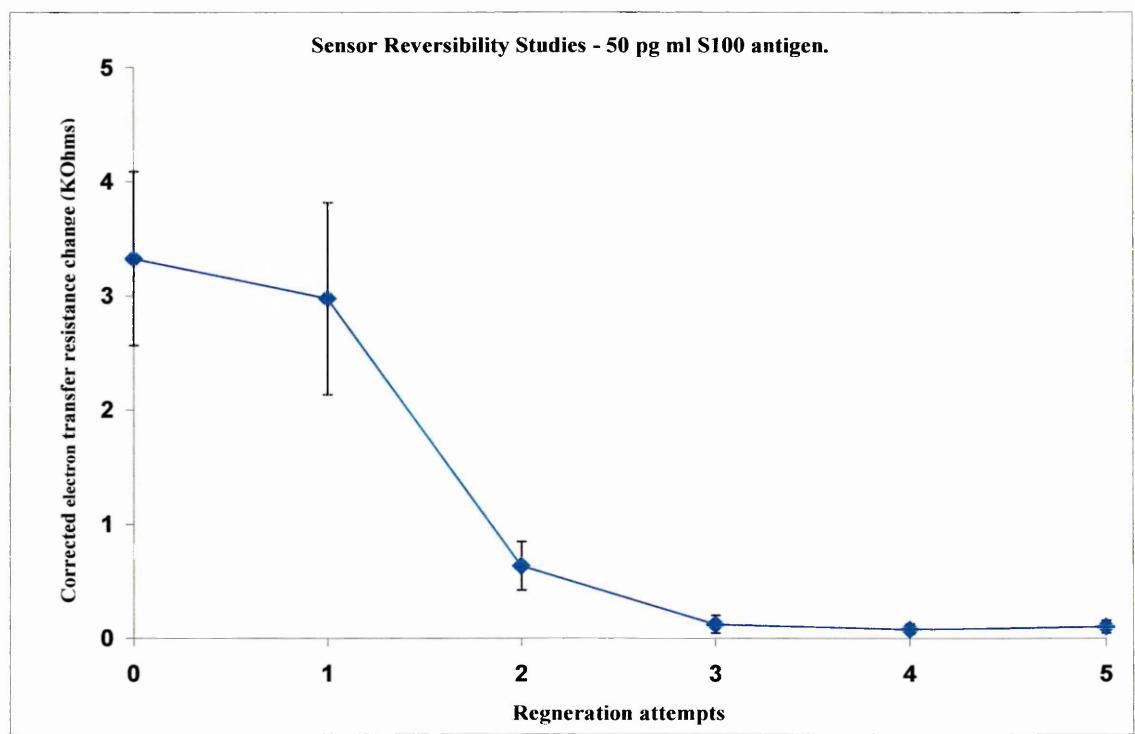


Fig 7.21: Recovery of sensor response following a number of regeneration attempts.

The results in Figure 7.21 indicate that negligible changes in ‘corrected’ electron transfer response at the saturation concentration of S-100 are encountered after the first

regeneration for the 5 sensor pairings. Both specific and non-specific sensors are showing reproducible behaviour.

For 50 pg ml⁻¹ S-100 the mean 'corrected' electron transfer change and the standard deviation were comparable to the initial response obtained. For one sensor regeneration the response was reversible.

After the first regeneration it is apparent that the 'corrected' electron transfer change has been dramatically reduced with a response approximately 15% that of the initial response. Following the initial regeneration and recovery of response there was a reduction in sensitivity. All subsequent regeneration attempts for the 5 sensor pairings show little variation from the baseline response. After the first regeneration the PANI-modified microelectrode array sensor surface remains intact, however, all antibody activity has been lost.

The reversible phenomenon observed after one sensor regeneration attempt can be attributed to the restoration of the availability of the binding sites of the antibody after a short-time acidic washing. Unfortunately, repeated washing of the sensing layer reduced the activity of the antibody, which can explain the decrease of the sensitivity of the sensors after the initial regeneration. Subsequent attempts to regenerate the surface show that further exposure to the acidic conditions removes all analyte recognition properties and the antibody no longer acts as the receptor component of the immunosensor.

The results are still significant in the fact that they demonstrate the potential for the development of the regeneration process. Further work should yield an optimum protocol for further, or perhaps continual, true reversibility. Greater initial response recovery over a greater number of regenerations would be highly beneficial. The phenomenon of reversible regeneration at immunosensor surfaces is a property that is rarely encountered and is a highly desirable property of these AS-100 immunosensors.

7.4.5 AS-100 immunosensor: Stability studies.

The stability of the AS-100-S-100 immunosensor system was assessed from storage in dry state, at 4°C, for a period of 12 weeks. An identical experimental protocol is employed for electrochemical impedance interrogation (section 3.5.5) and the ‘corrected’ electron transfer change was the analysis method used for assessing response recovery. Initially 18 matched sensor pairings of AS-100 (specific) and AIgG (non-specific) immunosensors are refrigerated at 4°C in sealed, sterilised vessels. Every 2 weeks a set of 3 matched sensor pairings is removed from storage and interrogated over the full S-100 antigen analyte concentration range from 1 to 300 pg ml⁻¹.

Figure 7.22 shows the data obtained at each time interval and the saturation concentration of 50 pg ml⁻¹ S-100 was chosen as a means of final evaluation of sensor stability and the mean change in ‘corrected’ electron transfer resistance response is shown plotted against storage time. The error bars indicate the standard deviation for the three sensor pairs.

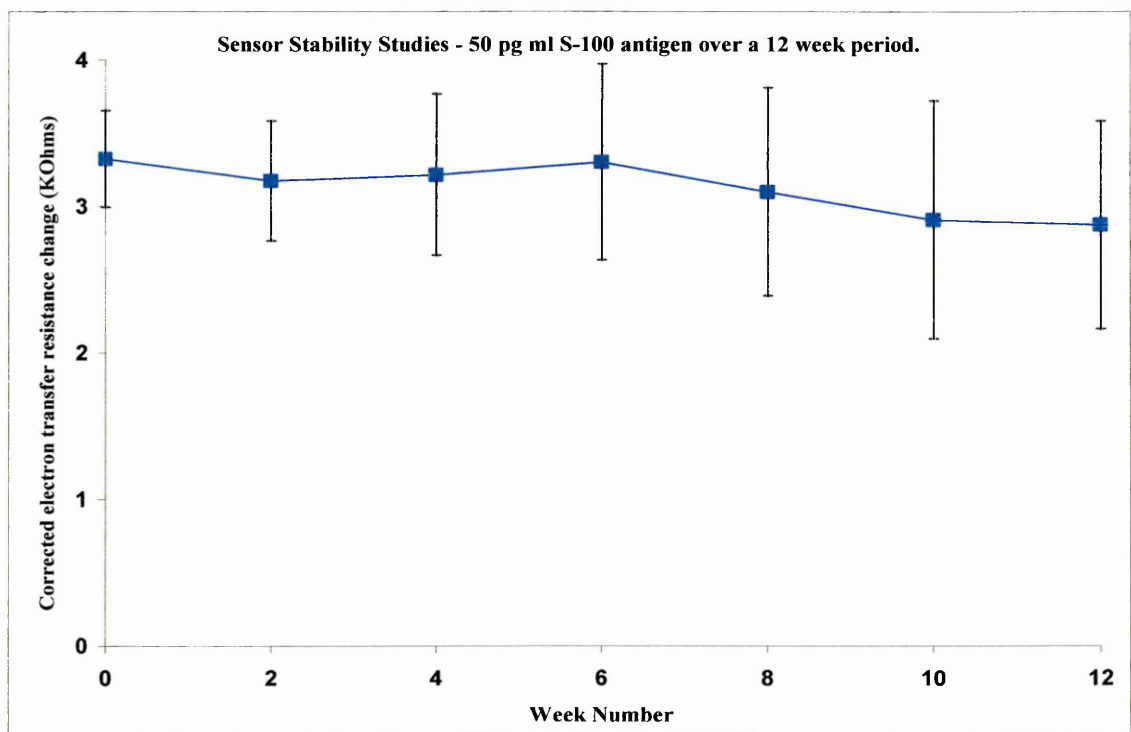


Fig 7.22: Corrected electron transfer resistance change at 50 pg ml⁻¹ S100. Stability study – Variation over 12 week period. (n = 3, error bars = SD).

Figure 7.22 shows that the sensor response is fully recovered after 12 weeks in storage with negligible difference from the initial response at $t = 0$. The activity of the antibody receptor biocomponent is unaffected by storage over time. The results lend themselves well to possible packaging and suggest a satisfactory shelf-life.

7.4.6 AS-100 immunosensor: Further non-specific studies.

Five differing protein analytes were added to the test solution and 10 sensor pairings were analysed over the full concentration ranges for the antibody / antigen system using the protocol described in section 3.5.5. Aside from the foreign species present in test solutions the experimental protocol was repeated in an identical manner. 10 ng ml^{-1} BSA, HSA, CA125 (tumor marker), NSE & PSA were added for S-100 sensing. In this case results did not differ significantly from the original calibration figures (Figure 7.19 and Figure 7.20). The mean 'corrected' electron transfer changes showed a variation of less than 5% for all concentrations and standard deviations that were comparable to the original.

These results demonstrate that the modified electrodes keep biosensing abilities after their exposure to target analyte in a test solution containing multiple foreign species. Thus, adsorption of BSA / HSA / CA125 / NSE and PSA and any non-specific interactions at the sensor surface do not proceed or do not affect significantly the biorecognition events (stable immunocomplex formation) and their impedance response. The matched AIgG sensor (non-specific) response does not alter so it is postulated that the HSA blocking during fabrication minimises non-specific effects almost totally. The AIgG sensor response is thus not due to the presence of the target analyte or the presence of foreign proteins. This control sensor must be responding to any other effects at the sensor surface that may be occurring such as changes in the polymer or effects from the solution itself on the sensor. In any case, the effects are always minor compared to the signal obtained when a sensor immobilised with a specific receptor antibody is exposed to its target analyte. Again these results are promising for prototype development as they suggest that sensing of target in 'real' samples is a plausible proposal.

7.4.7 AS-100 immunosensor: Conclusions.

The results obtained verify the fabrication techniques and experimental interrogation protocol as a viable approach towards the labelless sensing of markers for TIA and demonstrate the generic nature of the technology. The AS-100-S-100 immunosensor system gave a low non-specific response associated with the faradaic sensing of S-100. The AS-100 immunosensor was reversible for one regeneration attempt before the acid treatment has a detrimental effect on the performance of the immobilised antibody as a receptor biocomponent. The stability of the sensor assemblies yielded encouraging responses over time with only a slight loss of sensitivity after storage for twelve weeks at 4°C in dry state.

The AS-100 immunosensor gave a 'corrected' linear-type response from 1 to 50 pg ml⁻¹. The higher the S-100 concentration in the test solution the greater the number of stable antibody-antigen complexes formed at the sensor surface. The complex formation introduces a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration.

At concentrations of analyte above 50 pg ml⁻¹, the number of antibody-antigen couples formed begins to plateau as the sensor approaches saturation. The recognition entities, becoming occupied with analyte and active sites for further antigen binding, are no longer available. The limit of detection of the AS-100-S-100 immunosensor system was found to be 1 pg ml⁻¹ and there was an ability to discriminate at 10 pg ml⁻¹ S-100 analyte.

The low quantification levels and limits of detection for S-100 has illustrated the potential use of these immunosensor assemblies for the labelless detection of markers for TIA, with results spanning a concentration range of clinical significance for ischemic stroke diagnosis. At present, elevated levels of S-100 following TIA can be monitored from serum sample analysis via ELISA with detection limits of S-100 is 98 pg ml⁻¹ for quantification up to 6.3 ng ml⁻¹ (Cosmo Bio Co, Ltd). From optical immunoassay of

whole blood the detection limit is 250 pg ml^{-1} S-100 (Ettinger *et al*, 1999). S-100 immunosensors described here may be able to compete with these conventional methods when analysis of 'real' patient samples is performed, but offer advantages in terms of speed of response and the potential for use at point-of-care by non-skilled users. Non-specific studies have demonstrated the potential for operation in the presence of several foreign species without a detrimental effect on the sensitivity for the target analyte.

7.5 Faradaic impedimetric interrogation of PANI 'mushroom' microelectrode array sensors: ANSE immunosensor studies.

Neuron specific enolase is a recognised biochemical marker of brain disease and as such is an early indicator of transient ischemic attack. Rapid detection and quantification of NSE can help diagnose and differentiate stroke type and severity and can prevent further attacks of greater severity via the introduction of the appropriate thrombolytic therapy.

Following immobilisation of antibody, ANSE, according to section 5.3 impedance analyses were performed from 1Hz to 10,000Hz ($\pm 5\text{mV}$ amplitude perturbation) in pH 7.4 tris buffer, i.e. containing no antigen, as a baseline trace. This buffer solution did, however, contain a 50:50 mixture of $[\text{KFe}(\text{CN})_6]^{3-/4-}$, at a concentration of 10mM as redox mediator so as to perform faradaic impedance spectroscopy. The potential of the electrochemical cell is offset to the formal potential of the redox probe ($+0.12\text{V}$ vs. Ag/AgCl in tris buffer identified via amperometric cyclic voltammetry). Subsequently, ANSE doped sensors are exposed to a range of concentrations from 1 to 300 pg ml^{-1} NSE. NSE antigen was suspended in pH 6.8 tris buffers containing $[\text{KFe}(\text{CN})_6]^{3-/4-}$ cyanide at pre-prepared analyte concentrations of 1, 2, 3, 4, 5, 10, 20, 30, 50, 100, 200 and 300 pg ml^{-1} . The experimental protocol developed and described in section 3.5.5 is applied for the electrochemical impedimetric interrogation of ANSE immunosensors. A consistent sensor is used for all analytical concentrations and changes encountered are compared to the initial baseline trace (0 pg ml^{-1} NSE).

Results are presented in the form of Nyquist plots depicting the faradaic ($Z'_{(re)}$) and capacitive ($-Z''_{(im)}$) components of impedance and their variation throughout the recording of the ac impedance spectra.

7.5.1 ANSE immunosensor: Specific recognition of NSE antigen.

Electrochemical Impedance Spectra obtained for an ANSE-doped PANI mushroom microelectrode array sensor exposed to increasing NSE concentrations.

Figure 7.23 shows the specific response to NSE for a novel ANSE-immobilised, PANI modified, carbon microelectrode array sensor assembly. The concentration of NSE analyte represented is 1 to 10 pg ml^{-1} .

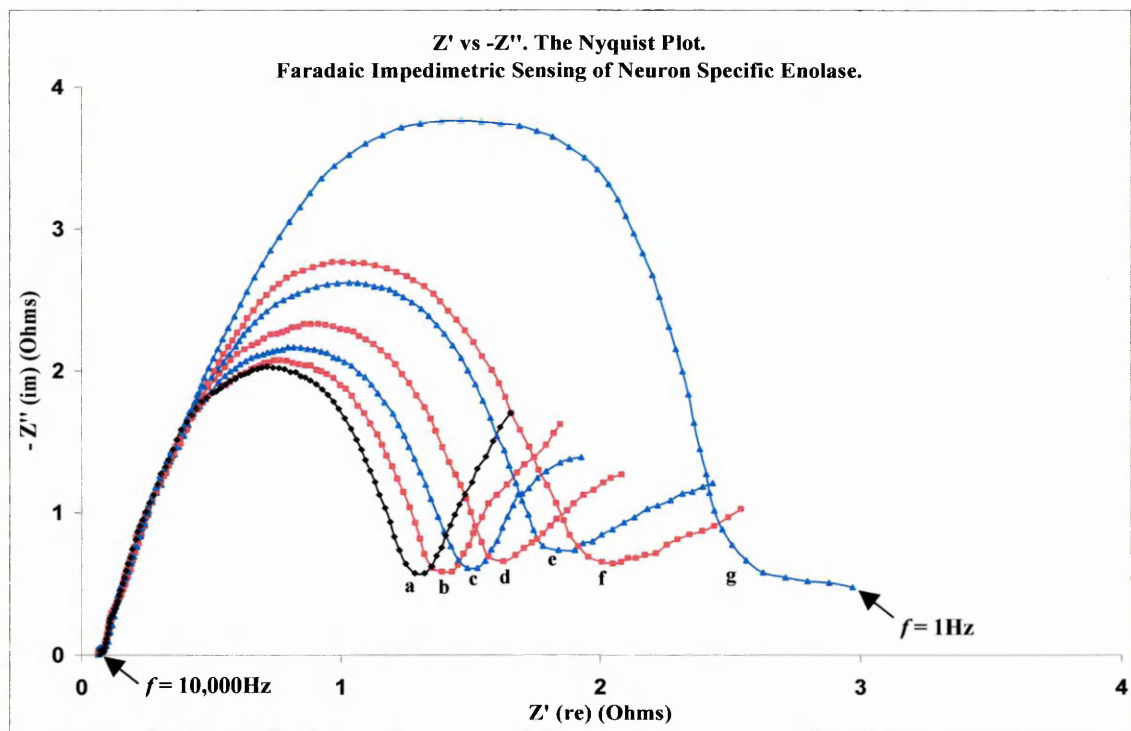


Fig 7.23: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel ANSE-doped PANI immunosensor following exposure to various concentrations of NSE. The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10 pg ml^{-1} NSE.

For the baseline trace, the $Z'_{(re)}$ component is seen to increase with decreasing frequency for the entire range of applied frequencies whereas the $-Z''_{(im)}$ component is seen to increase up to the characteristic frequency of each trace (the frequency at which the $-Z''_{(im)}$ value is at its maximum) before decreasing to the lower applied frequencies ($\sim 1\text{Hz}$).

In general, the impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow and the impedance is controlled by the interfacial electron transfer (Katz & Willner, 2003).

Figure 7.24 represents the specific response to NSE analyte at the higher concentrations of the experimental range up to 300 pg ml^{-1} NSE. Again the Nyquist curves follow the same general format as those in Figure 7.23.

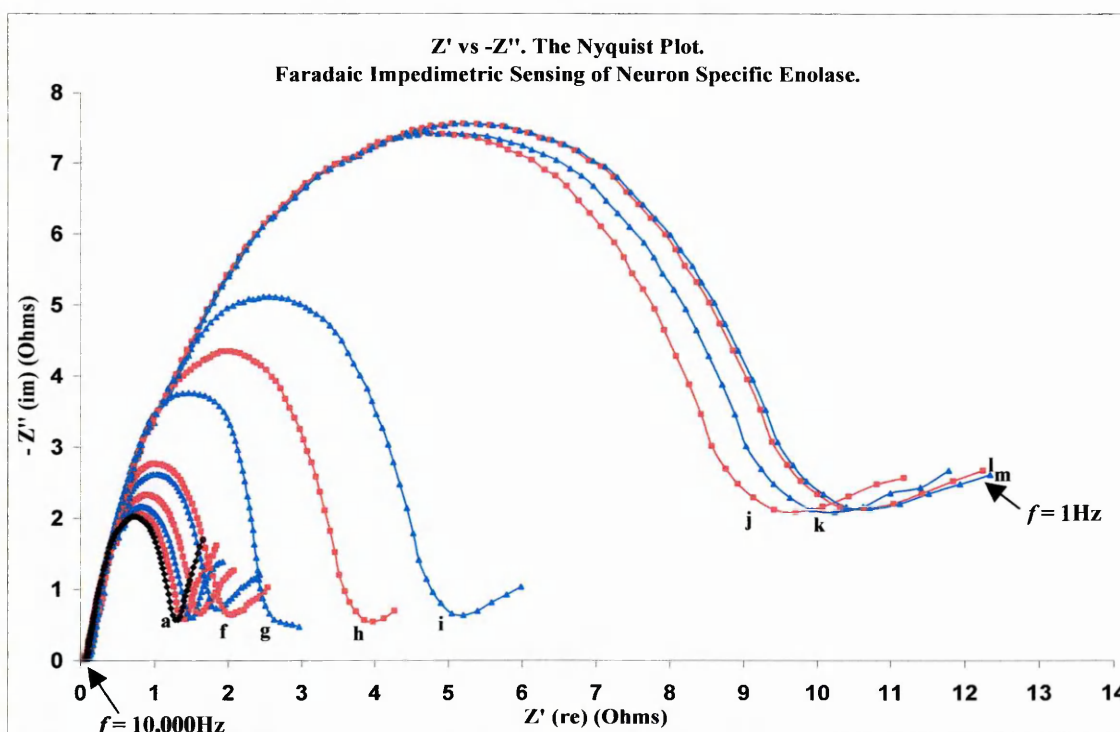


Fig 7.24: Typical Nyquist plot showing faradaic component ($Z'_{(re)}$) vs capacitive component ($-Z''_{(im)}$) for a novel ANSE-doped PANI immunosensor following exposure to various concentrations of NSE.

The concentration of antigen (pg ml^{-1}): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml^{-1} NSE.

A computer fitting of the experimental data to a theoretical model, represented by a simple equivalent circuit, is performed by the software provided with the frequency response analyser as data is extracted by the instrument. For a more thorough evaluation of the data obtained in Figures 7.23 and 7.24 the changes in the Nyquist curves are translated into electron transfer resistance changes to provide a clear and consistent format.

Experimentally determined and simulated values of electron transfer resistance were compared, showing a 100% correlation to two decimal places, and the relative electron transfer resistance changes from the baseline response at each concentration are plotted in Figure 7.25.

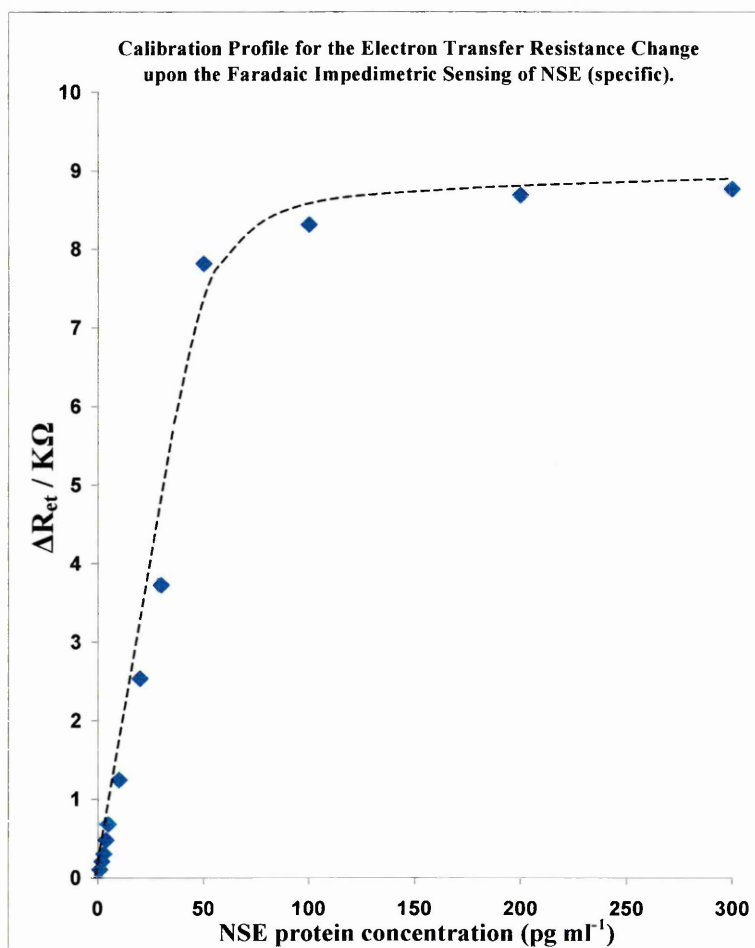


Fig 7.25: Calibration curve for the change in electron transfer resistance from the baseline response for a range of NSE antigen concentrations (specific).

Figure 7.25 depicts the electron transfer resistance change calculated with respect to the baseline trace (0 pg ml^{-1}). Equation 6.4 details the calculation of the electron transfer resistance change.

Figure 7.25 shows that the ANSE immunosensor gave a generally linear response to NSE analyte from 1 pg ml^{-1} to approximately 50 pg ml^{-1} NSE.

The electron transfer resistance increased with increasing antigen analyte concentration up to approximately 50 pg ml^{-1} .

The interaction of NSE with the electrode modified with ANSE resulted in significant insulation of the electrode reflected by the increase in the electron transfer resistance.

It is postulated that as antigen concentration was increased the number of antibody-antigen complexes formed also increased and this is reflected by the observed electron transfer resistance increase.

The insulation of the modified electrode upon formation of stable ANSE-NSE immunocomplexes hinders the electron transfer kinetics of the redox probe resulting in the increase of electron transfer resistance. The electron transfer resistance increases with increasing antigen concentration gave rise to a linear-type response from 1 to approximately 50 pg ml^{-1} . The sensitivity of the specific ANSE immunosensor was 1 pg ml^{-1} . There is negligible change from 50 pg ml^{-1} to 300 pg ml^{-1} suggesting that the immunosensor approaches saturation and stable antibody-antigen complex formation no longer relates to the analyte concentration.

Figure 7.27 represents the non-specific sensor response over the whole concentration range from 1 to 300 pg ml⁻¹ NSE antigen.

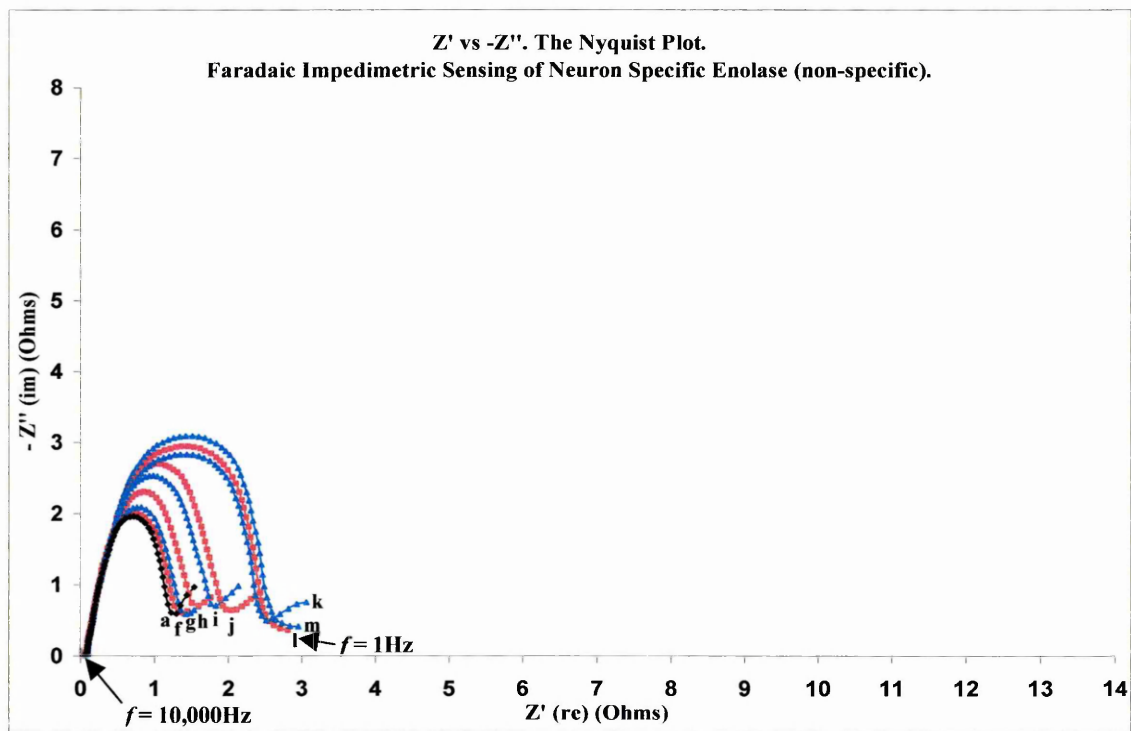


Fig 7.27: Typical Nyquist plot showing faradaic component ($Z'_{\text{(re)}}$) vs capacitive component ($-Z''_{\text{(im)}}$) for a novel AlG-doped PANI immunosensor following exposure to various concentrations of NSE. The concentration of antigen (pg ml⁻¹): a) 0 (baseline); b) 1; c) 2; d) 3; e) 4; f) 5; g) 10; h) 20; i) 30; j) 50; k) 100; l) 200 and m) 300 pg ml⁻¹ NSE. Refer to Fig 7.24 for direct comparison on same axes scale.

All impedimetric spectral traces obtained follow the same general form and similar shaped Nyquist curves are obtained, indicative of a surface-modified electrode system where the electron transfer is slow and controlled by interfacial electron transfer.

For clarity, Figure 7.28 shows the non-specific experimental data presented as the relative change in electron transfer resistance from the baseline trace (0 pg ml⁻¹ NSE) for each concentration analysed. Figure 7.28 has the same y-axis scale as Figure 7.25.

The electron transfer resistance values are extracted from the instrument and also independently calculated showing a 100% correlation to two decimal places.

The change in electron transfer resistance is calculated according to equation 6.4 with respect to the baseline trace at each experimental concentration.

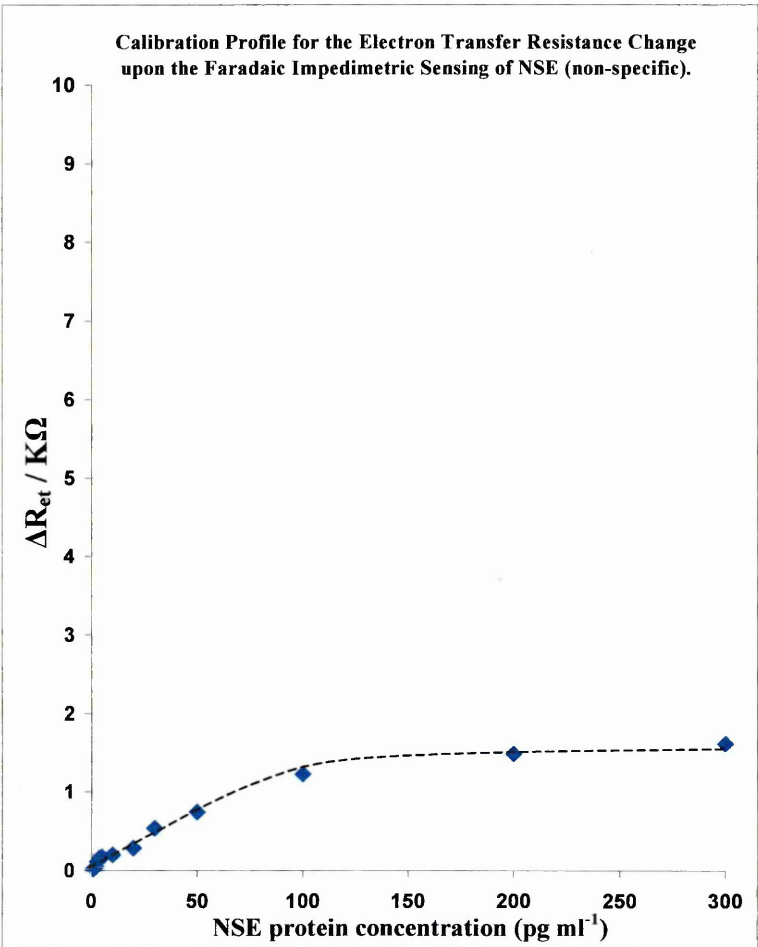


Fig 7.28: Calibration curve for the change in electron transfer resistance from the baseline response for a range of NSE antigen concentrations (non-specific). Refer to Fig 7.25 for direct comparison on same axes scale.

A linear-type non-specific response is observed from 1 to 100 pg ml⁻¹ NSE. Upon comparison of Figure 7.28 with Figure 7.25, it is apparent that approximately 10% of the APSA-immobilised sensor response encountered is, in fact, non-specific. The calibration curve tends towards a plateau at approximately 100 pg ml⁻¹ NSE suggesting that an optimum electron transfer resistance change from the baseline response has been reached.

7.5.3 ‘Corrected’ specific response: ANSE immunosensor.

To obtain a calibration profile, in terms of ‘corrected’ electron transfer resistance change for an ANSE immunosensor, accounting for any non-specific responses, the response obtained in Figure 7.28 (non-specific) was subtracted from those obtained in Figure 7.25 (specific) over the entire analytical concentration range. This matched sensor approach enables a true understanding of the ANSE-NSE immunosensor system. In total 10 ANSE-immobilised sensors and 10 corresponding AlgG-immobilised sensors were interrogated over the concentration range, according to section 7.5.1 and section 7.5.2 respectively, to allow an assessment of the reproducibility of the responses for this system. Results are presented in Figure 7.29.

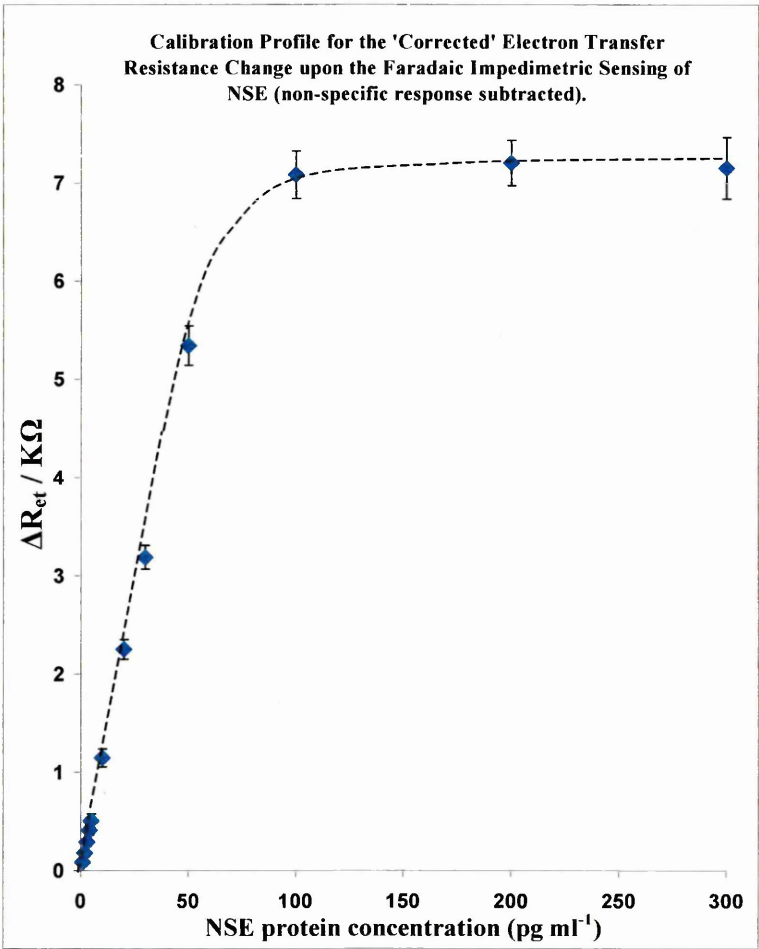


Fig 7.29: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of NSE antigen concentrations (non-specific response subtracted).
n = 10, error bars = standard deviation.

Figure 7.29 represents the ‘corrected’ profile for 10 replicate pairs of matched sensors. Identical experimental procedures were employed throughout investigations and all parameters were kept consistent. The mean electron transfer resistance response at each experimental analyte concentration was plotted. The error bars indicate the standard deviation obtained for the 10 matched sensor pairs from the mean ‘corrected’ values. The resultant response can be attributed to the interaction of ANSE with NSE analyte and arises from the effect on the electron transfer resistance due to the formation of antibody-antigen complexes at the sensor surface.

For clarity Figure 7.30 represents the calibration curve from 1 to 10 pg ml^{-1} NSE.

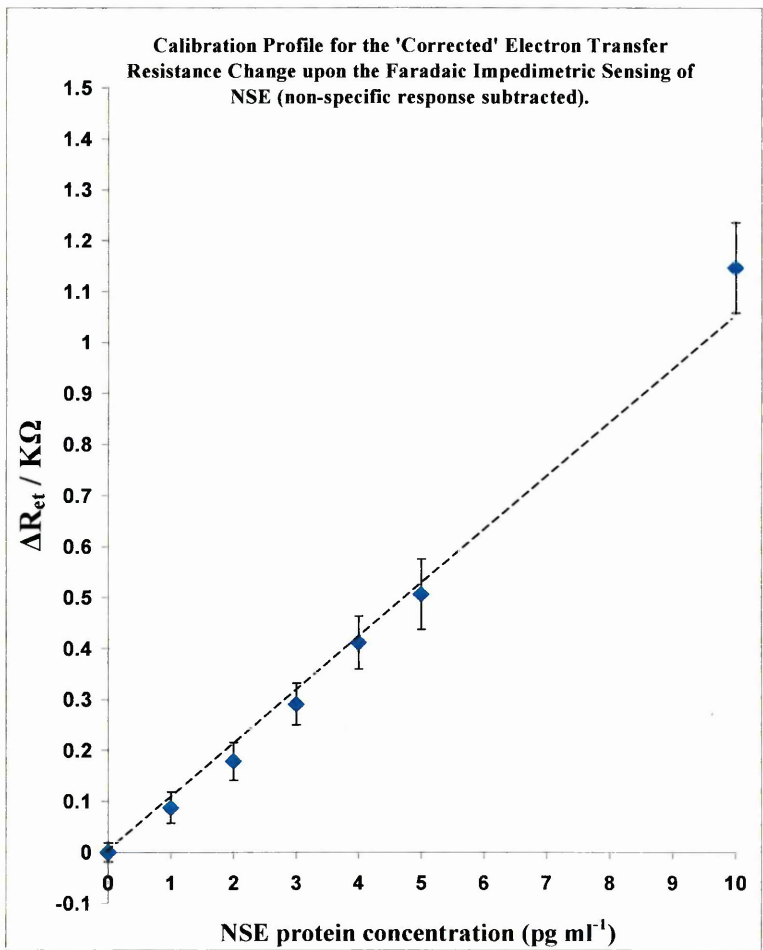


Fig 7.30: Calibration curve for the ‘corrected’ change in electron transfer resistance from the baseline response for a range of NSE antigen concentrations (non-specific response subtracted). n = 10, error bars = standard deviation.

At these concentrations, the responses in terms of electron transfer resistance change from the baseline trace (0 ng ml^{-1} NSE), were fairly linear. From Figure 7.29 a linear-type response was also observed from 1 to approximately 50 pg ml^{-1} NSE. At concentrations above 50 pg ml^{-1} , the electron transfer resistance change no longer relates to antigen concentration and the curve begins to tend towards a plateau. The sensor response approaches saturation and stable antibody-antigen complex formation is absolute.

The reproducibility of the 'corrected' response obtained from the standard deviation for 10 matched sensor pairings enables discrimination to be possible at 1 pg ml^{-1} NSE antigen analyte. The limit of detection of the ANSE immunosensor is 0.5 pg ml^{-1} NSE.

7.5.4 ANSE immunosensor: Reversibility studies.

5 ANSE-immobilised sensors and 5 matched non-specific AlG-immobilised sensors are exposed to the full interrogation procedure prior to reversibility investigations. Sensors were exposed to the full range of NSE antigen concentrations for 30 minutes at each concentration and in between concentrations a washing / flushing of weakly-bound or non-specifically adsorbed matter from the sensor surface was performed before the impedance trace is recorded in redox-containing buffer. Once the trace had been recorded for both ANSE & IgG-immobilised sensors at a particular concentration the experimental protocol was continued up to 300 pg ml^{-1} .

The 5 sensor pairs under investigation formed part of the 10 sensor pairings analysed to obtain the data in Figure 7.29 and Figure 7.30. A mean 'corrected' electron transfer change for each concentration was obtained with a standard deviation for the 5 pairs. After the regeneration treatment, the same experimental procedure was followed again to assess the recovery of the initial response in terms of 'corrected' electron transfer change over the concentration range.

If the Faradaic response can be restored the sensor has potential for reusability. 5 regenerations are attempted and the saturation concentration of 50 pg ml⁻¹ NSE is selected for detailed analysis.

In order to split the antibody-antigen complex and to regenerate the sensors, 0.1M HCl acidic buffer (pH 2.3) was applied for 3 separate 1 minute time periods. In between each acidic buffer exposure pH 7.4 PBS was used to rinse the sensor surface. Antigens are released from the sensor surface and antibodies are still bound when the ANSE antibody / NSE antigen immunocomplexes are disrupted. Finally the sensor was rinsed with 50ml PBS to produce the NSE-free sensors and the experimental EIS analysis could be repeated.

The results in Figure 7.31 indicate that negligible changes in ‘corrected’ electron transfer response at the saturation concentration of NSE are encountered after the first regeneration for the 5 sensor pairings.

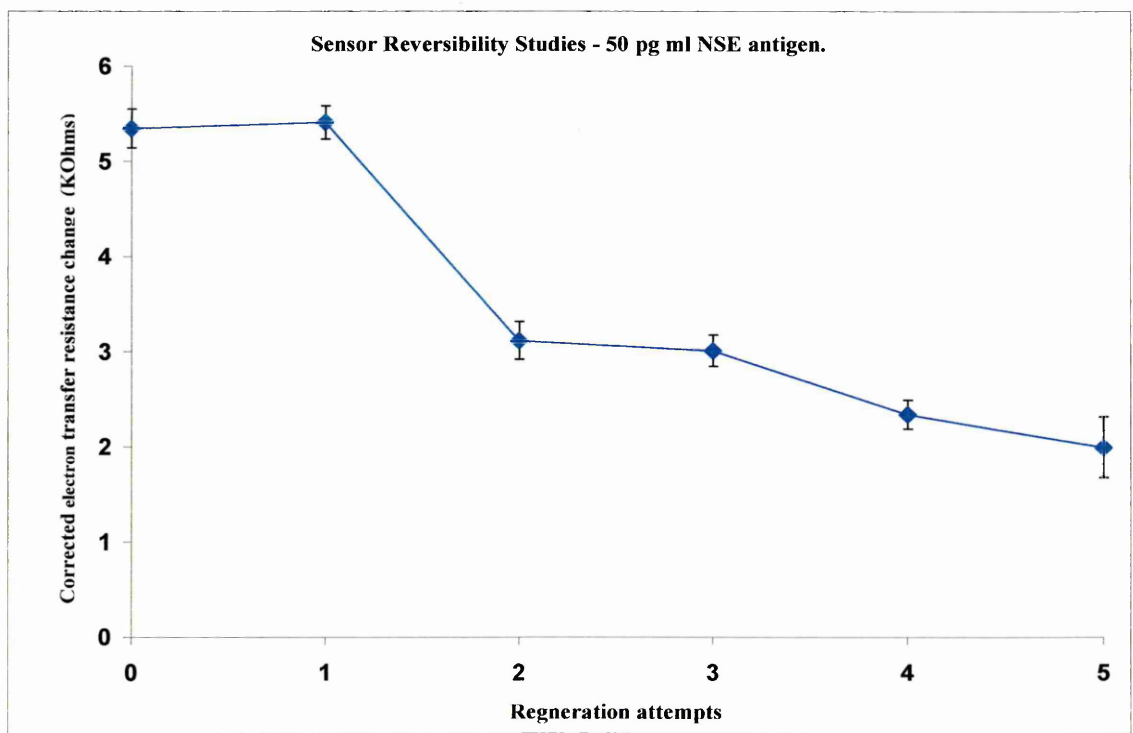


Fig 7.31: Recovery of sensor response following a number of regeneration attempts.

Both specific and non-specific sensors exhibited reproducible behaviour.

For 50 pg ml⁻¹ NSE the mean 'corrected' electron transfer change and the standard deviation are comparable to the initial response obtained. For one sensor regeneration the response is reversible.

After the first regeneration it is apparent that the 'corrected' electron transfer change has been significantly lowered with a response approximately 60% that of the initial response. Following the initial regeneration and recovery of response there is a reduction in sensitivity. All subsequent regeneration attempts for the 5 sensor pairings show a similar response of 50-60% of the original. After the first regeneration it is presumed that the PANI-modified microelectrode array sensor surface remains intact, however, not all antibody activity has been eradicated.

The reversible phenomenon observed after one sensor regeneration attempt can be attributed to the restoration of the availability of the binding sites of the antibody after a short-time acidic washing. Unfortunately, repeated washing of the sensing layer reduced the activity of the antibody, which can explain the decrease of the sensitivity of the sensors after the initial regeneration. Subsequent attempts to regenerate the surface show that further exposure to the acidic conditions, in this case, does not remove all analyte recognition properties and the antibody still acts as the receptor component of the immunosensor, although to a lesser degree.

The results are significant in the fact that they demonstrate the potential for the development of the regeneration process. Further work should yield an optimum protocol for further, or perhaps continual, true reversibility. Greater initial response recovery over a greater number of regenerations would clearly be highly beneficial. The phenomenon of reversible regeneration at immunosensor surfaces is a behaviour that is rarely encountered and is a highly desirable property of these ANSE immunosensors.

7.5.5 ANSE immunosensor: Stability studies.

The stability of the ANSE-NSE immunosensor system was assessed from storage in dry state, at 4°C, for a period of 12 weeks. An identical experimental protocol is employed for electrochemical impedance interrogation (section 3.5.5) and the ‘corrected’ electron transfer change was the analysis method for response recovery. Initially 18 matched sensor pairings of ANSE (specific) and AlG (non-specific) immunosensors are refrigerated at 4°C in sealed, sterilised vessels. Every 2 weeks a set of 3 matched sensor pairings is removed from storage and interrogated over the full NSE antigen analyte concentration range from 1 to 300 pg ml⁻¹.

Figure 7.32 shows the data obtained at each time interval and the saturation concentration of 50 pg ml⁻¹ NSE was chosen as a means of final evaluation of sensor stability and the mean change in ‘corrected’ electron transfer resistance response is shown plotted against storage time. The error bars indicate the standard deviation for the three sensor pairs.

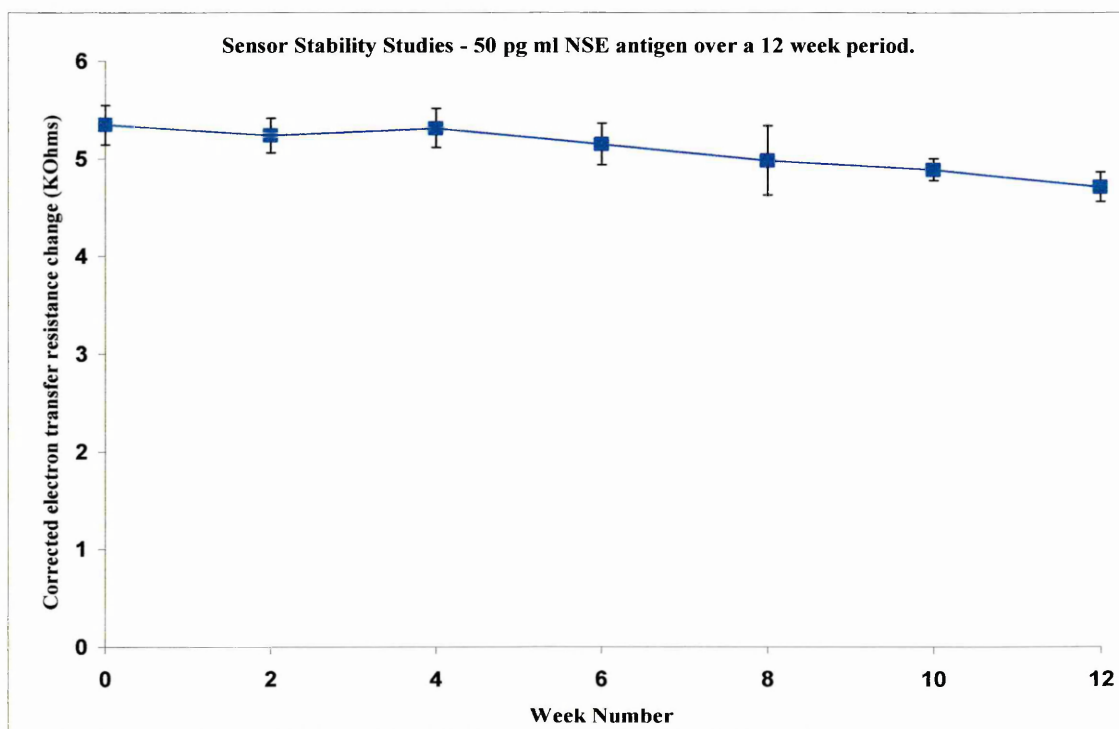


Fig 7.32: Corrected electron transfer resistance change at 50 pg ml⁻¹ NSE. Stability study – Variation over 12 week period. ($n = 3$, error bars = SD).

Figure 7.32 shows that the sensor response was fully recovered after 12 weeks in storage with negligible difference from the initial response at $t = 0$. The activity of the antibody receptor biocomponent is unaffected by storage over time. The results lend themselves well to possible packaging and suggests a satisfactory shelf-life.

7.5.6 ANSE immunosensor: Further non-specific studies.

Five differing protein analytes were added to the test solution and 10 sensor pairings were analysed over the full concentration ranges for the antibody / antigen system using the protocol described in section 3.5.5. Aside from the foreign species present in test solutions, the experimental protocol was repeated in an identical manner. 10 ng ml^{-1} BSA, HSA, CA125 (tumor marker), PSA & S-100 were added for NSE sensing. In these case results did not differ significantly from the original calibration figures (Figures 7.29 and 7.30). The mean 'corrected' electron transfer changes showed a variation of less than 5% for all concentrations and standard deviations that were comparable to the original.

These results demonstrate that the modified electrodes keep biosensing abilities after their exposure to target analyte in a test solution containing multiple foreign species. Thus, adsorption of BSA / HSA / CA125 / PSA and S-100 and any non-specific interactions at the sensor surface do not proceed or do not affect significantly the biorecognition events (stable immunocomplex formation) and their impedance response. The matched AlGg sensor (non-specific) response does not alter so it is postulated that the HSA blocking during fabrication minimises non-specific effects almost totally. The AlGg sensor response is thus not due to the presence of the target analyte or the presence of foreign proteins. This control sensor must be responding to any other effects at the sensor surface that may be occurring such as changes in the polymer or effects from the solution itself on the sensor. In any case, the effects are always minor compared to the signal obtained when a sensor immobilised with a specific receptor antibody is exposed to its target analyte. Again these results are promising for prototype development as they suggest that sensing of target in 'real' samples is a plausible proposal.

7.5.7 ANSE immunosensor: Conclusions.

The results obtained verify the fabrication techniques and experimental interrogation protocol as a viable approach towards the labelless sensing of markers for TIA and demonstrate the generic nature of the technology. The ANSE-NSE immunosensor system has a low non-specific response associated with the faradaic sensing of NSE. The ANSE immunosensor was reversible for one regeneration attempt before the acid treatment has a detrimental effect on the performance of the immobilised antibody as a receptor biocomponent. The stability of the sensor assemblies was encouraging with negligible loss of sensitivity after storage for twelve weeks at 4°C in dry state.

The ANSE immunosensor gave a 'corrected' linear-type response from 1 to 50 pg ml⁻¹. The higher the NSE concentration in the test solution the greater the number of stable antibody-antigen complexes formed at the sensor surface. The complex formation introduces a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration.

At concentrations of analyte above 50 pg ml⁻¹ the number of complexes formed begins to plateau as the sensor approaches saturation. The recognition entities become occupied with analyte and active sites for further antigen binding are no longer available. The limit of detection of the ANSE-NSE immunosensor system was found to be 0.5 pg ml⁻¹ and there was an ability to discriminate at 1 pg ml⁻¹ NSE analyte.

The low quantification levels and limits of detection for NSE has illustrated the potential use of these immunosensor assemblies for the labelless detection of markers for TIA as results are of clinical significance. Several other highly beneficial properties are also associated with the immunosensors. Currently ELISA permits the quantification of elevated levels of NSE in human serum following stroke from approximately 1 - 150 ng ml⁻¹ (μg L⁻¹) NSE (Canag Diagnostics, Diagnostic Automation, Inc).

7.6 Chapter 7 Conclusions:

In summary, the APSA-PSA system gave rise to a linear-type response from 1 to 100 pg ml⁻¹ PSA analyte with a limit of detection of 1.4 pg ml⁻¹ and the ability to discriminate at 5 pg ml⁻¹ following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. This is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was reversible for up to two regeneration attempts and the original response was fully recovered. Further attempts at sensor regeneration caused complete loss of PSA sensing. The stability of the sensors was encouraging and negligible sensitivity was lost after 12 weeks in storage.

There was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of PSA was also possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable.

The affinity-immobilised PSA immunosensor described exceeds the clinical requirements for PSA sensing for prostate cancer with the critical diagnostic region being from 4 to 10 ng ml⁻¹ (Polascik, 1999; Fernandez-Sanchez, 2004, Fernandez-Sanchez, 2005). The lower non-specific response, reversibility, stability and sensing in the presence of several foreign species suggest that PSA immunosensors described in this chapter offer a realistic diagnostic potential for the quantification of total prostate specific antigen in serum.

The AS-100-S-100 system gave rise to a linear-type response from 1 to 50 pg ml⁻¹ S-100 analyte with a limit of detection of 1 pg ml⁻¹ and the ability to discriminate at 10 pg ml⁻¹ following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte.

This is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was reversible for a single regeneration attempt and the original response was fully recovered.

Further attempts at sensor regeneration ultimately lead to a complete loss of S-100 sensing. The stability of the sensors was encouraging and negligible sensitivity was lost after 12 weeks in storage.

Again there was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of S-100 was also possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable.

The low quantification levels and limits of detection for S-100 has illustrated the potential use of these immunosensor assemblies for the labelless detection of markers for TIA, with results spanning a concentration range of clinical significance for ischemic stroke diagnosis.

Non-specific studies have demonstrated the potential for operation in the presence of several foreign species without a detrimental effect on the sensitivity for the target analyte. S-100 immunosensors may be able to compete with these conventional methods when analysis of 'real' patient samples is performed, but also offer advantages in terms of speed of response and the potential for use at point-of-care by non-skilled users.

The ANSE-NSE system gave rise to a linear-type response from 1 to 50 pg ml^{-1} NSE analyte with a limit of detection of 0.5 pg ml^{-1} and the ability to discriminate at 1 pg ml^{-1} following the electrochemical impedimetric interrogation of 10 matched sensor pairings;

one specific and one non-specific to the target analyte. Again this is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was reversible for a single regeneration attempt and the original response was fully recovered.

Further attempts at sensor regeneration caused ~50% loss of NSE sensing and after 5 regeneration attempts some degree of NSE sensing was still possible. These results suggest that an optimum protocol similar to the one employed may offer further reversibility. The stability of the sensors was excellent and negligible sensitivity was lost after 12 weeks in storage.

Again there was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of NSE was again possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable. The NSE immunosensors may provide quantification of NSE in human serum at sensitivity levels currently achieved by ELISA laboratory techniques. The immunosensors would offer a rapid interrogation approach in comparison and a hand-held device would enable point-of-care testing by non-skilled users.

In general, for the linear responses obtained for each immunosensor system, the higher the analyte concentration in the test solution the greater the number of stable antibody-antigen complexes are formed at the sensor surface. The stable antibody-antigen complex formation introduces a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration.

At respective specific concentrations of analyte the number of complexes formed begins to plateau as the sensor saturates, the recognition entities become occupied with analyte and active sites for further antigen binding are no longer available. For the APSA immunosensor the sensor surface approaches saturation at 100 pg ml^{-1} PSA, for the AS-100 immunosensor the surface approaches saturation at 50 pg ml^{-1} S-100 and for the ANSE immunosensor 50 pg ml^{-1} NSE causes saturation.

The results have demonstrated the suitability of EIS interrogation (section 3.5.5) as a viable approach towards the labelless detection and quantification of biochemical markers of stroke (TIA).

The results have also demonstrated the suitability of the immunosensors fabricated according to chapter 4, utilising the site-specific immobilisation techniques detailed in chapter 5 (section 5.3), as viable platforms with which to perform the labelless detection and quantification of biochemical markers of stroke (TIA).

It is suggested that the low sensitivity of the immunosensor systems is attained via the organisation of the recognition layer. Antibodies are specifically orientated during immobilisation and retain their full biological properties. The number of binding sites and their availability for analyte binding is greatly increased. The non-specific occurrences are minimised via HSA blocking and the specific response is not 'swamped' with noise. The PANI-modified microelectrode array sensors confer hemispherical diffusion profile behaviour to the immunosensors such that an enhanced and optimised sensor surface area is exposed to the test solution.

Chapter 8: General Conclusions.

8.0 General Conclusions.

The development of a rapid, labelless and reversible impedimetric immunosensor for the detection and quantification of stroke markers has been described. Focus has been drawn towards the diagnosis of biomedical markers involved in transient ischemic attack (TIA).

Chapter 4 details the development of a microelectrode array electrode platform. As a prerequisite to the production of microelectrode arrays via ultrasonic ablation, an electrode must first be coated with a thin insulating film of poly(*o*-phenylenediamine).

The electropolymerisation of poly(*o*-phenylenediamine) onto gold sputter-coated ground glass slide electrodes was characterised in neutral conditions to show a two electron transfer mechanism for the formation of a non-conducting, ultrathin film. This was then applied to screen printed carbon-ink electrode surfaces, which offer a less expensive route to the mass production of disposable sensors. As a consequence of the decreased electron transfer kinetics associated with carbon-ink host electrodes, the electropolymerisation process was refined by extending the polymerisation potential window from 0 to +800mV to 0 to +1000 mV (vs. Ag/AgCl), allowing the more anodic reactions to take place and so a more efficient passivation of the electrode surface to occur. A pre-polymerisation sonocation step (three 0.5 s pulses of ultrasonic energy) was identified as being required to remove trapped air from the electrode surface, thereby permitting intimate contact with the monomer solution and preventing disruption of the polymer layer.

The area under each peak produced can be related to the quantity of charge passed due to the faradaic reaction and can therefore be linked to the thickness of the formed film. The irreversible peak observed is seen to diminish in size with each successive cycle, as the working electrode becomes coated with more polymer, and therefore becomes insulated.

The electropolymerisation of *o*-phenylenediamine is a self-regulating process since poly(*o*-phenylenediamine) is an insulating polymer and thus the working electrodes became fully insulated after 20 potential sweeps, indicated by the reduction in area under the curve with each cycle (i.e. charge passed), and this limits the deposition of further polymer to regulate the final thickness of the film.

Insulation was confirmed by the lack of any electroactivity in a redox couple when cyclic voltammetry was performed following this electrode modification step.

Microelectrode array assemblies were produced initially via the ultrasonic ablation of poly(*o*-phenylenediamine) modified gold sputter-coated ground glass slide electrodes. Sensors were interrogated using cyclic voltammetry in a ferri/ferrocyanide redox couple to show sigmoidal behaviour and were found to offer good microelectrode array performance. Again, the techniques were transferred to the screen-printed carbon ink electrodes.

Novel microelectrode array sensors were successfully fabricated and evaluated via several techniques to confirm microelectrode-type electrochemical behaviour and to validate the fabrication steps on poly(*o*-phenylenediamine) modified screen printed carbon-ink electrodes. Reproducibility was found and the reduction in sensor variation was supported by several imaging techniques together with the development and optimisation of key experimental factors.

An industrial-scale ultrasonic tank was procured and a modulated frequency sweep introduced to enhance the uniformity of the ultrasonic field. A procedure for analysing the distribution of cavitation activity via the sonochemical luminescence of Luminol was developed and used to successfully identify regions of high and low cavitation activity within the ultrasound tank, these relating to energy focused by the positioning of the ultrasonic transducers and the asymmetry in the profile of the tank base.

The uniformity of the distribution of acoustic energy was then further enhanced through the development of an acoustic baffling system.

The results in this chapter have been published in *Biosensors and Bioelectronics* as part of a series focussing on the widespread applicability of microelectrode array sensors in the field of biosensor technologies (Barton *et al*, 2004).

For this programme the bio recognition entity (antibody receptor) was subsequently immobilised at the novel modified microelectrode array transducer within and at the surface of conductive PANI 'emeraldine' mushroom-like protrusions and also, as an alternative, attached to the PANI protrusion surface via non covalent affinity techniques. These immobilisation strategies were described in Chapter 5. The sensors were finally interrogated by electrochemical impedance spectroscopy for the application of immunosensors for markers of TIA such as NSE & S-100 β protein; the results for both immobilisation approaches were discussed in Chapters 6 and 7 respectively.

Biomolecule, in this case antibody receptors, were successfully immobilised within intimate proximity of the transducer surface of the novel microelectrode array sensors.

Polymeric protrusions emanate from the micron pore ablations at the microelectrode array surface conferring a hemispherical diffusion profile across the modified working electrode.

A simple and rapid one-step immobilisation protocol involving co-entrapment of biomolecule throughout electrogeneration of PANI within the matrix and at the surface was exploited for the creation of immunosensors for impedimetric investigations into the quantification of analyte specific to TIA diagnosis. Confirmation of PANI protrusion formation was achieved by conventional voltammetry and imaging techniques. These immunosensors created via conductive polymer entrapment (CPE) were initially investigated.

A more sophisticated and elegant strategy involving non covalent affinity attachment was also employed whereby orientation of receptor can be controlled to a greater extent to facilitate antibody-antigen interactions. QCM techniques were employed and confirmed that this non-covalent affinity immobilisation approach was successful.

The suitability of both immobilisation approaches for utilization within impedimetric immunosensors for stroke (TIA) was evaluated.

Immobilisation techniques discussed in section 5.2 and employed according to section 3.5.3.1 were interrogated as impedimetric immunosensors - and again results were reported in chapter 6.

Immobilisation techniques discussed in section 5.3 and employed according to section 3.5.3.2 were interrogated as impedimetric immunosensors and results were reported in chapter 7.

In summary, the *CPE*-immobilised ABSA-BSA system gave rise to a linear-type response from 1 to 50 ng ml⁻¹ BSA analyte with a limit of detection of 1.5 ng ml⁻¹ and the ability to discriminate at 5 ng ml⁻¹ following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. This was a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was employed to interrogate the binding of antigen analyte to the sensor surface.

The sensor system was found not to be reversible and the stability of the sensors was found to be unsatisfactory.

50% of the initial electron transfer resistance changes were found to be non-specific. Attempts to lower the antigen concentration quantification lead to only minor changes from the baseline response together with insufficient sensitivity to proceed to pg ml⁻¹ detection levels.

A linear-type response was observed for *CPE*-immobilised APSA immunosensors from 1 to 100 ng ml⁻¹ PSA together with a limit of detection of 0.7 ng ml⁻¹ and the ability to discriminate at the ng ml⁻¹ concentration level. This again was a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. 10 matched sensor pairings were analysed over the entire concentration range to assess the reproducibility.

Approximately 30% non-specific response was encountered during the control experiments leading to the 'corrected' response.

The APSA-PSA *CPE* immunosensor system was not found to be reversible and the stability of the sensors was found to be unsatisfactory.

Any attempts to reduce the analytical concentration range led to a loss of sensitivity for PSA since only very minor electron transfer resistance changes were encountered.

In general, for the linear responses, the higher the analyte concentration in the test solution, the greater the number of stable antibody-antigen complexes were formed at the sensor surface. The stable antibody-antigen complex formation introduced a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistances extracted from the spectra obtained increased accordingly with the antigen analyte concentration. At specific concentrations of analyte, the number of antibody-antigen couples formed began to plateau as the sensor saturates, the recognition entities became occupied with analyte and active sites for further antigen binding were no longer available. For the ABSA immunosensor, the sensor surface approached saturation at 50 ng ml⁻¹ BSA and for the APSA immunosensor the surface approached saturation at 100 ng ml⁻¹ PSA.

Although not ideal the results demonstrated the potential for EIS interrogation as a viable approach towards the labelless detection and quantification of biochemical markers of stroke (TIA).

It is postulated that a lot of the sensitivity of the *CPE*-immobilised immunosensor systems was swamped by the non-specific events and, thus, the organisation of the antibody recognition layer, including the receptors orientation and the availability of active sites, with the minimisation of non-specific events became the focus for the achievement of lower limits of detection and analytical concentration ranges of clinical relevance.

Although *CPE* is a rapid one-step procedure, simple entrapment antibody immobilisation techniques within and at the surface of the PANI mushroom protrusions did not allow for the successful elaboration of *CPE* immunosensors via electrochemical impedance spectroscopy. The antibody orientation was random and the spatial immobilisation is not well controlled. The poorly organised recognition layer caused a severe lack of sensitivity and this was thought to be the primary factor for the disappointing responses obtained.

Immunosensor fabricated via non-covalent affinity immobilisation techniques yielded far superior results in terms of suitability for quantification of stroke markers.

In summary, the APSA-PSA system gave rise to a linear-type response from 1 to 100 pg ml^{-1} PSA analyte with a limit of detection of 1.4 pg ml^{-1} and the ability to discriminate at 5 pg ml^{-1} following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. This was a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was again the technique employed to sense the binding of antigen analyte to the sensor surface.

In this case the sensor system was found to be reversible for up to two regeneration attempts and the original response was fully recovered. Further attempts at sensor regeneration caused complete loss of PSA sensing. The stability of the sensors was encouraging and negligible sensitivity was lost after 12 weeks in storage.

There was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of PSA was also possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable and there is the potential for development of these immunosensors as a diagnostic tool for the quantification of total PSA in human serum and the diagnosis of prostate cancer.

The AS-100-S-100 system gave rise to a linear-type response from 1 to 50 pg ml^{-1} S-100 analyte with a limit of detection of 1 pg ml^{-1} and the ability to discriminate at 10 pg ml^{-1} following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. This is a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was reversible for a single regeneration attempt and the original response was fully recovered.

Further attempts at sensor regeneration ultimately lead to a complete loss of S-100 sensing. The stability of the sensors was encouraging and negligible sensitivity was lost after 12 weeks in storage.

Again there was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of S-100 was also possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable.

The ANSE-NSE system gave rise to a linear-type response from 1 to 50 pg ml^{-1} NSE analyte with a limit of detection of 0.5 pg ml^{-1} and the ability to discriminate at 1 pg ml^{-1} following the electrochemical impedimetric interrogation of 10 matched sensor pairings; one specific and one non-specific to the target analyte. Again

this was a corrected response with the non-specific antibody sensor response (polyclonal human IgG) subtracted from the specific sensor response at each concentration. Faradaic EIS was the technique employed to sense the binding of antigen analyte to the sensor surface.

The sensor system was reversible for a single regeneration attempt and the original response was fully recovered.

Further attempts at sensor regeneration caused ~50% loss of NSE sensing and after 5 regeneration attempts some degree of NSE sensing was still possible. These results suggest that an optimum protocol similar to the one employed may offer further reversibility. The stability of the sensors was excellent and negligible sensitivity was lost after 12 weeks in storage.

Again there was a low non-specific response obtained suggesting that the novel immobilisation techniques and the HSA blocking served to minimise non-specific events. The sensing of NSE was again possible in the presence of several foreign species suggesting that 'real' sample analysis is conceivable.

In general, for the linear responses obtained for each *affinity*-immobilised immunosensor system, the higher the analyte concentration in the test solution the greater the number of stable antibody-antigen complexes are formed at the sensor surface. The stable antibody-antigen complex formation introduced a barrier to the electron transfer of the redox couple, also present in the test solution, at the formal redox potential and hence the electron transfer resistance extracted from the spectra obtained increases accordingly with the antigen analyte concentration. At respective specific concentrations of analyte the number of complexes formed began to plateau as the sensor saturates, the recognition entities became occupied with analyte and active sites for further antigen binding were no longer available. For the APSA immunosensor the sensor surface approached saturation at 100 pg ml⁻¹ PSA, for the AS-100 immunosensor, the surface approached saturation at 50 pg ml⁻¹ S-100 and for the ANSE immunosensor 50 pg ml⁻¹ NSE caused saturation.

These results demonstrated the suitability of EIS interrogation (section 3.5.5) as a viable approach towards the labelless detection and quantification of biochemical markers of stroke (TIA).

The results also demonstrated the excellent suitability of the immunosensors fabricated according to chapter 4, utilising the site-specific immobilisation techniques detailed in chapter 5 (section 5.3), as viable platforms with which to perform the labelless detection and quantification of biochemical markers of stroke (TIA).

The low sensitivity of the immunosensor systems was attained via the organisation of the recognition layer. Antibodies are specifically orientated during immobilisation and retain their full biological properties. The number of binding sites and their availability for analyte binding is greatly increased. The non-specific occurrences were minimised via HSA blocking and the specific response was no longer 'swamped' with noise. The PANI-modified microelectrode array sensors confer hemispherical diffusion profile behaviour to the immunosensors such that a greater sensor surface area is exposed to the test solution.

In essence the successful creation of a labelless and reversible immunosensor system for the detection of markers of stroke (TIA) in buffered test solution has been described.

Following publication of this thesis a series of papers describing the quantification of stroke (TIA) markers will be prepared.

Chapter 9: Suggestions for further work.

9.0 Suggestions for further work.

Future research relating to TIA immunosensor manufacture will benefit from further enhancement in the microelectrode array fabrication techniques. A number of routes may be investigated to enhance the uniformity of cavitation activity in the ultrasound tank. Primarily, the design of the ultrasound tank must be improved to provide more accurate control over variables (such as power, time and temperature), eliminate asymmetry in the final construction and to include a motorised transducer base which will help to homogenise the position of nodes and antinodes.

A more uniform and densely populated pore distribution at the poly(*o*-phenylenediamine) modified electrodes following ultrasonic ablation will permit greater biomolecule loading and increase the recognition potential of the immunosensors since every pore created results in the formation of protrusions following the subsequent PANI modification step.

Further optimisation of the attachment of biomolecules via non-covalent affinity approaches should proceed to permit greater biomolecule loading, control of orientation and increase the availability of antigen binding sites. The more stable antibody-antigen complexes that can be formed, the greater will be the potential for enhancing both sensitivity and working concentration response range prior to saturation.

Current experimental interrogation techniques would meet the need for rapid quantification; however, data analysis can be time-consuming. It would be beneficial to have an appropriate software algorithm written to perform the analysis as the impedance spectrum is recorded.

Investigations should also continue to include further biomolecule systems of interest.

Detection of analyte has been achieved in buffered test solutions and simulated solutions containing several foreign species.

Further work is also required to quantify 'real' patient samples as plasma or whole blood since point-of-care testing would be highly appropriate for the application of immunosensors of this type.

The development of a hand-held prototype diagnostic device for use at point-of-care is the ultimate goal for future development.

Chapter 10: References.

10.0 References.

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Chapter 11: Appendices.



Sonochemically fabricated microelectrode arrays for biosensors offering widespread applicability: Part I

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Abstract

A novel and patented procedure is described for the sonochemical fabrication of a new class of microelectrode array based sensor with electrode element populations of up to $2 \times 10^5 \text{ cm}^{-2}$. For some years it has been accepted that microelectrode arrays offer an attractive route for lowering minimum limits of detection and imparting stir (convective mass transport) independence to sensor responses; despite this no commercial biosensors, to date, have employed microelectrode arrays, largely due to the cost of conventional fabrication routes that have not proved commercially viable for disposable devices. Biosensors formed by our sonochemical approach offer unrivalled sensitivity and impart stir independence to sensor responses. This format lends itself for mass fabrication due to the simplicity and inexpensiveness of the approach; in the first instance impedimetric and amperometric sensors are reported for glucose as model systems. Sensors already developed for ethanol, oxalate and a number of pesticide determinations will be reported in subsequent publications.

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1. Introduction

Biosensor technology has developed into an ever expanding and multidisciplinary field since the Clark enzyme electrode was first reported (Clark and Lyons, 1962). Biosensors generically offer simplified reagentless analyses for a range of biomedical and industrial applications and for this reason this area has continued to develop into an ever expanding and multidisciplinary field during the last couple of decades. Since more than half of the sensors reported in the literature are based on electrochemical transducers (Meadows, 1996) and at the time of writing approximately 85% of the world commercial market for biosensors is for blood glucose monitoring (Newman et al., 2002), we will as the first model system of this sonochemically fabricated microelectrode

array biosensor, report a glucose oxidase based impedimetric sensor. We have already within our laboratory demonstrated the fabrication of enzymatic and affinity based sensors that lend themselves to interrogation by either (i) amperometric or (ii) impedimetric approaches. Enzymatic sensors have been developed for analytes including ethanol, oxalate and a series of pesticides. Affinity sensors have been developed using antibody/antigen and DNA hybridisation based approaches. These will all be reported in a series of further publications.

Microelectrodes offer advantages over conventional larger working electrodes within biosensors since they experience hemispherical solute diffusional profiles, and it is this phenomenon that can impart stir-independence to sensor responses, whilst also offering lowered limits of detection.

Individual microelectrodes offer very small responses and one approach for overcoming this problem is to use many microelectrodes together in the form of an array to allow a cumulative and so larger response to be measured.

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Microelectrode arrays may be fabricated by a number of approaches although techniques such as photolithography or laser ablation have to date proved cost prohibitive for the mass production of disposable sensor strips. We will within this paper describe a novel sonochemical fabrication approach (Higson, 1996) for the production of microelectrodes, that lends itself to the mass production of sensor arrays.

2. Experimental

Glucose oxidase from *Aspergillus niger* (80% protein, 132,000 units/g solid), was purchased from the Sigma Chemical Company (Poole, Dorset, UK), D-glucose, aniline, potassium hydrogen phthalate, disodium hydrogen orthophosphate 1-hydrate, sodium dihydrogen orthophosphate 12-hydrate, sodium chloride, and diaminobenzene dihydrochloride (all 'AnalaR' grade), were purchased from BDH (Poole, Dorset, UK). All chemicals were used without further purification.

Glucose oxidase was prepared using 250 units/ml, in distilled water for deposition at microelectrode arrays.

Anhydrous α -D-glucose solutions were prepared to a range of concentrations, from 1 to 40 mM, in pH 7.4 phosphate buffer.

A small volume, 2.7 ml, PTFE cell was milled for the co-deposition of aniline/enzyme. The cell held the glass slide working and counter electrodes at a fixed distance from each other, whilst the reference electrode was clamped overhead.

A Sycopel PCI-100 MK3 Potentiostat computer interface was used in conjunction with a 'Ministat Potentiostat', H.B. Thompson and Associates, (Newcastle-upon-Tyne) for all electrochemical studies: current/charge transients were recorded using a PC with dedicated software. AC impedance measurements were performed using an ACM Auto AC DSP frequency response analyser and potentiostat again linked to a PC.

A Philips XL30 FEG SEM was used for all scanning electron microscopy.

2.1. Electrode preparation

Polymer modified insulated electrodes were prepared on gold-coated glass slides as reported earlier (Myler et al., 1997).

2.2. Sonochemical ablation of polydiaminobenzene ultra-thin films

The polymer modified electrodes were then immersed in a beaker containing distilled water, and then sonicated for 1, 5, 10, 20, 30, 40, 50, 60 and 90 s using a Camlab Transsonic T460, 25 kHz sonic bath. This is set and calibrated by the manufacturer and we have verified this using a crystal microphone and recording the sound waves on a digital storage

oscilloscope. The sonic bath employed 12 transducers geometrically arranged and bonded to the base of the stainless steel tank housing.

2.3. Polymerisation of microelectrode arrays

For the polymerisation of glucose oxidase enzyme microelectrode arrays, a pH 4.5 phthalate buffer was first prepared using 0.2 M potassium hydrogen phthalate and 0.2 M NaCl. A 0.2 M aniline solution was then prepared in the phthalate buffer. Glucose oxidase, (500 units ml⁻¹) was prepared in distilled water, to avoid denaturing the enzyme. 2.7 ml of the buffer and enzyme preparation (1.35 ml each) were mixed in a small volume PTFE cell, immediately prior to the electrochemical coating procedure being performed.

Aniline/GOD was polymerised by potentially sequentially cycling for 5 min between -200 and +800 mV versus Ag/AgCl at 50 mV s⁻¹, film growths were terminated at -0.2 V. Immediately following polymerisation, the working electrode was submerged in pH 7.4 phosphate buffer to prevent enzyme denaturation.

2.4. Voltammetric techniques

Hydrodynamic voltammetric techniques were used to examine the stir-independent behaviour of the microelectrodes. Polydiaminobenzene coated electrodes, sonicated for 1, 10, 20, 30, 40, 50, 60, and 90 s, were placed in the glass stage within the cell along with a gold sputtered counter electrode and Ag/AgCl reference electrode. The electrodes were submerged within a 5 mM ferricyanide/phosphate buffer solution previously purged for 20 min with N₂; the working electrode was then polarised at -300 mV versus Ag/AgCl. A magnetic stirrer was used to agitate the solution. Current responses were recorded using a PC with dedicated software. We have determined the reproducibility of sensor responses to be <5% variability in a series of trials.

2.5. AC impedance analysis

A sealed glass cell assembly contained a gold sputtered auxiliary, a Ag/AgCl reference and an enzyme/polyaniline microelectrode array in pH 7.4 phosphate buffer solution previously purged with nitrogen for 20 min for the removal of oxygen prior to any electrochemical investigation. Serial additions of glucose were introduced to the cell giving 1, 5, 10, 20, 30 and 40 mM concentrations. AC impedance measurements were performed using an ACM Auto AC DSP frequency response analyser between the frequencies of 0.1 Hz and 10 kHz.

3. Results and discussion

We have within our laboratory previously shown that 1,2-diaminobenzene dihydrochloride may be electropolymerised

at conductive surfaces via a $2e^-$ process to form essentially defect free insulating polymer films of less than 100 nm thickness (Myler et al., 1997).

The rationale underpinning this work is that sonochemical ablation of thin insulating polymer films at electrode surfaces may expose localised areas, each of which can act as localised microelectrodes and collectively as a microelectrode array.

Many other workers have studied the immobilisation of enzymes (e.g. the oxidases and dehydrogenases) within conducting polymers such as polyaniline or polypyrrole for use within sensors (Foulds and Lowe, 1986; Shaolin et al., 1992). We will, in this paper, describe a technique that allows the co-deposition of glucose oxidase within the conducting polymer, polyaniline, at conducting microelectrode cavities to form “mushroom” shaped microelectrode protrusions.

The interrogation of enzymatic microelectrode sensors of this type is described by both amperometric or ac impedance approaches to demonstrate their versatility. Scanning electron micrographs of electrode surfaces are included at each stage of the electrode fabrication process.

1,2-Diaminobenzene was first electropolymerised on gold sputter coated ground glass slides as previously reported (Myler et al., 1997) and this is shown schematically in Fig. 1(a). Cyclic voltammetry for the electrodeposition of insulating polydiaminobenzene film, Fig. 1(b), shows that diminishing peak currents are seen as the electrode becomes progressively insulated by the polymer film as it is deposited at the electrode surface (Myler et al., 1997).

Scanning electron microscopy of a polydiaminobenzene coated gold substrate, Fig. 1(c), confirms that its surface is largely featureless and that the electrode is coated and so insulated by an essentially defect free polymer film of polydiaminobenzene. Charge integration calculations for the current passed during the electropolymerisation would suggest that the polymer film is ~ 30 – 40 nm in thickness, in close agreement with previously calculated and experimentally determined thicknesses (Myler et al., 1997).

Ultrasound (in the kHz range) passing through a solvent such as water causes thermal agitation and localised hotspots of up to several hundred to a few thousand K, which in turn gives rise to the formation of superheated vapour bubbles (Suslick, 1990; Taleyarkan et al., 2002). These bubbles are, however, cooled by the solvent at ambient temperature (in our studies 25°C) and asymmetrically implode with the ejection of micro-jets of solvent at speeds of up to several hundred ms^{-1} . These micro-jets may cause the shattering of hard brittle solids and this is exploited, for example, within medicine for the shattering of kidney stones. Soft solid surfaces such as polymers may, however, be ablated by such jets (Suslick, 1990).

Sonochemically fabricated microelectrodes were prepared via the ablation of polydiaminobenzene films using an ultra-sonic frequency of 25 kHz as shown schematically

within Fig. 1(d), for a range of differing time durations from 1 through to 90 s.

Scanning electron micrographs for a 60 s sonicated electrode assembly are shown in Fig. 1(e). These were chosen since these provided the clearest images of the cavitation at the polymer surface. There are several features of interest within these micrographs. Firstly the distribution of the pores is random since ultra-sonic cavitation is a chaotic process. It is also evident that almost all of the cavities are bimodal in size, possessing either $\sim 3\ \mu\text{m}$ ($\pm 1\ \mu\text{m}$) or sub-micron diameters. We believe that the smallest of the cavities observed are formed by the initial impact of the micro-jets of fluid (Suslick, 1990). These cavities are known to act as nucleation sites for further bubble formation (Suslick, 1990) and it is thought that the cavity grows as new bubbles implode within the confines of the original cavity. This process gives rise to a quantum enlargement in the diameter of a cavity. Since no larger pores are seen it is believed that the $3\ \mu\text{m}$ diameter pores no longer act as nucleation sites. In some instances there is evidence of a few pores joining to form dumbbell shaped cavities when two pores form in close proximity to each other, Fig. 1(e). Fig. 1(f) shows a close-up of the pores, showing how the polymer coating has been peeled back from the substrate.

3.1. Electrochemical characterisation

Electrochemical evaluation of microelectrode assemblies was performed for possible use within sensors or biosensors. Microelectrodes characteristically exhibit sigmoidal shaped cyclic voltammograms (Southampton Electrochemistry Group, 1985) for reversible solution bound redox couples such as $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, in contrast to the characteristic diffusion limited reversible voltammograms expected at a planar electrode surface.

Fig. 2 shows a series of cyclic voltammograms recorded at a scan rate of $50\ \text{mV s}^{-1}$ for a 1 mM solution of $\text{Fe}(\text{CN})_6^{3-}$ at (i) planar gold electrodes, the (ii) sonochemically fabricated microelectrode arrays and (iii) polydiaminobenzene coated gold electrodes as a control. The voltammogram corresponding to the plain gold slides reveals an approximate 59 mV peak separation as would be expected for a $1e^-$ diffusional controlled reversible reduction/oxidation process. Cyclic voltammograms for the polymer insulated electrode and the microelectrode array are also shown in the enlarged bubble caption of Fig. 2 for clarity. Very little voltammetric response is observed at the polydiaminobenzene coated electrode, confirming that it has been insulated. The microelectrode array electrodes yield sigmoidal shaped voltammetric profiles suggesting that all of the microelectrode arrays formed in this way were indeed exhibiting true microelectrode-like behaviour. Individual microelectrodes would be expected to see forward (reductive) and reverse (oxidative) current transients that would fall almost on top of each other with little evidence of double-layer charging

capacitance, however separate forward and reverse peaks displaying evidence of double-layer charging are observed in this case due to the very large pore density (115,000 microelectrodes cm^{-2}) that will give rise to a significant cumulative double-layer charging current.

3.2. Microelectrode population density and reproducibility of sensor arrays

It is important to understand in this context both how microelectrode population densities grow with increasing

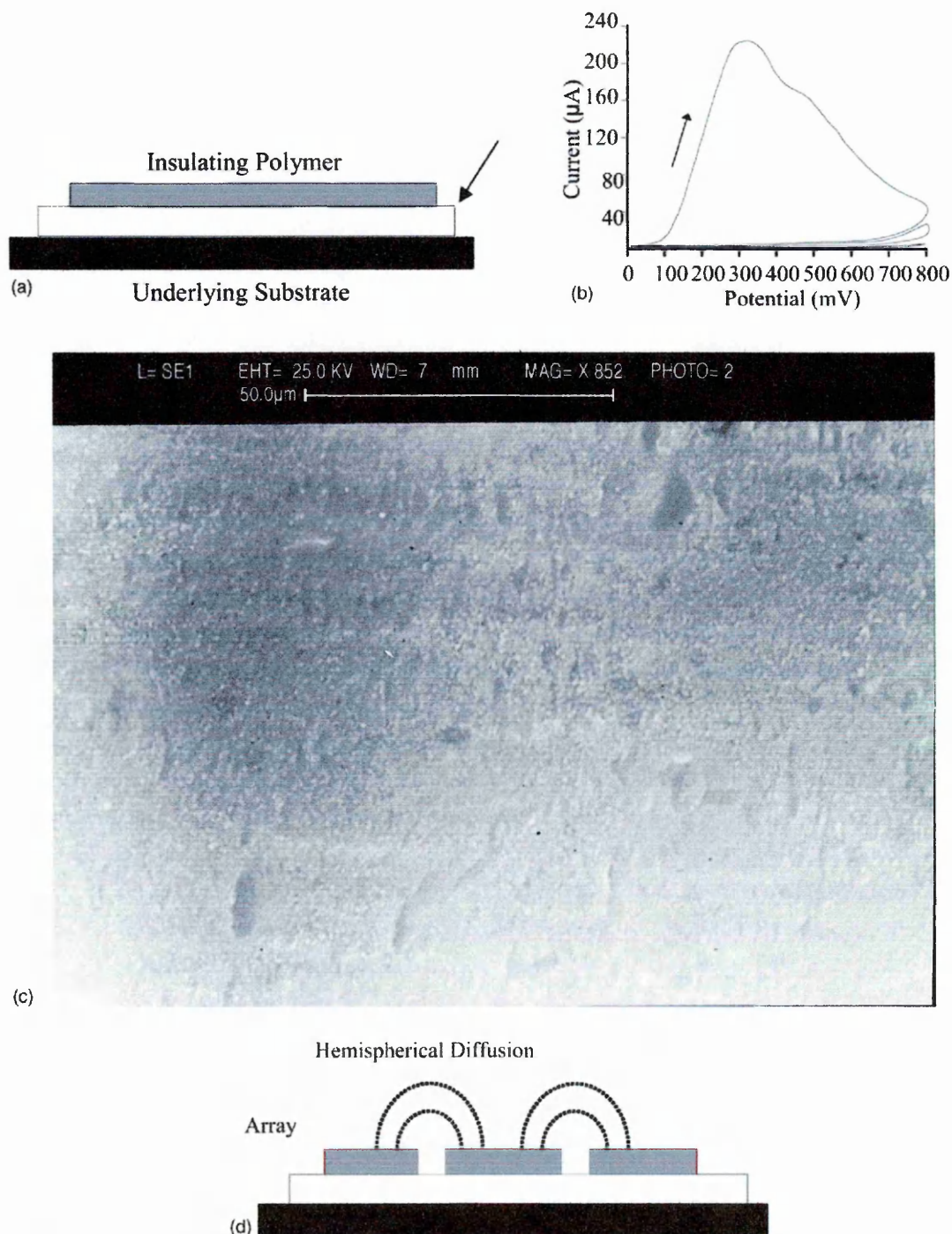


Fig. 1. Deposition of polydiaminobenzene to form an insulating film. (a) Schematic of polymer insulated electrode. (b) Cyclic voltammogram for polymerisation of 5 mM *o*-phenylenediamine dihydrochloride at a potential scan rate of 50 mV s^{-1} . (c) Electron micrograph of polydiaminobenzene insulated gold electrode surface. (d) Schematic of microelectrode array (e) Electron micrograph of sonochemically fabricated microelectrode array. (f) Close-up of sonochemically fabricated microelectrode array showing pore structure.

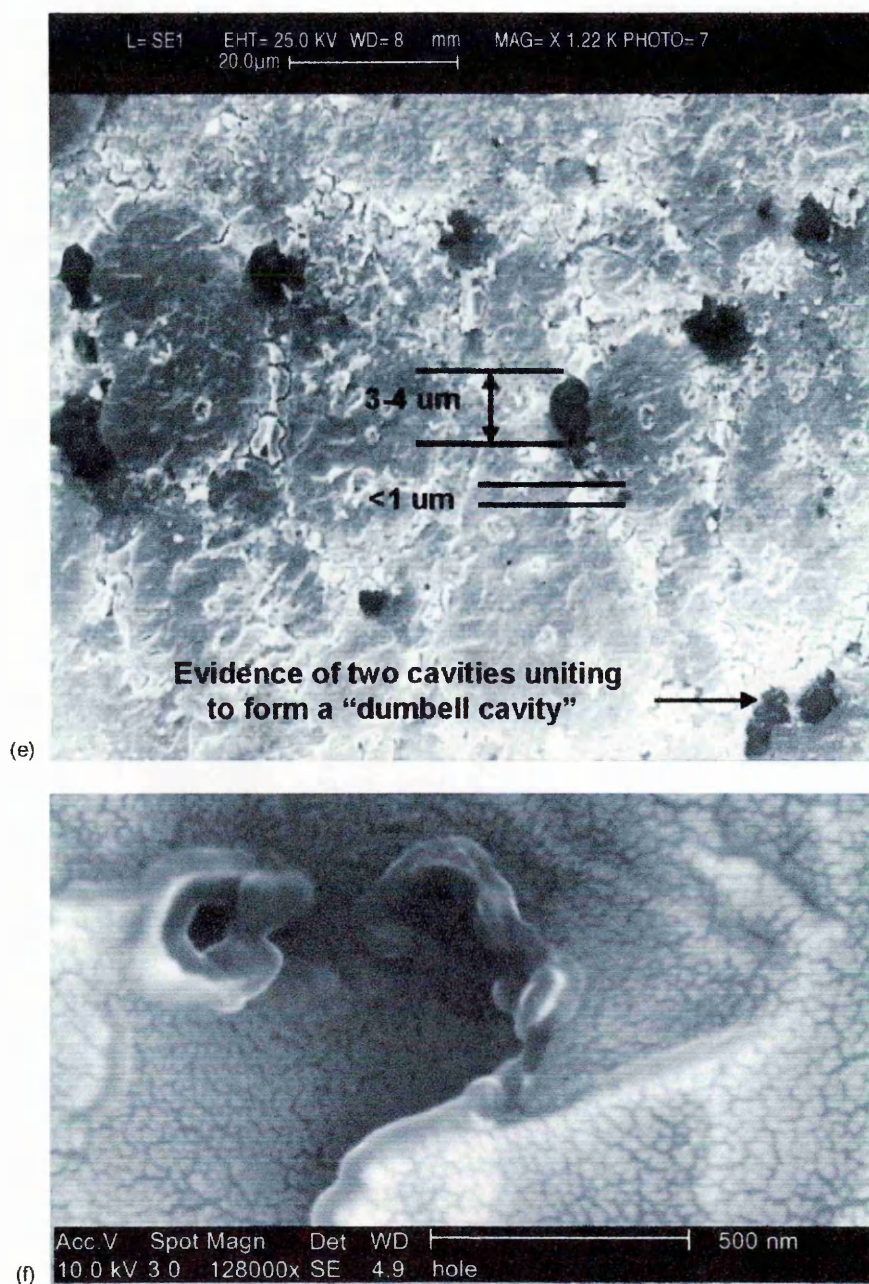


Fig. 1. (Continued).

sonication time as well as the reproducibility of their formation. Population density may be estimated via SEM imaging across a number of quantified cross-sectional areas. Fig. 3(a), shows that the calculated population density of the microelectrode arrays enlarges in a near linear manner as the sonication time increases from 1 through to 90 s. Coefficient of variance of the charge densities for arrays was found to be <2% (at an electrode state $E = -100$ mV versus Ag/AgCl for the reduction of 1 mM $\text{Fe}(\text{CN})_6^{3-}$).

3.3. Convective stir-independence of microelectrode arrays

The convective (stir-independent behaviour) of the microelectrode arrays was investigated since this is one of the most significant advantages that arrays could offer for use within sensors.

Amperometric responses of microelectrode arrays formed via a range of differing sonication times polarised at -600 mV versus Ag/AgCl to 1 mM $\text{Fe}(\text{CN})_6^{3-}$, were

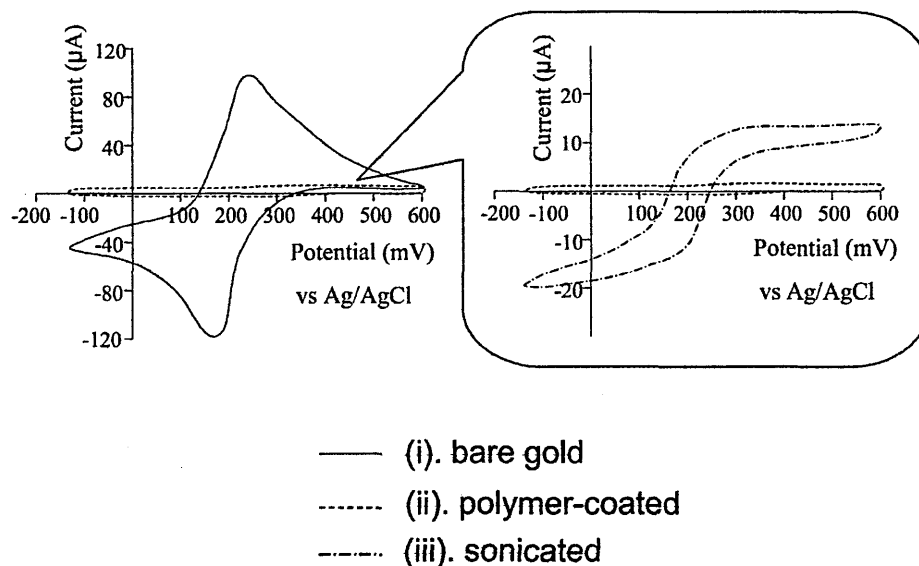


Fig. 2. Cyclic voltammetry of ferri/ferrocyanide couple at: (—) bare gold electrode, (---) polymer coated gold electrode and a 20 s sonochemically fabricated microelectrode array (— · —). Scan rate 50 mV s^{-1} .

recorded in both unstirred (quiescent) and stirred solutions. If the microelectrodes were to become too closely packed together, eventually hemispherical diffusion profiles would begin to overlap and ultimately all microelectrode-like behaviour would be lost.

The calculation of the percentage change in amperometric response in a quiescent and a stirred solution for a given electrode allows us to assess when microelectrode behaviour begins to be lost, as microelectrode populations increase. The performance of electrodes prepared via different son-

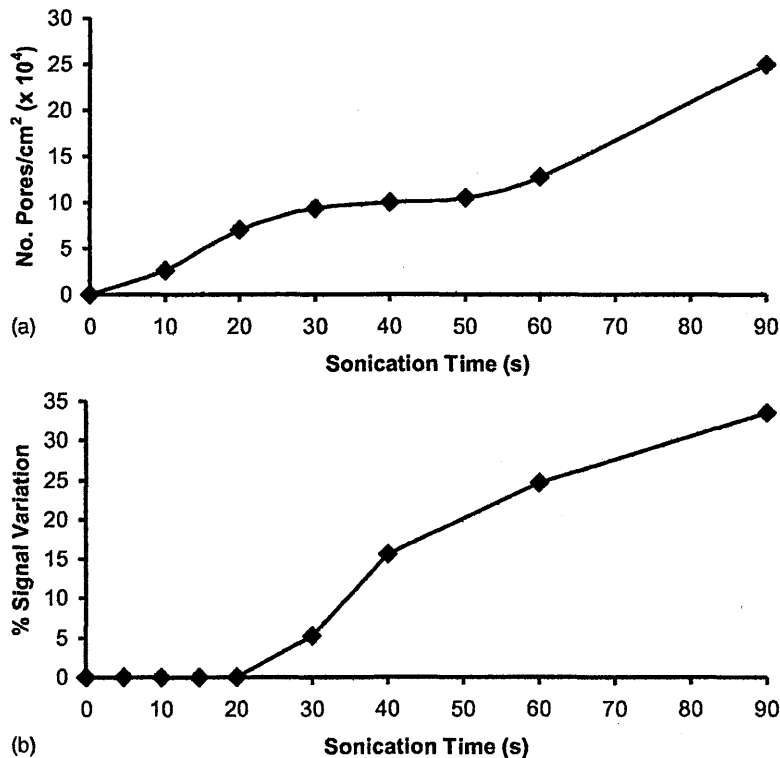


Fig. 3. (a) Population densities of sonochemically formed pores and (b) stir-dependence/independence of microelectrode arrays of these system with differing sonication times.

ication times, Fig. 3(b) shows the percentage change in amperometric responses for electrodes when stirring is introduced (in excess-via the use of a mechanical stirrer) in comparison to the response for the same electrode in a quiescent (unstirred) solution.

Microelectrode arrays prepared via 20 s or less sonication show no change in signal response to the reduction of Fe(CN)_6^{3-} , confirming that under these conditions the microelectrode arrays indeed show true stir independence in their response behaviour. Microelectrodes sonicated for 30 s, give rise to a ~5% increase in response upon stirring the solution—and this variation increases progressively to a value in excess of 30% for electrode arrays prepared via 90 s sonication. It is therefore clear that for electrodes prepared via 30 s or longer sonication, that the microelectrode arrays population density reaches a value where the hemispherical

diffusional profiles of the individual microelectrodes begin to overlap and that this effect progressively leads to the loss of stir-independent performance.

3.4. Electropolymerisation of enzyme containing polymer protrusions in microelectrode arrays

Aniline solutions containing glucose oxidase were electropolymerised on microelectrode surfaces to form enzyme containing protrusions at each of the microelectrode cavities of the array as shown schematically, Fig. 4(a). Fig. 4(b), shows cyclic voltammograms recorded for the electropolymerisation of aniline from phosphate buffer containing glucose oxidase. The voltammogram is characteristic of an irreversible deposition with successive voltammograms progressively showing smaller peak currents (Foulds and Lowe,

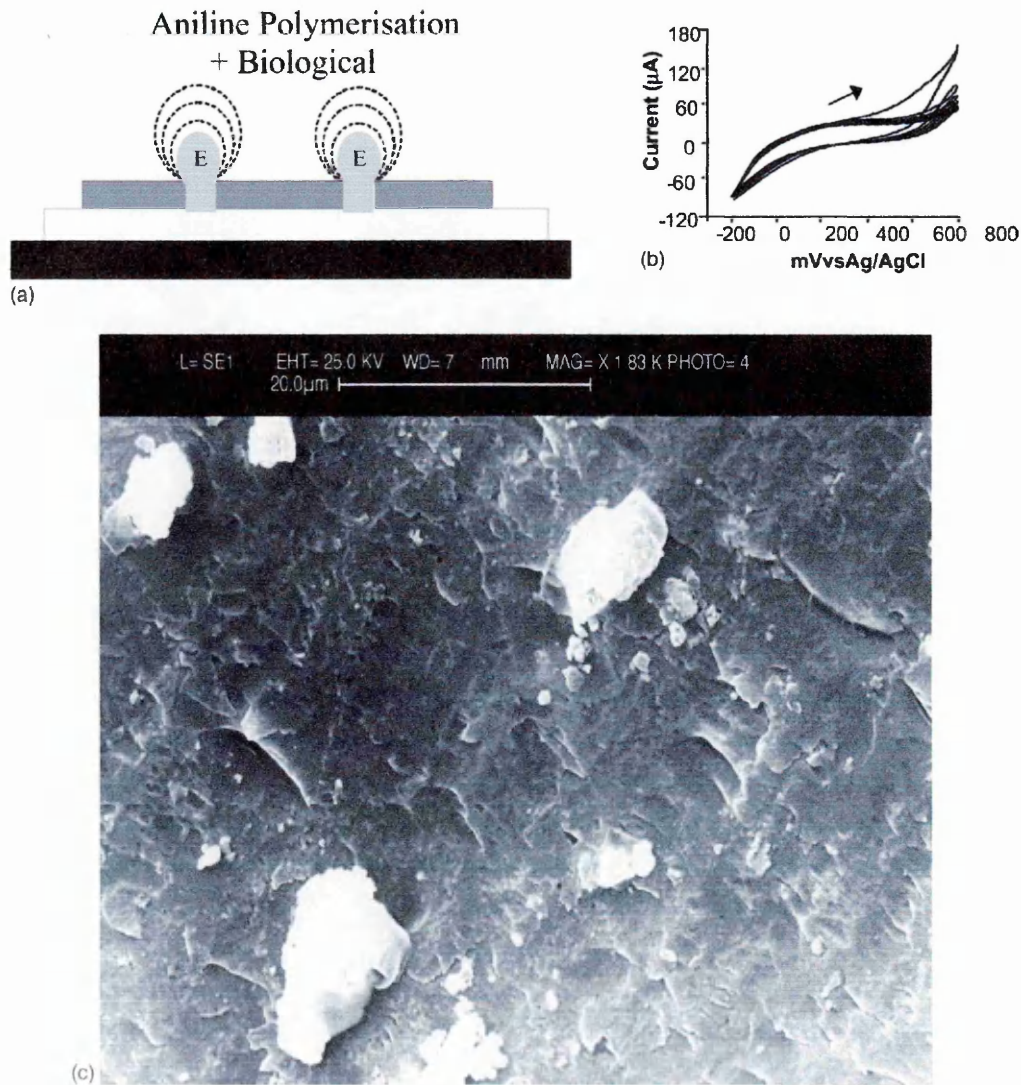


Fig. 4. Generation of enzyme containing-polyaniline protrusion microelectrode arrays. (a) Schematic of sonochemically fabricated polyaniline/enzyme microelectrode array. (b) Cyclic voltammetry (50 mV s^{-1}) for electropolymerisation of polyaniline and co-entrapment of glucose oxidase. (c) Scanning electron micrograph of sonochemically fabricated polyaniline enzyme microelectrode array.

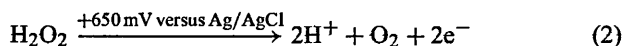
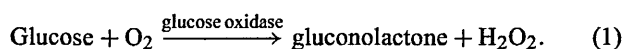
1986, Shaolin et al., 1992). Scanning electron microscopy, Fig. 4(c), of these arrays following the electrodeposition of polyaniline shows that polymer protrusions are indeed formed via growth of the polymer from the sonochemically fabricated pores. Several interesting points should be noted from the electron micrograph of Fig. 4(c). Firstly it can be seen that all of the microelectrode cavitations appear to be filled with polyaniline, including the very smallest of the $\sim 0.1 \mu\text{m}$ diameter pores. This shows all ablative cavitations of the insulating polymer film lead to exposure of the underlying conductive surface, since aniline could not be electropolymerised without access to the underlying conducting surface.

3.5. Sensor interrogation approaches

Enzymic microelectrode arrays of this type may interrogated by both (i) amperometric and (ii) impedimetric approaches. Microelectrode arrays prepared by 20 s sonication were used throughout, since these offered the largest response whilst retaining stir-independent properties.

3.5.1. Demonstration of amperometric interrogation

Enzyme electrodes were polarised at +650 mV versus Ag/AgCl for the interrogation of glucose responses as a first demonstration of the enzymic microelectrode array described in this paper. Glucose oxidase catalyses the production of H_2O_2 in the presence of glucose and oxygen, Eq. (1). This approach allows for the interrogation of the sensor based on the amperometric monitoring of H_2O_2 , Eq. (2).



The calibration curve of Fig. 5(a), clearly demonstrates that an amperometric enzymatic sensor may be produced (with stir-independent responses) that could be exploited for a variety of applications in which various flow conditions may be encountered.

3.5.2. Demonstration of impedimetric interrogation

Impedimetric measurements for enzyme electrode sensors were recorded in the plane of the working microelectrode array $\rightarrow$ analyte $\rightarrow$ solution $\rightarrow$ electrode. Since the solution impedance contribution will affect the final response of any sensor, defining and controlling the inter-electrode spacings of the working microelectrode array and counter electrode would clearly be crucial for all electrochemical investigations. A glass stage was therefore designed, to hold the working microelectrode array a fixed distance of 10 mm from the counter electrode within an analyte solution, with this assembly being designed so as to be held within a commercial Methrohm[®] glass cell as previously reported (Myler et al., 1997).

AC impedance spectra were first recorded for glucose oxidase/polyaniline sonochemically fabricated microelectrode arrays across a range of frequencies from 0.1 through to 10,000 Hz upon $\pm 5 \text{ mV}$ ac excitation (0 V bias) (Teasdale and Wallace, 1993), in order to characterise electrochemical redox behaviour and sensor performance for a range of differing concentrations of glucose within O_2 saturated conditions, Fig. 5(b). This bias potential allows monitoring of the impedance (conductivity) of the polymer without interference from, for example, the oxidation of H_2O_2 or biological interferents such as ascorbate. It is clear that AC Bode impedance spectra show a clear trend towards lowered impedances upon increasing concentration of glucose, with lowered impedances also occurring at higher frequencies.

Control experiments were also performed by recording impedance spectra for enzyme free polyaniline microelectrode arrays (not shown for clarity) and showed minimal impedance changes to differing concentrations of glucose, confirming that the sensor responses observed within Fig. 5(b) were indeed enzymatic in nature.

It is firstly clear that increasing concentrations of glucose give rise to lowered impedances, via the enzymic activity of glucose oxidase.

A plot of total impedance versus frequency is shown in Fig. 5(b) for a microelectrode exposed to 20 mM glucose with a corresponding Nyquist phase angle plot. For simplicity, only these plots are shown, since similar profiles are seen for all of the glucose concentration ranges studied. Fig. 5(c) shows a straight line profile of the real Z' versus the imaginary Z'' components with a slope close to unity, indicative of a diffusion controlled reversible process, where the phase angle (θ) profiles seen within Fig. 5(b) would appear to be dictated by the capacitance of the polymer film in conjunction with the double-layer contribution within the circuit.

Until now, we have only considered how recorded impedance values, or components thereof, vary under differing conditions, as opposed to how percentage changes in impedimetric behaviour vary even though the total impedance measured may be dominated by the background impedance of the circuit. Percentage changes of microelectrode array glucose sensors across a range of glucose concentrations as a function of frequency under aerobic conditions are shown within Fig. 5(d). Percentage impedances are reported relative to the corresponding impedance at 0 mM glucose at a particular frequency, as shown in Fig. 5(d). These percentage changes may be plotted with respect to the analyte concentration in the form of a calibration plot, Fig. 5(e) clearly demonstrating this approach for interrogation.

The most prominent feature of this data is that impedance minima are observed at frequencies between 10 Hz and 100 Hz. Similar responses are seen under anaerobic conditions (not shown for clarity), although the percentage changes in response are not so pronounced. We have already shown that the impedance values are modulated by enzyme catalytic behaviour, although the nature of the impedance changes have not yet been considered.

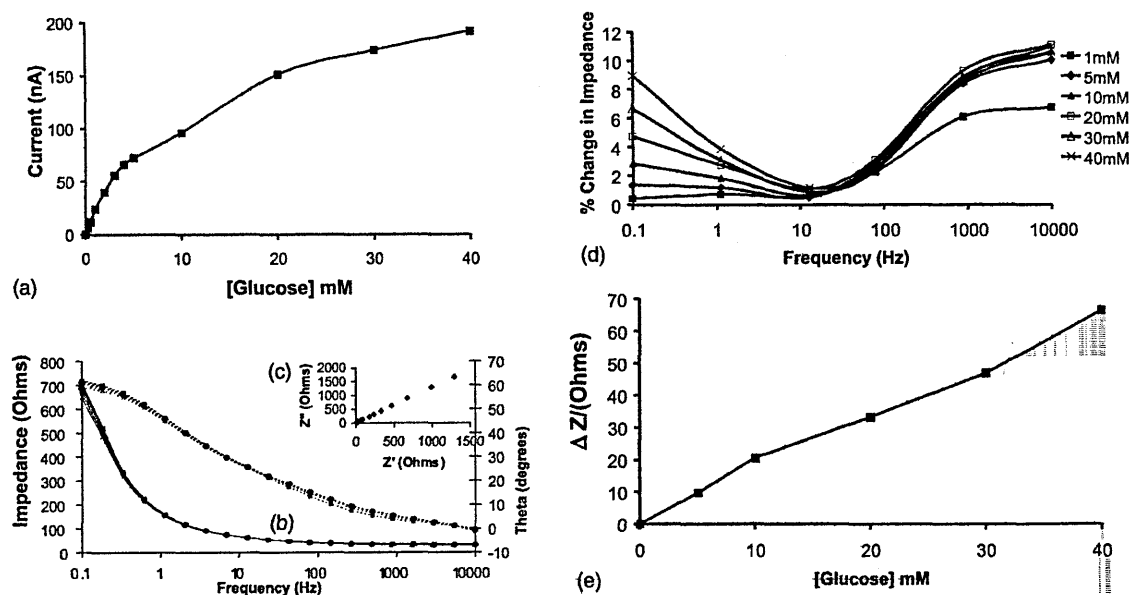


Fig. 5. (a) Amperometric glucose calibration curve for polyaniline/glucose oxidase microelectrode array. (b) Total impedance (solid line) and phase angle (dashed line) of microelectrode arrays immersed in glucose/phosphate buffer solutions. Concentration of glucose: 0 mM (■), 1 mM (◆), 5 mM (▲), 10 mM (*), 20 mM (□), 30 mM (Δ), 40 mM (×). (c) Complex plane plot for a microelectrode array in 20 mM glucose. (d) Percentage change in impedance for microelectrode arrays in various concentrations of glucose vs. phosphate buffer. Concentration of glucose 1 mM (■), 5 mM (◆), 10 mM (▲), 20 mM (□), 30 mM (Δ), 40 mM (×). (e) Calibration plot of Impedance change vs. glucose concentration for a 20 s sonicated GOD microelectrode.

At this stage the possible mechanisms by which the conductivity of the polyaniline may be modulated should be considered. Polyaniline has three differing forms (Eggins, 1996).

The first mechanism by which glucose oxidase may give rise to redox modulated conductivities within the polyaniline involves the catalysed production of H_2O_2 (Eggins, 1996). Previous workers (Cooper and Hall, 1992) have shown that in aerobic conditions, H_2O_2 would be expected to play a major role in the modulation of the impedimetric behaviour of polyaniline. The responses observed under anaerobic conditions could clearly not be accounted for by such a mechanism, since without a supply of molecular oxygen as an electron acceptor from the enzyme, H_2O_2 could not be produced. Since we have established that the impedimetric responses are due to enzyme catalytic behaviour, it follows that electron donation from the enzyme must be occurring by some other mechanism. Since the sensor responses under aerobic conditions are always greater than those observed under anaerobic conditions, it is therefore probable that the generation of H_2O_2 in aerobic conditions might contribute to this behaviour—even if additional mechanisms are involved in the overall response.

A number of other possible mechanisms to explain anaerobic glucose oxidase responses have also been proposed by other workers. One of the most controversial of these is via a possible direct electron transfer occurring between the active site of enzyme and the polymer (Cooper and Hall, 1992). The distance through which electrons would have to traverse would be in excess of 1.3 nm (<http://www-biol.paisley.ac.uk>,

academic website containing enzyme database.) and would on first reflection appear to be prohibitive. Other workers have shown, however, that electron tunnelling can occur across distances that had previously been thought impossible via complex pathways of unsaturated and delocalised bonds together even with some saturated bonds and free space (Moser et al., 1992; Onuchic et al., 1992).

It should not be forgotten that gluconolactone may be produced under anodic conditions so long as a surrogate electron acceptor for the enzyme is provided. Gluconolactone readily hydrolyses to form gluconic acid and this may easily protonate the polymer allowing another route for altering the conductivity of the polymer.

Skinner and Hall (1997) have previously shown that gluconolactone produced under anaerobic conditions may give rise to conductivity changes within the polymer via interaction of gluconolactone with the emeraldine base to form a zwitterion complex that itself may be readily oxidised through to the perigraniline form of the polymer.

While this behaviour would account for anaerobic impedimetric responses for our sensors to glucose, it would be difficult to fully explain why the response profiles we have observed exhibit minima at frequencies of approximately 10 Hz to 100 Hz. We believe that this behaviour could be linked to the hydrogen bonding of water to the imine centre of the polymer. These effects have been extensively studied and while these findings cannot be fully described here due to space limitations, they will form the basis of a subsequent publication.

3.6. Applications of sonochemically fabricated sensors

The focus of this report has been to show that sonochemical ablation of thin-polydiaminobenzene film coatings that insulate planar electrode surfaces can be exploited as a novel approach for the fabrication of microelectrode arrays. It was, at the outset, our intention to exploit these arrays as templates within a range of differing sensors and biosensors.

Enzymatic sensors of this type have so far been developed for ethanol, oxalate and a range of pesticides as well as DNA hybridisation and affinity antibody/antigen based sensors, all of which will be reported in forthcoming publications. These enhanced sensitivities and lower limits of detection have been made possible via the hemispherical diffusional profiles these microelectrode arrays experience.

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Label-free and reversible immunosensor based upon an ac impedance interrogation protocol

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Abstract

We report the fabrication of a label-free and reagentless immunosensor based on the direct incorporation of antibodies into conducting polymer films along with a subsequent ac impedimetric electrochemical interrogation. Model sensors of this type were prepared by electrochemically polymerising conducting polypyrrole films containing anti-BSA at the surface of screen-printed carbon electrodes. Films containing chloride or anti-human IgG as counter-ions were used as controls. An ac measurement protocol was used to determine the impedance of the electrodes when immersed in water or analyte solutions. A selective and reversible binding of analyte to the electrode could be monitored electrochemically and studies are reported in detail relating analyte concentrations to bulk impedimetric measurements, the real component, the imaginary component and the phase angle of the responses. The results of this study showed detectable and reversible antibody–antigen interactions could be measured and mainly affected the Faradaic behaviour of the electrode. BSA could be detected with a linear response from 0 to 75 ppm.

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Keywords: Immunosensor; ac Impedance; Bovine serum albumin; Polypyrrole; Detection

1. Introduction

Since Yalow and Berson in 1959 [1] established the principle of immunoassay, a wide range of novel assay designs for many analytes have been described. Immunological techniques offer benefits in terms of cost and ease of use compared to more traditional techniques such as mass spectrophotometry and gas liquid chromatography. An immunosensor format that does not rely on the use of a secondary label as a detection principle, e.g. an enzyme or radioisotope, could lead to simpler immunoassay formats and ideally lower times of detection. The rapid measurement of analytes of clinical significance such as disease markers could in turn permit ear-

lier intervention, which in a medical setting is frequently of utmost importance.

Antibodies bind solely to their antigens, usually via a complex series of multiple non-covalent bonds with no newly generated molecules, electronic reactions or exchanges accompanying the reaction to aid detection. The incorporation of antibodies into conducting polymer films was first reported by John et al. [2]. Their technique of dc pulsed amperometric detection has been transferred to other analytes, including *p*-cresol [3], thaumatin [4] and polychlorinated biphenyls [5], and it is from here that the basis of the work described in this communication was developed. Recent work has showed that antibody-containing films can be used to detect luteinising hormone [6,7] and that similar films when interrogated by impedance spectroscopy can be used as sensors for biotin and DNA [7]. Mammary tumour-associated glycopro-

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tein has been detected using impedance techniques where the corresponding monoclonal antibody GP1D8 was directly and spontaneously immobilised onto the gold surface of the electrodes used for measurement [8]. Both anti-rabbit IgG and anti-human IgG have been immobilised onto functionalised polypyrrole films and interrogated by impedance spectroscopy by Ouerghi et al. A linear range of 600–900 ng ml⁻¹ was reported for the anti-rabbit IgG–polycyanoethylpyrrole film [9] whilst a much lower detection level at 10–80 ng ml⁻¹, with a limit of detection down at 10 pg ml⁻¹ was reported for the anti-human IgG–biotin polypyrrole film [10]. Other work by Sadik et al. has developed a method of differential impedance spectroscopy which can detect presence of antigens such as cyanazine down to 0.01 ppm [11].

Previous work by this group [12,13] has studied the incorporation of anti-BSA into polypyrrole films and their use to detect BSA by dc pulse amperometry. However, in the case of BSA, there were problems with electrical double layer effects that interfered with signal measurement and led to difficulties in interpreting the data. Therefore, in the present work, a new protocol will be described in which the films will be monitored using an ac impedimetric interrogation of an antibody-containing polymer film for constant excitation.

In a previous report [12] we unambiguously showed that by electrically stimulating a conducting polymer containing an antibody, it is possible to follow the electrical relaxation of the polymer by recording current transients after antibody–antigen associations were occurring, i.e. compared to a polymer/antibody system not exposed to antigen.

In this paper we are elaborating on this concept by attempting to continually stimulate the polymer/antibody system by means of a ac waveform perturbation; we further report on how the resolution of the ac response may be related to the origin of the gross ac impedimetric response. We were the first to show and explain the true origins of the results first derived by Sadik et al. [4] how this interrogation approach could be made reproducible [12], we now report findings relating to the nature of the ac responses observed by ourselves and others [7].

ac impedance could in theory allow the current to be monitored at any particular phase angle with respect to the polarising potential. In this model, if the current was monitored directly in phase with an alternating polarising waveform potential, the current would reflect and thus allow the determination of the value of the resistive component within the circuit. In a similar manner, if the current was monitored at a phase angle of 90° ahead of the polarisation potential, it would allow the calculation of the value of the capacitive current.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise stated. Concentrated sulphuric acid was purchased from Fisher

Scientific UK (Leicestershire, UK). Bovine serum albumin (BSA), pyrrole, sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate, platinum gauze, platinum foil, antibody conjugates and sheep anti-BSA antibodies were supplied by Sigma–Aldrich Company Ltd. (Dorset, UK). Deionised water was obtained from a Millipore Milli-Q purification system. Pyrrole was double-distilled under vacuum in batches of approximately 10 ml using a Quick Fit micro-distillation apparatus. After distillation it was placed in a darkened container and deoxygenated by bubbling nitrogen through the solution. Pyrrole solution was stored in a sealed container at –20 °C and was warmed to room temperature before use.

Screen-printed carbon electrodes with a working electrode area of 9 mm² were purchased from Gwent Electronics Materials Ltd. Electrodes were printed on alumina substrates in sheets of 20.

2.2. Preparation of 50 mM phosphate buffered saline (PBS) pH 7.4

PBS was prepared by dissolving 965 mg disodium hydrogen phosphate, 330 mg sodium dihydrogen phosphate and 4.25 g sodium chloride into 500 ml of distilled water.

2.3. Preparation of screen-printed carbon electrodes and electrochemical cells

Individual screen-printed carbon electrodes were immersed into 50 mM PBS, pH 7.4, with an Ag/AgCl reference electrode and a platinum gauze auxiliary electrode.

2.4. Polypyrrole/anti-BSA films for impedance analysis

Distilled pyrrole (173 µl) was stirred gently in deionised water 5 ml for 5 min until dissolved after which 150 µl of sheep anti-BSA was added and thoroughly mixed into the solution. All pyrrole/anti-BSA films were grown as described [9,10] earlier in a three-electrode cell using a galvanostatic protocol of 1 mA cm⁻² for 10 min.

2.5. Impedance analysis

Using a stationary cell in a three-electrode format, the pyrrole films were exposed to increasing concentrations of BSA dissolved in distilled water. A baseline of the impedance was obtained by placing the antibody loaded electrode into distilled water. In these series of experiments, there were three types of polymer deposited namely:

- Polypyrrole deposited with potassium chloride (as a control);
- Polypyrrole deposited with anti IgG (as a control);
- Polypyrrole deposited with anti-BSA.

Working electrodes were coated using each of the three approaches, placed into the three-electrode cell and exposed to

an ac waveform generated by an Gill AC (ACM Instruments) frequency response analyser. Each electrode was scanned between frequencies of 1 kHz down to 1 Hz between +500 and +100 mV versus Ag/AgCl; each electrode was allowed to equilibrate for 1 s and 50 points were taken per test with each electrode being scanned four times. This represented the closest parameters to the original pulsed amperometric investigations reported previously [9,10].

3. Results and discussion

3.1. Detection of bovine serum albumin

ac impedimetric responses of antibody loaded films may be presented in one of two ways:

- (a) as a Bode plot which displays total impedance versus frequency and indicates the overall result or
- (b) as a Nyquist Plot which displays either:
 - the real versus imaginary impedance, or
 - the phase angle versus frequency.

Representative plots will be shown, although for clarity not all the impedance spectra will be included.

3.2. ac impedimetric analysis of anti-BSA-loaded polypyrrole films upon exposure to BSA

Initial examination of the total impedance of each of the films at various concentrations of BSA and across the entire frequency range studied shows that a clear calibration curve can be observed when the BSA antibody-containing film is exposed to increasing concentrations of BSA (Fig. 1). The greatest impedance changes were observed at the highest frequencies studied (i.e. at 1 kHz) and consequently data obtained at this frequency will be used for all subsequent discussions. Total impedances were recorded across a BSA concentration range of 0–100 ppm for an anti-BSA loaded polypyrrole coated electrode (Fig. 1).

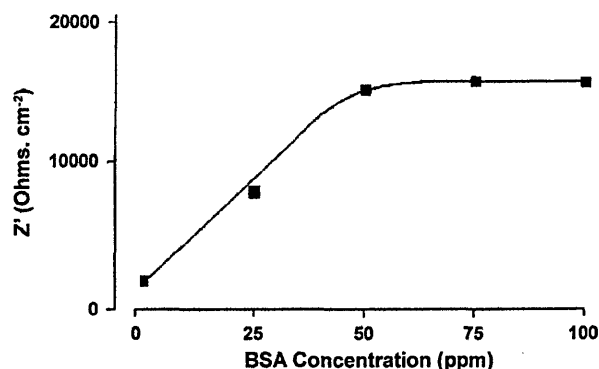


Fig. 1. Calibration curve of BSA concentration at 1 kHz. Calibration curve generated as a result of exposure of an anti-BSA loaded polypyrrole film to increasing concentrations of BSA. The curve displays total impedance (at 1 kHz) against BSA concentration.

In all cases a minimum of five replicate measurements were recorded on separate electrodes. The ac responses recorded (total ac impedance, real and imaginary components) gave R.S.D. values of <5%; these error bars are not displayed since these would not be distinguishable from the symbols used to plot data points.

It can be clearly observed that the calibration curve (Fig. 1) for BSA is similar in profile to the calibration curve we obtained and previously reported where an identically deposited BSA antibody loaded polymer electrode was exposed to varying BSA concentrations, excited and interrogated via a pulsed polarisation of the electrode [12,13]. Control films of (a) polypyrrole deposited with potassium chloride and (b) polypyrrole deposited with IgG (as described in Section 2), both showed decreases in impedance on exposure to increasing concentrations of BSA.

It would be expected that the impedance of any electrode could be affected when exposed to additional ions that might diffuse in to and then change the conductivity of the polymer [11]. The antibody loaded polypyrrole films appear to show signs of a possible plateau in response to the analyte at higher concentrations. However, it is apparent that this effect is occurring at a much lower concentration for the chloride and IgG electrode surfaces than that of the BSA antibody loaded film. In this context it is probable that the BSA antibody loaded polypyrrole film is providing specific recognition sites in comparison to the two films that do not contain BSA antibodies. Saturation of any of the films in all of these cases appears to lead to a decrease in total impedance observed. It is possible that the excess analyte could be giving rise to an alternative conduction pathway, which in turn could be reducing the total impedance within the cell. Similar results have been observed elsewhere for anti-IgA containing electrodes [11].

3.3. Investigations into the individual components of impedance

It is important to differentiate between the individual components of the total impedance of the cell so that the capacitive and Faradaic components of the composite impedimetric response may be identified and quantified. The initial components of interest were the real and imaginary or the Faradaic and capacitive components respectively, and the easiest way to observe the effects of these components was via analysis of a Nyquist plot. A classical Nyquist plot for a circuit containing both double layer capacitive and uncompensated resistive components in series as represented by Randles equivalent circuit (Fig. 2a), will display a semicircle representing the capacitive component and a straight line with a slope of 45° that indicates the contribution that the resistive component makes to the total impedance of the circuit (Fig. 2b).

From the Nyquist plots, the effects of the BSA concentration on the real and imaginary components of an anti-BSA loaded polypyrrole film can be observed. Before BSA was

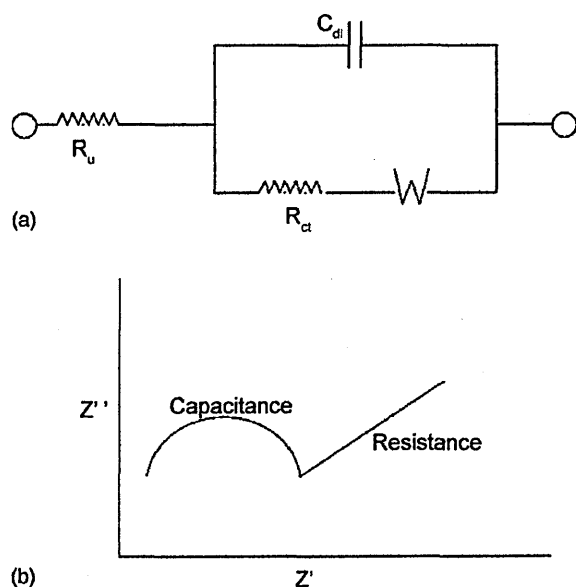


Fig. 2. (a) Randles equivalent circuit: model of a simple electrochemical cell which depicts the electrode reaction occurring in these experiments where C_{dl} is the double layer capacitance, R_u the solution resistance, R_{ct} the charge transfer resistance and W is the working impedance; (b) a classical Nyquist plot: a diagrammatic idealistic Nyquist plot displaying the classical semicircle of capacitance and the straight line of the resistance for a circuit containing both capacitive and resistive components in series.

added to the electrode, the impedance for the circuit was recorded with a Nyquist plot in this system being shown in Fig. 3. However, it was noticed that after the addition of BSA into the cell, the Nyquist plots become extremely difficult to interpret and that while both the imaginary and real components increase, the increase in the real component dominated the total increase in the impedance. It follows that the increase in the real component indicates in this situation that the Faradaic component of the impedance accounted for the larger increase in the impedance observed.

If the real and imaginary components against BSA concentration are plotted individually for replicate measure-

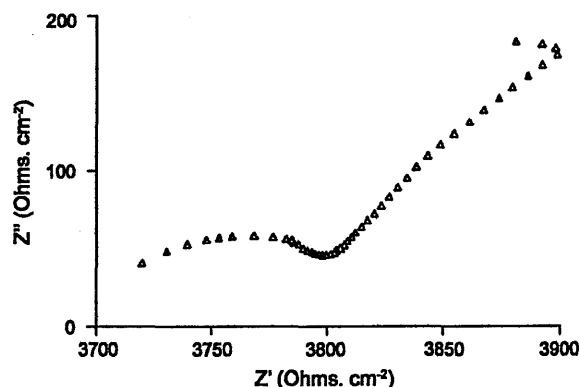


Fig. 3. Nyquist plots of polypyrrole films loaded with BSA antibody in the absence of BSA. Nyquist plot displaying the relationship between the real and imaginary components of a polypyrrole film loaded with BSA antibodies in the absence of BSA.

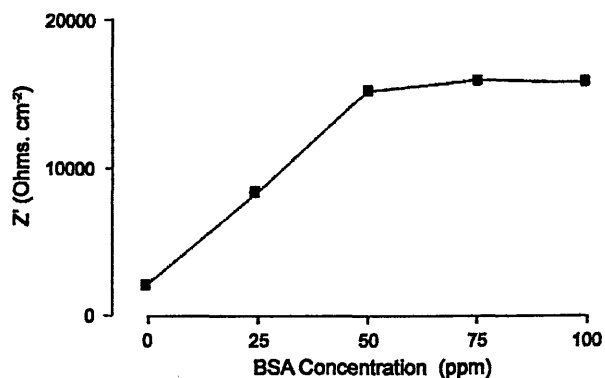


Fig. 4. The mean Faradaic component from 10 frequencies against BSA concentration. The graph displays the mean "real" component of the total impedance obtained at 10 different frequencies (from 1 Hz to 1 kHz) against several BSA concentrations.

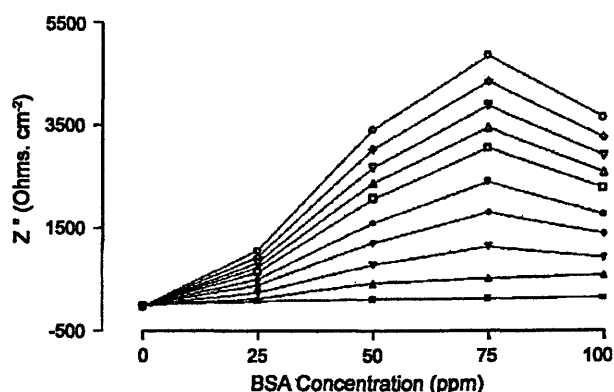


Fig. 5. Capacitive component at several alternative frequencies against BSA concentration. Imaginary component of the total impedance obtained at 10 different frequencies (from 1 Hz to 1 kHz) are plotted vs. several BSA concentrations. Frequency: 10 Hz (■), 100 Hz (▲), 200 Hz (▼), 310 Hz (◆), 410 Hz (●), 560 Hz (□), 640 Hz (△), 750 Hz (▽), 860 Hz (◇), 1 kHz (○).

ments at 10 different frequencies from a BSA antibody loaded film, it can be seen that the real component yields data that is frequency independent and offers far greater reproducibility in comparison to the imaginary contribution (Figs. 4 and 5, respectively). The coefficient of variation for each of the BSA concentrations of 0, 25, 50, 75 and 100 ppm for the real and imaginary components can be seen in Table 1.

The data plotted again displays a decrease in the capacitive component at higher BSA concentrations whereas the

Table 1
Coefficient of variation for the real and imaginary components

BSA concentration (ppm)	Coefficient of variation	
	Real component	Imaginary component
0	0.38	-512.06
25	2.33	61.34
50	2.35	63.45
75	3.99	63.57
100	2.99	60.40

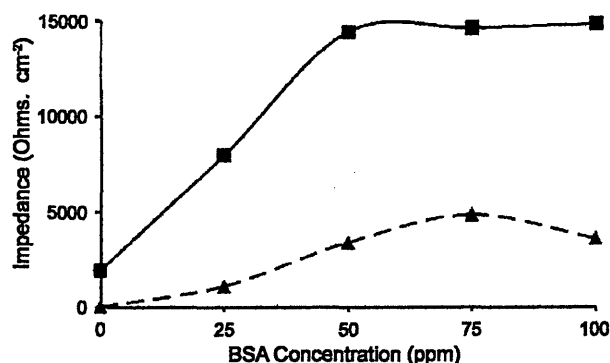


Fig. 6. Capacitive and Faradaic impedance changes of anti-BSA loaded polypyrrole to BSA at 1 kHz (capacitive (imaginary) response (▲), Faradaic (real) response (■)).

Z' component tends towards a plateau. These observations are to be expected in such a situation, as a change in the capacitive component is indicative of frequency dependent changes in the conductivity of the polymer film due to factors such as double layer charging effects. Since it is believed that saturation of the polymer surface would result in alternative conduction pathways being followed, it is possible this data could also suggest that these pathways might be largely capacitive in nature. It can also be observed that the imaginary component of the impedance dips above BSA concentrations of 75 ppm and that the total impedance follows a similar trend.

3.4. Comparison of resistive and capacitive responses

Fig. 6 shows the comparison between the capacitive and Faradaic components of the impedance. As can be seen, the effect of BSA concentration is much greater on the Faradaic component of the film rather than its capacitance.

Further analysis of the components of the total impedance reveals that for the films loaded with BSA antibodies, the phase angle decreases as the concentration of the BSA increases (Fig. 7). However, for the control films that were

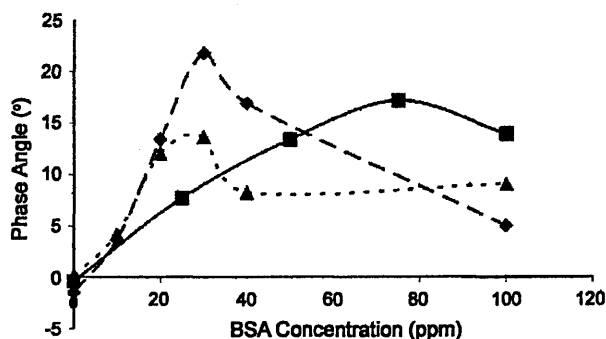


Fig. 7. The effect of BSA on the phase angle. Calibration curve expressing the relationship between phase angle and BSA concentration for an anti-BSA loaded electrode. Polypyrrole film containing: anti-BSA (■), anti-IgG (▲), Cl^- (◆).

deposited with either IgG antibodies or chloride as the counterion, the phase angle decreases until a concentration of approximately 25–30 ppm BSA after which it increases again. These observations of the phase angle correspond to similar observations made within the capacitive component which was to be expected and supports the possibility that an alternative conduction pathway is formed upon saturation of the polymer film thus leading to decreases in the total impedance of the film at the higher BSA concentrations.

The nature of the Faradaic response cannot at this stage be speculated on, although control experiments have confirmed this to be related to an affinity binding of the antigen and antibody at the polymer modified electrodes.

4. Conclusions

As an alternative to the pulsed amperometric detection system, ac impedance studies were investigated as a method to interrogate the antibody-containing electrodes to allow differentiation of the individual capacitive and Faradaic components in the form of Nyquist complex plane impedance plots. In this way calibration profiles for the detection of BSA were demonstrated for BSA antibody-containing polymers.

It has been found that the Faradaic real component of the impedimetric response acts as the dominant component of the ac impedimetric response of anti-BSA loaded polypyrrole films on exposure to increasing concentrations of BSA.

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