Development of Novel Techniques for Monitoring Anti-oxidant Thiols

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Abstract

The clinical exploitation of physiological biomarkers could yield considerable improvements in diagnosis and treatment providing their measurement can be conducted speedily and preferably at the point of care. The project has sought to investigate the development of new methods that could allow such measurements to be made. The various biomarkers that could be exploited as the basis of a general "index" of physiological wellbeing have been identified and their potential clinical merit critically appraised. Anti-oxidant sulphur compounds (cysteine, glutathione, sulphite) were selected as potential targets on the basis of their physiological role in protecting the body from damage by free radicals. The variation in their concentration within biofluids is widely acknowledged as a useful diagnostic gauge as to the degree of oxidative stress that an individual may be experiencing. The main problem, from a clinical perspective, is the lack of a suitable procedure for monitoring their concentration speedily at, or by, the patient.

A brief assessment of the various electroanalytical options (encompassing voltammetric, amperometric and potentiometric methodologies) available for the detection of the sulphur anti-oxidants has been conducted. A potentiometric detection strategy was found to offer numerous advantages and a novel indicator family based on quinone interaction adopted and forms the foundation of the work presented herein. The reaction mechanism has been elucidated and the analytical applicability of the system

investigated using a variety of techniques – covering both chromatographic, spectroscopic and electrochemical methodologies.

The system has been characterised in terms of selectivity, sensitivity and its efficacy for the quantification of thiol containing pharmaceuticals and various biofluids (urine and plasma) has been demonstrated. The simplicity of the detection methodology is shown to markedly contrast alternative thiol detection strategies. The transfer of the technology to a mass production format through the adoption of screen print electrode formats has been achieved. A series of clinical trials were performed and the efficacy of the approach and the underlying technology format demonstrated. The results have been corroborated using standard techniques and the routes through which the system can be adopted within mainstream biomedical environments highlighted.

An alternative sensor system based on a composite polymer laminate approach was also investigated as a route through which prototype sensors could be speedily prepared and which would be more accessible to general chemistry laboratories. A new approach to the detection of sulphite – based on the quinone system – was used as the principal detection system to allow the system to be evaluated and proof of principle demonstrated. The fabrication methodology adopted has been found to provide a highly versatile option for the construction of polymer film electrodes.

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Chapter 1

Introduction to Decentralised Testing

Abstract

There has been a steady move within the research literature to development of tests and devices that can be used outside the laboratory – usually by non specialist personnel. The transfer of conventional instrument to a format that portable, reliable and accurate is a considerable challenge. The basic rationale behind the pursuit of such systems and the issues that need to be addressed in their development is critically assessed and their potential influence and impact on goals of the present project are highlighted.

1.0 Introduction

There is an increasing effort to design and develop new approaches to the construction of decentralised tests and sensors. These are essentially analytical devices which can perform a rapid analysis of a given sample and provide the user with immediate feedback as to the concentration of a particular analyte. The term decentralised refers to the fact that they can be used out side the normal laboratory environment and by users with varying scientific background. Ideally, they should be portable, robust and provide a quick and simple measurement that doesn't require any sample manipulation by the user. The principal benefit is that they avoid the time delays associated with conventional lab based analysis (indicated in Figure 1.1) and means that the results can be acted upon much sooner.

The core aim of the project detailed herein has been to investigate the development of such sensors for the analysis of biomarkers that can be associated with oxidative stress – principally small molecule anti-oxidant compounds (cysteine, homocysteine, glutathione, ascorbate and sulphite). The remit was to investigate new detection strategies for such molecules and then attempt to transfer what could be considered lab technology to a format that could be used by appropriate clinical staff – whether in the home or in the hospital ward. The most common examples of

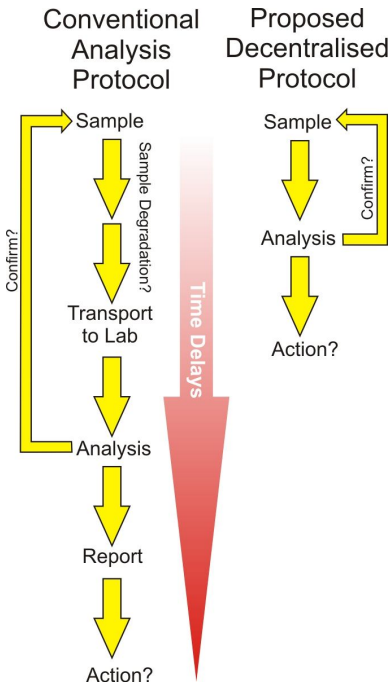

Figure 1.1

decentralised testing sensors are glucose monitors used by diabetics. There is however an almost endless spectrum of applications in which they could be used beyond the biomedical area - water quality meters for environmental monitoring or portable gas sensors for those working in the industrial sector and these have been extensively reviewed [1-5]. Many of the problems in the development and transfer process are common to all – irrespective of application or context but there are many more problems that need to be overcome in the design and construction of such systems. Before considering the main objectives and results obtained from this project, it is necessary to consider the background to the development of such sensors and outline the core issues that they must address.

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1.1 Types of Sensor

Any analytical device capable of being used outside a normal chemistry laboratory falls under the heading of a decentralised test. This could be a thermometer, blood pressure meter, urine diptest, cholesterol test, a nitrate meter for soil quality or a sensor to detect toxic gas. The group can be further divided into “Point of Care” or “Near Patient Testing” devices or Water or Soil Quality tests or Gas Sensors which refer to those devices used predominantly for biomedical, environmental or industrial purposes respectively. There is often a degree of overlap between the different headings as advances in the underlying technology of one area can often be translated or adapted to fit within the other. The main area where they differ is in attempting to make one device sensitive and selective to one particular analyte in a particular application context. The

basic rationale behind the test systems irrespective of context is shown in Figure 1.2. For example, it can be important to be able to measure nitrate in both biomedical and environmental situations but while the labelling system may be common to both it is not generally possible to take a test designed for one area and use it in the other. The sample considerations can be different depending on the context (ie blood being substantially different in composition from soil) and thus another level of sophistication must be added which tailors the basic test to the demands of a particular sample.

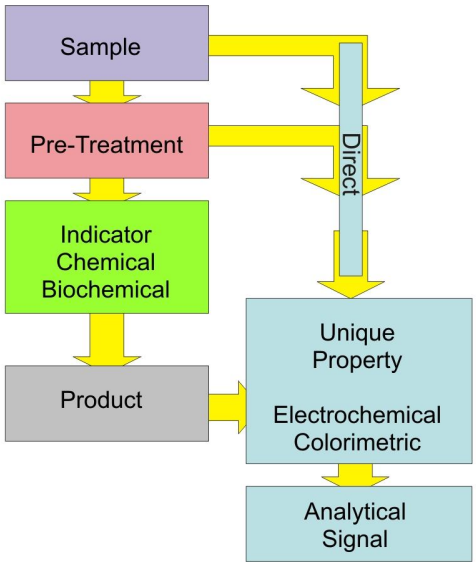

Figure 1.2

Identifying the analyte from all the other species that may be present within the sample matrix is however the central issue – without selectivity the test is useless – and this issue forms much of the foundation of the present thesis. Much research is targeted at developing the chemistry so that the analyte is selectively transformed into something else which has a "unique and measurable" property with little or no interference from the other components. In many cases, this requires the design of a chemical indicator that

will readily and selectively react with the analyte or the use of a biological agent (enzyme or antibody) that can selectively act upon that particular substrate. A key issue is that the labelling is done with minimal sample preparation and hence little interaction by the user beyond simply adding the sample as indicated in Figure 1.3

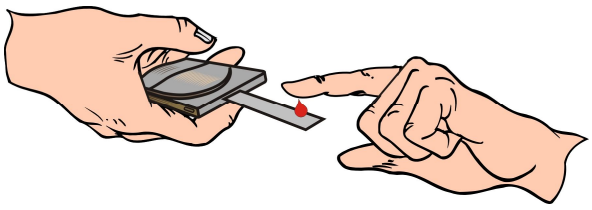

Figure 1.3

It is also possible to design the sensor such that it acts directly upon the analyte – avoiding the need for any additional reactions. This approach is less common and usually exploits the inherent differences in either the spectroscopic or electrochemical properties of the molecules. In the latter case, it can sometimes be possible to either oxidise or reduce the target analyte and achieve an unambiguous signal. The electrodeposition and stripping of transition metal ions (typically Cu^{2+}, Pb^{2+}, Cd^{2+}) being the most common application [4,5]. The optimisation of the chemistry on the sensing surface to enhance the electrochemical properties is another area of high interest to both the analytical community and also to those in materials science. Direct measurement by spectroscopic analysis is much more complex due to the "broadness" of the spectra and the inevitable overlap of different species creating a highly ambiguous signal. As such, spectroscopic analysis tends to be used in conjunction with indicator systems where the reaction leads to production of a wholly new absorption band in a region of the spectrum where there is no existing signal.

1.2 Biomedical Screening

Point of Care Testing (POCT) or Near Patient Testing (NPT) is an area which has expanded considerably in recent years – partly due to public concerns over individual well being and partly through government initiatives to increase patient participation in their own health management [6]. At present, the market is estimated to be worth several billion pounds and liable to grow as the technology improves and becomes a reliable alternative to conventional lab based analysis [7]. Every NHS hospital has a POCT department – but a multitude of commercial sensors can also be found in local pharmacies – glucose, cholesterol and pregnancy tests have been available for many years – a summary of the more common systems that are available are indicated in Table 1.1. It is conceivable that drug testing kits will be available over the counter soon as they can already be bought through the internet.

POCT device	Test Analyte	Diagnostic	Ref
Bilirubinometers	Bilirubin	Liver function	8, 16
Blood glucose meter	Glucose	Diabetes	9, 12
Blood gas analyser	Oxygen Carbon Dioxide pH	Lung, metabolic and kidney problems	10, 13,14
Cardiac enzyme analysers	Creatine Kinase (CK) Troponin Myoglobin	Heart Attack Markers	11, 15
Cholesterol meter	Lipoproteins and triglycerides	Heart Disease	10, 13
Drug testing devices	Cannabinoids Cocaine / Amphetamines Barbiturates Opiates	Drug abuse	4,12
HbA1c analysers	Glycosolated haemoglobin (HbA1c)	Diabetes	4,12

Table 1.1

While many of the commercial systems analyse only one analyte (ie glucose or cholesterol) it would be much better if a number of tests could be performed on a single sample. This is already available to an extent through the use of dipstick urine tests kits [12]. When you take a urine sample to your GP, the nurse will screen for a range of different markers (protein, glucose, nitrate, nitrite etc). The main benefit of this being that the more common biomarkers – known to be indicative of a problem could be quickly assessed. The range of analytes however tends to be very limited and usually regarded as the "usual" suspects – protein or nitrate/nitrite in urine being indicative of renal malfunction and/or infection. There are however difficulties associated with this "screening approach" in that more analytes must ideally be tested to give a better, more rounded biochemical picture of patient well being but such tests require a more careful design of the sensor and better miniaturisation of the sensing components. The outcome of the labelling or enzyme reactions need to be highly selective to prevent "cross talk" where one reaction leads to a positive result being recorded for another – resulting either in a false positive or an artificial amplification of the concentration of the other analyte

1. 3 Technological Evolution - Spot Test Measurements

The earliest and simplest POCT designs were and still are based on a simple colour change and would involve wet chemistry [12]. A drop of the sample would be added to a small volume of the indicator in a reaction vial and (ideally) the development of colour noted after a preset period. This could be a simple yes/no reaction in which the change would signify a positive result. More commonly, a graduated colour chart would be

provided in which gross changes in colour could be associated with particular concentration ranges. If the target analyte is present – the indicator binds – resulting in the formation of a "product with a unique property" with the magnitude of change assumed to be proportional to the concentration of the analyte present in the sample. The systems are typically designed / selected to obey Beers Law but using the eye in place of a spectrometer. A calibration chart consisting of blocks of colour representative of a given "analyte range" will normally be included to which the test is compared by visual inspection. There are a number of problems associated with this approach:

1. colour variability of the actual sample (background colour)
2. chemical variability of the sample (pH)
3. availability of the analyte (complexation or covalent binding)
4. presence of interferences (false positives)
5. colour perception when comparing response to chart
6. sensitivity (broadness of the different concentration bands ie 200 ppm steps)
7. transport of the test materials and setting up the reactions
8. disposal of the reaction contents

A test strip with an indicating reagent immobilised within an absorbent pad was the next step in the evolution of spot tests – the most common example being the urine dipstick test that is performed in GP surgeries (Table 1.2). A drop of the aqueous sample is added to the strip and the colour allowed to develop. This has a number of procedural advantages (operational simplicity and reagentless nature of the test being the more

important) over the reaction vial approach but the interpretation and quantification issues remain.

Sample / Test	Test Analyte	Ref
Urine Strip	Glucose Ketones Blood (in urine) Protein Nitrite pH Urobilinogen Bilirubin	12

Table 1.2

1.4 Technological Evolution – Electronic Systems

Electronic sensors – whether based on colorimetric or electrochemical detection methodologies have emerged in recent years as the next stage in the decentralisation of diagnostics [1-5]. Many of the issues of selectivity, sensitivity, accuracy and reproducibility still remain but they offer procedural advantages of being largely reagentless (requiring only the addition of the test sample) and provide a digital "readout" of the analysis result. Removing the ambiguity of ascribing a colour to a given chart is a large component of the uncertainty of the measurement and the numerical value provided by the electronic systems can be considered a significant step forward. The project detailed herein sought to develop the foundations of a test that could provide this but utilise an electrochemical detection methodology and thereby overcome some additional issues related to sample/matrix colour.

1.5 Sensors – Recognising the Analyte

There is no strict definition of a "biosensor" - a fairly diverse literature base exists covering a vast number of designs and applications. There are however two potentially distinct categories: sensors that simply measure a biological component (the remit of the present project) and sensors that utilise a biological component for the actual measurement process. The latter however, need not be applied solely to the measurement of a biological component within a traditional biofluid but can be applied within a context that nature never intended. An example of a non medical application is typified by the use of sulphite oxidase for the measurement of sulphur dioxide used as food preservative or for air quality measurements. The generic structure of a biosensor, irrespective of the previous classification generally consists of two main components – the recognition element and the transducer [1-5,13]. The former can be biological or purely synthetic in nature. The description of the latter can be equally vague in terms of possible material (carbon, metal, alloy or semiconductor) but must be conductive to some degree as it is responsible for converting the signal from the recognition element into a form which can be electronically quantified, processed and presented to the user.

The two central components (recognition element and transducer) must be intricately wired together. This can be problematic in practice – especially in the case of biological recognition elements such as enzymes and antibodies. The protein shell in biocomponents acts as a fairly good insulator and, as such, there is a danger that the binding of the target analyte to the receptor may effectively go unnoticed [4]. A number of strategies have evolved to facilitate the conversion of target-receptor recognition into

a measurable signal that can be read by the transducer [13]. The nature of the approach taken depends strongly on the nature of the recognition element being employed. In the case of enzyme systems, the signal conversion can be achieved by monitoring the consumption of a cofactor, the production of byproducts or by introducing electron shuttles that can facilitate charge transfer between the biocomponent and the transducer. These three approaches are summarised in Figures 1.4 A-C [3-5]. The current generated or consumed as a consequence of the enzymatic reaction and relayed through the base substrate (transducer) can then be related to the concentration of the target.

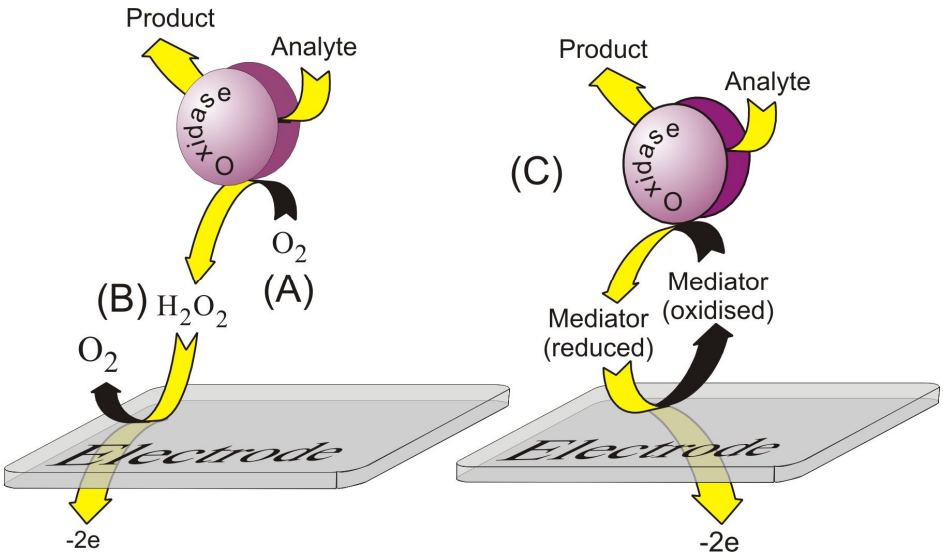

Figure 2.4

The sensor, as a whole, relies upon the specificity of the enzyme reaction to confer selectivity. Immunoassays differ from the enzymatic systems in that there is normally no inherent change – chemical or electrochemical – upon the binding of the

target. The interaction is typically a non covalent binding event between antibody (the recognition element) and antigen (the target analyte) [3,4]. The combination of antibody with an enzyme however has led to the evolution of the amperometric immunoassay. These can take a number of formats both homogeneous and heterogeneous. The basic scheme for the latter is shown in Figure 1.5 and can take the form of a sandwich assay or modified Enzyme Linked Immuno-Sorbant Assay (ELISA). The latter normally produces a chromophore in response to antigen binding but the enzyme substrate can be manipulated to produce an electroactive agent that can be measured at the transducer [3,4].

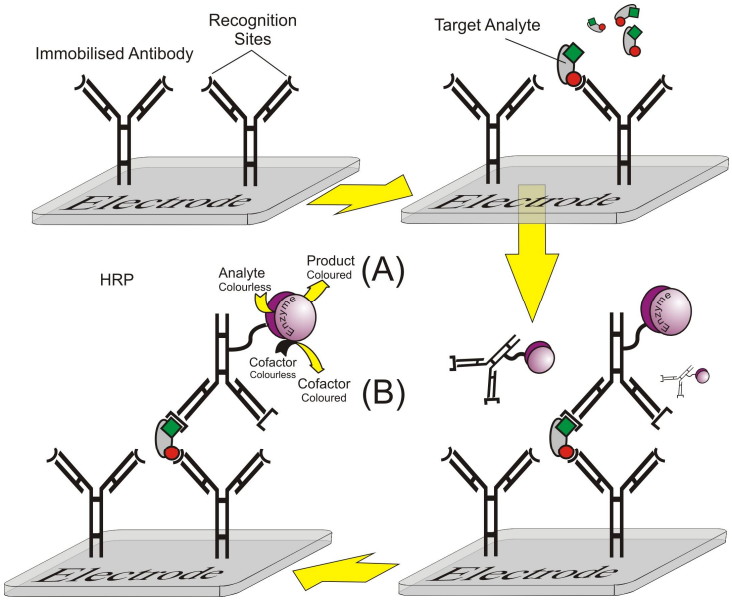

Figure 1.5

Chemical receptors function in much the same way as the enzyme systems in that they attempt to selectively screen out the target analyte and, either create a wholly new electrochemical signature, or modify the target's existing electrochemical profile such

that the other components do not interfere. This is the methodology that is followed in the work detailed here and is based on the proposed reaction of a quinone molecule with the target biomarker – typically small molecule anti-oxidants. The core reaction scheme is highlighted in Figure 1.6 and relies upon the selective nucleophilic addition of anti-oxidant thiols to the indicator [14, 15]. In doing so, there is a change in the redox balance of the system and this can be measured (our unique signal). The magnitude of the change then being related to the concentration of the quinone indicator.

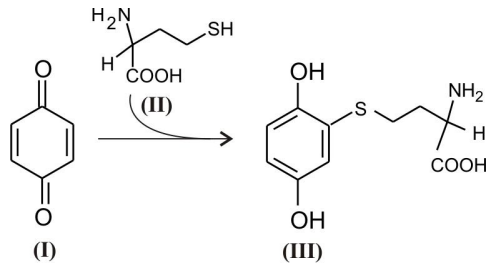

Figure 1.6

A number of different systems have been investigated and a more detailed overview of their role, action and incorporation as a sensor is given in subsequent chapters. The rationale behind using this approach over the biosensor approaches outlined in Figure 1.4 and 1.5 relate to the relative simplicity of the chemistry but also the fact that there are a range of relatively inexpensive commercial quinone systems available (in contrast to one or two enzyme systems or immunoassays – whose costs can be prohibitive). The availability of a "molecular library" could give more freedom in terms of sensor design but also in terms of response characteristics (selectivity, sensitivity etc). The basic scheme shown in Figure 1.6 highlights the simplest system but this could, in principle be "engineered" using conventional organic synthesis (if not

already available off the shelf) with substituent groups that can impart steric or electronic properties that enhance the reaction selectivity or the "unique-ness" of the analytical signal – by changing either the spectroscopic profile to a region of the spectrum where there is no interference from matrix components or for enhancing the electrochemical signal [14,15].

1.6 Sensors – Integration

Ultimately, the aim would be to develop a disposable test strip (as indicated in Figure 1.3) which would be a single shot measurement. This avoids any issues over contamination or safety implications – especially when dealing with biological fluids such as blood. The sensor substrate/transducer can take many forms whether metal, carbon or composite in nature and each has its own limitations and advantages and different electrode substrates can elicit significantly different responses from a given analyte. There is an extensive literature base on the material science that underpins the optimization of electrode substrate but much is beyond the scope of the present study. In this case our investigations were mainly restricted to carbon based systems as it can be viewed as the most flexible of these substrates as a consequence of the variety of physical forms it can take. There is a diverse range of chemical properties (or functionalities) that can allow sensor enhancements through a surface modification. In this respect, carbon was viewed as a significantly more flexible diagnostic system.

Glassy carbon (GC), carbon aerogels, carbon fibre (CF), carbon felts, reticulated vitreous carbon (RVC) or graphite have all been used in a great number of analytical

techniques [16]. Highly orientated pyrolytic graphite (HOPG) offering edge plane (eppg) and basal plane (bppg) morphologies are frequently used to explore the more fundamental side of electron transfer processes but can be of considerable value in electroanalytical systems. The basal and edge plane possess different properties (Figure 1.7) with the latter tending to exhibit considerably faster electrode kinetics and, as a consequence, possesses the potential for greater detection sensitivities [17-20].

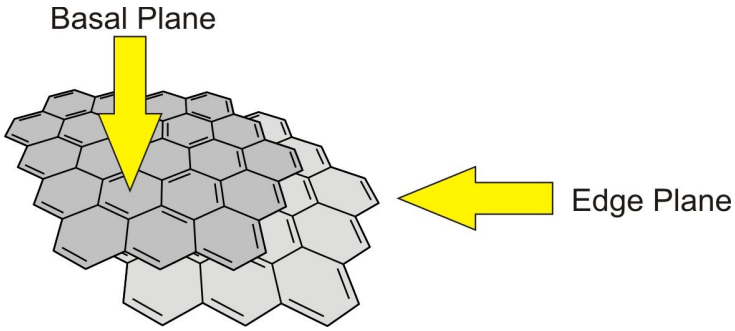

Figure 1.7

The edge plane sites on carbon nanotubes (CNTs) are also thought to be a major contributor to the high electron transfer rates and electrocatalytic effects observed in such systems [17, 20]. Glassy carbon is however the most commonly employed electrode material in electroanalysis and is easily adapted for use in the detector designs and in most cases served as the benchmark electrode through which it was possible to compare the different "disposable systems" needed for transfer of the technology to a POCT format.

Composite electrodes typically consist of two or more components which together form the bulk of the base substrate. The composition of this group can be incredibly diverse depending on definition but, in general, the field can be sub divided into carbon

paste [21-25] and screen printed [26-30] systems. Despite the different formats and processing requirements – both share a common methodology in which carbon particles are bound together by a binder. The great strength of this approach is the fact that the composition - and hence properties - of the electrode can be easily manipulated by altering one or more of the components in the paste or ink or by the addition of a catalyst or other modifier during the initial mixing phase.

Carbon paste electrodes typically rely upon carbon particles being held together by an impregnated organic liquid phase that is compacted into a holder and can then be used in much the same way as a conventional solid electrode and likewise can be used in much the same way as a commercial glassy carbon or HOPG electrode [21-25]. The paste electrodes can be rapidly constructed with little expense beyond the initial cost of the components. As such, they can provide a prototyping function that allows the lab based evaluation / production of modified electrodes. Operationally, the paste electrodes can have the advantage of providing a low background compared to solid graphite and noble metal electrodes. They are also renewable in that, upon fouling, the surface can be regenerated by polishing [21,25]. There is clearly the potential for a huge number of variations in the preparation – the form of carbon used, the nature of the binder and presence of modifiers or catalysts. The binder can take the form of a simple mineral oil (nujol or paraffin) [21], wax or epoxy [31], polymers [32] or ionic liquid [33].

Screen printed or pad printed electrodes are the mass production capable form of composite electrode sensor systems and are generally inexpensive to fabricate when considering large scale processing in which the costs of individual electrodes can be expected to fall dramatically as the batch sizes increase. Access to such production

facilities and the large volumes of the reagents required can however be prohibitive – especially when considering the incorporation of expensive biological reagents (cf carbon paste systems). Mass manufacture does however open an avenue to the production of disposable electrodes that can overcome the fouling and surface deactivation effects that repeated analysis can bring. The batch production of near identical sensors can therefore minimise the reproducibility problems associated with the regeneration of conventional electrochemical detectors. Pad printing in contrast – offers similar properties to the screen printed system but on a more prototyping scale [34]. The basic production processes behind the two systems are considered in the next chapter.

Screen printed electrodes were selected as the format of choice for the latter stages of the project as it was viewed as the material most likely to be readily extrapolated to a commercial format and met most of the criteria required for the disposability and portability. The screen printed sensors used in the course of the studies present here were supplied from a commercial company and were not modified to any extent. Their use was solely to demonstrate that the substrates could be used as the basis of a disposable system.

1.7 Overall Project Aim

The project was a multi-disciplinary investigation encompassing aspects of analytical, biomedical and materials science and there are aspects of all three within the subsequent chapters. A detailed introduction is given to each particular section – covering the background to the existing literature and outlining the proposed developments. The

core research path was to identify suitable indicators, assess the transfer to a disposable format and evaluate the potential clinical efficacy of such a system compared to conventional laboratory based analysis.

1.8 Project Objectives

1. Assess the electrochemical and spectroscopic properties of various quinone molecules as potential labels for detecting low molecular weight anti-oxidants.
2. Identify candidate molecules that can be exploited as the basis of a selective potentiometric detection system for core biomarkers (cysteine, homocysteine, glutathione and sulphite).
3. Evaluate the responses obtained at various electrode substrates (macro, micro and disposable).
4. Investigate the possibility of using novel composite electrode substrates as an alternative to screen printed formats.
5. Assess the response of sensors to authentic clinical samples and compare with standard laboratory procedures.

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Chapter 2

Experimental Details and Methodologies

Abstract

The materials, system configurations and methods considered to be general or core procedures and used throughout the project are briefly described. More specific details relating to specific experiments can be found under the Experimental Section within each chapter.

2.0 Chemicals and Instrumentation.

The reagents utilised all were of the highest grade available and no further purification was carried out. All solutions were prepared on a daily basis using deionised water from an Elgastat (Elga, UK) water system. Electrochemical measurements were conducted using computer controlled potentiostats: μAutolab (Eco-Chemie, Utrecht, Netherlands) and AWE-10 (Sycopel, Newcastle, UK),. Unless specified otherwise, the solutions consisted of Britton-Robinson buffer (acetic, boric and phosphoric acids - each at a concentration of 0.04 M and adjusted to pH 7 through the addition of sodium hydroxide). Potassium chloride (0.1M) was added to all buffer solutions in order to define the reference potential. Solutions of sulphite were degassed to preparation.

2.1 Potentiometry

Potentiometry is one of the simplest and the cheapest electrochemical techniques and can be used for analytical applications. Potentiometry is based on the measurements of potential difference between two electrodes operating under zero current. The basic cell setup used in the present project is indicated in the schematic shown in **Figure 2.1**

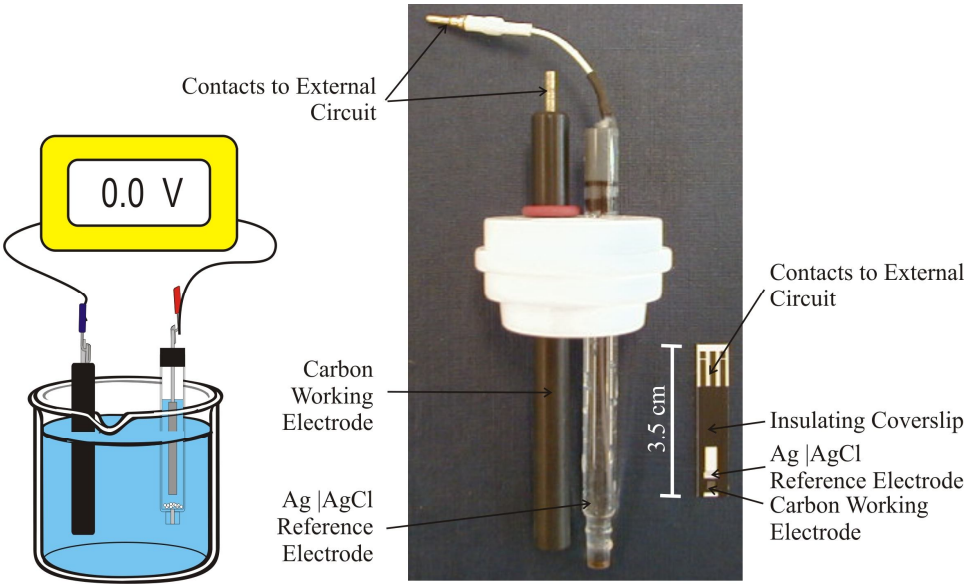

Figure 2.1 Cell setup and electrodes used in the project

One electrode has a known and unchanged electrochemical potential and is regarded as a reference electrode. The reference electrode, which was used for this project was silver/silver chloride reference electrode (Ag/AgCl, 3M Cl^-) and its components/construction is highlighted in **Figure 2.2**. The second electrode in an electrochemical cell is a working or indicating electrode and was either glassy carbon, screen printed carbon (shown in **Figure 2.3**) or carbon polymer laminate.

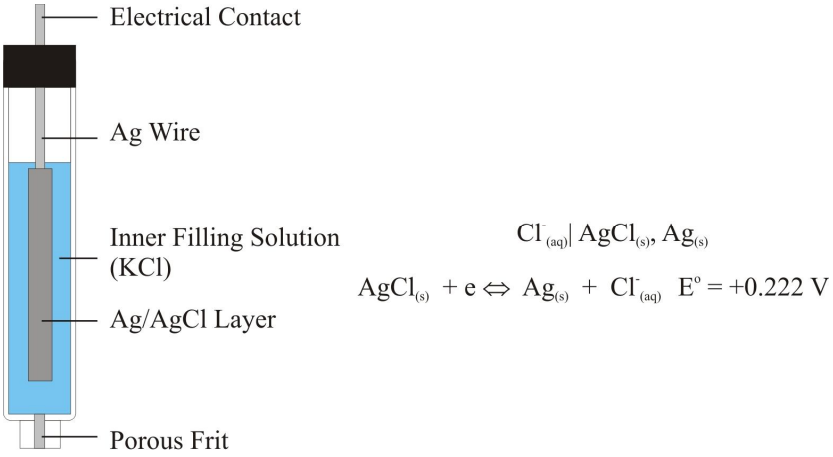

Figure 2.2 Silver/Silver Chloride reference electrode

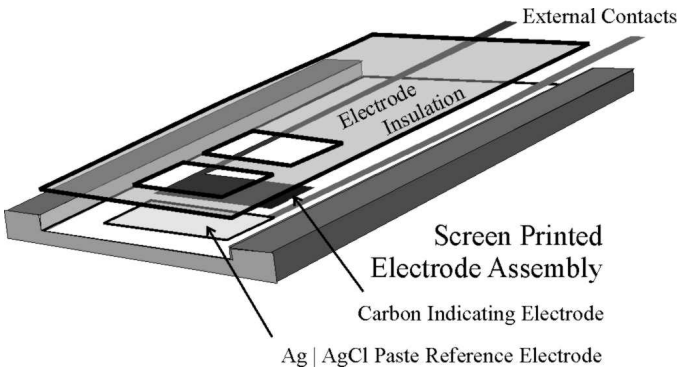

Figure 2.3 Schematic of the screen printed electrode components used throughout the project

The overall chemical reaction, taking place in the cell can be described by the means of two processes – oxidation and reduction. The difference in potential between the two of them is the emf of the cell. The electrochemical process taking place in the cell can be described by the Nernst equation [1,2]

$$aOx + n\bar{e} \leftrightarrow bRed$$

$$E = E^{o} - \frac{RT}{nF} \ln \frac{a_{red}^{\ b}}{a_{ox}^{\ a}}, \text{ where}$$

E is the electrode potential;

E^o is the standard electrode potential;

R is the gas constant 8.314 $JK^{-1}mol^{-1}$;

T is temperature in Kelvin;

n is the number of electrons transferred to the electrode during reaction;

F is Faraday constant 9.649×10^4 C mol^{-1}

a_{ox} *and* a_{red} are activities of oxidized and reduced species.

Prior to addition of the analyte, a steady response towards naphthoquinone itself is obtained. After addition of the analyte, the potential drops significantly and then reaches a plateau. The analytical signal is taken from there. Depending on the system under investigation, the magnitude of the drop in potential is proportional to the analyte concentration and through using several additions of the analyte calibration data can be obtained.

2.1.1 Cell Operation and Mass Transport

Mass transport to the electrode can occur through three basic processes [2]:

- Diffusion – Movement across a concentration gradient
- Migration – movement of charged particles within an electric field
- Convection – mechanical movement caused by stirring or by fluid flow

In the subsequent chapters the two main processes to be considered are diffusion and convection. Migration is effectively eliminated through the addition of relatively high concentrations of inert salts to serve as supporting electrolyte (typically 0.1M KCl), the intention being to dissipate the effects of an electric field. In addition, buffering agents are normally included to provide optimal conditions for the quinone systems. In the case of the potentiometric measurements, the large majority of measurements were done under steady state which relates to controlled convection – normally by a magnetic stirrer fixed at a given rate – where an appropriate quinone indicator is effectively titrated with a known amount of a target analyte.

2.1.2 Potentiometric Assay Protocol

The experimental set up shown in **Figure 2.1** was used throughout much of the work and is reported in the following chapters. The basic procedure involved placing the electrodes within a cell containing 10mL of an appropriate Britton-Robinson buffer containing 0.1M potassium chloride as the supporting electrolyte. A known amount of a given quinone indicator would be added (typically 0.5mL of a 5mM solution of the quinone prepared in either methanol or acetone). The potentiometric response would be recorded over a period of 5 or 10 minutes during which the solution would be stirred magnetically at a constant rate. Additions (ie known amounts of ascorbate or thiol biomarker) would be made to the solution and the change in the chronopotentiometric profile recorded as shown in **Figure 2.4**.

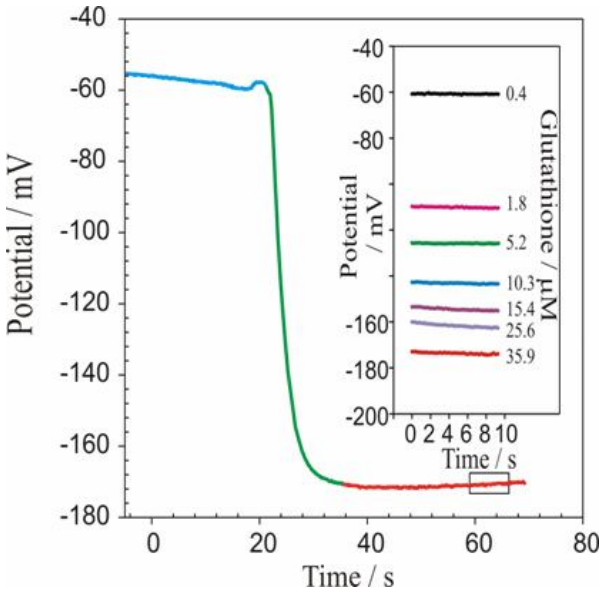

Figure 2.4 Typical chronopotentiometric response of a quinone indicator to an addition of analyte (glutathione) recorded under "steady state" at a glassy carbon electrode.

The procedure was later modified when using the screen printed electrodes – as shown in **Figure 2.1** they contain a solid state Ag/AgCl reference and the complete assembly replaced the bulky macro electrodes. The change in the potential – before and after addition of the target biomarker was then used to form the basis of a calibration graph or to assess the level of interference.

2.2 Voltammetric Investigations

In principle, the voltammetric measurements can be conducted using the same basic electrode configuration as that indicated in **Figure 2.1** In general however, a third electrode is introduced to act as the counter (auxiliary) electrode which effectively serves to protect the reference electrode such that it registers a constant potential. Voltammetric methods involve monitoring the current resulting from a faradaic process occurring at the electrode as a consequence of scanning a potential range. The basic rationale is to induce either an oxidation or reduction process to occur at the electrode surface and through using a number of diagnostic tests – information about the system can be obtained – this can include the ease of oxidation/reduction, electron transfer kinetics, reaction mechanism elucidation and homogeneous reaction kinetic data[2,3].

The operation of the cell involves controlling the potential of the working (or indicating electrode) relative to the reference electrode. The current is passed between the working and the counter electrode and as such there is negligible current passing through the reference. In contrast to the potentiometric system outlined in section 2.2,

the voltammetric investigations are generally carried out under diffusion control – static solution.

Two different measurement methodologies were conducted: cyclic voltammetry and squarewave voltammetry. The former is normally used for investigative purposes[2,3] whereas the latter is essentially a quantitative analytical technique[4]. The core concepts of each are described in the following sections.

2.2.1 Cyclic Voltammetry

Cyclic voltammetry involves monitoring the current resulting as a function of potential and is generally called a triangualer waveform. Given a simple one electron oxidation process:

$$X_{(aq)} \underset{k_{red}}{\overset{k_{ox}}{\rightleftharpoons}} Y_{(aq)} + e$$

and follows the format detailed in **Figure 2.5**. The potential is swept at a constant "scan" rate between the start (E_1), the first vertex (or return point,E_2) and the end potential (E_1).

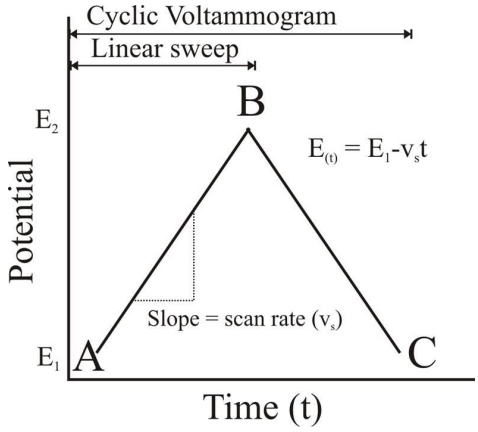

Figure 2.5. Triangular waveform characteristic of cyclic sweep voltammetry

A typical profile obtained is indicated in **Figure 2.6** and a more detailed breakdown of the various stages in the measurement given in **Figures 2.7-2.9**

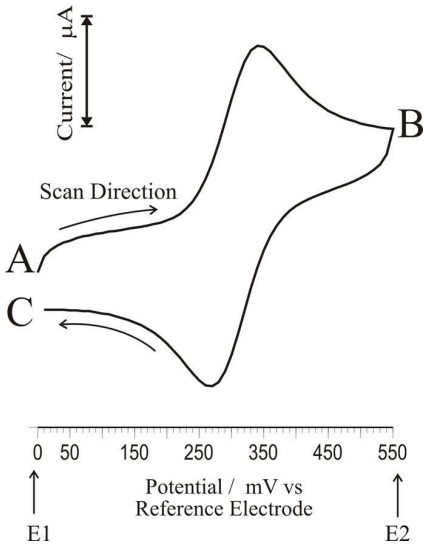

Figure 2.6. Typical voltammetric profile expected for a reversible one electron system.

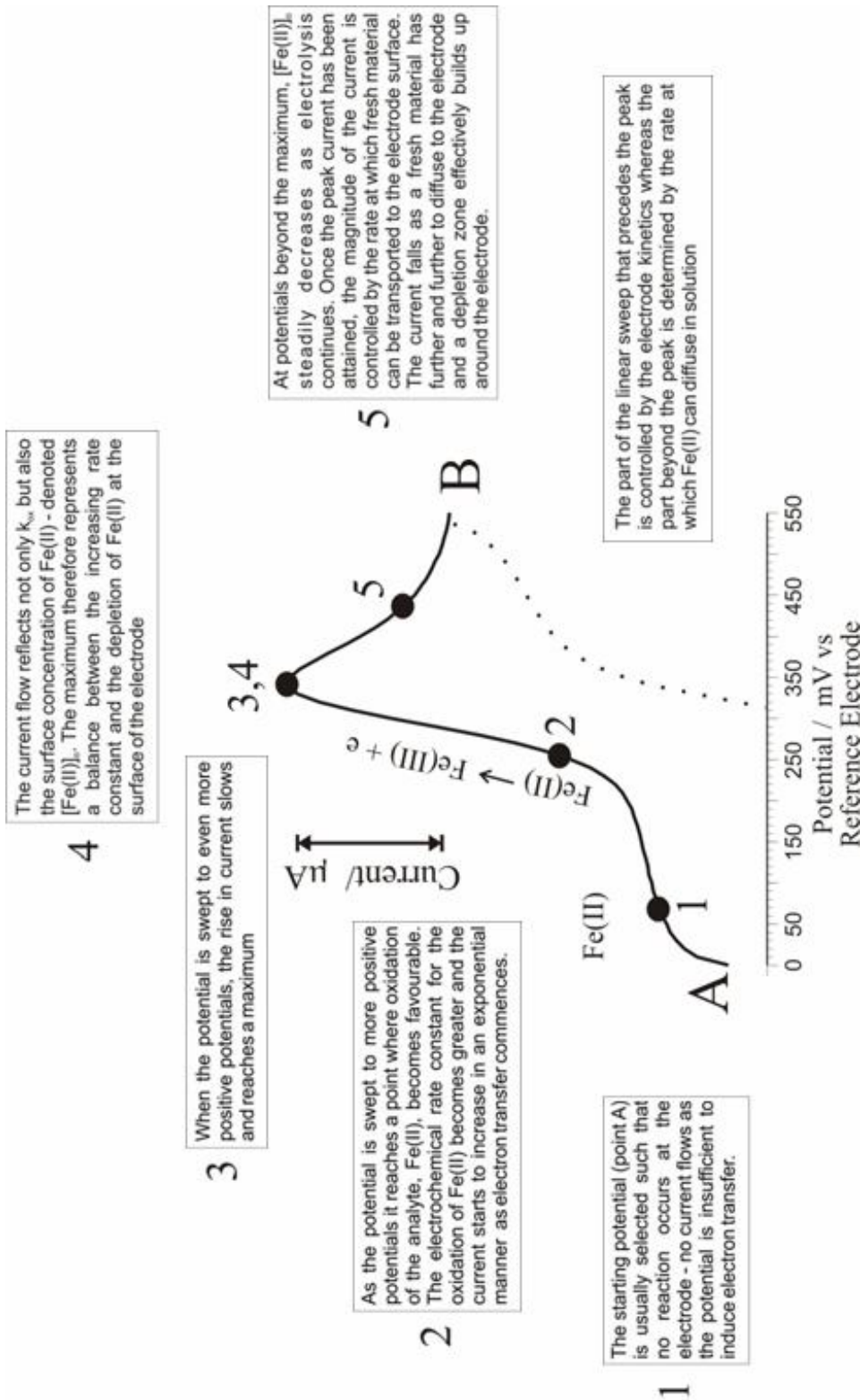

Figure 2.7 Main stages in the anodic sweep process

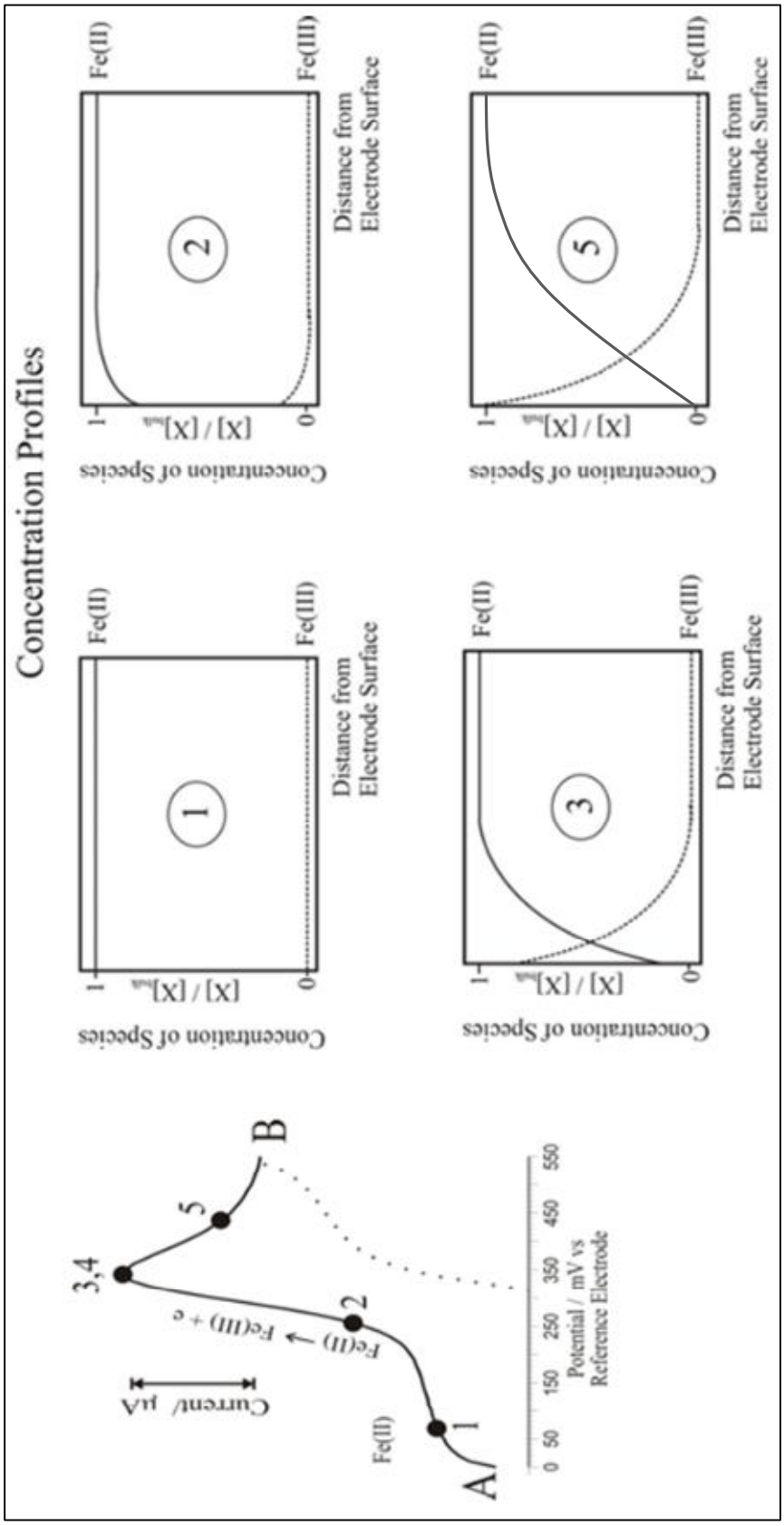

Figure 2.8 Concentration profiles expected in the anodic sweep process

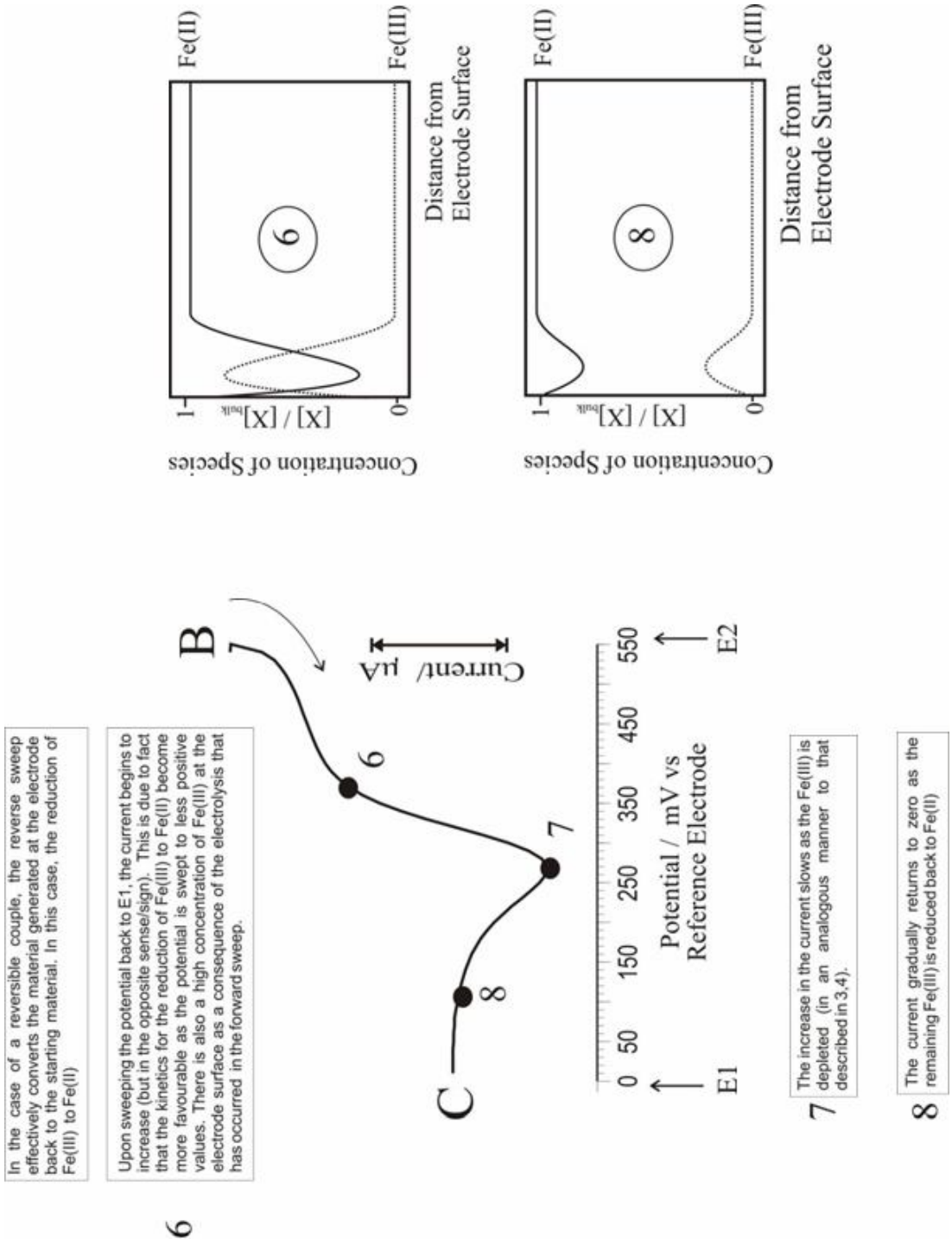

Figure 2.9 Concentration profiles expected in the cathodic sweep process

2.2.2 Square Wave Voltammetry

Square wave voltammetry has become increasingly popular as an electroanalytical technique and possesses a number of advantages over cyclic sweep and the other pulse techniques. A square wave is essentially superimposed on top of a staircase potential as shown in **Figure 2.10**.

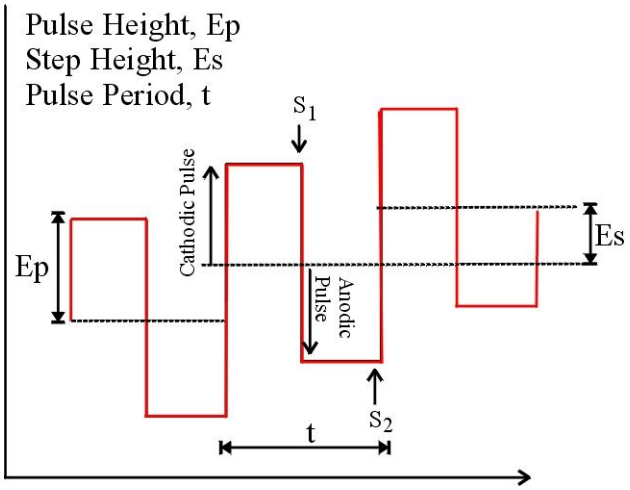

Figure 2.10. Typical waveform employed in Squarewave voltammetry [4]

If we are probing a reduction process then the reverse pulse (in this case the anodic one) re-oxidises the product formed in the initial reduction pulse. The polarographic signal is the difference between the two currents (**Figure 2.11**) and is larger than that observed in other pulse techniques such as differential pulse polarography (DPP). This is due to the fact that the reverse current is not observed in DPP. For reversible reactions - SQW can increase the sensitivity by a factor of 5 when compared to DPP.

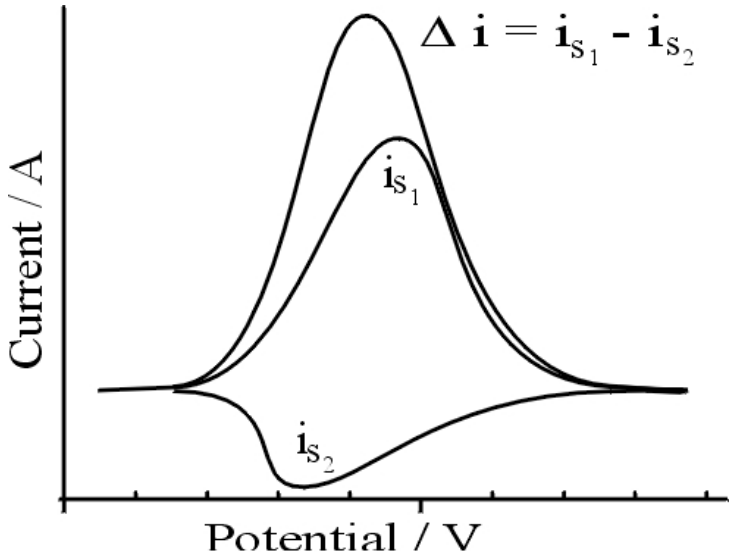

Figure 2.11 Typical "peak shaped" voltammetric profile expected in SQW voltammetry [4]

Another advantage lies in the speed with which the analysis can be carried out. An entire SQW polarogram can be recorded in less than 10 ms and as such can be used as detectors in LC systems

2.3 Spectroscopic Assays : Ellman's Test

Ellman's test is the conventional spectroscopic technique for quantitatively measuring thiol (RSH) concentration. This is traditionally conducted by adding 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to the sample of interest[5]. Any molecules with reduced thiol functionality will react with the DTNB *via* the substitution mechanism illustrated in **Figure 2.12**. The reaction produces one equivalent of 5-thio-2-nitrobenzoic acid (TNB), which under alkaline conditions is an intense yellow chromophore that can be detected spectroscopically using its

absorbance at 412 nm. The reaction is specific to thiol (RSH) molecules but is unable to distinguish between them.

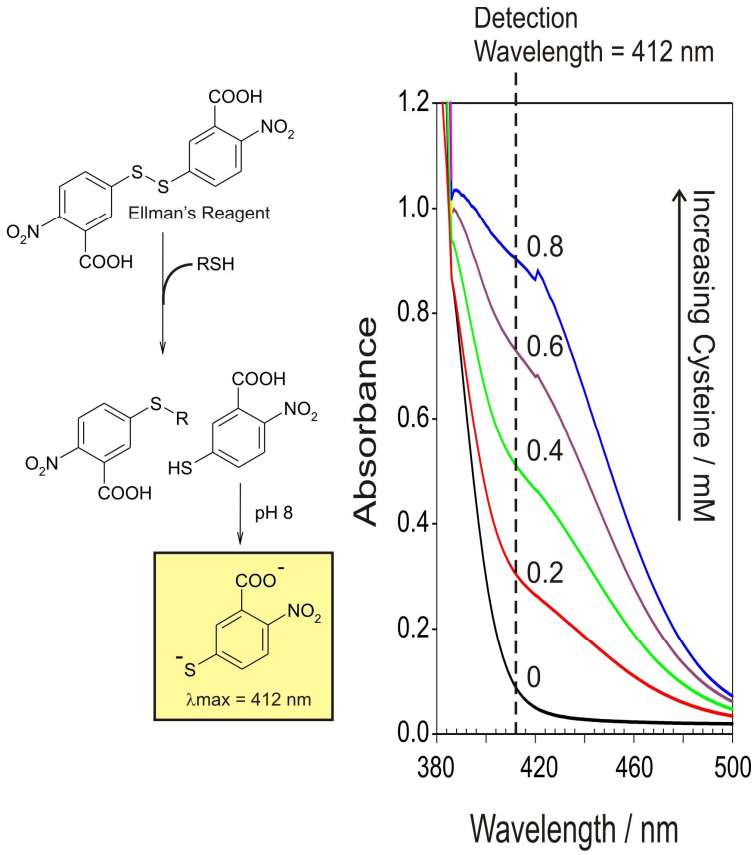

Figure 2.12 Ellman Assay reaction scheme and typical colorimetric responses

In this project, Ellman's reagent solution (1mM) in pH 8 BR buffer was used. In the cuvette normally were 1.9 ml of Ellman's reagent and 100uL of analyte. The calibration curve typically follows the form Abs=0.62[GSH/mM]-0.005, though it is important to note that new calibration data were collected for each new investigation. The concentration of the sample can be determined from the intensity of absorption using the Beer-Lambert Law. This test was used to validate electrochemical measurements.

2.4 Clinical Trials

Clinical trials involving nondiabetic control subjects and well controlled (HbA1c<7.5%) type-2 diabetic subjects ranging from 32-67 years of age were conducted. Samples of blood were obtained from the participants and analysed within three hours of extraction. Blood was collected in heparinised gel permeation vaccutainers and then centrifuged at 3000 rpm for 10 minutes. The plasma was withdrawn and used immediately for both electrochemical and spectroscopic analysis.

2.5 Sulphite Measurement

The Association of Analytical Chemists (AOAC) have long held a standard reference method for sulphite measurement derived from studies by Monier and Williams and involves a combination of distillation and end point titration [6]. There have been numerous refinements over the years to adapt the basic methodology to particular applications but it still retains a common core. In general, the sample is refluxed in acid (ie 0.5M HCl) to liberate sulphur dioxide (**Eq 1**). This is typically done under nitrogen flow with the carrier gas bubbled through a 3% peroxide solution. The oxidation of the sulphur dioxide gas to sulphate yields an acidic solution (**Eq 2**) that is subsequently titrated with standardised hydroxide and the initial concentration of sulphite estimated.

$$SO_3^{2-} + 2H^+ \rightleftharpoons SO_2\uparrow + H_2O \quad \text{Eq 1}$$

$$SO_2 + H_2O_2 \longrightarrow SO_4^{2-} + 2H^+ \quad \text{Eq 2}$$

Caution must be exercised when performing the basic procedure as it is prone to false positives whereby the transfer of volatile acids (ie from wine) under the reflux conditions can lower the pH in the receiving flask with the subsequent acid-base titration leading to an over estimation of sulphite content [7-9]. A number of modifications have attempted to counter such deficiencies and have focused on improving the selectivity of the detection process rather than the initial separation. Iodometric titrations rely upon the direct redox interaction with the liberated sulphite / sulphur dioxide and are largely insensitive to the acidic components carried over from the distillation process [10, 11].

2.6 References

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3. A.C. Fisher, "Electrode Dynamics" Oxford Primer
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8. H.J. Kim; K.R. Conca, M.J. Richardson, JOAC Intl, 1990, 73, 983
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11. D. Lowinsohn, M.Bertotti, Food Addit. Contam., 2001, 18, 773

Chapter 3

Electroanalytical Detection of Oxidative Stress Biomarkers: A Preliminary Assessment

Abstract

The clinical exploitation of physiological biomarkers could yield considerable improvements in diagnosis and treatment providing their measurement can be conducted speedily and preferably at the point of care. The present chapter has identified the key species involved in oxidative stress processes and has investigated the various electrochemical methodologies that could be employed to facilitate their quantification. The results are critically assessed with regard to their suitability for transfer of the technology from a laboratory based format to one that could be employed in a point of care testing device.

3.0 Introduction

It is well known that pathological events occurring as a consequence of disease and injury can alter the concentrations of specific biomolecular species within physiological fluids [1]. The viability of utilising the concentration of extracellular components as a diagnostic aid in the clinical management of disease and injury prognosis has been demonstrated in numerous instances and have included premature atherosclerosis, cancer, diabetes, sepsis, liver disease, cataracts, Alzheimer's and Parkinson's diseases [1-3]. It must be noted however that the adoption of such procedures within routine clinical settings is often hampered by the specificity and complexity of the techniques required to extract the necessary information. Referral of the appropriate sample to a central laboratory will invariably be the only option available to the clinician but the time delays incurred can prompt questions over the relevance of the information once it has been received. The possibility of sample degradation and the generation of artefacts can also lead to ambiguous results [4-6]. It has become clear that the practical exploitation of such biomarkers could be dramatically improved were the attendant clinical staff given access to devices that can permit near patient testing.

The development of discrete devices possessing sufficient selectivity, sensitivity and robustness to operate directly upon compositionally variable physiological samples is however a considerable challenge. The situation is further complicated, as outlined in **Chapter 1**, by the need to deliver a single, unambiguous signal that can be interpreted by operatives with little or no analytical experience. The resolution capabilities of either liquid chromatographic [7] or capillary electrophoretic [8] techniques currently represent the most effective methods of quantifying such compounds in complex media. However, neither technique is appropriate to near-

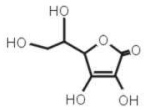
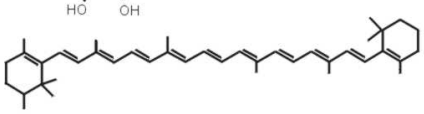
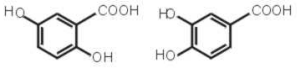
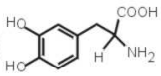
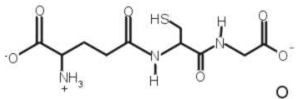
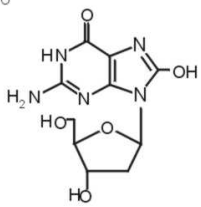
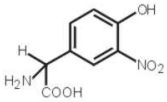
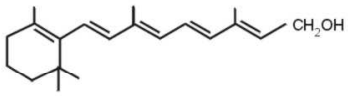
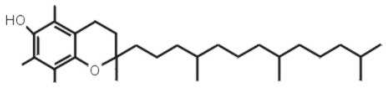
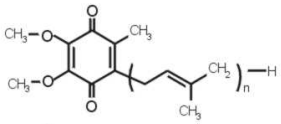
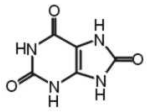
patient monitoring with both requiring a considerable degree of user expertise. There is clearly a role for simpler techniques and assay systems. The ability of electrochemical methodologies to fulfil such demands has long been suggested with the advantages of portability and operational simplicity contrasting the more elaborate demands levied by the separation techniques [9]. Providing selectivity can be retained, these would appear to be particularly suited for routine screening applications given the increasing maturation of micro-engineered instrumentation and the increasing availability of disposable electrode systems [10].

Acquiring selectivity at electrochemical detectors however can be problematic, particularly so for the detection of the small molecules that are commonly identified as potential biomarkers. Considerable gains in performance have been achieved in recent years through improvements in material design and by the introduction and continued evolution of biosensing technologies [11, 12]. Reviews covering the various electroanalytical techniques have been compiled [12, 13] but in most cases the treatments focus on the intricacies of the methodological and technological developments that have occurred in recent years. There is a need however for a broader perspective in which the salient aspects of these innovations can be presented within the context of their application to the near patient testing (POCT) of physiological biomarkers. This chapter has collated such information with the aim of providing a concise research summary that identifies those species that are capable of direct electroanalytical detection, considers the practicalities of conducting such measurements and critically assesses the clinical implications of their application within Point of Care contexts.

3.1 Disease and Illness Biomarkers

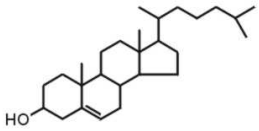
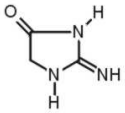
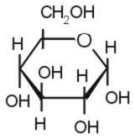
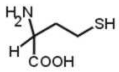
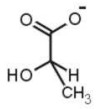
There is a large range of molecules whose physiological concentrations could be profitably exploited in clinical diagnosis but the majority are largely inaccessible to conventional electroanalytical methods. The biomarkers highlighted in the following discussion are those that are readily amenable to direct or enzymatic electrochemical quantification. It is possible to place potential biomarkers into two broad groupings that reflect their capacity for being used as either a general indicator of oxidative stress [14-37] or whether they are associated to specific conditions such as diabetes or heart disease [38-45]. General indicators of oxidative stress are identified within **Table 3.1** with those possessing a more explicit connection to particular conditions highlighted in **Table 3.2**. The typical concentration ranges within which they are found in physiological fluids and the tendency for the concentrations of particular species to be either depleted or elevated as consequence of the disease process is also indicated. The diagnostic implications of changes in the physiological concentration of a biomarker molecule will obviously depend on the specificity of the latter to a particular condition. If we consider diabetic patients as an example, glucose concentration will be routinely monitored and actively used to manage the disease on a daily basis with changes in the biomarker almost exclusively associated to that condition. It has been demonstrated in recent years however that the onset of diabetic complications can also lead to a depression in the concentration of reduced thiol species (typically glutathione) [3]. This occurs through their reaction with free radicals (reactive oxygen (ROS) or reactive nitrogen (RNS) species) generated as a consequence of the underlying hyperglycaemic condition. Monitoring such species in

addition to glucose could therefore serve to complement clinical control. A degree of caution however must be exercised in the diagnostic exploitation of such species.

Biomarkers	Conditions	Reference Range	Ref
Ascorbic Acid 	CD, D, DS, N ↓	30-150 μM	14,15
Carotenoids 	C, CD ↓	0.3-0.6 μM	16,17
Dihydrobenzoic Acids 	EOH ↑	13-113 nM (2,3) 0.19-1.48 μM (2,5)	18,19
Dihydrophenylalanine (DOPA) 	EOH ↑	5-8 nM	20
Glutathione 	C,CD, D, N ↓	2-6 μM	15,22-24
8-Hydroxy-2-Deoxyguanosine 	DS, H, N ↑	9-15 nM (urine)	4-6, 21,25-28
3-Nitrotyrosine 	MS, N ↑	0.2-2.3 nM	29,30
Retinol 	CD, N ↓	1-2.5 μM	14,17
Tocopherols 	CD,DS, N ↓	15-40 μM	17,31,32
Ubiquinones 	CD, D ↓	0.4-1.0 μM	14,21,33-35
Uric Acid 	CD, D, N ↑	160-450 μM	21,36,37

[C] Cancer, [CD] Cardiovascular Diseases,[D] Diabetes and associated complications, [DS] Down Syndrome,[H] Hepatitis/Aids, [L] Leukemia, [MS] Multiple Sclerosis, [N] Neurodegenerative Diseases, [EOH] Endogenous OH radical production

Table 3.1. General Biomarkers for Oxidative Stress

Biomarkers		Principal Condition		Reference Range	Ref
Cholesterol		CD	↑	<200mg/dL	11-13,38
Creatinine		R	↑	70-124 μM	39-41
Glucose		D	↑↓	2-5 mM	9-13,2,42
Homocysteine		CD	↑	5-15 μM	43,44
Lactate		CD	↑	0.4-1.4 mM	11-13,45

[CD] Cardiovascular Diseases,[D] Diabetes and associated complications, [R] Renal Diseases

Table 3.2. Condition Specific Biomarkers

Changes in glutathione concentration (along with numerous other biomarkers) can reflect a more general physiological response that may be exacerbated by the diabetic condition but are not inherently linked to it. Thus, inflammation resulting from another underlying disease or injury (i.e. arthritic processes) may similarly lead to a depletion of glutathione within physiological fluids [2, 3]. Oxidative stress is principally associated with the damage inflicted upon cellular and vasculature components (typically lipids, proteins and nucleic acids) by ROS and RNS. While oxidative challenge is a routine occurrence within physiological systems, a variety of antioxidants within the plasma and cellular structures serve to minimise the deleterious effects. A prolonged assault on the physiological defence mechanisms

through injury will however result in the depletion of specific compounds and in the generation of molecular debris. It is little surprise to find that a significant proportion of the biomarkers identified within **Table 3.1** are common antioxidants with the remainder being species that arise from the attack of ROS / RNS on endogenous amino acids (i.e. tyrosine) or purine bases/nucleosides (typically guanine/guanosine).

3.2 Electrochemical POCT Methodologies

Selectivity is the main issue facing the development of POCT devices and various strategies have evolved within the electroanalytical community to counter such problems. Ion selective electrodes (ISE's) have traditionally been associated with single analyte detection and rely upon the design of membranes incorporating immobilised host molecules that have an affinity for particular analytes. These systems are widely used in biomedical contexts for the quantification of inorganic ions (typically Na^+, K^+, Ca^{2+}, Cl^-, F^-, NO_2^-, NO_3^-) and their integration / miniaturisation and implementation within sensing applications have been widely reviewed [46, 47]. It must be noted that most of the compounds presented within **Table 3.1** however are largely inaccessible to direct determination by ISE technologies due to the lack of a suitable host molecule but they can be quantified by amperometric or voltammetric techniques. These approaches promote a reaction at the electrode surface through the imposition of a potential that will be sufficient to oxidise or reduce the functional groupings within the target analyte. The resulting current will be proportional to the concentration of species undergoing reaction and forms the basis of the analytical signal.

Amperometric technologies are commonly employed in the design of liquid chromatographic detectors (LC-ED) where a single potential is applied to the working (indicating) electrode [7]. Voltammetric approaches are more versatile (although require more complex instrumentation) in that they sequentially scan a range of potentials [9]. As different chemical functionalities can often undergo reaction at different potentials, voltammetry therefore allows the possibility of resolving individual redox processes and offers multi-analyte detection. There are many variations and refinements to these basic approaches and their application to the direct analysis of physiological biomarkers is considered in more detail in the following sections.

3.3 Experimental Details

All reagents were of the highest grade available and used without further purification. All solutions and subsequent dilutions were prepared daily using deionised water. Electrochemical measurements were conducted using a μAutolab computer controlled potentiostat (Eco-Chemie, Utrecht, Netherlands). An initial cell volume of 10 cm^3 was generally used and the measurements recorded at a temperature of $20^oC \pm 2^oC$. A three electrode assembly was used throughout and was comprised of a glassy carbon working electrode (3 mm diameter, BAS Technicol, UK), a platinum wire counter electrode and a silver / silver chloride reference electrode (3M chloride, BAS Technicol, UK). Rotating disk experiments were conducted using the electrode configuration detailed above and a BAS RDE-1 system operating at 5000 rpm at a fixed potential of +0.4V was utilised for the steady state amperometric measurements. Square wave voltammetry was also conducted using the RDE glassy carbon electrode

(25 cm^3 cell volume, Frequency 50 Hz, Initial Potential +0.5 V, Final Potential -0.5 V, Step Potential 0.00105 V, Amplitude 0.00495 V) with each scan conducted in triplicate with the electrode polished (1 μm alumina, Buehler, UK) and sonicated between each measurement. Screen printed electrodes were prepared using commercially available systems (Lifescan Ltd) similar to those used for commercial glucose sensing strips and consisted of a two electrode assembly (1mm^2 carbon and a Ag|AgCl paste electrode). Unless specified otherwise, the electrochemical solutions consisted of Britton-Robinson buffer (acetic, boric and phosphoric acids - each at a concentration of 0.04 M and adjusted to pH 7 through the addition of sodium hydroxide) containing 0.1M KCl.

3.4 Results and Discussion

3.4.1 Direct Voltammetric Detection

In ideal circumstances, the redox transition of target analyte should reside within a region where there are no interferences from other electroactive species. In reality, the compositional complexity of physiological systems and the various interactions between the redox components will naturally pose a significant problem for the accurate quantification of most analytes. The challenge of acquiring such selectivity is highlighted in **Figure 3.1** where the individual square wave voltammograms of common physiological components (0.5 mM, pH 7) recorded at an unmodified glassy carbon electrode are compared. While square wave voltammetry is widely recognised as one of the most sensitive techniques available to the electroanalyst and known to provide superior peak resolution, it can be seen that many of the signals will still overlap. The development of an inexpensive POCT device will

generally favour the utilisation of instrumentation that imposes a single potential rather than employ scanning techniques. Were amperometric devices targeted at glutathione, it is clear that large positive potentials (greater than +0.6V) would be required to oxidise the thiol component.

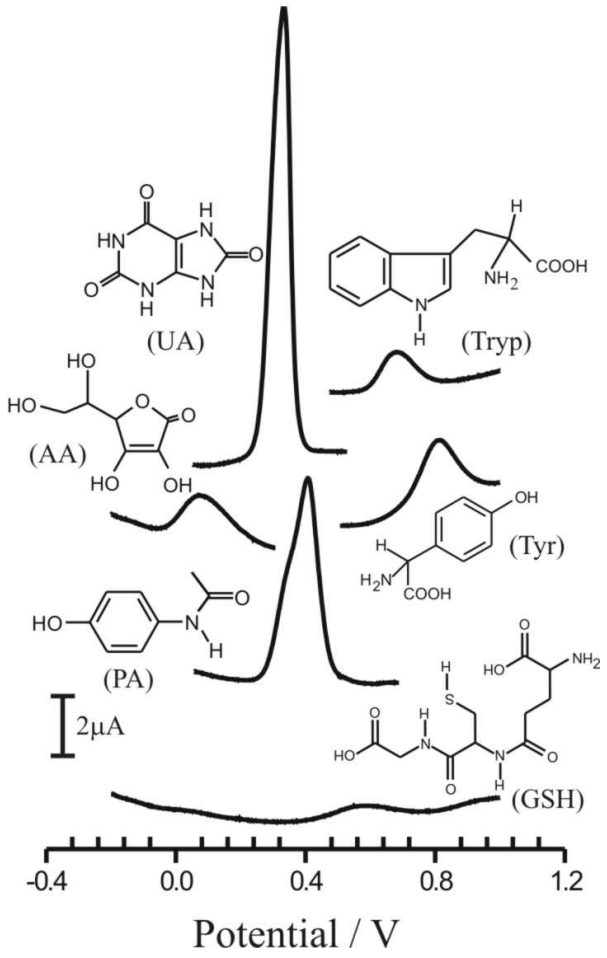


Figure 3.1. Individual squarewave voltammograms of ascorbic acid (AA), glutathione (GSH), paracetamol (PA), tryptophan (Tryp), tyrosine (Tyr) and uric acid (UA) at a glassy carbon electrode in pH 7 buffer. Each analyte was present at a concentration of 0.5mM.

As a significant proportion of the other electroactive species highlighted within **Figure 3.1** are oxidised at lower potentials than the thiol, these would also be expected to contribute to the current and would effectively lead to the glutathione concentration being grossly overestimated.

The situation can be further complicated by the interactions of the various species with particular electrode substrates. Thus, the adsorption of matrix components can lead to deterioration in the electrode response through simply blocking access to the substrate or, as in the case of uric acid, lead to a considerable enhancement in the signal. This behaviour can be heavily dependent upon the nature of the underlying electrode material. This is shown in **Figure 3.2** where the electrode responses of the previous analytes at disposable screen printed electrodes are compared.

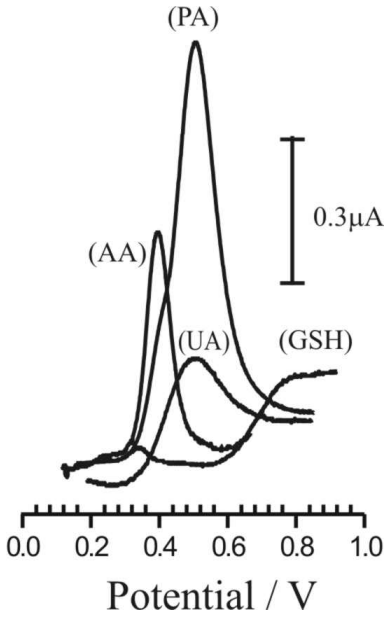


Figure 3.2. Individual squarewave voltammograms of ascorbic acid (AA), glutathione (GSH), paracetamol (PA) and uric acid (UA) at a screen printed carbon electrode in pH 7 buffer. Each analyte was present at a concentration of 0.5mM.

The experimental conditions were identical to those used to obtain the scans presented in **Figure 3.1** yet the peak resolution is severely compromised and the relative signal intensities (particularly urate) found to be significantly different. While carbon electrodes were used in both cases, the disposable strips are essentially a composite material (incorporating binders and processing reagents necessary for screen print application) and, as such, exhibit a reduced electrical conductivity when compared with glassy carbon [10]. The heterogeneity of the deposited electrodes will also provide a markedly different surface chemical composition and hence a different electrode response is inevitable.

3.4.2 Electrocatalytic Systems

Electrode performance can be improved however through the introduction of electrocatalysts and their application is typified in the analysis of the antioxidant thiols [47]. The basic mechanism through which these systems operate is detailed in **Figure 3.3A** with cyclic voltammograms detailing the ferrocyanide catalysed oxidation of cysteine to cystine shown in **Figure 3B**. In the absence of the thiol, the voltammogram (solid line) details the oxidation of ferrocyanide to ferricyanide at +0.22V on the forward sweep with the corresponding reduction observed at +0.18V on the reverse sweep. Upon adding cysteine to the solution, the electrogenerated ferricyanide is chemically reduced back to ferrocyanide. This is subsequently re-oxidised at the electrode and, as this occurs within the timescale of the electrochemical experiment, the magnitude of the oxidation peak is increased through the recycling of the ferrocyanide at the electrode substrate.

The electrochemical properties of cysteine are very similar to those of glutathione with both possessing sulphydryl (RSH) functionalities that exhibit slow electrode kinetics. The application of an electrocatalyst therefore enhances the detection of the thiol by increasing the detection current but also enables the detection to occur at operating potentials which are less positive than those required for the direct oxidation of the target. Interference that could arise through the oxidation of other matrix components can therefore be minimised.

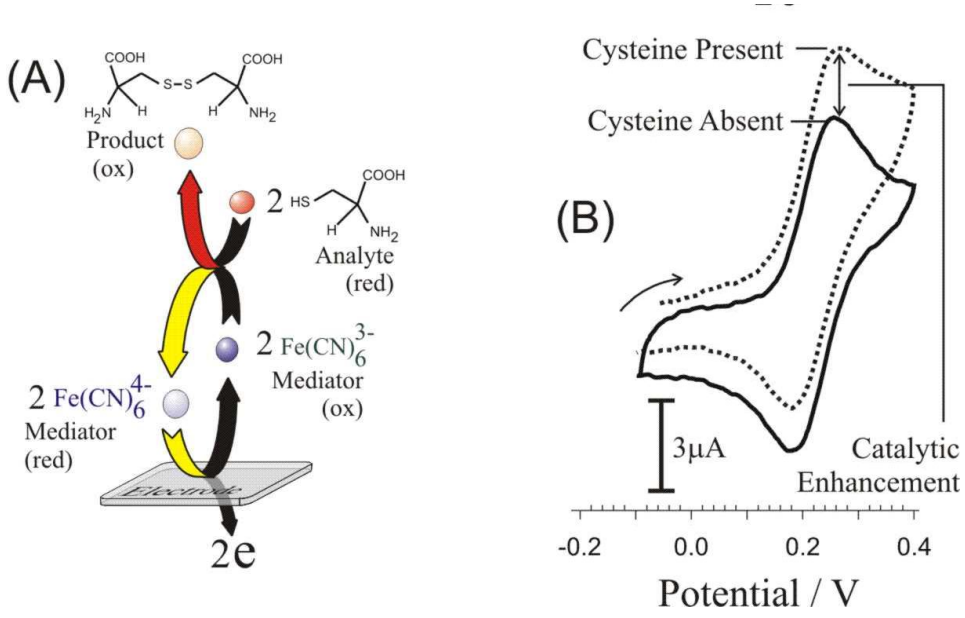


Figure 3.3 (A) Schematic of a typical electrocatalytic mechanism. (B) Cyclic voltammograms detailing the response of ferrocyanide in the absence of cysteine (solid line) and the catalytic response when cysteine is added to the solution (dotted line). Voltammetric profiles recorded in pH 7 buffer at scan rate of 50mVs^{-1}.

Early implementations of this approach simply involved the addition of the catalyst to the assay solution but there have been numerous refinements where it can be effectively confined to the electrode surface either by direct chemical modification or polymer entrapment. Coating the electrode surface with polymeric films can also serve to minimise interference through providing a molecular sieving action that can discriminate by means of electrostatic interactions or through size exclusion [9, 11, 12]. Nafion® has been extensively exploited in electroanalytical applications with the anionic nature of the film proving particularly valuable when applied to the exclusion of ascorbate. Cationic polymers such as poly(vinylpyridine) and polypyrrole have also been used but chiefly as a means of encapsulating an anionic redox catalyst (such as ferrocyanide). More recent developments have focused on the use of poly(phenylene diamine) films which, although neutral, have been shown to preclude most of the common electroactive interferences [9-13].

The advent of screen printed electrode (SPE) technologies however have considerably eased the process of electrode modification with the catalyst either incorporated within the ink prior to printing or simply deposited as an additional layer [9, 10]. These processes are readily amenable to mass manufacturing practices with the technological and commercial viability of the approach exemplified by the success of electrochemical glucose monitors. The ease of production, low cost and their inherent disposability makes this substrate one of the most likely candidates upon which a POCT device would be constructed. The development of microfluidic systems such as those based on photolithographic “Lab-on-a-Chip” approaches will inevitably rival the SPE systems through offering the possibility of chromatographic (electrophoretic) resolution. The facilities and techniques required for the production of such devices however remains prohibitive and, as such, many of the current

electroanalytical strategies are focused on the utilisation of SPE technologies and these are likely to remain dominant in the short term.

It is clear that the antioxidants will be more readily accessible to direct electroanalytical detection by virtue of their inherent redox capabilities. Individual quantification can be problematic, as shown in **Figure 3.1** and **Figure 3.2**, as each will interfere with the determination of the other. Ascorbic acid is arguably the easiest to detect as it possesses the lowest oxidation potential of the group and therefore it could be conceivable to build an amperometric device where the majority of the current will originate from the oxidation of ascorbate. A similar case can be put forward for the detection of uric acid. While ascorbate will interfere, the magnitude of the urate response at glassy carbon is such that the contribution to the signal will be minimal. The use of disposable electrodes would however remove this advantage but, as seen from **Table 3.1**, this analyte is liable to be present at substantially greater concentration. A problem that that will inevitably be encountered is the discrepancy between plasma and cellular concentrations of the target analytes and any device must reconcile the nature of the sample matrix. For example: the concentration of glutathione within the erythrocytes is significantly higher than in plasma and it is likely that any POCT device would struggle to quantify the thiol within the latter. Were it possible to lyse the red blood cells in the course of the measurement then the concentration would be substantially increased and likely to fall within the range of electroanalysis.

The carotenoids [17, 26], retinoids [14, 17] and tocopherols [17, 31, 32] are other well known antioxidants and, having been established as prime indicators of oxidative stress, their concentrations are routinely assayed within clinical research. The low micromolar concentrations of these species within extracellular fluid

however can severely test the sensitivity of conventional electroanalytical techniques. As yet, there are no available enzyme systems specific for these species and their detection is reliant upon direct oxidation at the electrode. Potentials in excess of +0.6V are generally employed but their application to untreated physiological fluid has been compromised by a lack of selectivity and would inevitably encounter significant interference from the matrix constituents. Their large molecular sizes effectively preclude the polymeric molecular sieving at the electrode that can be accomplished for smaller targets. Similar problems arise with the detection of ubiquinone / ubiquinol couples [36, 37].

The reliable detection of biomarker depletion will be complicated by natural variations in the endogenous concentrations. Confidence in the measurement and in the subsequent diagnostic outcome will therefore be dependent upon significant shifts in the concentration of the target analyte. This may severely test the detection limits achievable at POCT devices. A better approach is to exploit biomarkers that are generated as a consequence of injury or underlying disease. Uric acid is one of the few antioxidants whose concentration is increased as a consequence of the disease but this reflects its biochemical role in purine metabolism more than its free radical scavenging capabilities. Increased concentrations of urate can occur through overproduction of purine nucleotides and are symptomatic of stroke, pre-eclampsia, gout, Lesch-Nylan syndrome and leukaemia [36, 37]. Dihydrophenylalanine [20], nitrotyrosine [29, 30] and 8-hydro-deoxyguanosine [4-6, 21, 25-28] all arise as a consequence of free radical attack and are characteristic of oxidative stress processes. The dihydrobenzoic acids could also be classed along with this group but they are slightly anomalous in that they arise through the attack of hydroxyl radical on salicylic acid (derived from previously administered acetylsalicylic acid) [18,19].

While the attack of ROS on tyrosine results in the dihydrophenylalanines [20], RNS attacks produce nitrotyrosine [29, 30]. In contrast to the oxidative process used for quantification of nearly all the biomarkers outlined in **Table 3.1**, nitrotyrosine can be detected through the reduction of the nitro group to the nitroso and ultimately to the corresponding amine. The main drawback to the detection of this analyte is the low concentration (typically nM) within the plasma. Quantitative reduction is also hampered by potential interference from the reduction of dissolved oxygen (to the radical anion) and which is likely to be present at high concentrations relative to the nitrotyrosine. It should be noted that ROS/RNS will also attack protein bound tyrosine residues but these are largely inaccessible to conventional electrochemical methods.

A similar problem arises with the attack of ROS on guanosine residues within DNA/RNA to yield 8-hydroxyguanosine. This is by far the most common biomarker used to assess oxidative stress but its accessibility to electrochemical quantification is severely impeded through incorporation within the macromolecular structure of the nucleic acid. Standard procedures employ various digestion methods to release the nucleoside prior to quantification by LC-ED detection but these would be inappropriate to POCT [4-6]. It must be noted that the reliable analysis of the modified guanosine residue even within a laboratory environment can be problematic with the extraction procedure producing highly variable results when inter-laboratory comparisons are made [4-6]. Quantification of the guanosine nucleoside within urine [28] is an alternative source but again problems of selectivity and sensitivity arise as the concentrations are liable to be in the nanomolar range.

3.4.3 Electrochemical Biosensors

The requirement for accessible redox inter-conversion directly at the electrode substrate provides the greatest restriction in terms of candidates as the majority lie beyond the normal operating windows ascribed to aqueous electrochemistry. Typical examples have been highlighted in **Table 3.2** where, with the exception of homocysteine, they are largely unresponsive to conventional electrochemical interrogation. The use of enzymes however can extend the range of accessible analytes and also serve to improve the selectivity of the device through exploiting the specificity of biological recognition [9-13]. The protein shell surrounding the enzyme active site effectively insulates the redox process from direct electrical contact with the underlying electrode and their re-activation is usually accomplished through the donation of electrons to its "natural mediator". However, the enzyme reaction can be exploited as the basis of an electrochemical sensor through a variety of routes. The first couplings of enzymes with electrochemical detection involved monitoring the production or consumption (cf **Figure 1.4A and 1.4B**) of the reaction components [12]. Thus, in the case of oxidase enzymes (i.e. glucose, cholesterol, lactate) hydrogen peroxide generated from the enzymic reaction can be monitored through oxidation at the electrode. Alternatively, the consumption of molecular oxygen, required to regenerate the enzyme redox centre, can be used as indirect indicator of the target analyte concentration. More recent developments have focused on the use of artificial redox mediators (**Figure 1.4C**) to effectively shuttle electrons between electrode and enzyme active site [10-13] and the process is effectively an extension of the catalytic cycle shown in **Figure 3.3**. The principal advantage provided by this approach is that

it obviates the natural variation in oxygen tension that can arise in physiological systems.

Irrespective of the detection mechanism used, the processes can still be prone to interference from other electroactive species that can be oxidised at the potentials required to detect the reaction by-products or facilitate the recycling of the mediator. An amperometric POCT device for glucose would therefore simply fix the potential of the working electrode at +0.35V with the resulting current being related to the concentration of glucose. The more glucose present – the higher the current and it is this approach that underlies many of the commercial glucose sensors that are currently available. Problems arise however when attempting to detect low concentrations of glucose in the presence of high levels of antioxidants (typically ascorbate and urate) as these can lead to an over-estimate of the glucose concentration. The electrode response would still appear to indicate the presence of glucose thereby leading to a false reading.

Multi-enzyme assemblies have become more common and can be employed to remove interferences, release bound analyte or to facilitate the indirect electrochemical evaluation of a non redox enzyme reaction [11-13, 48]. Common examples are the co-immobilisation of ascorbate oxidase alongside the principal target oxidase enzymes (glucose, lactate etc.) to counter the interference. The determination of total cholesterol will generally require the inclusion of cholesterol esterase as the analayte will be predominantly found as the ester within plasma and hence will be inaccessible to the cholesterol oxidase enzyme that will ultimately be responsible for generating the analytical signal. The ability to couple enzymes together to form a synergistic assembly is further highlighted in the case of creatinine. Creatininase does not possess an electrochemically addressable redox centre but it can be utilised as the

basis of an amperometric system through co-immobilisation with two other enzymes – creatine aminohydrolase and sarcosine oxidase. These feed from the stepwise production of reaction intermediates (**Figure 3.4**) with the terminal acceptor, sarcosine oxidase, producing peroxide that can be detected at the electrode.

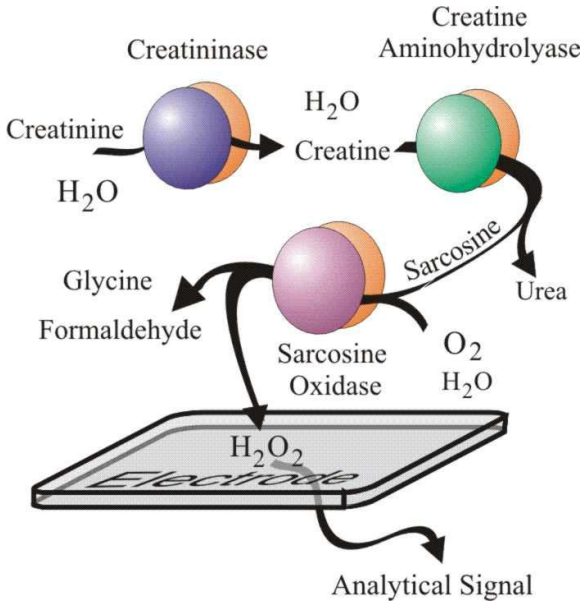


Figure 3.4. Schematic representations of multi-enzyme electrode employing product detection as the analytical signal through which the target analyte concentration can be quantified.

It is important to note however that it is not a requirement for the enzyme to possess a distinct redox centre providing that the reaction products can elicit an electrochemical response. This is exemplified in the creatinine enzyme electrode systems where changes in local pH brought about by the enzyme reaction are harnessed to generate a potentiometric signal [39]. These systems can therefore be coupled to a simple pH transducer and have been successfully incorporated within ion selective field effect transistors [39, 46, 47]. As the electrode is essentially passive, interference from the other electroactive species is avoided and clearly contrasts the amperometric and voltammetric approaches.

3.5 Conclusion

Medical authorities and private health providers are increasingly encouraging patients to be proactive in the clinical decision and management processes. This can be of particular importance for patients with metabolic conditions such as diabetes and hyperlipidaemias where patient lifestyle can have a telling influence on long term outcome. Experience has shown that patients are motivated to learn and pick up on new developments, particularly when they can be provided with prompt feedback on their condition. POCT devices would therefore fulfil an important role in diabetes, lipid and haematology clinics, renal dialysis units, and clinical biochemistry laboratories. It could also be envisaged that the opportunity for home testing and the consequent active participation in the disease management may provide the patient with an extra incentive to comply with medical advice. The latter is exemplified by the availability of commercial glucose and cholesterol monitors whose introduction has effectively revolutionised clinical treatment for the respective conditions. A similar case could therefore be made for biomarkers that are sensitive to underlying oxidative stress that, more often than not, may well go undiagnosed until clinical complications arise.

At present, screen print technologies represent the most viable route through which electroanalytical POCT can occur. Their availability, low expense and inherent disposability are advantages that have proven to be particularly apt within clinical contexts given the concerns over potential contamination and transmission of biological agents. The electrochemical basis from which near patient monitors can be devised have been outlined and their application to the monitoring of common physiological biomarkers assessed. Despite electrochemical detection being routinely

employed for the LC quantification of the latter, the development of discrete, non enzymatic, sensing devices is however likely to be restricted to a few compounds - principally ascorbate, urate and glutathione – on the basis that these species offer detectable concentration ranges for which selectivity can be secured. It is likely that improvements in biosensing technologies and innovative coupling of enzyme systems may significantly broaden the range of targets and yield further improvements in selectivity.

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Chapter 4

Preliminary Investigation of Quinone – Thiol Biomarker Interactions

Abstract

The reaction of benzoquinone with the principal biomarkers (ascorbate, cysteine and glutathione) has been investigated using potentiometric techniques. The specificity of this reaction for thiol functionality has been briefly assessed and the nature of the electrode response has been elucidated. The system has been characterised in terms of selectivity, sensitivity and the efficacy of using the system for the quantification of thiol containing pharmaceuticals has been demonstrated. The simplicity of the detection methodology is shown to markedly contrast alternative thiol detection strategies. The transfer of the technology to a mass production format through the adoption of screen print electrode formats has been achieved and the efficacy of the approach demonstrated. Recovery experiments using captopril and penicillamine in tissue culture as a model analyte system were performed. The reaction stoichiometry and possible products were investigated using spectroscopic and chromatographic methods. The development of a potential Point of Care Testing system for cysteine was briefly assessed through the development of a novel tablet system.

4.0 Introduction

Anti-oxidants such as ascorbate, cysteine and glutathione are known to play a crucial role in the prevention of free radical induced oxidative damage and there are numerous instances in which being able to monitor their concentration within the body would be highly beneficial [1-4]. The depletion of endogenous anti-oxidants within plasma and whole blood/lysate is known to be an indicator of oxidative stress and it could be envisaged that were a clinician, or indeed a patient, able to routinely monitor antioxidant status then preventative action could be implemented before the manifestation of physical symptoms. At present, analysis of blood or any other biofluid for anti-oxidant concentration requires the referral of the samples to a specialised central laboratory however it is clear that the practical exploitation of such biomarkers however will depend upon the realisation of devices that can permit near patient testing by the attendant clinical staff. The portability and instrumentational simplicity inherent in the construction of electrochemical sensors could offer a path through which such demands could be met. The aim of the present and following chapters has been to investigate the electrochemical capability of using quinone as selective markers for the quantification of ascorbate and thiols and to develop an analytical protocol that could form the basis of either a voltammetric or potentiometric sensor. A brief review of the biomedical significance and technologies presently available is present beforehand such that a critical comparison can be made with the methodology being proposed.

4.1 Glutathione

Glutathione (GSH), L-y-glutamyl-L-cysteine-glycine, is the most abundant non-protein thiol and is the predominant thiol "target" in the present project. It is the most important intracellular thiol antioxidant which protects the cell from oxidative stress. It is a cofactor for glutathione peroxidase (GPx) which acts as a defence mechanism against peroxides, preventing accumulation of Reactive Oxygen Species (ROS) and hence preventing cell injury.

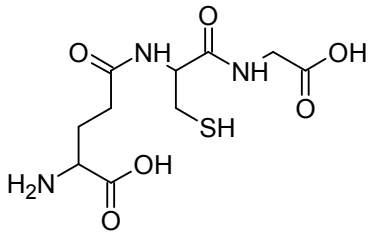


Figure 4.1 Chemical Structure of Glutathione.

Reduced glutathione (**Figure 4.1**) is regenerated by Glutathione reductase (GR) an NADPH dependent enzyme. Glutathione plays a role as a cofactor for the glutathione peroxidase (GPx) family. Glutathione peroxidase is present in a number of tissues and catalyses GSH-dependent reduction of H_2O_2 [5]. Glutathione peroxidase takes part in the biosynthesis of prostaglandins and in the regulation of prostacyclin formation. GSH peroxidase consists of four protein subunits, each of which contains one atom of selenium. It has long been known that Se is an essential nutrient. Se-deficient animals have markedly decreased GSH peroxidase activity. The reduced form of GSH peroxidase appears to contain selenocysteine selenol (-SeH) at the active site [5]. Apart from its role

as a cofactor for the glutathione peroxidase family, GSH is also involved in many other metabolic processes, including ascorbic acid metabolism, maintaining communications between cells [6] and generally preventing protein –SH groups from oxidizing and cross-linking. It also appears to be involved in intracellular copper transport [7]; GSH can chelate copper ions and diminish their ability to generate free radicals, or at least to release radicals into solution[8]. GSH is a radioprotective agent and a cofactor for several enzymes in different metabolic pathways, including glyoxylases [9], and enzymes involved in leukotriene synthesis. Glutathione also plays a role in protein folding and degradation of proteins with disulphide bonds, such as insulin (the first step in insulin removal is cleavage of disulphide bridges linking the two peptide chains). Glutathione can react *in vitro* with $OH^{\bullet}$, HOCl, peroxynitrite, $RO^{\bullet}$, $RO_2^{\bullet}$, carbon-centred radicals and $^1O_2$.

4.2 Detection Strategies

There are a lot of methods for the determination of thiols, which suggests that none of them is totally satisfactory. In general, most methods for thiol detection can be divided into several groups:

1. Enzymatic
2. Spectroscopic methods
3. Electrochemical methods
4. HPLC methods

5. Biosensors

Enzymatic Methods

One example of an enzymatic assay is horseradish peroxidase, immobilized on a rotating disk, in the presence of hydrogen peroxide catalyzed the oxidation of catechols, whose electrochemical reduction was detected on glassy carbon electrode surface [10]. The method is based on Michael addition reactions of thiols with catechols yielding thioquinone derivatives, decreasing the peak current obtained proportionally to the increase of its concentration. Cysteine was used as the model thiol-containing compound for the study. The highest response for CSH was obtained around pH 7. This method could be used to determine CSH concentration in the range 0.05-90 μM and GSH concentration in the range 0.04-90 μM. The only disadvantage of this was that authors did not examine how their method would work with biological fluids. Two amperometric biosensors with immobilized horseradish peroxidase were developed for thiols screening based on biosensor signal inhibition. The horseradish peroxidase was retained either in magnetized nanoporous silica microparticles or in a carbon paste [11].

Spectroscopic Methods

There are a large number of such methods and some of them are summarised in **Table 4.1.** Typically these methods are based on Ellman's reagent, but recently a new method has been reported[12]. A simple colorimetric method for the determination of cysteine and homocysteine using azo dyes containing an aldehyde group has been

developed. The reactions are shown below and are capable of proceeding under physiological pH.

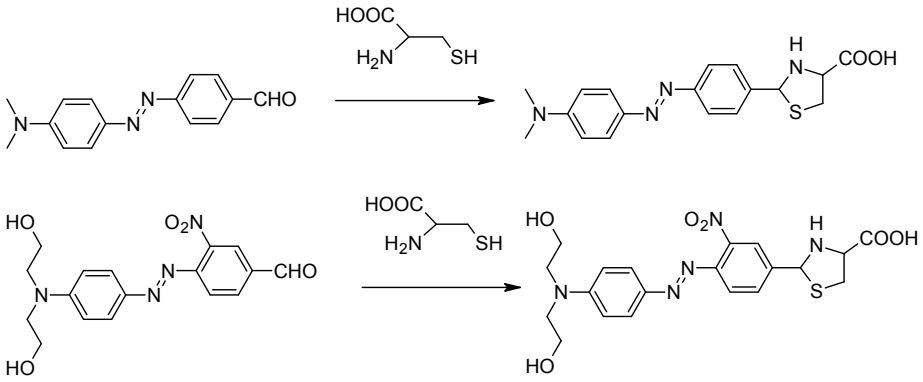


Figure 4.2

The authors investigated the interferences and discovered that the method is insensitive to GSH and to a range of amino acids whereas the indicator changes colour from pink to yellow when cysteine or homocysteine is present.

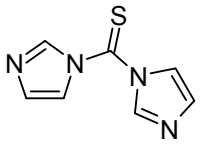
	Technique	Label/method	Sample	Detection limit	Ref.
1	Spectrofluorimetric method	5-maleimidyl-2-(*m*-methylphenyl) benzoxazole (MMPB)	Human blood, pig's liver and heart	0.1nM (GSH)	13
2	Spectrofluorimetric method	Zn(II)-8-hydroxyquinoline-5-sulphonic acid complex	Human blood serum	0.01μM(Cys); 2μM (GSH)	14
3	Spectrofluorimetric method	$[Zn(cyclen)(lumazine)]^+$	water	50μM GSH	15
4	Spectrophotometric method	Thiocarbonyldiimidazole 	Dietary supplement	2-μM Cys	16
5	Spectrofluorimetric method	fluorescein/rhodamine attached to diaminophenyl disulfide	*Escherichia coli,* Zebrafish embryos	Method works for 1 to 10mM	17

Table 4.1Electrochemical Methods.

Detection of thiols based on electrochemical methods has been of great interest in recent years. The Ricci group[18] has recently reported a new screen printed electrode modified with Prussian Blue (ferric hexacyanoferrate). The amperometric detection at +200mV showed the best results for thiocholine and cystemine and the method was proposed as an electrochemical alternative to Ellman's test for total thiol estimation. The Ellman's test was adapted for an electrochemical detection of thiols. The detection range was 2- 120 μM for cysteine and the method can be transferred to the SPE[19]. Also a renewable three-dimensional chemically modified carbon ceramic electrode containing Ru [(tpy)(bpy)Cl] PF_6, constructed by a sol–gel technique, was used for cysteine and glutathione detection [20] The detection limit and sensitivity is 1 μM, 5 nA/μM for L-cysteine and 1 μM, 7.8 nA/μM for glutathione. More comprehensive surveys of electrochemical methods were reviewed recently and the principal detection systems have been summarised in **Table 4.2** .

	Technique	Electrode	Sample	Detection limit µM	Ref.
1	Direct voltammetric measuremens	Edge plane pyrolitic electrode	N-acetylcysteine, CYS, HCYS, GSH in tissue growth media	2.1	21
2	Direct voltammetric measuremens	Glassy carbon rotating disk	CYS in borate buffer, pH 9.2 in the presence of Cu^{2+}	0.0005	22
3	Voltammetry with redox mediator	Boron doped diamond electrode with octacyanomolybdate (IV) as mediator	CYS in borax buffer, pH 10	0.3	23
4	Voltammetry and 1,4 Michael addition	Boron doped diamond electrode		4	24
		Hydroquinone sulfonic acid 2,3 dimethylbenzoquinone	CYS in phosphate buffer	6	
5	Square Wave Voltammetry	Glassy Carbon Electrode /Catechol	tissue culture medium		25
			CYS	6	
			HCYS	7	
			GSH	1	
6	Voltammetry	Saturated calomel electrode	GSH in phosphate buffer	0.94	26
7	Amperometric sensors used as post column detectors	Boron doped electrode	Britton-Robinson buffer, pH2		
			GSH	1.4 nM	27
			HCYS	1nM	28
8	Amperometric detection after separation by capillary electrophoresis	Mixed-valence ruthenium cyanide modified carbon fibre array microelectrode	Urine from patients with kidney stone		29
			CYS	3	
			HCYS	2.5	
9	Amperometric detection after separation by capillary electrophoresis	Mercury coated gold amalgam microelectrode	GSH	21nM	30
10	Amperometric detection after HPLC	Glassy carbon modified with Pd-IrO_2	Rat brain microdialysate		31
			GSH	2	
			CYS	0.5	
11	Amperometric detection in a bulk solution	Sol-gel glass encapsulating pyrroloquinone-modified	CYS	18nM	32
			GSH	26nM	
12	Amperometric detection in a bulk solution	Carbon ceramic electrode modified with Nafion and $[Ru(bpy)(tpy)Cl]PF_6$	Phosphate buffer CYS	0.1	33

Table 4.2

The methods can be divided into several core methodologies:

1. Direct voltammetry;
2. Voltammetry with redox mediators/catalysts
3. Post column amperometric sensors;
4. Amperometric sensors used in a bulk solutions.

Numerous electroanalytical strategies have been employed to aid the quantification of thiols with the majority employing voltammetric methodologies [34-41]. Direct oxidation of the target analytes at bare electrodes however has largely been superseded by the use of electrode modifiers in combination with pulse techniques such as square wave voltammetry to extract greater selectivity and sensitivity from the analytical procedure [34,35]. The modifiers are usually metallophthalocyanines [36-38] and are either immobilised at (or indeed in) the electrode substrate or added to the analysis solution as the basis of an assay. The various merits and limitations possessed by these approaches have been reviewed [34] but it is worthwhile to note that few of the techniques are sufficiently robust to offer a facile route towards point of care analysis. Some of the reported methods are based on 1,4 Michael nucleophilic addition to quinone compounds or catechols [34]. In the present work, the intention has been to study the interaction between the oxidised form of the quinones and thiols.

4.3 Proposed Methodology

The operational simplicity common to amperometric and potentiometric systems and the maturity of the technology required for data extraction and analysis could be more accessible to non expert staff than voltammetric systems. It is important to note that neither amperometric nor potentiometric thiol detection features highly in the scientific literature and at present there are no commercial selective electrodes or sensors for thiol moieties. Potentiometric thiol detection has largely centred around the indirect determination of silver ion at silver sulphide electrodes or through redox interactions with iodine [34]. Both approaches are susceptible to significant interference from common physiological constituents. As such, they are only of value in simple matrices or where the sample components can be resolved as a consequence of column or flow injection separation. Neither approach is appropriate in the present context and there remains a need for the development of simple yet selective protocols for the thiol determination.

The interaction of a hydroquinone / benzoquinone redox couple with thiols could however provide the foundations of a generic strategy for the selective amperometric or potentiometric determination of the latter. There are three possible mechanisms through which quinone species could interact with reduced thiols and these are highlighted in **Figure 4.3**.

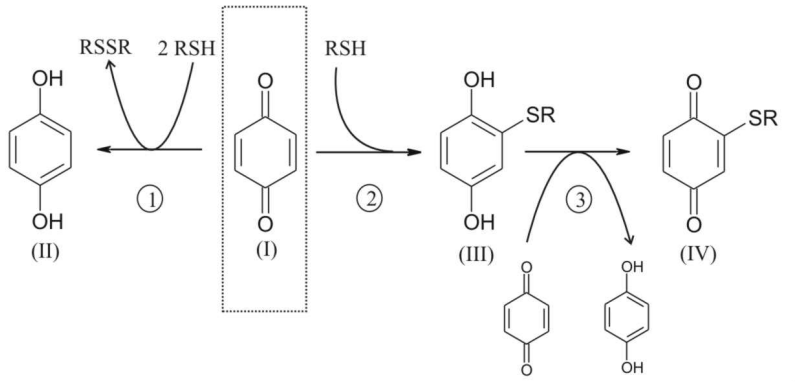


Figure 4.3

The quinone component (**I**) of the redox couple could interact through simple redox inter-conversion of the thiol to the corresponding disulphide (**Path 1**) and hydroquinone (**II**) derivative. Alternatively, the quinone could react through a nucleophilic 1,4-Michael addition to form the corresponding reduced thiol conjugate (**III**) shown in **Path 2**. As mentioned previously, the voltammetric analysis of the latter has been investigated and the electrocatalytic oxidation to the quinoid form (**IV**) exploited in a number of analytical contexts [34,38]. The amperometric and potentiometric responses however look at the bulk solution reaction and operates over a greater timescale. Thus, there is a possibility that secondary reactions can occur (**Path 3**) in situations where excess quantities of the quinone (**I**) are present and result in the chemical oxidation of the reduced conjugate (**III**) to the oxidised form (**IV**). Interpreting changes in the redox composition of the benzoquinone / hydroquinone couple would therefore require knowledge of which path is followed. Successful implementation of the approach would also require that one predominates such that the response characteristics are of a magnitude to allow an unambiguous assessment to be made by biomedical staff.

4.4 Experimental Details

All reagents were of the highest grade available and used without further purification. All solutions and subsequent dilutions were prepared daily using deionised water from an Elgastat (Elga, UK) UHQ grade water system with a resistivity of not less than 18 MΩ cm and were refrigerated when not in use. Electrochemical measurements were conducted using a μAutolab computer controlled potentiostat (Eco-Chemie, Utrecht, Netherlands). An initial cell volume of 15cm^3 was generally used and the measurements recorded at a temperature of 20^{o}C ± 2^{o}C. A two electrode assembly was used throughout and comprised of a glassy carbon (GC) indicating electrode (3 mm diameter, BAS Technicol, UK) and a silver / silver chloride reference electrode (3M chloride, BAS Technicol, UK).

The glassy carbon electrode was polished with 1 μm alumina powder (Buehler, UK) and sonicated in deionised water for 2 minutes prior to use. Unless specified otherwise, the electrochemical solutions consisted of Britton-Robinson buffer (acetic, boric and phosphoric acids - each at a concentration of 0.04 M and adjusted to pH 7 through the addition of sodium hydroxide). The electrodes were immersed in buffered solutions and stirred magnetically at a constant rate throughout. The potential difference between the electrodes was continuously monitored and additions of the analyte initially added at 60 second intervals. Greater time intervals were however required as the quinone component was gradually consumed (discussed in the following section).

4.5 Results and Discussion

The potential response of a glassy carbon electrode operating under zero current in pH 7 buffer towards an equimolar mixture of benzoquinone / hydroquinone (130 μM) is shown in **Figure 4.4**. Upon adding aliquots (16 μM) of glutathione to the solution, the potential was found to decrease and can be ascribed to the thiol reacting with the quinone indicator. The consequent change in the oxidised/reduced ratio of the indicator couple leads to the change in the electrode potential.

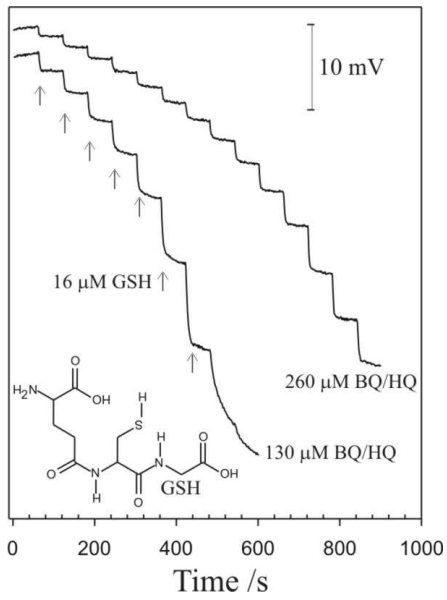


Figure 4.4. Potentiometric response of glassy carbon towards increasing additions of cysteine (CSH) in the presence of equimolar mixtures of benzoquinone (BQ) / hydroquinone (HQ).

No appreciable change in the electrode response was observed to increasing concentrations of the thiol (16 μM additions) in the absence of the quinone and serves to confirm the efficacy of employing the indicator reagent. The potential-time profile shown

in **Figure 4.4** effectively represents the titration of the quinone by the incremental addition of thiol. It can be seen that the acquisition of a stable signal takes progressively longer as the concentration of the indicating component decreases with the end point marked by a sustained decrease in potential. The failure to reach a plateau after the addition of excess thiol can be attributed to the fact that under such conditions there are no defined redox couples. The predominant species will be the reduced forms of the thiol conjugate and hydroquinone.

Increasing the concentration of the quinone was found to increase the detection range but at the expense of the response sensitivity. This is highlighted in **Figure 4.4** where the potentiometric profiles for the addition of glutathione to 130 and 260 μM benzoquinone are compared. The increase in the detection range is principally due to the availability of a larger reservoir of indicator. The decreased sensitivity can be accounted for on the basis that the addition of a defined quantity of thiol will obviously have a smaller impact on the BQ / HQ ratio (and hence electrode potential) for the higher, 260 μM, indicator concentration than for a similar addition to the 130 μM BQ / HQ. The selectivity of the approach towards other thiol species was assessed through examining the response to cysteine. The potentiometric profile obtained in the presence of 260 μM quinone is shown in **Figure 4.5.**

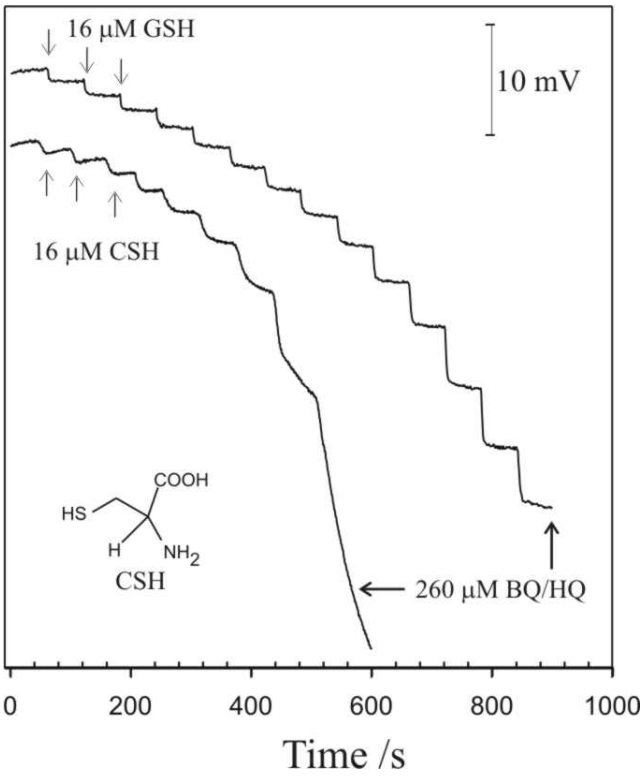


Figure 4.5. Potentiometric response of glassy carbon towards increasing additions of cysteine (CSH) in the presence of an equimolar (260 μM) mixture of benzoquinone (BQ) / hydroquinone (HQ). The response to glutathione (GSH) under identical conditions has been included for comparison.

The response to glutathione under analogous conditions has been included for comparison. It can be seen that the reaction of cysteine with benzoquinone reaches the end point significantly before that of glutathione. Repeated measurements indicated that while glutathione reacts in a 1:1 reaction stoichiometry with the benzoquinone indicator, the response to cysteine was consistently 1 thiol:2 quinone. The responses to other

sulphydryl thiols (N-acetylcysteine, captopril and penicillamine) were found to mirror that of glutathione and invariably exhibited a 1:1 reaction stoichiometry (not shown).

The experimental responses of 260 μM BQ/HQ to increasing additions of cysteine were compared against the theoretical potentials predicted from the modification of the BQ / HQ ratio under the various pathways outlined in **Figure 4.3**. The results are compared in **Figure 4.6** and it can be seen that there is a significant deviation from the calculated values were **Path 1 (Figure 4.6A)** being followed. Similar deviations were observed when working on the assumption that the reaction terminates at **Path 2 (Figure 4.6B)**. A much closer match was however found using the prediction that the reaction progresses through both **Path 2** and **Path 3 (Figure 4.6C)**.

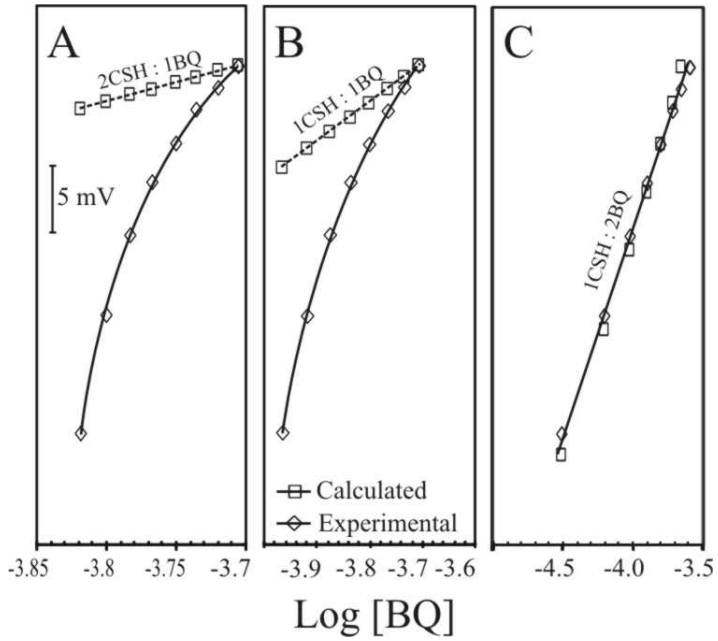


Figure 4.6. Potentiometric response of glassy carbon towards increasing additions of glutathione in the presence of equimolar mixtures of benzoquinone / hydroquinone.

In the presence of excess BQ, the reduced conjugate is chemically oxidised with the production of hydroquinone. This would effectively remove half of the BQ indicator stock and hence would appear to justify the 1:2 stoichiometry observed in **Figure 4.5**. It does not however explain the discrepancy in the responses to glutathione and cysteine.

The 1:1 stoichiometries observed for the majority of thiol-BQ interactions can be explained if we assume that the resulting conjugate has a redox potential that is similar to that of BQ/HQ. A similar assumption was made when attempting to rationalise the use of BQ-S-Protein interactions for electrospray mass spectrometry [39]. In the present context we can assume that the following occurs:

$$BQ / HQ + RSH \rightarrow HQ + HQ\text{-}SR \qquad \textbf{Eq 1}$$

$$BQ + HQ\text{-}SR \rightarrow HQ + BQ\text{-}SR \qquad \textbf{Eq 2}$$

In the initial stages of the titration, BQ will be in a greater surplus than the BQ-SR and it can be assumed that each addition of thiol is effectively scavenged by BQ. The electrode potential will therefore be determined by the relative concentrations of BQ and HQ. However, as the BQ is gradually depleted, a mixed potential will arise as BQ-SR and HQ-SR begin to co-exist. Continuing the titration to the end-point produces a second process:

$$BQ\text{-}SR + RSH \rightarrow HQ(SR)_2 \qquad \textbf{Eq 3}$$

At this point – there are no viable redox couples and hence the loss of a stable redox potential. The sacrifice of the BQ in **Eq 2** yields a similarly reactive component that continues to react with the thiol and which exerts an influence on the electrode potential.

The overall effect is the ratio of thiol to BQ becomes 1:1 as observed in the titration profile. The proposed mechanism is corroborated by comparing the experimental response for GSH - BQ (260 μM) interactions with the calculated values. The electrode responses are detailed in **Figure 4.7** and while they match at the beginning of the experiment where there is a large excess of BQ, they begin to deviate significantly as the BQ is gradually consumed and the BQ-SR / HQ-SR couple emerges.

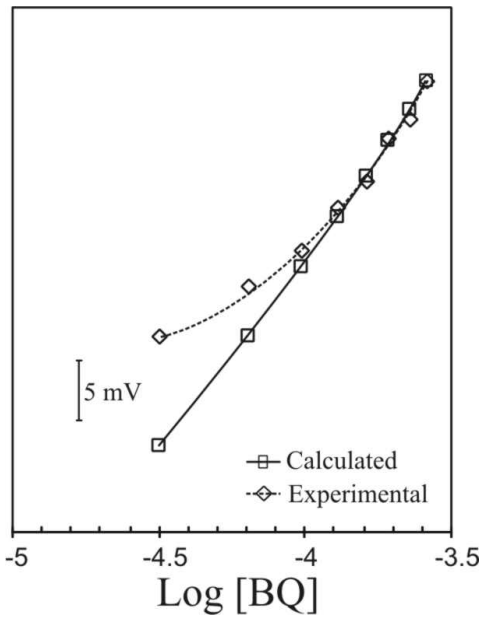


Figure 4.7 Potentiometric response of glassy carbon towards increasing additions of glutathione in the presence of equimolar mixtures of benzoquinone / hydroquinone.

The anomalous electrode behaviour observed with cysteine is due to the fact that the BQ-SR undergoes an intramolecular cyclisation reaction [40,41] that effectively prevents it from reacting with a second RSH molecule. The reaction occurs through the interaction of the alpha amino group with the quinone carbonyl. The direct incorporation

of the alpha amino group of the cysteinyl residue in the GSH tripeptide in the amide bridge precludes it from undergoing such reaction and hence the 1:1 stoichiometry. This is corroborated when we consider that N-acetylcysteine also exhibits a 1:1 stoichiometry and where the amino group is similarly blocked.

The selectivity of the approach was briefly assessed through examining the responses to other physiological components. No interference in the electrode measurement was observed when aliquots (typically 16 μM) of cysteic acid or lysine were added to the hydroquinone / benzoquinone mixture (**Figure 4.8**). The addition of N-acetyl cysteine was found to elicit a response analogous to that observed with glutathione. This is an important indicator as it essentially confirms that the reaction is indeed progressing through the attack of the SH group rather than the alkyl amino functionality and is supported by the failure of lysine (an amino acid containing two alkyl amine groups) to provoke a response. While amino groups can possess considerable nucleophilic character – the pKa of the alkyl amine group is typically between 10-11 (compared with a pKa of 8 for the SH group) [40,42]. The lone pair of electrons on the nitrogen will be protonated at pH 7 and thereby unable to participate in the nucleophilic addition reaction. This does however raise a limitation of the methodology as it restricts detection to samples of neutral pH. This was confirmed through examining the electrode response in acidic solution (not shown). No response was observed with increasing additions of glutathione (16 μM) in weakly acidic (pH 3) solution and can be attributed to the increasing protonation of the RS-H group. Alkaline conditions must also be avoided

as, while a response can be obtained, the stability of the signal will be compromised by the attack of hydroxyl anions.

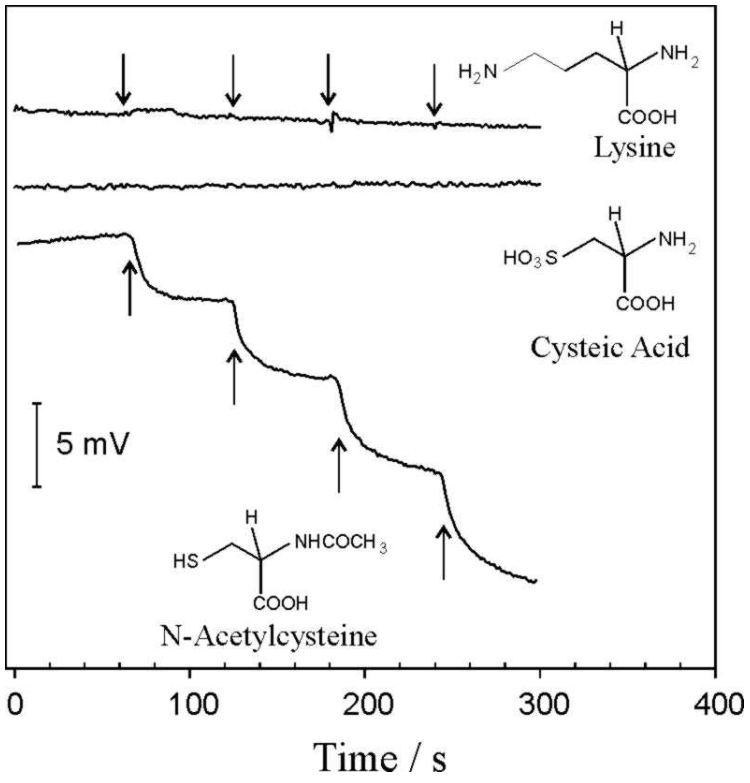


Figure 4.8. Influence of various test components (16μM additions) on the electrode response observed with a 130 μM benzoquinone/hydroquinone redox couple.

A major consideration that needs to be addressed is the response to ascorbic acid. The ubiquity of this agent within physiological fluids and its increasing inclusion within many of the over-the counter (OTC) health supplement preparations would obviously pose a problem to systems relying upon simple redox interactions. The potential interference posed is highlighted in **Figure 4.9** where the response of the BQ/HQ system to increasing additions of 16 μM ascorbate is detailed. The inset diagram within **Figure 4.9** confirms that in a mixture of equimolar (16 μM) thiol / ascorbate – the change in the

electrode potential will be significantly amplified by the presence of the latter leading to a gross over-estimation of the thiol concentration.

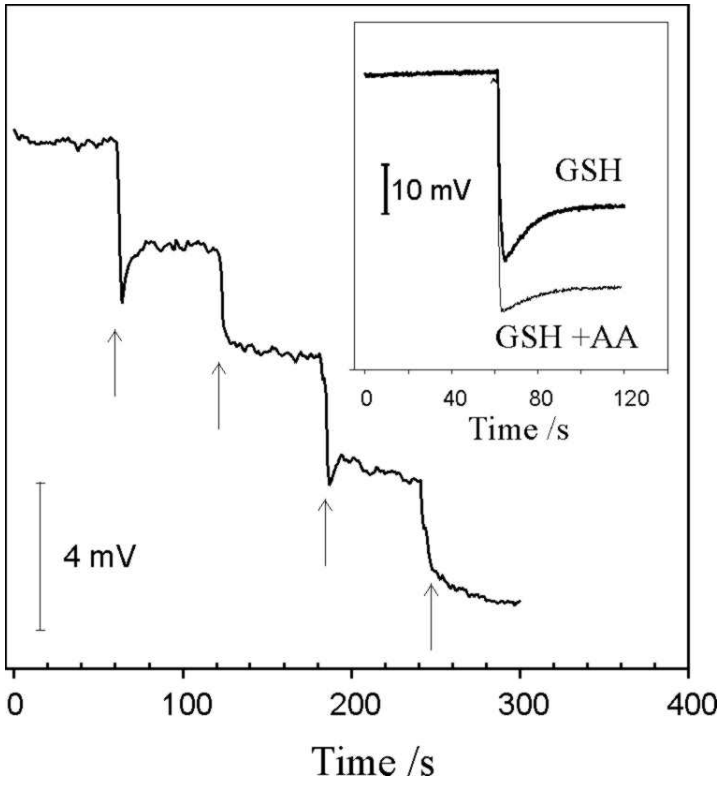


Figure 4.9. Electrode response to increasing additions of ascorbate (16μM) in the presence of 130 μM benzoquinone indicator. Inset: Influence of equimolar ascorbate (16μM) on the glutathione response.

The interaction between ascorbate and the quinone indicator operates purely through a simple redox exchange, whereas thiol species promote a change in the redox status as a result of direct chemical modification. It could be anticipated that the use of a naphthoquinone (NQ) system could alleviate the problem as the oxidising power of the former will be significantly less than that of benzoquinone system and hence, would hopefully prove ineffectual in interacting with the ascorbate. Although, it should still be

susceptible to the nucleophilic addition of the thiol. The electrode responses of a 1 mM NQ system to ascorbate (16 μM) and glutathione (16 μM) are compared in **Figure 4.10**.

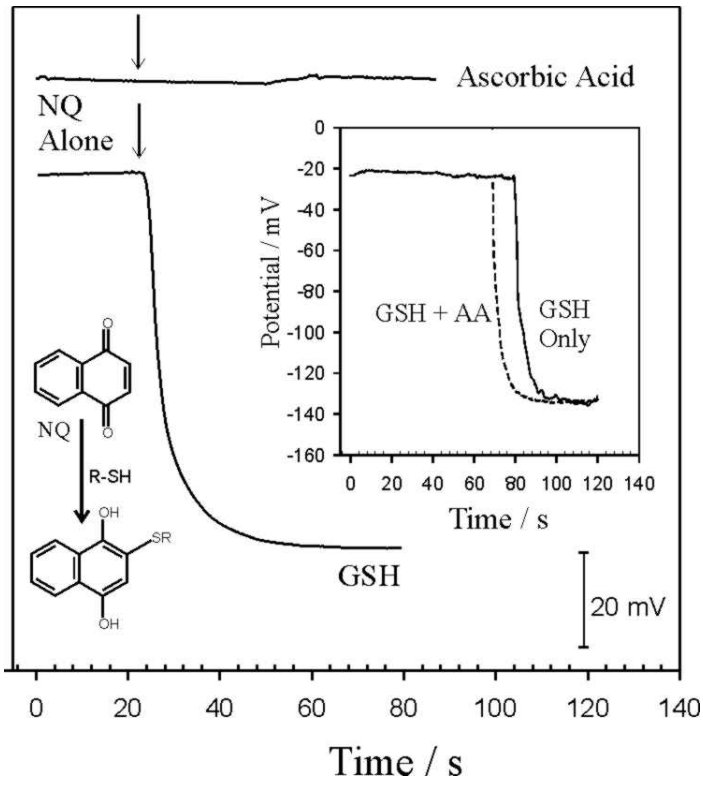


Figure 4.10 Electrode response of a 1mM napthoquinone (NQ) indicator to ascorbate and glutathione (both 16 μM, pH 7). Inset: Influence of equimolar ascorbate (16μM) on the electrode response to glutathione

While there is effectively no response to ascorbate, the potential drops dramatically upon the addition of glutathione and quickly stabilises. The absence of interference observed with the NQ indicator is further corroborated in the inset diagram in **Figure 4.10**. The response to equimolar glutathione and ascorbate (16 μM) are compared with that obtained solely with glutathione and markedly contrasts the results obtained with benzoquinone (**Figure 4.9**).

The detection system described thus far has utilised bulk carbon – silver-silver chloride electrode assemblies and has examined the electrode responses from a purely investigative viewpoint. The analytical efficacy of the approach would however demand a more rigorous appraisal. It could be expected that were such systems employed within an authentic matrix (physiological or pharmaceutical), electrode fouling or passivation at the carbon indicating electrode would impact considerably on the analytical performance. The transfer of the technology to a disposable system could be envisaged as the base electrode substrates have been exploited in a variety of sensing applications. These are invariably used in an amperometric/voltammetric detection mode [43-46] and as yet, the suitability of such platforms for potentiometric methods is unproven.

The response obtained at these substrates to 1 mM NQ before and after the addition of various aliquots of Captopril (a common thiol drug for treating blood pressure and heart failure) are shown in **Figure 4.11A**. The profile is similar to that observed with glutathione with sharp response times that quickly stabilise (typically within 20-30s) and allow the extraction of a consistent analytical signal which can be related to the Log of the analyte concentration (**Figure 4.11B**).

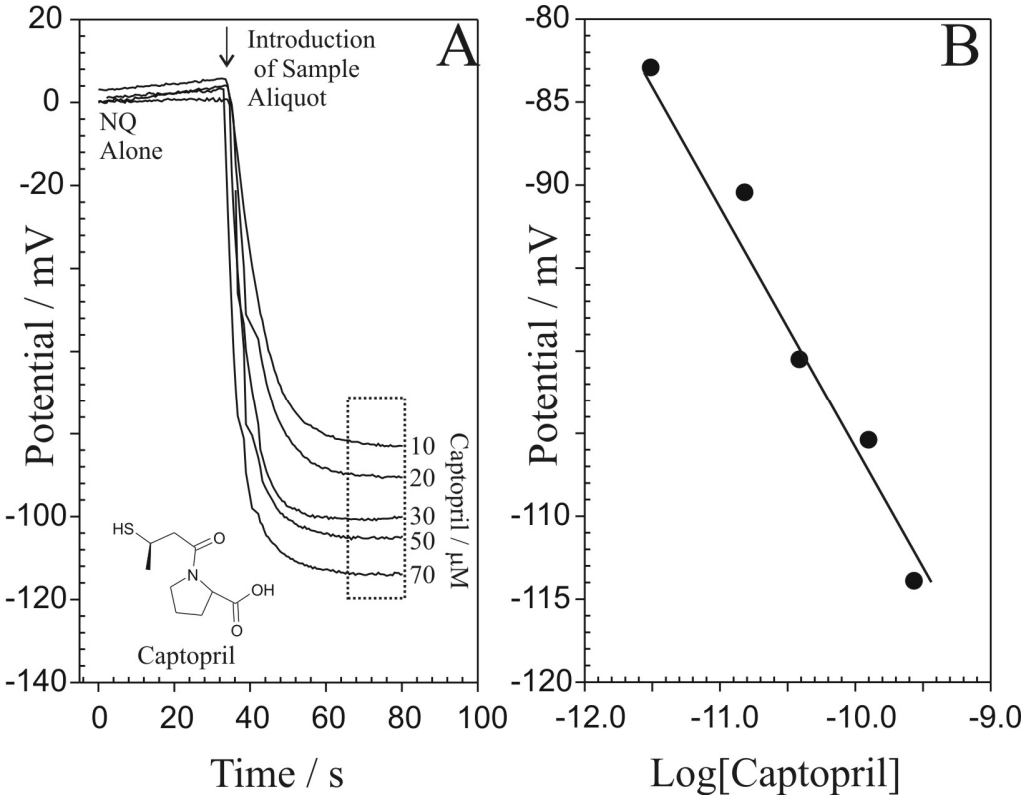


Figure 4.11. A) Electrode response of individual SPE strips to increasing concentrations of Captopril in the presence of a NQ indicator (1mM, pH 7 saturated with KCl). B) Corresponding calibration graph used in the recovery experiments for Captopril

The main limitation of the strip approach is that chloride must be added to the solution in order to define the reference potential. Ordinarily this would pose few problems for simple, well defined, systems. The analysis of an "unknown" sample containing a variable amount of chloride (as is common with biological samples) would clearly lead to erroneous assessments were a calibration approach adopted. The introduction of additional chloride would lead to an alteration in reference potential and, hence the overall cell potential difference. One remedy is to saturate the test and

calibration solutions with chloride ion – effectively nullifying the influence of any additional, sample induce, chloride component. The results detailed in **Figure 4.11A** were obtained using a 1mM NQ (pH 7) solution saturated with KCl and show that the inclusion of massive amounts of chloride does not hamper the electrode response.

In order to test the validity of the approach, recovery experiments employing (60 μM) captopril and (35 μM) penicillamine dissolved in tissue culture medium were conducted. The tissue medium has a defined composition of common mono-molecular physiological constituents and 0.12 M chloride. The matrix therefore served to test the accuracy of the indicator system through the potential – chemical interference of the constituents and the chloride induced potential reference shift. The test solution consisted of 1mM NQ dissolved in pH 7 buffer saturated with KCl. The responses were compared against a calibration set of the particular analyte prepared in 1mM NQ - KCl saturated buffer. Recoveries of 95% (3.5%RSD, N=3) for Captopril and 97% (2.7%RSD, N=3) pencillamine confirm the efficacy of the strip detection system.

The reaction stoichometry of the cyclisation reaction was probed further using altenative quinone indicators and through employing liquid chromatographic analysis. It had been noted in some preliminary investigations examining the response of different quinone species that cysteine interacts with 2,6-dimethylbenzoquinone yielding a sparingly red soluble precipitate after about 40 minutes whereas when it reacts with GSH the colour of solution remains yellow. It was initially envisaged that this finding would give an opportunity for the development of a new analytical spectroscopic method, which allows differentiation between the two thiols. The spectroscopic profiles of the reaction

between 2,6-dimethylbenzoquinone and cysteine and glutathione under identical conditions are compared in **Figure 4.12**. The maximum absorbance of the CSH product is at 527nm, which corresponds to a pink colour. It was thought that 40 minutes reaction time at the room (25^{o}C) temperature might be unacceptably long for an analytical assay, and therefore the reaction was thermostated at 50^{o}C and reaction time was reduced to 20 min.

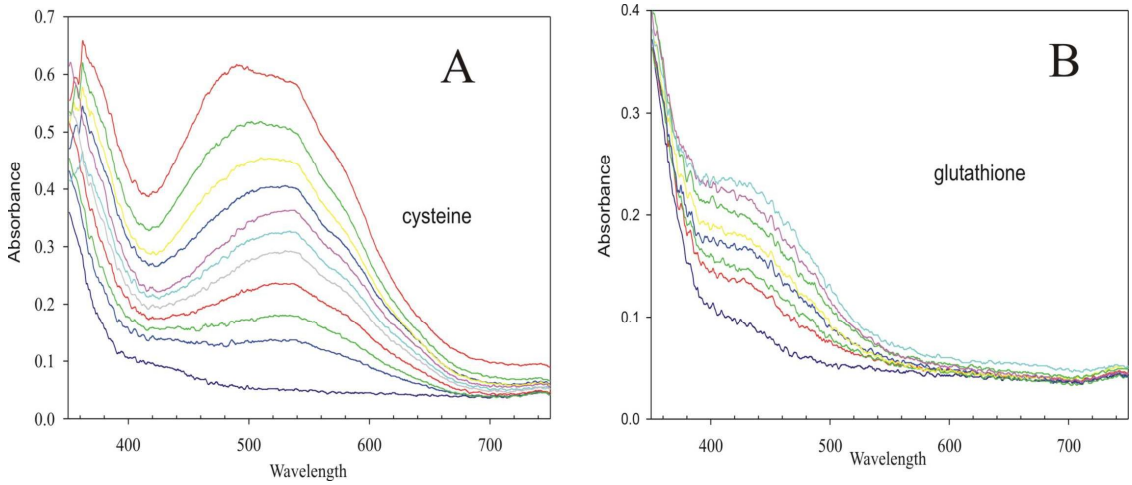


Figure 4.12 Spectroscopic profile of the reaction between 2,6-benzoquinone and cysteine B. Spectroscopic profile of the reaction between 2,6-benzoquinone and glutathione

The system was also investigated to find out whether 2,6-dimethylbenzoquinone forms any coloured compounds with acetylcysteine, lysine or ascorbic acid. None gave rise to the characteristic pink colouration with no response recorded at all for lysine and ascorbate. N-Acetylcysteine did however yield a profile consistent with that obtained for glutathione and the corresponding spectra are highlighted in **Figure 4.13**.

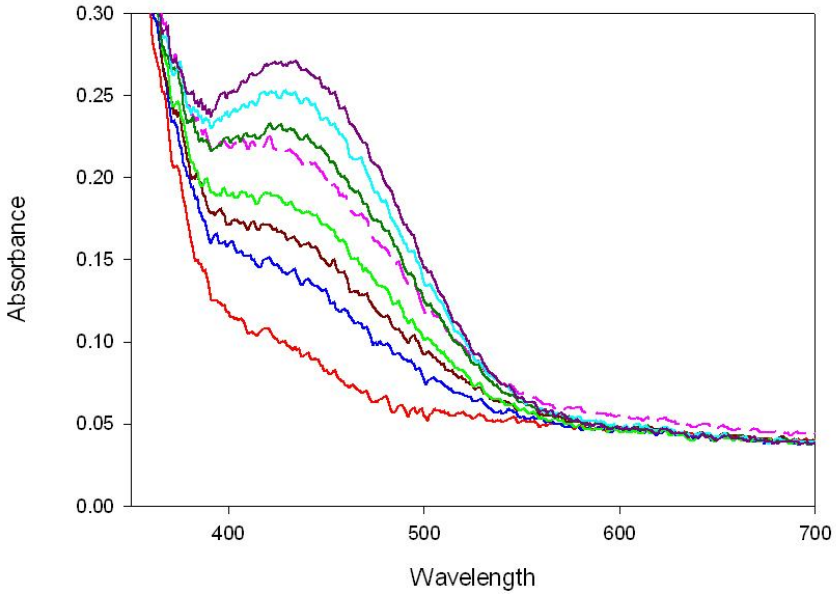


Figure 4.13 Spectroscopic profile of the reaction between 2,6-benzoquinone (1mM) and acetylcysteine (50-200μM)

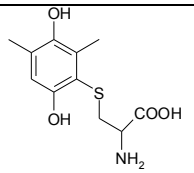
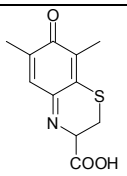
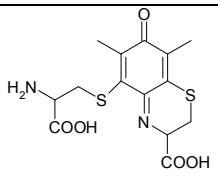
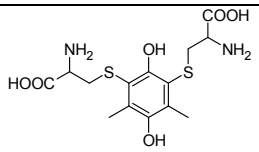
However, for a robust spectroscopic technique molar absorbtivity should be high, which can be explained by Beer-Lambert law. In case of the 2,6-dimethylbenzoquinone-cysteine product, the calculated ε for 527nm is 179 $M^{-1}cm^{-1}$ which is significantly lower than preferred for analytical analysis and much lower than that of Ellman's reagent – the standard thiol assay.

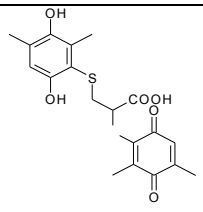
The reaction of 2,6-dimethylbenzoquinone with albumin was also investigated. Albumin is the most abundant plasma protein. It consists of a single polypeptide chain of 585 amino acid residues and contains 17 disulfide bonds. Plasma concentration of albumin ranges from 35 to 50 g/l in healthy subjects[47] One of the most important properties of

Alb is that it has unbounded cysteine in the 34 position, hence Alb constitutes most of plasma protein thiols[47,48]. It was found that no change of colour was observed when Alb was added to 2,6-dimethylbenzoquinone. This is a very positive sign as the technique can form the basis for differentiation between low molecular mass thiols and protein thiols in plasma and serum.

It would appear that the reaction between cysteine and 2,6-dimethylbenzoquinone does not stop after the simple 1,4-Michael addition. Considering the fact that the pink colour occurs only after forty minutes of incubation, it is possible to suggest that a follow up reaction takes place. In order to investigate the reaction route and find the product responsible for the pink colour – a series of LC-MS experiments were performed. The assumption was that reaction can go not only through Michael addition but that a Schiff base can also be formed. Another possibility is a second nucleophilic addition through the nitrogen of the cysteine pendant. In an attempt to better understand the mechanism of the reaction and interpret the results obtained by LC-MS experiments, the list of most probable products of the reaction has been created and is given in **Table 4.3.**

Table 4.3 Possible products of the reaction between 2,6-dimethylbenzoquinone and cysteine

Number	Product
1	 Molecular Weight =257.31
2	 Molecular Weight =237.28
3	 Molecular Weight =237.28
4	 Molecular Weight = 376.45

5	 Molecular Weight =376.43

It was an open question, however, if polymerisation should be taken into account, so the above table should not become endless. Unfortunately, further LC-MS experiments confirmed that polymerisation does take place. From experiment to experiment masses of the products varied from 257 to 586 which suggests the polymerization can take place under the conditions of the reaction. However, the major handicap in analysing products of the reaction was precipitation of the product from the reaction medium after approximately two hours of incubation depending on concentration of the solution. A typical example of LC-MS chromatogram and mass spectrum of the same sample are shown in **Figure 4.14**. As such, it is yet premature to entirely eliminate the possibility of polymerisation and the formation of products different from the ones given above. The experimental work in this direction is under way and this also includes using other techniques such as NMR.

The reaction between 2,6-dimethylbenzoquine and cysteine should be considered as a promising method for spectroscopic cysteine detection. On the one hand, this reaction is slow and often results in formation of multiple products which are difficult to characterize. These products tend to polymerize and degrade with time and therefore the reaction is not the best performer as far as a rapid analytical detection method is

concerned. On the other hand, the reaction does appear to offer unique advantages in terms of speciation. The pink colour of the products from the reaction medium gives an unmistakable indication of the species involved and provides a rare simplicity of a yes or no answer. In this respect the advantages of the method should not be underestimated. While the exact mechanism of this interaction is yet to be completely elucidated, the work has thus far succeeded in moving a few steps forward to a better understanding of the reaction profile.

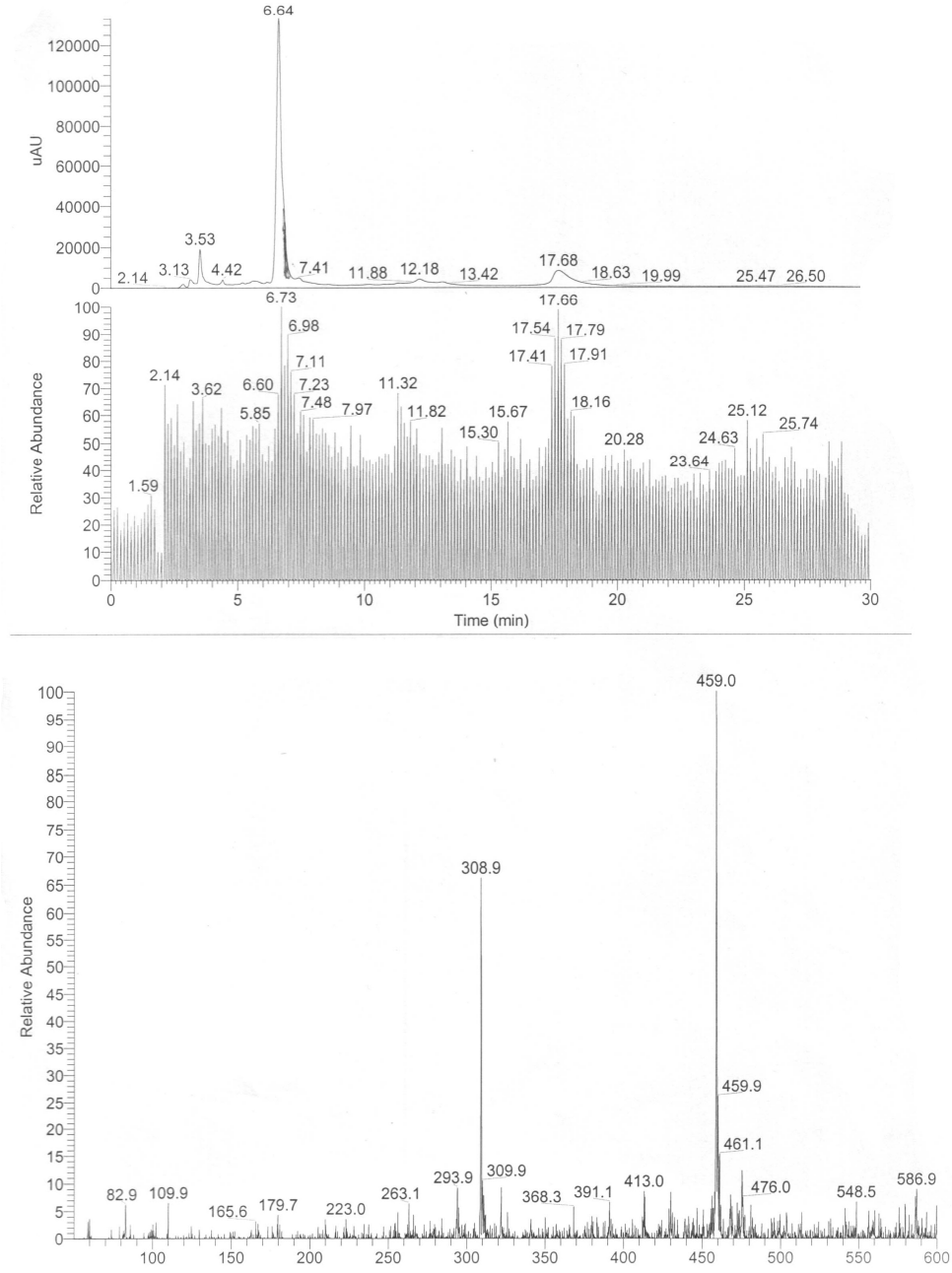


Figure 4.14 Typical results of LC-MS run. Mobile phase Acetonitrile-water 1:1 v/v

4.6 POCT Cysteine Assay Development

The use of 2,6-Dimethylbenzoquinone as a means of quantifying cysteine through the spectroscopic approach and its applicability as a potential foundation from which a point of care test for cysteine could be developed was briefly assessed. The basic rationale was to prepare a series of detection tablets – containing buffer and quinone indicator components. These would be mixed as the powder – compressed through a die press assembly to yield the tablet. It was anticipated that the addition of the tablet to a solution containing cysteine would result in the formation of a pink colour which may be suitable for a semi quantitative assessment of cysteine concentration. The potential application related to assessing cystine for urinary stone detection. While cystine will not react with the quinone it could be hydrolysed prior to the addition of the tablet or the reagents could be incorporated within the tablet. The first stage in the development process however was to evaluate the viability of the quinone as indicator.

The die assembly is shown in **Figure 4.15** and consists of a series of stainless steel plates that interlock to yield the holes into which the powder can be introduced, pressed and released – much in the same way as a conventional KBr disc die.

Figure 4.15. Quinone-Tablet Die Assembly

The tablets (shown in **Figure 4.15**) were typically placed into an eppendorf tube into which was introduced a solution of known cysteine concentration. The tablet dissolved – upon which the buffer components and indicator were released into the solution with the latter reacting with the cysteine. The colour was then assessed after a period of 30 minutes. The typical results from a test solution containing no cysteine and one with cysteine are shown in **Figure 4.16**. The solution containing the quinone (RHS, **Figure 4.16**) developed into a red colour – slightly darker than that previously observed in the initial spectroscopic investigations.

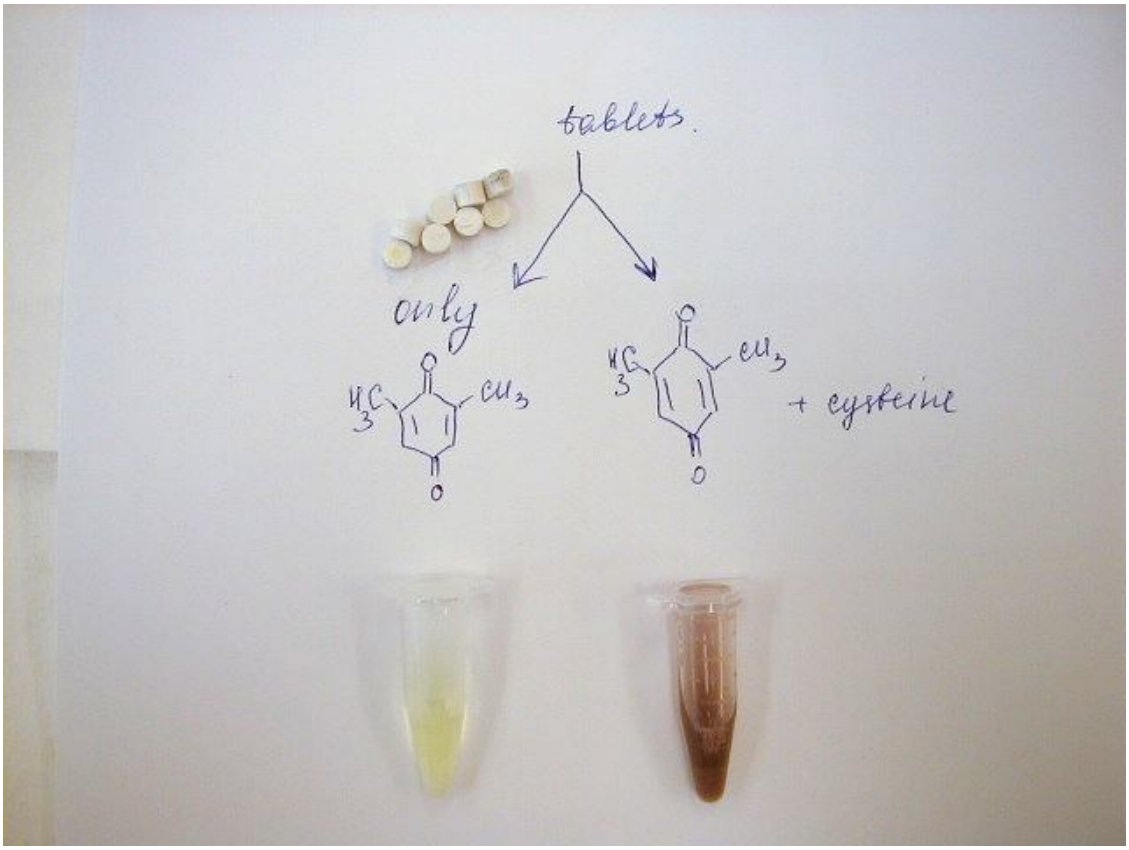


Figure 4.16 Response of the quinone tablet to a blank buffer solution (Left) and the cysteine test solution (Right).

A calibration series was then constructed to evaluate the applicability of the test beyond a simple yes/no system to one which could give at least a semi-quantitative measure of cysteine. The results are shown in **Figure 4.17** for cysteine solution covering the range 0-0.4mM cysteine.

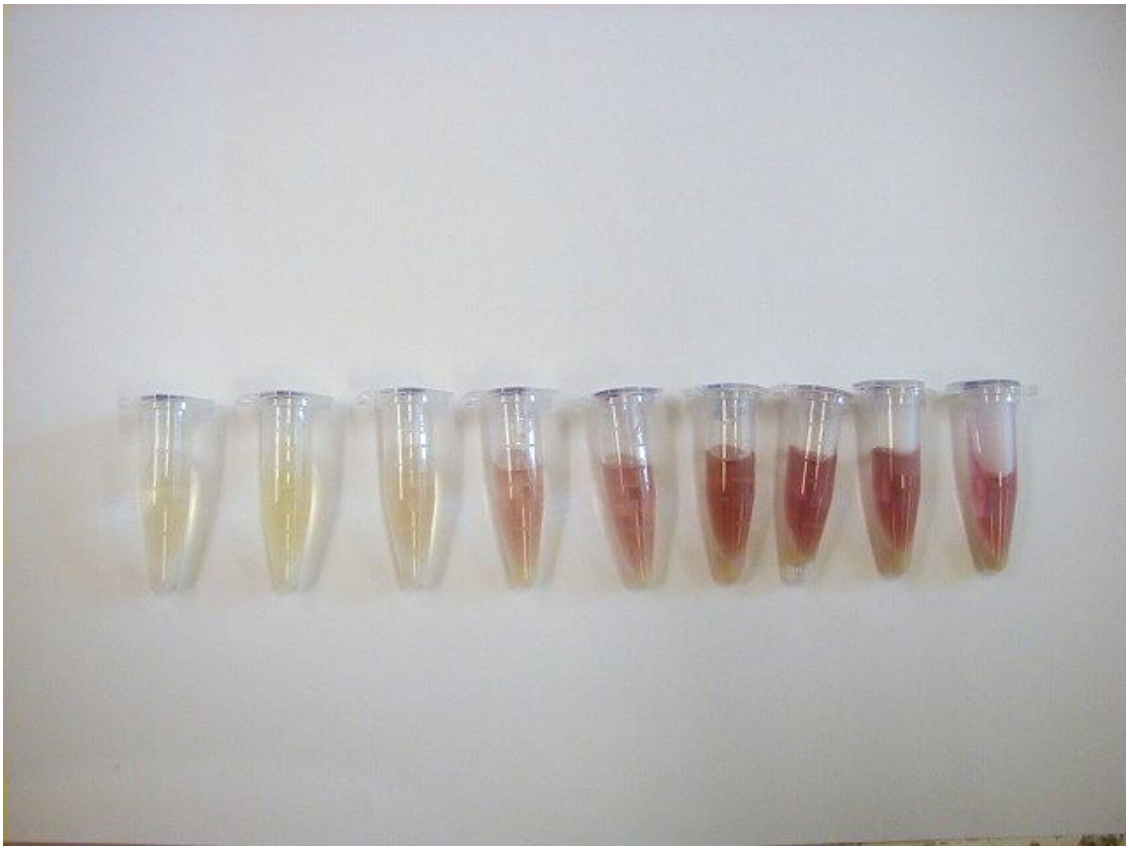


Figure 4.17 Quinone Tablet calibration series.

It is clear that the quinone-tablet can provide a semi-quantitative measure of cysteine and is equivalent to the dipstick test that are currently available for other analytes. There are obvious limitations – sensitivity and the ambiguities of visual colour differentiation (identified in **Chapter 1**) remain and it is for these reasons that the further development of the test was not continued. The core aim of the thesis was to develop electrochemical methods that can overcome the colour issues but the system does represent one of the first attempts to provide a simple and easily performed POCT thiol test.

4.7 Conclusion

The ability to utilise a simple potentiometric system for monitoring the concentration of reduced thiols has been demonstrated and an assessment of its selectivity presented. The protocol required to conduct the experiments is procedurally simple and can be conducted using inexpensive equipment that contrast the more elaborate requirements of the chromatographic procedures. The insensitivity of the approach to sample colour also provides an advantage over conventional spectroscopic procedures (Ellman's reagent) with detection limits that are comparable. The data can be analysed using traditional potentiometric approaches and thus amenable to operatives with a basic chemical background and contrasts the voltammetric procedures previously applied to the quinone systems. The exploitation and viability of the methodology within untreated physiological fluids remains speculative but it could be anticipated that the low instrumentational requirements of the system will encourage the adoption and stimulate the further development of the assay. The ability to transfer the technology to a disposable strip format opens up the analytical options and also highlights the possibly of using the base, screen printed, substrates for potentiometric measurements within more generic applications.

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Chapter 5

Development of disposable potentiometric sensors for the testing of plasma thiol concentrations

Abstract

The reaction of naphthoquinone with physiological thiols has been investigated as the basis of a NPT strategy for the measurement of the latter. Screen printed carbon electrode assemblies provide an inexpensive and inherently disposable platform for the detection methodology. The key analytical parameters underpinning the selective and sensitive (0.4uM-1mM) determination of reduced thiol (RSH) have been assessed and the clinical efficacy of the approach demonstrated through its application to the analysis of human plasma. The results have been corroborated using standard techniques and the routes through which the system can be adopted within mainstream biomedical environments are highlighted.

5.0 Introduction

Sulphydryl thiols are known to play a crucial role in the prevention of free radical induced oxidative damage, however there are occasions where the increased production of the latter can overwhelm physiological defences [1-4]. The hyperglycaemic condition brought about by diabetes is a particularly apt example where there is often a corresponding increase in the concentration of reactive oxygen species (ROS). The subsequent perturbation of the free radical: antioxidant ratio within the tissues can significantly raise the patient's susceptibility to secondary complications such as nephropathy, neuropathy and retinopathy[1,5-7]. Inflammation induced depletion of plasma thiols (glutathione and cysteine) is well established[3-5] yet the biomedical exploitation of such information has been the preserve of dedicated research groups. Widespread adoption of such approaches within routine clinical settings could obviously bring significant benefits to the management of the disease and aid the identification of those patients at risk of developing further complications. Given the ubiquity of the disease, there is a considerable need to develop near patient testing protocols that can facilitate the quick assessment of an individual's wellbeing by the attendant clinical staff.

At present, blood thiol analysis requires the referral of the sample to a specialised central laboratory and this will invariably be the only option available to the clinician. The time delays incurred will tend to negate the immediacy of the diagnostic advantage originally presented with the inevitable aerial degradation of the sample leading to ambiguous results. Electrochemical sensors are widely used in the routine, near patient testing (NPT) of glucose and it could be envisaged that similar technology could be used

to assess thiol concentrations and, indeed, provide a complementary analytical assessment. The extrapolation of the underlying glucose methodology to thiol moieties however, is hindered by the absence of a redox enzyme that can provide the selectivity and sensitivity required. Chemical sensing approaches have been investigated[8-10] but few possess the simplicity required for the mass manufacturing of disposable sensing assemblies – a key requirement in the pursuit of a viable NPT device.

One approach that may meet such demands is based on the selective reaction of thiols with a quinone indicator. While the chemistry underpinning the indicating reaction is established and has formed the basis of several voltammetric methods[10-11], the efficacy of the approach has yet to be demonstrated within biological fluids. This communication seeks to address this point but, in contrast to voltammetric investigations, the analytical signal is derived from a potentiometric perspective. The adoption of a potentiometric detection system could offer a considerable step forward in terms of the procedural and instrumental simplicity necessary for its adoption within NPT contexts. The underlying detection route is detailed in **Figure 5.1** and involves the selective nucleophilic addition of physiological thiols to a quinone indicator (**I**) leading to the production of the corresponding quinone-thiol conjugate (**II**).

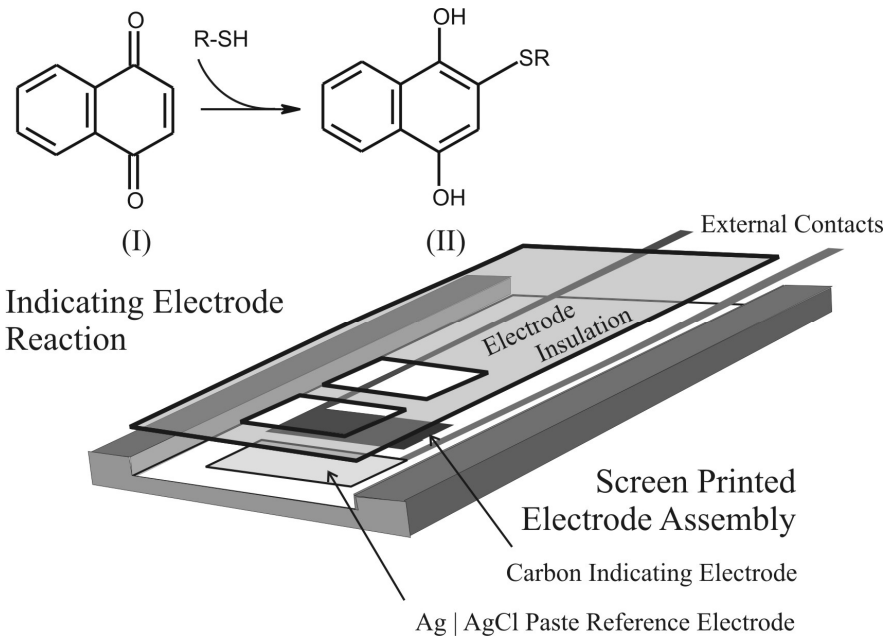

Figure 5.1. Schematic of the screen printed electrode assembly and the reaction methodology behind the detection of sulphydryl thiols

The alteration in the redox balance is then detected at a screen printed electrode assembly. The latter offers a cheap measurement option with the disposable nature of the technology obviating the concerns over reproducibility and contamination that are liable to arise in the analysis of clinical samples. The present investigation examines the selectivity of the detection mechanism and assesses the viability of the technology to the detection of total plasma thiol.

5.1 Experimental Details

All reagents were of the highest grade available and used without further purification. All solutions and subsequent dilutions were prepared daily using deionised water from an Elgastat (Elga, UK) water system and were refrigerated when not in use. Electrochemical measurements were conducted using a Sycopel AWE-10 computer controlled potentiostat. Preliminary investigations were conducted with an initial cell volume of 10 cm^3 with the measurements recorded at a temperature of $20^oC \pm 2^oC$. Disposable two electrode screen print assemblies were used throughout and the design is highlighted in **Figure 5.1**. The basic sensing strip (3 cm x 0.5 cm) comprised of a carbon working electrode ($1mm^2$) and a silver / silver chloride reference electrode deposited using conventional screen print methodologies. Unless specified otherwise, the solutions consisted of Britton-Robinson buffer (acetic, boric and phosphoric acids - each at a concentration of 0.04 M and adjusted to pH 7 through the addition of sodium hydroxide). Potassium chloride (0.1M) was added to all buffer solutions in order to define the reference potential. The standard notation for the electrochemical cell operating under zero current is:

$$(Ag, AgCl \mid Cl\,(0.1M) \parallel NQ\,(1\ mM) \mid C).$$

A clinical trial involving 4 non-diabetic control subjects and 4 well controlled (HbA1c<7.5%) type-2 diabetic subjects, 3 male and 1 female, ranging from 32-67 years of age was conducted. Samples of blood were obtained from the participants and

analysed within three hours of extraction. Blood was collected in heparinised gel permeation vaccutainers and then centrifuged at 3000 rpm for 10 minutes. The plasma was withdrawn and used immediately for both electrochemical and spectroscopic analysis. The latter was conducted using established Ellman's Assay protocols with the calibration and analysis data taken at 412 nm[12].

5.2 Results and Discussion

The steady state response of a screen printed carbon electrode assembly to 1mM naphthoquinone (NQ) in pH 7 buffer is shown in **Figure 5.2A**. Upon the addition of an aliquot of glutathione (25 μM), the electrode potential falls dramatically and can be attributed to the nucleophilic addition of the thiol to the quinone indicator as outlined in **Figure 5.1**. The nucleophilic reaction creates a disparity in the relative concentrations of oxidised / reduced species such that the potential at the indicating carbon electrode is forced to change to accommodate the corresponding change in solution redox state.

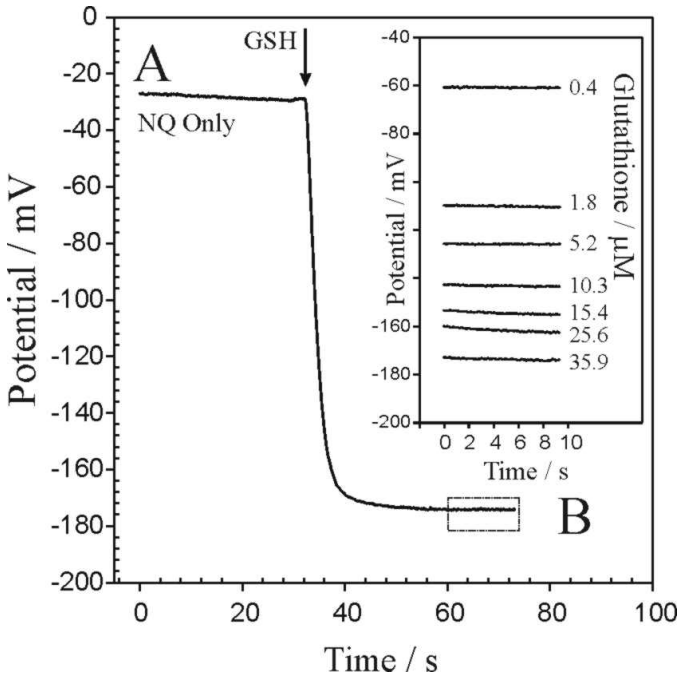

Figure 5.2. Response of a SPE assembly obtained with a solution containing 1 mM NQ (pH 7) before (A) and after the addition of 25 μM glutathione. Inset: Steady responses of the SPE / NQ detection system to increasing concentrations of glutathione

Increasing the concentration of thiol added increases the amount of reduced conjugate produced and hence the magnitude of the change in electrode potential is increased. The electrode potential stabilises at a point dictated by the relative concentrations of oxidised and reduced forms of the indicating quinone and by the resulting quinone-thiol conjugates (**Figure 5.2B**). The analytical signal that forms the basis of our technique is taken from a 10 second potential-time window typically recorded two minutes after the addition of the thiol. This is shown in the inset diagram in **Figure 5.2** where the steady state responses to various concentrations of glutathione are displayed. The recorded potential is averaged and used to compile the calibration data from which an unknown sample could then be assessed. The relationship is logarithmic ($E = -20.99 \log [RSH] - 95.85$, $R^2 = 0.97$, $N = 7$) and provides a detection range (0.4 μM - 0.1 mM) that is more than adequate for quantifying extra-cellular and total thiol concentrations[1-7].

The mechanism underpinning the potentiometric response of a simple benzoquinone-thiol system has been previously determined [11-13] and a similar approach was envisaged for the NQ system advocated here. Spectroscopic analysis was used to probe the reaction stoichiometry (**Figure 5.3**) and corroborate the addition mechanism. Naphthoquinone was essentially titrated with glutathione with the oxidised form of the NQ-SR conjugate producing an absorption band at 420nm - analogous to that observed with benzoquinone [13]. While the reaction scheme detailed in **Figure 5.1** outlines the production of the reduced form of the quinone-thiol conjugate, the presence of oxygen within the sample and buffer test solution will oxidise the former to NQ-SR

and hence the coloured profile. As excess thiol is added, the intensity of the absorption peak at 420nm decreases as the second molecule attaches to NQ-SR to form NQH-$(SR)_2$.

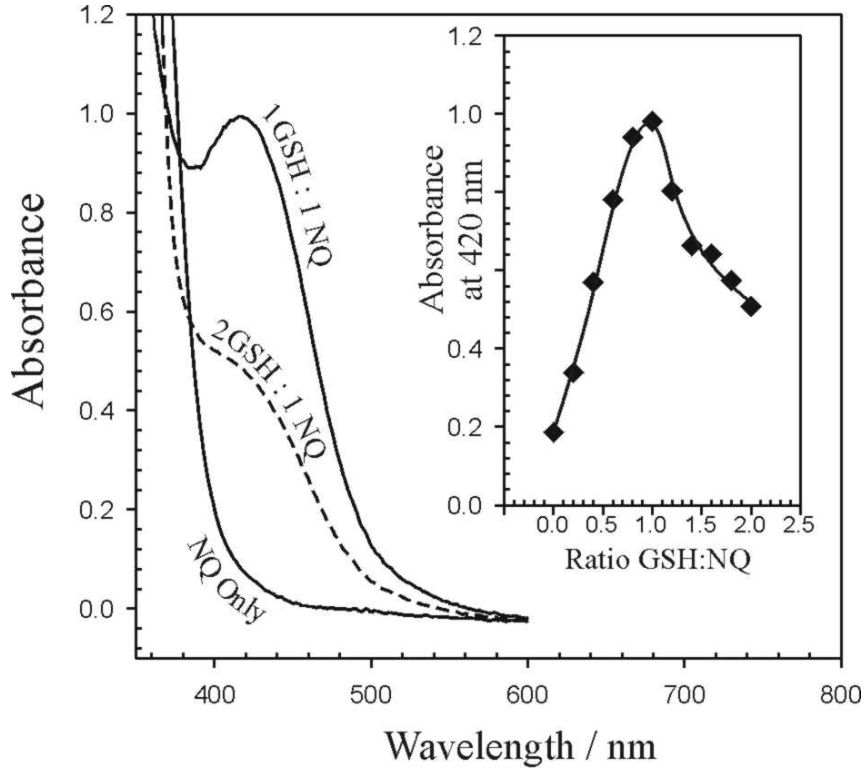

Figure 5.3. Spectroscopic analysis of the titration of NQ with glutathione in pH 7 buffer

It must be reiterated that the quinone system has the potential to react with any RSH moiety and, indeed, similar responses were observed with cysteine (not shown). The indicator is therefore limited to the detection of total reduced thiol and in many ways serves as an electrochemical equivalent of Ellman's Reagent[12]. The selectivity of the approach was however assessed through investigating the interaction of the indicating quinone with key physiological components. The principal concern was that other

functionalities may react directly with the indicator through nucleophilic addition, effectively mimicking the response obtained from the thiol moieties and thereby leading to the erroneous amplification of the analytical signal. The potential nucleophilicity of amino functionalities within the amino acids and associated peptides combined with the sheer number liable to be present within biofluids will clearly be the chief threat to the system. Lysine was chosen as a model interferent, given its possession of two alkyl amino groups, and the electrode response to increasing additions of the aminoacid (25 μM aliquots) are shown in **Figure 5.4A**.

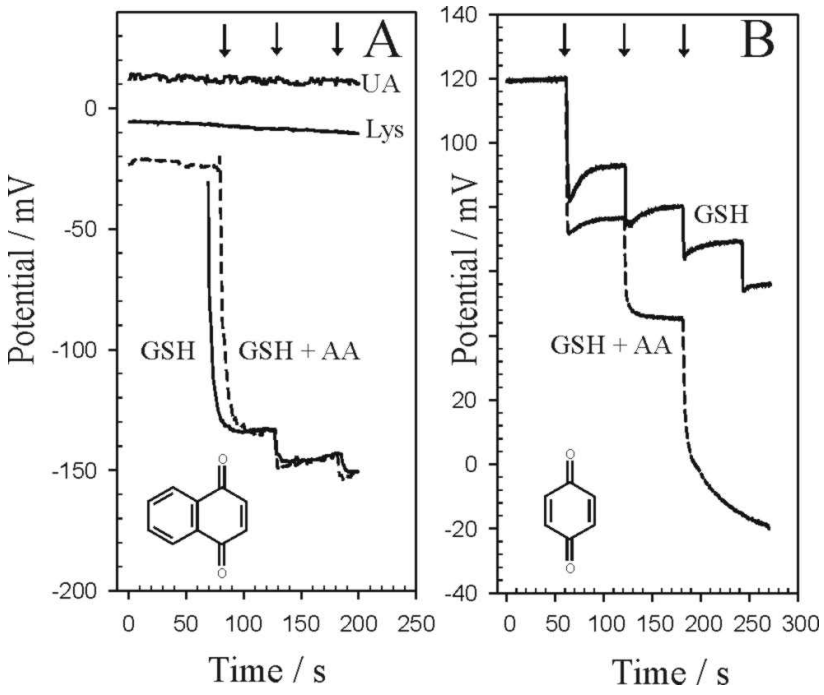

Figure 5.4. Response of SPE/NQ (**A**) and SPE/Benzoquinone (B) systems to the addition of aliquots (25 μM) of glutathione (GSH), ascorbate (AA), urate (UA) and lysine (Lys) in pH 7 buffer

No change in the electrode potential was observed and it can be concluded that through conducting the measurement at pH 7, the nucleophilic character of the alkyl amino groups (pKa ~10) will be substantially diminished. The operation of the system within tissue culture medium was also assessed as such media provide a complex matrix containing most of the monomolecular species that are liable to be encountered within biological fluids. The successful operation of the indicating system provided some evidence of the robustness of the approach with calibration data mirroring those detailed in **Figure 5.2** obtained. Ascorbate and urate were however omitted from such tests. As they represent the prime interferences that traditionally hamper electroanalytical investigations, an independent appraisal of each was required. The electrode response to a solution containing 1mM NQ before and after the addition of urate (25 μM aliquots) is shown in **Figure 5.4A**. Similar responses were observed when ascorbate was added and suggested little or no interference. This was confirmed where the electrode response to 1mM NQ after the addition of a solution containing 25 μM glutathione and 50 μM ascorbate (**Figure 5.4A**, dotted line) was found to match the profile of the control glutathione (**Figure 5.4A**, solid line).

The potentiometric responses obtained with other quinone derivatives (benzoquinone, 2,6-dimethyl and 2,6-dimethoxy derivatives – discussed in Chapter 4) was also assessed but, while they were found to respond to thiols, there is a considerable interference from ascorbate. This is highlighted in **Figure 5.4B** where the previous experiments conducted with NQ were repeated with 200 μM benzoquinone. It can be seen that the response to the mixture of 25 μM glutathione / 50 μM ascorbate (**Figure 4B**, dotted line) is significantly greater that that observed with 25 μM glutathione alone

(**Figure 4B**, solid line) and hence would present a significant error. The properties of the NQ/NQH_2 couple are significantly more reducing than ascorbate and, as such, the latter poses little interference through simple redox transitions.

The analytical viability of the approach to the analysis of real samples was assessed through the determination of total plasma thiol (PSH). Thus far, the majority of experiments have utilised glutathione as a model analyte through which the response of the devices could be characterised and assessed. The underlying assay will however respond to all available RSH groups – whether free (cysteine, homocysteine etc) or bound within a protein (ie albumin). The indicator is solution based and the response recorded at the electrode reflects the change in redox status occurring throughout the sample and is not localised to the sensor surface. The actual analytical signal within a real sample will therefore reflect total reduced plasma thiol (PSH) concentration rather than one particular component.

The basis of the measurement involves the addition of an aliquot of plasma (50 μL) to a buffered solution (10 mL, pH 7) containing the NQ indicator. The subsequent change in electrode potential would then be compared against a calibration set (prepared from pH 7 buffered solution) and the results validated against an established spectroscopic protocol. The electrode response obtained at the disposable SPE assembly is shown in **Figure 5.5** and was found to exhibit a potential-time profile analogous to those shown in **Figure 5.2**.

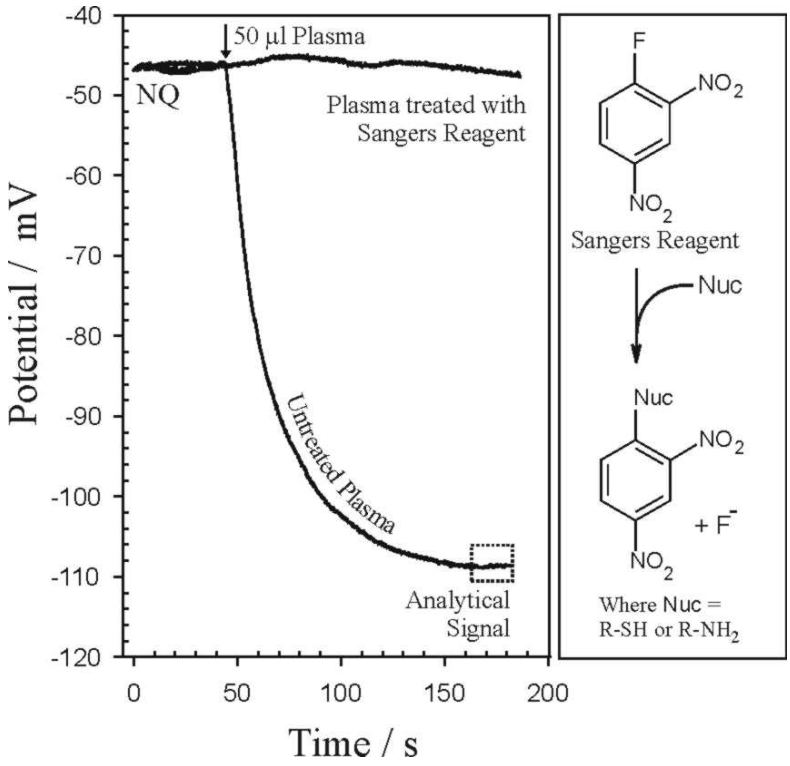

Figure 5.5. Electrode response of SPE/NQ system towards human plasma before and after treatment of the latter with Sanger's Reagent.

The results obtained from a clinical study involving 4 control and 4 diabetic patients are detailed in **Table 5.1.** These are based on three replicate measurements with the relative standard deviations for the potentiometric and spectroscopic protocols being below 2% and 7% respectively. The reproducibility of the technique is also noteworthy given the composite nature of the SPE electrodes. The concentration of PSH was found to be in agreement with previous studies examining the influence of ROS on the concentration of thiol antioxidants[14] and serves to corroborate the viability of the approach.

Non Diabetic Controls	1	2	3	4
		PSH / mM		
Potentiometric NQ/SPE	0.57	0.60	0.64	0.54
Ellmans Reagent UV-Vis	0.52	0.65	0.58	0.56
Diabetic	1	2	3	4
		PSH / mM		
Potentiometric NQ/SPE	0.55	0.47	0.54	0.56
Ellmans Reagent UV-Vis	0.57	0.50	0.64	0.46

Table 5.1.

Confirmation that the process does indeed go through the nucleophilic addition of the thiol was obtained through repeating the potentiometric assay with plasma that had been pre-treated with Sanger's reagent (2,4-dinitrofluorobenzene). This will effectively scavenge all nucleophilic species present within the sample and thereby remove the source of our analytical signal as shown in **Figure 5.5.** These results can also be used as further evidence of the insensitivity of the NQ indicator to other anti-oxidants and its selectivity for thiol functionality. The former would have remained largely unaffected by the addition of the nucleophile scavenger.

The main issue with the previous approach is that the NQ is unable to provide speciation information regarding the various contributions that the low and high molecular weight thiol components make to the PSH concentration. The latter is widely

recognized as having clinical merit[1,4,7] but there is considerable debate as to whether mono, macro or total thiol respond in equal measure to the onset of physiological stress [7,15-17]. At present, the protocol outline previoulsy will mean that the major contributor will undoubtedly be albumin. The next stage was to determine if the basic procedure could be adapted to differentiate between the mono and macromolecular components. The ability to differentiate between the different components would normally require a more elaborate liquid chromatographic procedure and hence require referral of the sample to specialist laboratories. This section presents a more integrated strategy for plasma thiol analysis that provides the clinician with ready access to a procedure that builds upon the SPE work and which could distinguish between these groups. This could thereby offer the possibility of facilitating a better understanding of the PSH/RSH metabolomics.

The basic strategy is outlined in **Figure 5.6** and is highlighted by the passage of cysteine (I) through a centrifugal filter packed with a 2,2'-dithio(bis)benzothiazole (II) indicator as first described by Chahine et al[18]. The latter is insoluble within aqueous solution and serves as a densely packed particulate filter. In principle, the disulphide should react with free sulphydryl thiols through a mechanism analogous to that observed with Ellman's Reagent (ER). This should result in the formation of the mixed disulphide (III) and the release of the mercaptobenzothiazole anion (IV).

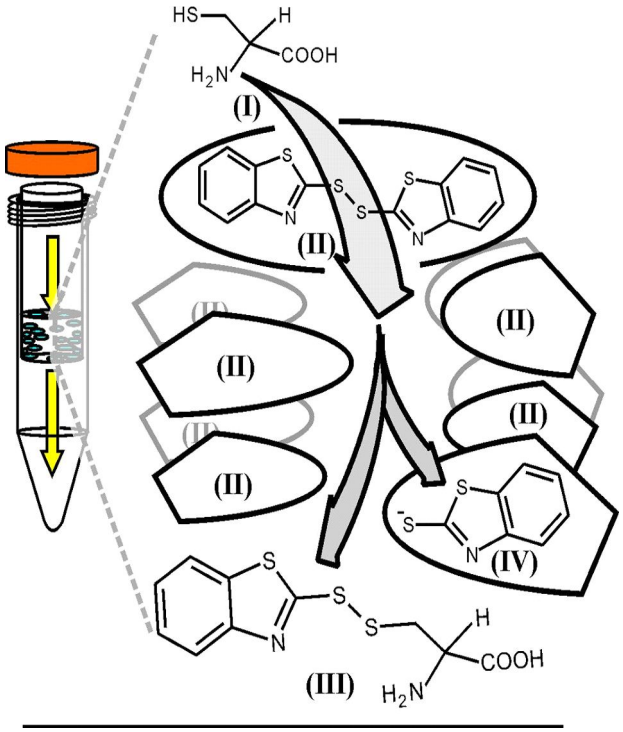

Fig 5.6. Centrifugal Filter Device (CFD)

An assessment of the percentage conversion of the free thiol into the mercaptobenzothiazole (MBT) conjugate was conducted and compared with the results previously reported (employing a before and after spectroscopic methodology). The recovery of the thiol constituents was found to be 98 +/- 2 % (based on 20 mM cysteine) which is consistent with the previous report[18]. The uv spectroscopic profiles for additions of CSH (40 μM), GSH (40 μM) and bovine albumin (0.82 mg/L) are shown in **Figure 5.7**. Significantly, the passage of albumin did not lead to any change in the absorption profile from that of the corresponding unfiltered control. The molar absorptivities (CSH, λmax = 312nm, $\varepsilon = 275$ mol^{-1} L cm^{-1} and GSH, $\varepsilon = 365$ mol^{-1} L cm^{-1}) are significantly less than that

of ER (λmax = 412nm, ε = 14150 mol^{-1} L cm^{-1}). and has been attributed to the lack of the nitrothiolate chromophore.

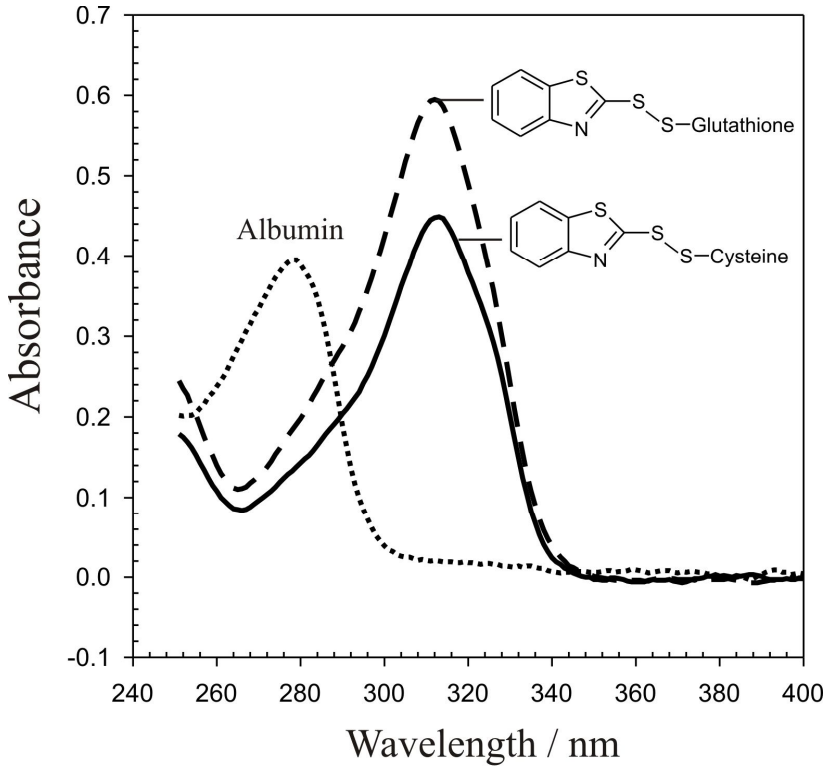

Figure 5.7 UV spectra Spectroscopic profiles of CFD processed MBT conjugates compared with that of an albumin sample.

The main advantage lies in the selective reaction of the reduced thiol group with the filter material. The 2,2'-dithio(bis)benzothiazole packing material is essentially insoluble and disulphide exchange is dependent upon the target reacting directly with the solid packing as it is forced through the filter by centrifugal action. It has been assumed that the buried thiol functionality within albumin – the predominant source of reduced thiol within plasma - would prevent reaction. While such groups are accessible to freely diffusible

derivatisation agents such as ER or naphthoquinone, it could be anticipated that the protein backbone would serve as a steric barrier to reaction in the present case. It was anticipated that the assay would therefore provide a basis for speciation studies that are generally unattainable without having to resort to conventional lab based chromatography techniques.

The absence of the MBT spectroscopic profile from the CFD processed albumin, **Figure 5.7**, indicates that the protein did not react with the filter material. A series of albumin solutions (0.02 to 0.82 mg/L) were passed through the modified filter at a slow scan speed (3000 rpm) and the filtrates then analysed for thiol through performing standard ER assays. The recovery of ER reactive albumin was was found to be 96 +/- 5 % (RSD = 3 %, N = 3 based on 0.82 mg/L AlbSH) . This was consistent with previous reports on the use of the mercaptobenzothiazole packing material[18] and again confirms that the buried thiol present within the albumin does not react to any appreciable extent.

Previous investigations have utilised the spectroscopic signatures of the filtrate but in this case, our interest lay with attempting to adapt and transfer the basic strategy to incorporate a SPE within the core of the centrifugal device in order to provide an integrated thiol system. As before, the addition of the reduced thiol functionality to the complementary thiazole component should serve to aid solubilisation and, as such, is preferentially released into the filtrate. A squarewave voltammogram detailing the response of a glassy carbon electrode towards the MBT-CSH conjugate (0.1mM CSH, pH7, post CFD) is detailed in **Figure 5.8**. A well defined peak is obtained (similar responses were obtained with GSH and HCSH – not shown). The main problem with the volatmmetric approach – as indicated in **Chapter 3** is that the peak potential lies within a region (+0.35V vs Ag|AgCl) that is susceptible to interference from other plasma constituents (cf ascrobate

and urate voltammograms). It would be difficult to ascribe the peak to the MBT-CSH within a complex sample.

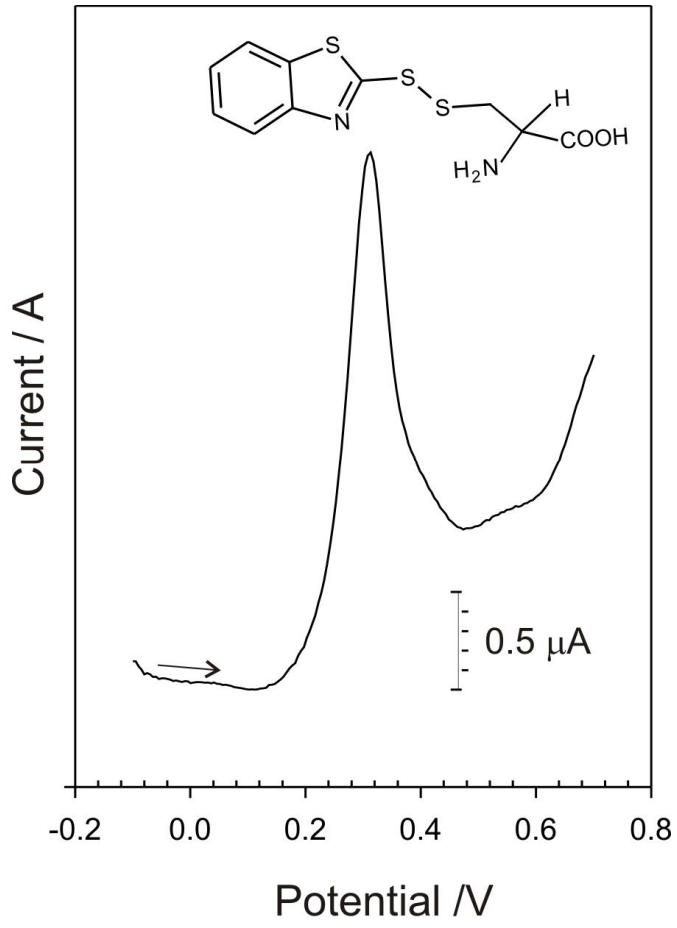

Figure 5.8 Squarewave voltammograms detailing the response of a glassy carbon electrode towards MBT-CSH (0.1mM, pH 7, post CFD processed)

The potentiometric approach is clearly a much more viable approach – simplicity, selectivity and sensitivity all factoring in as advantages. The problem however is that while cysteine, glutathione and homocysteine have been shown to react with the thiazole to form the corresponding conjugate (III) (indicated in **Figure 5.6**) the thiol group is bound through the disulphde link – neither is available for reaction with the naphthoquinone and hence no potentiometric response would be observed. The albumin however does not react to any appreciable extent with the filter material and as such the buried sulphydryl thiol will still be reactive towards the solution based naphthoquinone and will form the quinone-thiol conjugate. The latter could however then be detected through the same potentiometric procedure as previoulsy outlined – the main difference being that it should only be the macromolecular species which are being quantified.

Given that the centrifugation step requires two weight balanced tubes then it should be possible to construct a simple protocol that allows, in principle, a quick and simple speciation study. In addition, it allows the development of a sensitive potentiometric method of directly quantifying albumin. This could be done through having one centrifugal filter set to detect albumin – as described above – and a complementary filter device without benzothiazole packing set to determine total thiol. Subtraction of the former from the latter should provide the contribution of low molecular weight thiols – such as cysteine and glutathione. The basic scheme is shown in **Figure 5.9.**

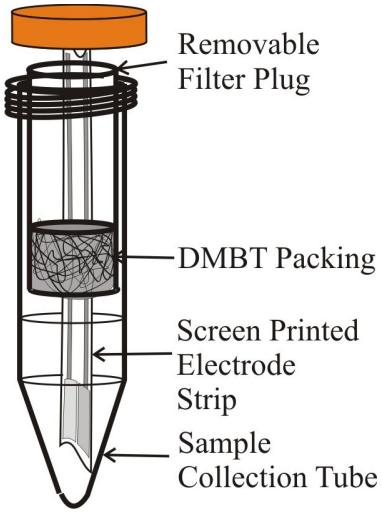

Figure 5.9 Schematic of the CFD Filter – SPE Assmbley.

The clinical efficacy of the system for discriminating between albumin, low molecular weight thiols and other physiological components was then assessed through examining the plasma albumin profiles of four volunteers (3M, 1F). Plasma samples (300 μL) were passed directly through the filter without any pre-treatment. The samples were processed in duplicate (two samples being required to balance the centrifuge). One CFD tube was analysed using direct uv spectroscopic analysis in accordance with previous studies and as indicated previously (**Figure 5.7**). The absence of any appreciable absorbance within the plasma at 312 nm would therefore allow any increase at this wavelength, subsequent to CFD processing, to be attributed to the MBT conjugates. The magnitude of the latter providing an immediate estimation of low molecular weight thiol concentration. However, there is an inherent limitation to this approach in that the spectroscopic properties of the conjugate will differ depending on the nature of the amino

acid substituent. This is evident from the molar absorptivity data presented previously for the GSH and CSH species. The initial results are detailed in **Table 5.2**. Ellman analysis of the plasma before filtration yields the total plasma thiol concentration (PSH) with the subsequent filtrate providing albuminSH - given that the low molecular weight species will have reacted to form the ER inactive MBT conjugates – and hence allows the two groupings to be distinguished as previoulsy reported by Chahine[18].

The amount of albumin can be obtained with more certainty through reaction with ER after the passage of plasma through the modified filter device. This is increasingly being viewed as an important diagnostic in its own right[15-17] as the standard adsorptive dye methodologies such as bromocresol green (BCG) assays that are currently used can be subject to interference[19-22]. The approach advocated here could allow a more reliable alternative to the conventional analysis – especially if coupled to the electrochemical detection. The clinical viability of the potentiometric assay was assessed and the results obtained from the centrifugal system compared with both the ER assay and the more conventional BCG test and are detailed in **Table 5.2**.

Controls	M1	M2	M3	F2
Electrochem after CFD /gL^{-1}	35	43	39	46
ER Assay after CFD / gL^{-1}	33	45	45	42
BCG / gL^{-1}	33	42	42	39

Table 5.2. Plasma albumin concentrations assayed using the solid state filter (CFD) compared with the bromocresol green (BCG) dye adsorption method.

5.3 Conclusions

The ability to utilise a simple potentiometric system for the monitoring of reduced thiol species has been demonstrated. The response is sufficiently sensitive and selective to deal with the complex matrix provided by human plasma and compares well with the traditional spectroscopic protocol. While the numbers involved within the clinical trial are not sufficiently large such that the non diabetic and diabetic group responses can be compared formally, the results clearly demonstrate the validity of the approach for the analysis of plasma from both groups. The adoption of the electrochemical approach is not restricted to those with access to screen printed electrodes as the basis of the methodology has been previously demonstrated using conventional, bulk carbon electrodes.

The key advantage posed by the SPE assembly advocated here however, is that it demonstrates how the technology could ultimately be adapted for use within NPT / point of care contexts. The system is inherently less expensive and easier to use than the spectroscopic procedures and utilises a portable instrumentation platform that is simpler than that employed within commercial glucose meters. While there are obvious obstacles to the implementation of the current system within whole blood – electrode fouling being the prime candidate, the basis of the approach has been shown to be robust. Further refinement of the system is needed before it is truly viable but it could be envisaged that polymer membrane technology could address this. The detection strategy and

instrumentation needed to perform the measurements is readily accessible thus facilitating wider uptake and further development of the technology.

The CFD system is clearly a verastile assay that can be used to effect the speedy, accessible, and accurate speciation of physiologically significant thiol groupings. It can thus aid the differentiation between mono and macromolecular species and provides distinct routes through which both can be easily quantified. The solid state differentiation capabilities presented by the disulphide could also be exploited in a variety of other formats and is not limited solely to the centrifugal separation exploited here. It could serve as an selective coating in sensors or a pre or post column derivatising step in LC applications. It is clear however that the protocol presented thus far provides a novel, robust and generic foundation from which this further work can be pursued.

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Chapter 6

Design of a Carbon Composite Sensing Assembly for the Selective Potentiometric Monitoring of Sulphite

Abstract

The thermal encapsulation of carbon loaded polyethylene and polycarbonate films within a polyester laminate has been evaluated as sensing substrates for the detection of sulphite. The approach is based on the redox reaction of sulphite with a quinone indicator with the resulting change in potential recorded at the carbon-polymer composite electrodes. This represents a new approach to sulphite sensing that has been found to be free from most matrix interferences and provides a detection sensitivity (40uM) that is well below the legislative limit for sulphite. The electrochemical and mechanical properties of the sensing structures are assessed and the analytical viability of the approach critically evaluated through examining the response to sulphite within a commercial wine sample. The fabrication methodology adopted has been found to provide a highly versatile option for the construction of polymer film electrodes.

6.0 Introduction

In previous chapters, conventional screen printed electrodes were assessed as the base substrate. In the present section, the use of large area carbon composite films are investigated as a potential alternative to the fabrication of disposable electrode sensing assemblies. More-over the detection of sulphite is the predominant target analyte rather than the sulphydryl thiols. The conductive properties of carbon based polymers have found numerous applications in recent years – particularly as a shielding material for the packaging and handling of electronic components susceptible to damage from electrostatic discharge [1-3]. The conductive nature of such composites could, however, form the basis of a versatile and potentially inexpensive substrate for electroanalytical applications.

While a large number of studies have focused on the effect of polymer formulation and carbon dispersibility on the electrical properties of the resulting materials[3-7], few studies have examined the interfacial electrochemical properties. The ability to modify the properties of carbon loaded polymers through varying the composition of the processing blend could be expected to similarly alter the chemical and physical characteristics of the proposed sensing platform[8-11]. When combined with the processing and moulding flexibility offered by such materials, it could therefore be envisaged that numerous advantages over traditionally rigid carbon based electrodes – even those based on screen printed electrodes would arise. This could effectively establish a new class of sensing material capable of being specifically tailored to

particular applications[8, 12, 13]. The present chapter has therefore sought to assess the potential applicability of such material to potentiometric sensing applications and to critically evaluate the performance of the resulting devices for use in the analysis of authentic samples.

The determination of sulphite was selected as a key application for which our sensing system would be developed and was chosen on the basis that it is a key sulphur metabolite (in keeping with Chapters 4 and 5) and that there are, as yet, no commercial sensors available for the measurement of this analyte. In addition, there has been an increasing interest in the application of this anion within the agri-food communities but there has also been increasing medical interest in the potential clinical effects that it can induce. Before discussing the practicalities of the sensor design and development, a summary of the chemistry of sulphite is presented and a critical comparison of the various detection strategies that have already been pursued is considered.

6.1 Sulphite Role and Application

Sulphite (SO_3^{2-}) within both the food and medical sectors has been of considerable interest in recent years as concerns over its influence in a number of medical contexts have increased [14,15]. While it is widely used as a preservative (E220-228) to prevent bacterial growth [16-21], concern over its widespread incorporation within commercial products (mainly dried fruit and wine) has led to legislation being imposed in many countries. While not restricting its use, these have tended to require more elaborate labeling on the packaging where concentrations exceed 10 ppm. As such, there has been renewed interest in the development of new techniques that can aid the monitoring of sulphite levels in food and drink. The main driving force behind such studies has been health and safety and legal / consumer regulation but there is also a drive to develop technologies that would allow small scale food producers to ensure proper compliance with the imposed legislation.

6.2 Preservative Action of Sulphite

The main food science interest in sulphite lies in its reducing properties. These are well established and play an important part, along with ascorbate, in the anti-oxidant defence that minimises the spoiling of food products that would occur if the products were left exposed to air [16-21]. Sulphites, in their various formulations, can be found in a diverse number of products that include: processed meats; wines, fruit juices; jams and jellies; dried, tinned and pickled fruits; shell fish and processed food products where the

product lifetime of fats needs to be prolonged. The concentration of sulphite can vary considerably from one product to another and will be dependent on the nature of the product, economic considerations and the subsequent processing procedures [22-26]. The role of the preservative in most pre-packaged fruit and vegetable produce (particularly those that have been cut or sliced) and shellfish is largely to increase shelf life through preventing the browning reactions that lead to the discolouration of the produce and which can have a negative impact on consumer perceptions[16-21, 26].

The nucleophilic capabilities of the sulphite anion also play a role in maintaining food quality through the inhibition of non enzyme, Maillard type browning reactions [16-21]. The condensation of amine functional groups (from free amino acids or protein) with the aldehyde of reducing sugars leads to the corresponding N-substituted glycosylamines. These intermediates can then undergo a variety of rearrangements and degradations that ultimately result in the nitrogenous polymers that provide the characteristic brown colour[16-21]. Sulphite additives add to carbonyl functionalities (**Figure 6.1**), effectively removing the sites at which amines can attack and thereby inhibits the non enzymatic browning. PPO is the chief enzymatic protagonist that contributes to browning and spoilage [7,8] and the enzyme action is summarised briefly in **Figure 6.2**. The oxygen mediated sulphite has a dual action in that it acts to directly inhibit the enzyme Polyphenol oxidase (PPO). This enzyme works by converting the phenolic derivatives to the highly reactive o-quinone intermediates (**Figure 6.2**) which subsequently promotes a cascade of reactions leading to the formation of undesirable coloured products. The sulphite also chemically reduces the o-quinone to the more stable 1,2-dihydroxybenzene

(III), thereby terminating the browning reaction at an early stage. The ability of sulphite to prevent the further oxidation of polyphenolics whether through enzymatic or chemical means is thought to be an especially important factor in wine production. However its high prevelance within such products has coincided with a rise in health concerns [14,15].

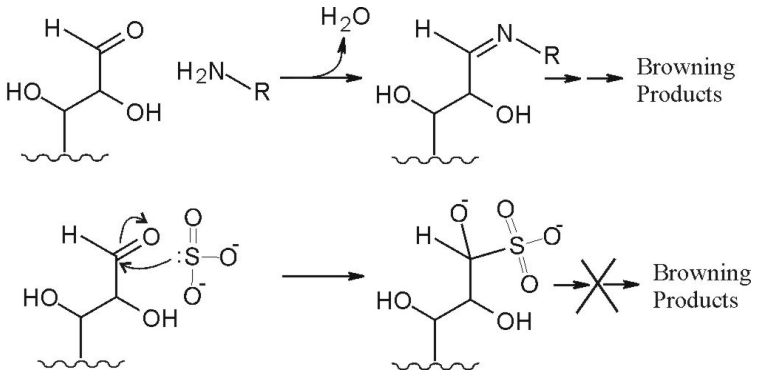

Figure 6.1. Reaction schemes highlighting the onset of non enzymatic (Maillard) browning and preservative action of sulphite.

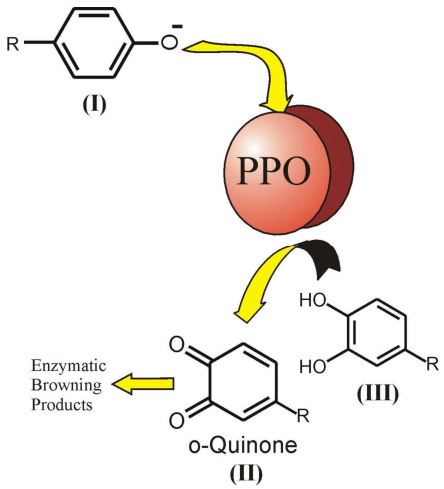

Figure 6.2 Enzymatic browning reaction scheme

As sulphite is a sufficiently powerful nucleophile, the reaction proceeds without the need for any acid or base catalysis [26] and it must be acknowledged that within any sample, it will be present in both free and bound forms. The reaction of sulphite with disulphide bonds (R-S-S-R) provides another route through which the chemical removal of the anion occurs [27]. The process results in the cleavage of the disulphide to yield free sulphydryl thiol (RSH) and the corresponding sulphinic acid ($RSSO_3^-$) [27]. Such processes are widely exploited in bread products where the sulphite induced cleavage of the disulphide can condition (effectively weaken) the dough prior to baking [26].

6.3 Clinical Relevance

The adverse health effects of sulphur dioxide inhalation are well established and numerous studies have investigated the association of air pollution with occupational and environmental lung diseases [28-32]. Sulphur dioxide has been shown to lead to an inflammation of the airways as a consequence of neutrophil activation and is directly implicated in the bronchoconstriction and general aggravation of asthmatic conditions [14, 15,33]. Public concerns over the latter has led to labeling requirements relating to the inclusion of sulphite within food and drink products. Recent investigations designed to assess the susceptibility of asthmatics to sulphites within wine, however, have failed to elucidate the molecular trigger directly responsible for the asthmatic response to sulphite[15] and much of the medical evidence remain contentious.

While the mechanism through which sulphite acts remains a matter of debate, its presence associated with neutrophil activation, has been characterised by the sulphite induced release of reactive oxygen species (principally H_2O_2) and chemotactic factor (IL-8) [34,35]. Neutrophils from human and animal sources have also been shown to spontaneously produce sulphite in response to stimulation from bacterial endotoxins and points towards an ability to participate in the mediation of antimicrobial and pro-inflammatory reactions [36]. Sulphite can arise from a variety of normal physiological and biochemical sources – mainly through the natural metabolic cycling of sulphur containing amino acids. Mammalian tissues possess sulphite oxidase enzymes that convert sulphite to the less toxic sulphate and tightly regulate the systemic sulphite concentration [37, 38]. The normal plasma concentration in healthy adults depends heavily on diet and lifestyle factors and can range from 0.1 μM to 10 μM. In one study, a group of volunteers possessing basal plasma sulphite ranging between 0.4 and 1.2 μM were found to have almost a ten fold increase 1 hour after the consumption of red wine (200mL containing 320mg of sulphite) [38].

It has been found that ingestion of excessively large amounts of sulphite can however increase the concentration to over 100 μM but the body will normally act rapidly to counter such increases[38]. Abnormally high sulphite concentrations can be sustained in patients suffering from renal complications but it is unclear as to whether such increases lead to further complications or is simply a result of reduced renal clearence. It was found that the transient increase in sulphite through massive oral intake did not lead to any significant adverse physiological effects. Recent studies investigating

the *in vitro* and *in vivo* nature of oral sulphite supplementation has shown that the anion can exert an anti-oxidant effect and prevents lipid peroxidation and, in contrast to much of the media concern, could actually have an important beneficial action against inter and intra cellular oxidative stress [39].

6.4 Detecting Sulphite

Measuring sulphite has traditionally faced difficulties of selectivity and sensitivity – especially when dealing with complex matrices (irrespective of whether they are food or of biomedical origin). The use of the classical techniques – principally titration based as those described in Chapter 2 - requires considerable skill on the part of the analyst to ensure accuracy [39-43]. Routine screening, which is increasingly demanded by industry and overseen by the food standard authorities, would ideally require the use of techniques suitable for high throughput analysis – especially within high volume quality assurance environments. It is unlikely, however, that a small scale food producer will possess the necessary chemical expertise or have the investment capacity for the purchase, operation and maintenance of such systems.

Electroanalytical techniques have long been suggested as a solution given the low cost of the instrumentation and the promise of user accessibility through simple reagentless sampling. They also offer more potentially favorable economics of their operation compared with the more demanding consumable and maintenance costs associated with ion chromatography systems [44]. The latter is evidenced by the

disposable screen print systems routinely used by diabetics for home glucose monitoring [45,46]. Such reasons are partly the driving force behind the research being presented within this Chapter.

Sulphite is chemically very flexible from an analytical viewpoint and can be reduced or oxidised [39]. It should therefore be readily amenable to electrochemical detection. There are a number of colorimetric sampling systems but there are no commercial electrochemical sensors for sulphite – in any form and serves to strengthen our interest in the development of new sensing approaches.

Historically, electrochemical detection has also been employed as a simple modification to the classical titration method and operates by means of quantifying acidified sulphite using amperometry [47], coulometry [48] or differential pulse polarography [49]. The first approach has been assessed as a postcolumn detection system in ion-chromatographic systems. This is widely recognised as a more effective approach for routine sulphite determination allowing direct quantification of liquids – minimising sample preparation and largely removes the need for the time consuming distillation process [22, 24, 42, 43, 50-52]. This is deemed to be more sensitive than the titration – through the combination of chromatographic resolution of the components and the simplicity (and potential clarity) of the signal obtained from the electrode assembly.

While there are a number of liquid chromatographic (LC) techniques for the determination of sulphite [22, 24, 42, 43, 50-52], flow injection analysis (FIA) however

has tended to predominate in recent years [22, 24, 53-55]. In contrast to conventional LC, the more simple FIA systems exploit the chemical properties of sulphite to enable resolution between it and other interferences (usually ascorbate). Dual channel systems employing gas diffusion cells or membranes (silicone or PTFE) are commonly used to separate the sulphite from the initial sample stream[22, 53-55]. The acidification of the latter generates sulphur dioxide which permeates through the polymer film into the accompanying stream whereby it can then be quantified using either amperometric [22, 53-55] or potentiometric [56-58] detection systems. All of these approaches require elaborate instrumentation and user expertise and could involve substantial running costs.

6.4.1 Amperometric and Voltammetric Systems

The oxidation of sulphite to sulphate tends to be the main method of electrochemical detection and has been studied at a range of electrodes that include platinum [53,59], gold [60], carbon [51,61] and metal oxide electrodes [54, 62]. Well defined and quantifiable oxidation processes can be obtained at most electrodes and provide an instrumentally simple route through which amperometric detectors (as evidenced by the incorporation of such with LC and FIA detector systems). Detection limits achievable at bare, unmodified electrodes, irrespective of substrate material, tend to be in the low micro-molar range which is normally sufficient for monitoring both endogenous and exogenous sulphite. The sensitivity and the potential for integrating the detector with LC and FIA autosampler systems has, in many cases, displaced the titration as the standard method.

One of the problems associated with such processes is the potential fouling of the electrode which leads to a loss in sensitivity and compromises the reproducibility of the method [50,52]. This can be a consequence of either sample constituents or the products of the oxidation process itself which adsorb onto the electrode and poison/foul the detector surface – especially at gold electrode which have an affinity for sulphur species. Pulsed amperometric detection (PAD) techniques have been employed in an effort to minimise the loss in sensor performance through imposing multi-step waveforms that serve to clean the electrode in situ [50,52].

The large overpotential required to obtain the oxidation signal can induce significant interference, particularly with amperometric systems, where the oxidation of other components will artificially amplify the current that would otherwise be attributed solely to sulphite. This issue was highlighted previously in Chapter 3. Ascorbate tends to be the main interference when considering electroanalytical detection and as it is almost universally found, at least to some extent, in most biological matrices and poses the biggest problem. The situation is made worse by the fact that ascorbate is frequently used alongside sulphite as a preservative within many food products [26].

Wine, irrespective of origin or type, provides an added complication through the presence of high concentrations of polyphenolics [63-66]. These can also undergo oxidation at similar potentials to the sulphite and will inevitably create a degree of ambiguity in measuring the magnitude of the sulphite signal. The presence of phenolics

are an integral part of wine and provide a highly variable interferent which will depend strongly on origin, type and processing and storage history.

6.4.2 Electrode Modification

Modifiying the electrode surface to reduce the potential required to initiate the oxidation of sulphite, thereby minimising the opportunity for unwanted electrode processes (i.e. ascorbate, polyphenolic oxidation) to contribute to the analytical signal has been one approach to solving the problem. There are two general approaches: the use of metal complexes or the use of biological agents. The former is the more common (possibly as a consequence of the reagent economics and construction simplicity) and a variety of complexes have been assessed. These include metallohexacyanoferrate films (Cu, Ni) [67,68], ferrocenes [69], iron phenanthrolines [70,71] and metallophthallocine / prophyrin macrocyles (Ni, Fe, Co) [72-74]. Such systems tend to be used as solution based mediators [69,70] but there have been a number of attempts to immobilise them on the electrode as mono or multilayer films or incorporate them within the body of composite electrode materials such as sol gels [75-78]. The reduction of sulphite has been suggested as a potential detection route but there are few extensive analytical investigations relating to the applicability of the system directly within complex media.

The organometallic complexes can significantly enhance the current response to sulphite and often succeed in shifting the over-potential for sulphite oxidation to less positive potentials such that the oxidation of polyphenolics could be avoided.

Unfortunately, almost all still remain positive of ascorbate oxidation and it could be expected that sample pre-treatment will still be required to eliminate this important interference. Their use as FIA or post column detectors may have considerable benefits but must be critically weighed in terms of the added complexity of the system. The simple electro-deposition of metals (Cu, CuO and Pt) onto carbon substrates have been shown to enhance the detector performance [51,54,62] and could present a more accessible option for the non specialist operator than the more elaborate complexes.

6.4.3 Amperometric Biosensors Applications

The selective conversion of sulphite to sulphate can be achieved with a high degree of selectivity through the use of enzymes (sulphite oxidase) [27, 56, 79-85] and microbes (thiobacillus sp) [86,87]. In general, the biocomponent can be coupled to conventional electrode substrates and the analytical signal derived from monitoring peroxide oxidation [79, 80], oxygen reduction [81,86,87] or the regeneration of electron transfer mediators [81-85] as indicated previously in Chapter 1. The oxidation of the peroxide byproduct is often regarded as the simplest approach but, like direct sulphite oxidation, suffers from the need for large overpotentials. It could be anticipated that the enzyme selectivity would be compromised by ascorbate oxidation at the underlying electrode substrate – much in the same way as the direct oxidation of the analyte.

Coating the electrode with a polymeric film (polytyramine, polydiaminobenzene) onto which the enzyme is then placed has been shown to retain the selectivity[27, 79]. In

this instance, the polymer acts as a permselective barrier allowing only the peroxide to reach the electrode. In contrast to most polymer-enzyme systems, the enzyme is not entrapped within the film but simply attached to the upper surface. The film serves to screen out the peroxide *after* the enzyme reaction.

The main limiting factor in unmodified systems is that a large potential is required to oxidize the peroxide and can induce unwanted electrode processes (co-existing interferences) that will contribute to the analytical signal. This polymer route is therefore often employed to improve the selectivity towards peroxide – which typically means preventing access of most other species to the underlying electrode substrate. The peroxide byproduct can also be reduced and with the cathodic potentials avoiding the unwanted oxidation of ascorbate and polyphenols. The main problem that has prevented the adoption of the approach has been large negative overpotential required to initiate the reduction processes. This can incur oxygen interference and, as mentioned previously, the competing processes will lead to errors. The introduction of various surface modifiers capable of catalysing peroxide conversions such that the operating potential of the sensor can be minimized have proven to be more successful. Common examples are Prussian Blue [88-90] and other mixed metal hexacyanoferrates [91], and electrodeposited metals and alloys (typically Ir / Cu / Pd / Ru [92-94]) and carbon nanotubes [95-97].

The enzymatic process consumes oxygen and this can be monitored through the electrochemical reduction of oxygen and is the predominant methodology employed when using microbial agents [86, 87]. The advantage of this route is that cathodic

potentials are employed that avoid the unwanted oxidation of the matrix interferences. The disadvantage lies in the sensor being dependent upon ambient dissolved oxygen concentrations and the fact that the analytical signal is derived from a decreasing signal rather than the positive offset provided by peroxide oxidation. The acquisition of a stable signal is also problematic with the latter providing faster response times. There have been a number of alternative systems – e.g. potentiometric sensing of oxidase released peroxide on a field effect transistor based sensor[56]. The obvious advantage is the microfabrication opportunities afforded by such technology and the inherent capacity for mass manufacture.

The dependence on molecular oxygen can be removed through the use of electron mediators. These also remove the peroxide byproduct and can allow operating potentials that are significantly less than those required to oxidise either peroxide or sulphite. Typical examples are TTF-TCNQ conducting salts[82], cytochrome c[83,84] and ferro / ferricyanide[85]. The latter has been used in screen printed systems with an operating potential of +0.3V (rel. Ag|AgCl). The influence of ascorbate was not studied but the technology does highlight an important step forward when considering the transferability of such systems to small scale food producers. The underlying technology is essentially the same as that adopted by commercial glucose meters and could provide an easily accessible route through which sulphite analysis could be speedily conducted by non specialist staff. The capability for mass production and the inherent disposability of the sensing strips require little capital outlay or maintenance costs.

6.4.4 Potentiometric Techniques

Potentiometric systems have been the main focus of the present project and ion selective electrodes (ISEs) are widely employed in the food industries for monitoring a range of both anions and cations [98,99]. As yet there are no commercial sulphite variants. One of the main issues has been the lack of suitable ionophores that are sufficiently selective for sulphite. In principle, ISE techniques should provide an excellent basis for monitoring sulphite with the passive sensing mechanism freeing the measurement from the interference effects of ascorbate, urate and the polyphenolics. Interference from other anions is the main problem (particularly perchlorate and salicylates). ISE systems based on macrocyles such as calixarenes [100] have been investigated but most approaches have focused on mercury complexes [101,102]. The mercury complexes have been shown to function more through redox interactions than specific complexation [102].

Classical redox indicators have traditionally been used, though mainly as an alternative to the more classical Monier-Williams titrations (chapter 2) with sulphite oxidation by iodine providing the potentiometric signal [39,58]. The main difficulty lies in the reducing properties of ascorbate, which like sulphite, will reduce iodine. Unless gas distillation permeation is used to separate out these two components [57,58], interference is highly likely and is complicated by the fact that both are liable to be present in biological samples.

6.5 Proposed Detection Methodology

The proposed electrode design was based on the thermal encapsulation of the film within a pre-patterned laminate with a copper track facilitating connection to the external recording device (potentiostat). The approach and the detection methodology are highlighted in **Figure 6.3**. The direct redox interaction of sulphite with quinoid indicators would be used as the analytical basis of the detection system. Reduction of the indicator by sulphite would lead to a change in redox status of the analysis solution as the quinone (**I**) component is converted to the corresponding hydroquinone (**II**).

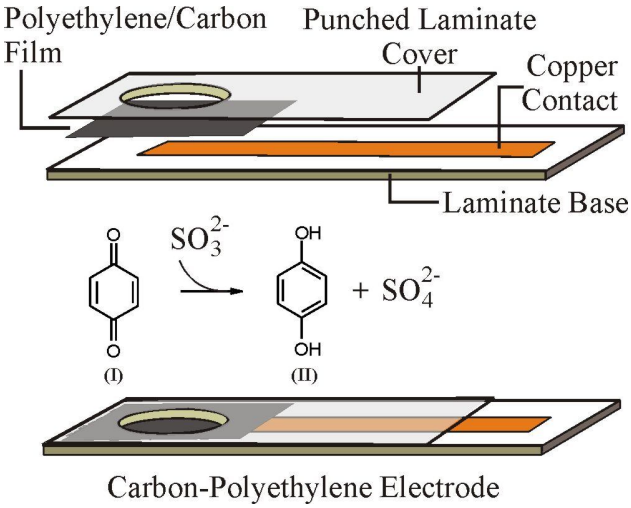

Figure 6.3. Schematic of the electrode lamination process and reaction detection methodology

The subsequent change in the oxidised/reduced indicator ratio thereby induces a change in the measured potential difference. Increasing sulphite will lead to an increase in the reduction of the quinone component and hence the greater the change in electrode potential. The magnitude of the latter can be related to the concentration of sulphite. Commercial carbon loaded polyethylene and polycarbonate films were selected as the base substrates for our investigation and compared against the responses of conventional glassy carbon electrodes.

While there are numerous alternatives to these substrates, our rationale in the present instance was that these materials would serve as immediately accessible references to which subsequent studies could be compared. The basic design represents an inexpensive means through which "Film" electrodes could be speedily constructed and it was anticipated that they could serve as a basis for the production of disposable sensors for ad-hoc sensing applications. It was therefore necessary to assess the efficacy of the laminate assemblies for single shot and repeat measurement of sulphite (as our model analyte) but also to determine the operational robustness of the proposed designs.

6.6 Experimental Details

Reagents and Materials: All reagents were of the highest grade available and used without further purification. All solutions and subsequent dilutions were prepared daily using deionised water from an Elgastat (Elga, UK) deionised water system and were refrigerated when not in use. Polyethylene (0.08mm thick, 10^5 Ohm cm) and Polycarbonate (0.014 mm thick, 100 Ohm cm) films were purchased from Goodfellow and used as received. Lamination pouches (Rexel UK) were a commercial stationary variety with a film thickness of 75 μm each side. Copper Shielding tape (100 μm thick, adhesive backed) was obtained from RS electronics.

Instrumentation: Electrochemical measurements were conducted using a μAutolab computer controlled potentiostat (Eco-Chemie, Utrecht, Netherlands). An initial cell volume of 20cm^3 was generally used and the measurements recorded at a temperature of $20^oC \pm 2^oC$. A two electrode assembly was used throughout and was comprised of either a glassy carbon (GC, 3 mm diameter, BAS Technicol, UK) or carbon loaded polyethylene (or polycarbonate) laminate (CPE) indicating electrode and a silver / silver chloride reference electrode (3M chloride, BAS Technicol, UK). The glassy carbon electrode was polished with 1 um alumina powder (Buehler, UK) and sonicated in deionised water for several minutes prior to use. Unless specified otherwise, the electrochemical solutions consisted of Britton-Robinson buffer (acetic, boric and phosphoric acids - each at a concentration of 0.04 M and adjusted to pH 7 through the addition of sodium hydroxide). Sulphite solutions were prepared daily using deionised

water as the diluent. The electrodes were immersed in buffered solutions stirred magnetically at a constant rate throughout. The potential difference between the electrodes was continuously monitored and additions of the analyte (or potential interferent) typically added at 60 second intervals.

6.7 Results and Discussion

6.7.1Fabrication of Laminates

Carbon loaded polyethylene film or polycarbonate was thermally sandwiched between sleeves of a pre-punched resin-polyester lamination pouch. Electrical connection to the carbon film was made through the presence of a strip of copper shielding tape. The adhesive backing on the tape was anchored to the laminate resin rather than to the polyethylene film as it was found that the former can prevent electrical contact between copper and the carbon black particles. Electrical contact was made directly through physical contact of copper onto the polyethylene film.

6.7.2 Laminate Characterisation

The integrity of the lamination process was chiefly assessed through scanning electron micrographic investigation of the morphology of the polyethylene-resin-polyester interface. In general, the lamination process provides a coherent seal between the sensing polyethylene layer and the insulating polyester sheath as shown in **Figure 6.4A**. Closer examination of the interface, **Figure 6.4B**, reveals microscopic imperfections. This can be attributed largely to the fabrication process where the periphery of the hole within the pre-punched laminate cover is microscopically ragged and hence the resin layer does not sufficiently contact the polyethylene film to form a complete, non porous, barrier. This is supported in **Figure 6.4B** where the thermal

lamination process has induced the melting of the resin from the polyester layer but the separation from the polyethylene is such that the resin effective by drips onto the latter.

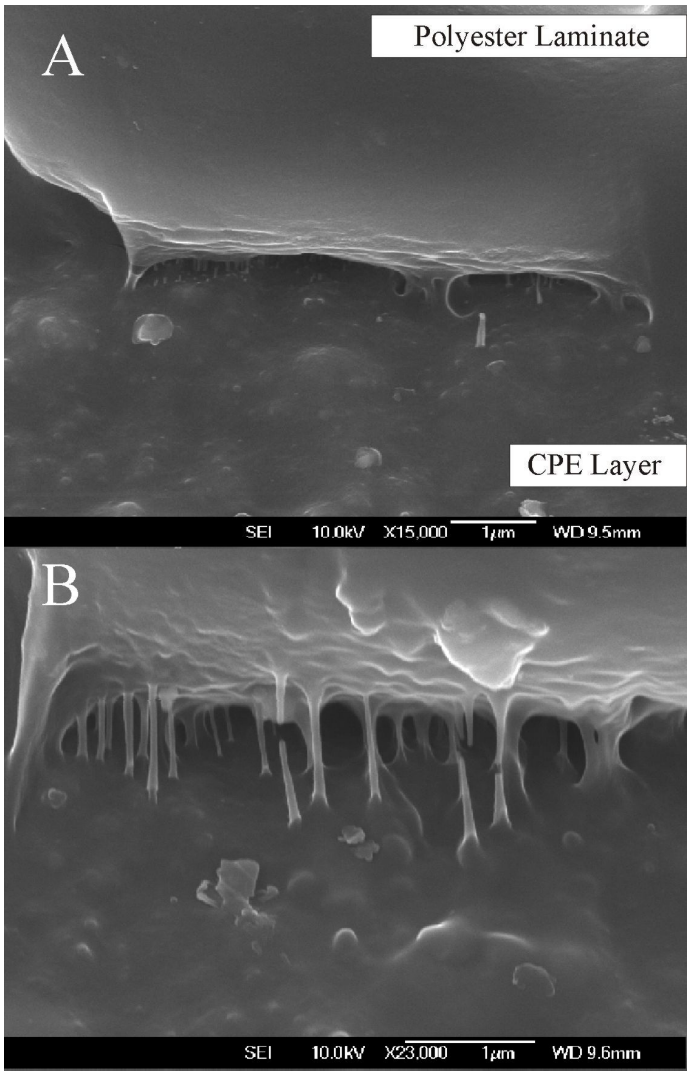

Figure 6.4. A) SEM of the polyethylene-polyester laminate interface. B) Microporosity of the incomplete resin sealing at the interface.

The apparent porosity however is confined to the interface and the macroscopic integrity is not compromised. This was corroborated by the absence of any observable copper electrochemistry (assessed by cyclic voltammetry) that would have arisen had the permeation of solvent to the metallic conducting tract occurred.

The surface of the polyethylene film (**Figure 6.5A**) is relatively featureless, as could be expected, but there is a degree of micro-roughness indicating the presence of the carbon particles. A more visible assessment of the carbon distribution within the film was achieved through the electro-deposition of silver (deposited at -1V for 600s from a stirred 1 mM $AgNO_3$ / 0.1M HNO_3 plating solution). The greater atomic mass of the silver provides better SEM contrast and the presence of submicron silver clusters across the surface of the polyethylene film can be seen in **Figure 6.5B**. The electro-deposition of the silver also provides an indication of the distribution of electrochemically accessible and hence interfacially active carbon particles – the latter providing the core reaction centres for our proposed sensing device.

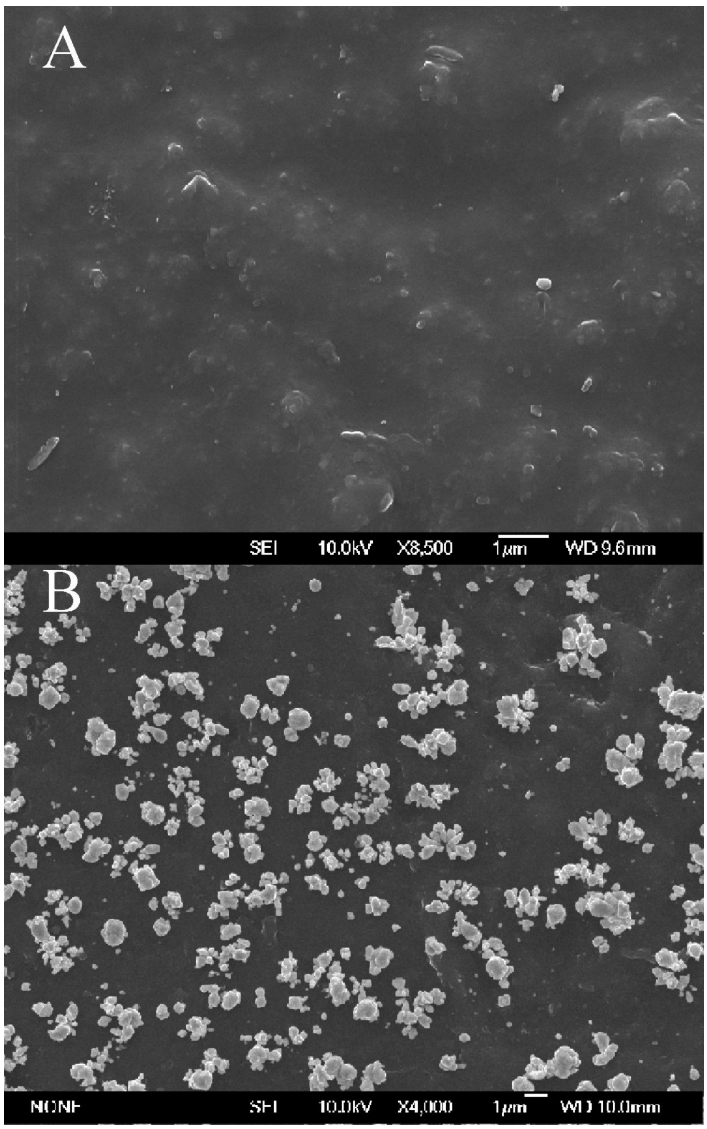

Figure 6.5. A) SEM of the bare carbon loaded polyethylene film and B) after the electrodeposition of silver

6.7.3. Sensor Characterisation

The potentiometric response of the polycarbonate loaded carbon composite electrode to increasing sulphite (83 μM) in the presence of an equimolar benzoquinone/hydroquinone (133 μM, pH 7) indicating couple is shown in **Figure 6.6A**.

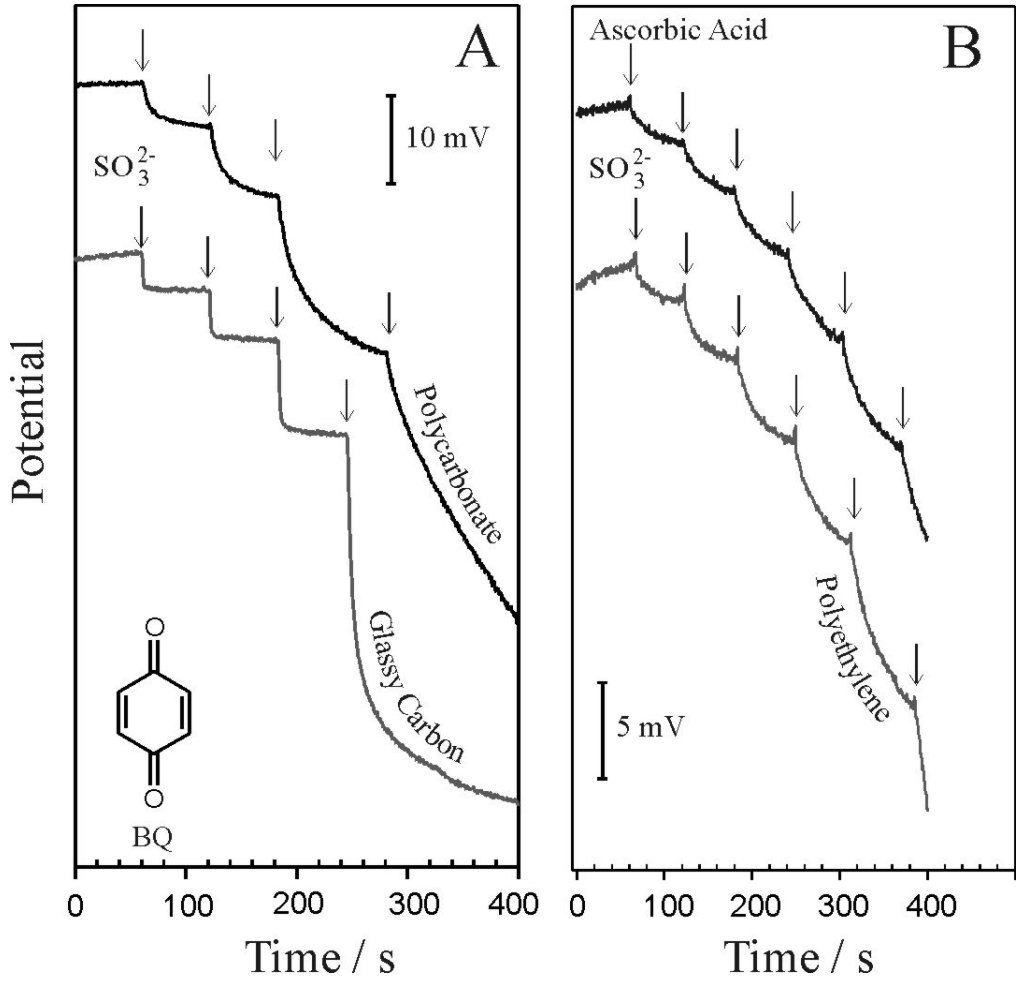

Figure 6.6. A) Potentiometric responses of polycarbonate and glassy carbon electrodes to increasing sulphite concentration (83 μM additions) in the presence of equimolar HQ/BQ (133 μM, pH 7) indicator. B) Response of the polyethylene- HQ/BQ indicator system to increasing sulphite (83μM additions) and ascorbic acid (16 μM additions)

The profile is analogous to that observed with a commercial glassy carbon electrode and serves to highlight the potential efficacy of the substrate for potentiometric analysis. The response of the polyethylene electrode is shown in **Figure 6.6B**. The response profile is significantly poorer than that of either glassy carbon or polycarbonate composite and reflects the lower conductivity of the system. Nevertheless, a discernible response to increasing sulphite can be observed. The electrode / indicator system was however also found to respond to ascorbic acid, **Figure 6.6B**, and highlights a clear deficiency in protocol selectivity. The quest for a sensor for the determination of sulphite ion within wine samples would obviously require the analytical system to be insensitive to ascorbate. This can be remedied simply through changing the indicator to a naphthoquinone (NQ) system. Although the underlying mechanism is essentially the same, NQ is a stronger reductant than ascorbate and while weaker than sulphite, should therefore only respond to the latter. This is confirmed in **Figure 6.7** where the potentiometric responses of the carbon-polyethylene electrode to sulphite and ascorbate in the presence of NQ (1 mM, pH 7) are compared. There is a significant change in the electrode potential upon the addition of the sulphite anion – the magnitude of which is dependent upon the concentration of the latter. Consecutive additions of ascorbate (as per **Figure 6.6B**) however did not yield any significant change in the electrode potential and confirms the selectivity of the approach.

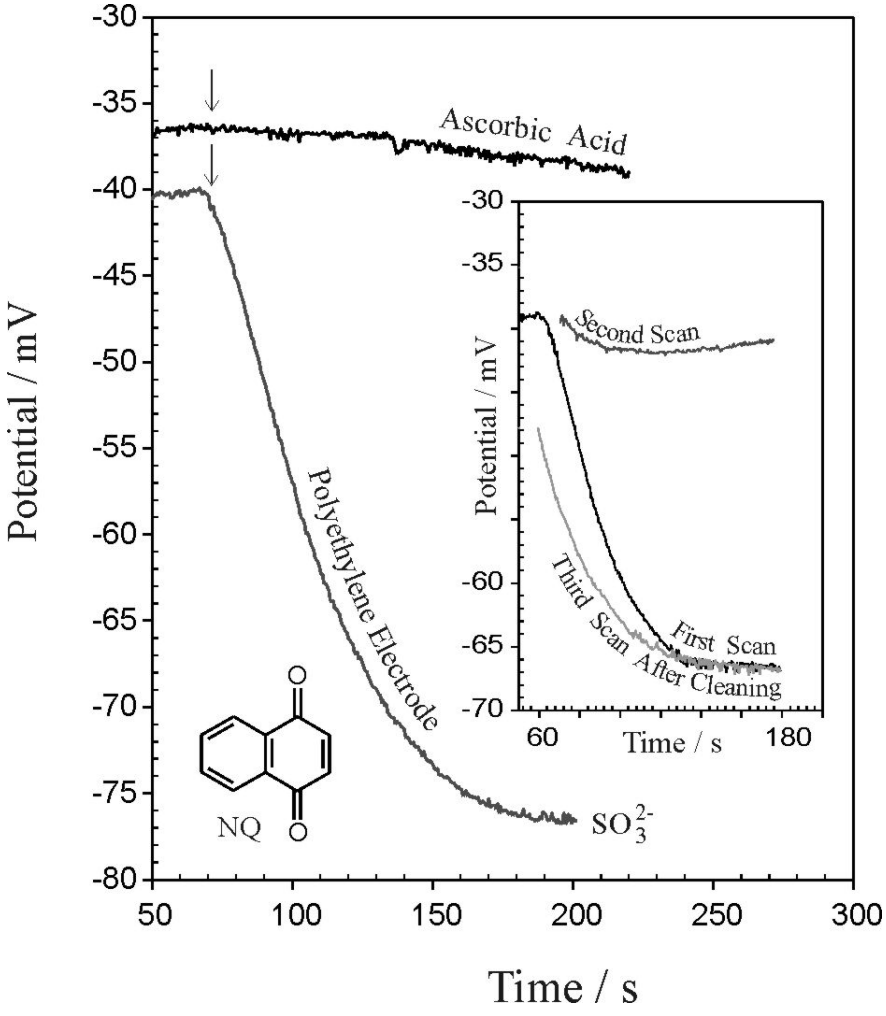

Figure 6.7. Potentiometric responses of a polyethylene-NQ (1mM, pH 7) system to sulphite (250μM) and ascorbate acid (150 μM). **Inset:** Effect of repeated measurements on the response profiles of the polyethylene electrodes and response recovery after cleaning with acetone.

The electrode did however require regeneration between each sulphite measurements. Repetitive additions of sulphite (83 uM) to the NQ indicating solution led to a successive decrease in the electrode potential eventually leading to the total loss in response as highlighted in the inset figure within **Figure 6.7**. This behaviour contrasts the responses obtained at glassy carbon electrodes (not shown) where repetitive measurements can be obtained without recourse to polishing the surface. The loss in sensitivity could be attributed to the hydro-NQ product, arising from the sulphite reduction, adsorbing upon the carbon surface. As indicated in the silver-SEM studies (**Figure 6.4B**), the active surface area of the CPE electrode will be substantially less than that of the GC electrode. It must also be noted that the differing surface chemistries between the two electrode substrates will play a part, and it is plausible to suggest that the hydro-NQ moieties may have a greater affinity for adsorption to the embedded carbon black particles. Confirmation that adsorption was the key factor in the loss of sensitivity was obtained through the regeneration of the CPE electrode by simple immersion in acetone for several minutes. The response was returned to that level obtained upon the first exposure to the NQ – sulphite solution.

An interesting point to note is that the polyethylene laminate assembly is stable to acetone with no degradation in the mechanical properties of either the composite sensing surface or the polyester-resin encapsulant. The polycarbonate composite system exhibited similar behaviour but the use of acetone, while having no effect on the laminate, was found to irreversibly compromise the conductivity of the sensing surface with a complete

loss of response observed on subsequent scans. The polycarbonate electrodes could however be regenerated through soaking in ethanol.

The application of the polycarbonate laminate electrode to the analysis of an authentic sample was assessed through examining the response to wine samples spiked with various concentrations of sulphite. A typical response to the preservative within a red wine sample is shown in **Figure 6.8** and effectively mirrors that obtained with the polyethylene system in **Figure 6.7**.

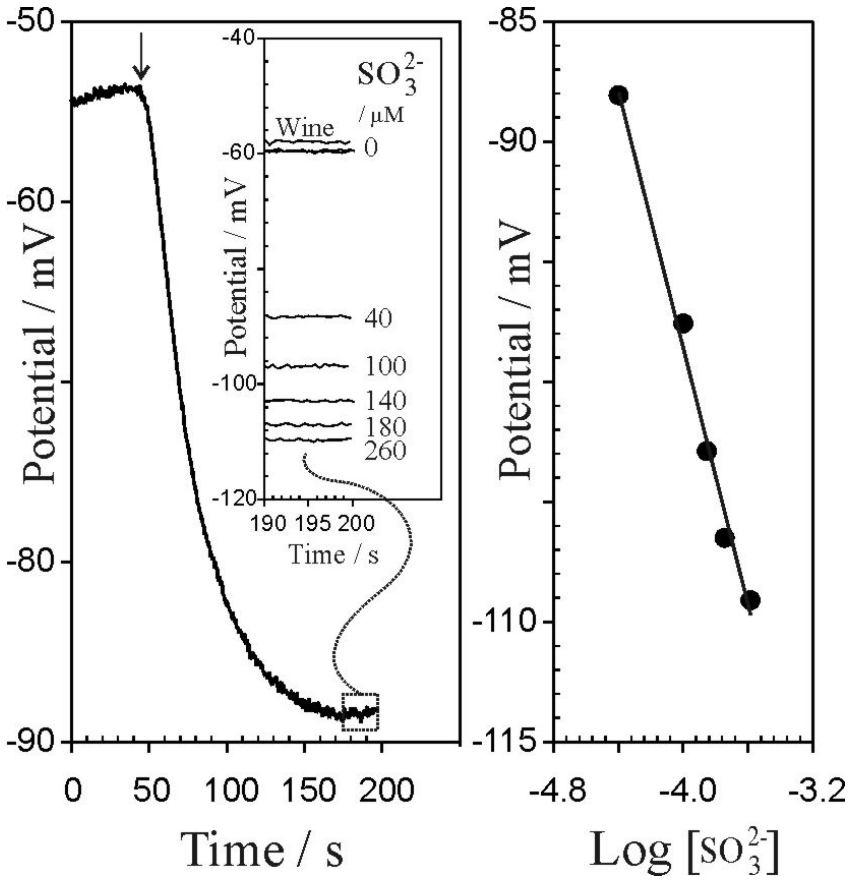

Figure 6.8. Response of a polycarbonate-NQ system to wine spiked with increasing concentrations of sulphite (40-260 μM).

The responses obtained to increasing sulphite are shown within the inset diagram in **Figure 6.8** and represent the plateau region (taken at 190s) where the redox indicating system has equilibrated and hence the change in potential is effectively constant. Three separate (baseline) measurements of the wine sample (effectively zero added sulphite) have been included and it can be seen that they are all in close agreement and confirm the reproducibility of the approach. The reproducibility (RSD) of the signal at low sulphite concentration (40 μM) was found to be 5.0 % (based on three replicate measurements). The electrode potentials measured were found to follow a log relationship (E = -26.84 log [Sulphite] – 205.95, R^2 = 0.99, N=5) in accordance with that expected for a conventional potentiometric measurement. The efficacy of the analytical approach presented is confirmed when the potentiometric responses to sulphite in the absence of the quinone indicator were assessed. There was essentially no correlation between measured potential and sulphite concentration and was confirmed by the fact that the RSD at 40 μM sulphite was found to be 138%. The absence of a defined redox couple results in a series of highly variable and essentially meaningless responses.

6.7.4. Laminate Modification and Optimisation

The polycarbonate laminate has been shown in the previous sections to be a versatile substrate through which sensors could be fabricated. One limitation of the approach is that there is a very limited range of materials available commercially. The great strength of the approach however is that it should in principle be possible to

produce custom formulations within a conventional laboratory. The dispersion of carbon particles within a polycarbonate matrix should enable films to be cast which reproduce the characteristics of the commercial variety and through the variation of the blend – potentially improve upon the response. This aspect was briefly investigated to assess the ease with which such a strategy could be transferred or adopted and thereby satisfy the assumption that it could be used for the speedy production of adhoc prototype sensors.

Various loadings of carbon particle (1-2 micron diameter) were homogeneously dispersed in a polycarbonate solution (polycarbonate granules – dissolved in dichloromethane) and the resulting mixture cast onto glass substrates. The solvent was then allowed to evaporate. The resulting film was then carefully peeled from the base layer and mounted in the laminate assembly indicated in **Figure 6.3**. The polymer layer was modified further using a novel laser etch technique – the intention being to remove some of the polymer matrix, expose more of the carbon and increase the oxygen moieties on the carbon such that electron transfer could be enhanced.

Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were used to investigate the physical characteristics of both the non-lasered/unmodified polycarbonate electrode and the laser-etched carbon composite electrode. This was important to determine the effect laser ablation has on the electrode surface and how it enhances the electrochemical characteristics, especially in comparison with the non-lasered polycarbonate electrode.

Scanning electron microscopy was used to assess the morphological features associated with the unmodified “cast” carbon-polycarbonate composite film (Figure 6.9) and the changes resulting from laser anodsiation / modification (Figure 6.10 and Figure 6.11). The image generated for the non-lasered polycarbonate composite electrode appears on initial inspection to be very similar to the images generated for the laser-etched carbon composite electrode, however there is a major difference as with the laser-etched carbon composite electrode the surface is less homogeneous, appearing rougher compared to the non-lasered polycarbonate electrode which has a more planar surface. This is expected as with the laser-etched carbon composite electrode the top layer of polycarbonate has been removed, to expose more of the underlying carbon layer; therefore the surface appears less homogeneous.

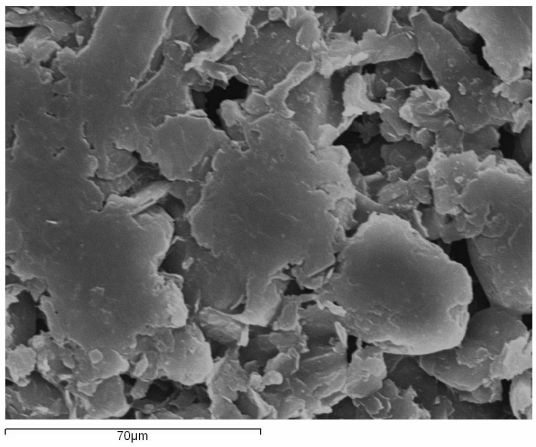

Figure 6.9: Non-lasered polycarbonate electrode (800 magnification, 20kV)

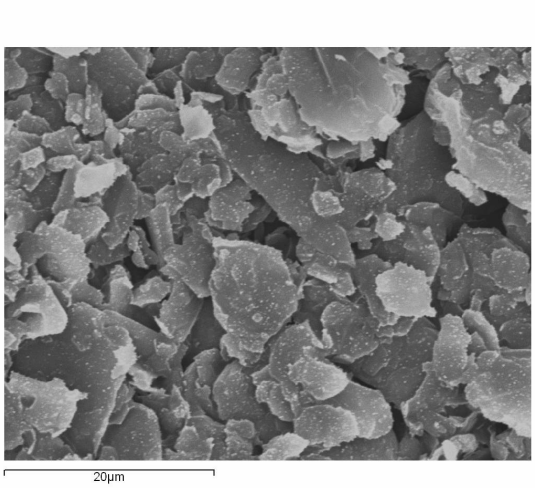

Figure 6.10: Laser-etched carbon composite (magnification 2300, 20kV)

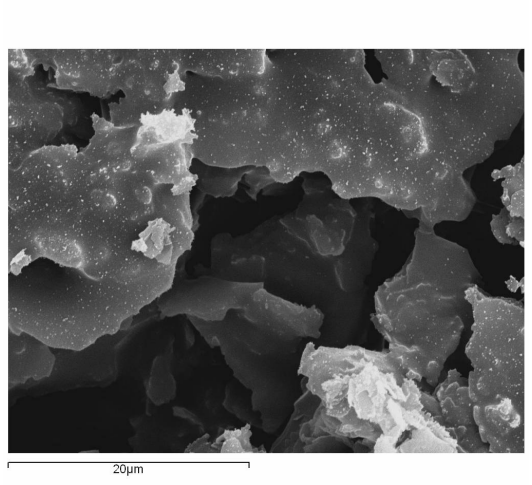

Figure 6.11: Laser-etched carbon composite (magnification 2700, 20kV)

Conductive Atomic Force Microscopy (cAFM) is a relatively new technique, allowing the user to simultaneously map the topography and current distribution of the sample surface. It is used with resistive samples to characterise conducting variations. Conventional AFM methodologies are normally only capable of mapping the topography

of a surface and would provide no information on the current distribution or conducting variations. Conductive AFM was used to demonstrate the variations in surface conductivity of the non-lasered polycarbonate electrode and laser-etched carbon composite electrode, the results are shown in **Figure 6.12** and **Figure 6.13** respectively. The topography image, showing the surface composition shows the difference in conductivity between the two electrodes, in **Figure 6.12**, the non-laser polycarbonate electrode, the surface appears relatively smooth and very little of the image showing high conductivity. In comparison the topography image in **Figure 6.13**, the laser-etched carbon composite electrode, shows an uneven surface, with the majority of the image showing the highest conductivity. The greater variation in surface topology is consistent with the SEM images and can be attributed to the laser ablation fracturing the surface of the polycarbonate film. The 'tip current' images show the current distribution within the two electrodes, with again the laser-etched carbon composite electrode indicating a higher current distribution, however neither electrode shows a high current distribution. This is expected as the carbon particles are coated with polycarbonate, which acts as an insulting mesh, therefore the carbon particles exist in a sea of polymer.

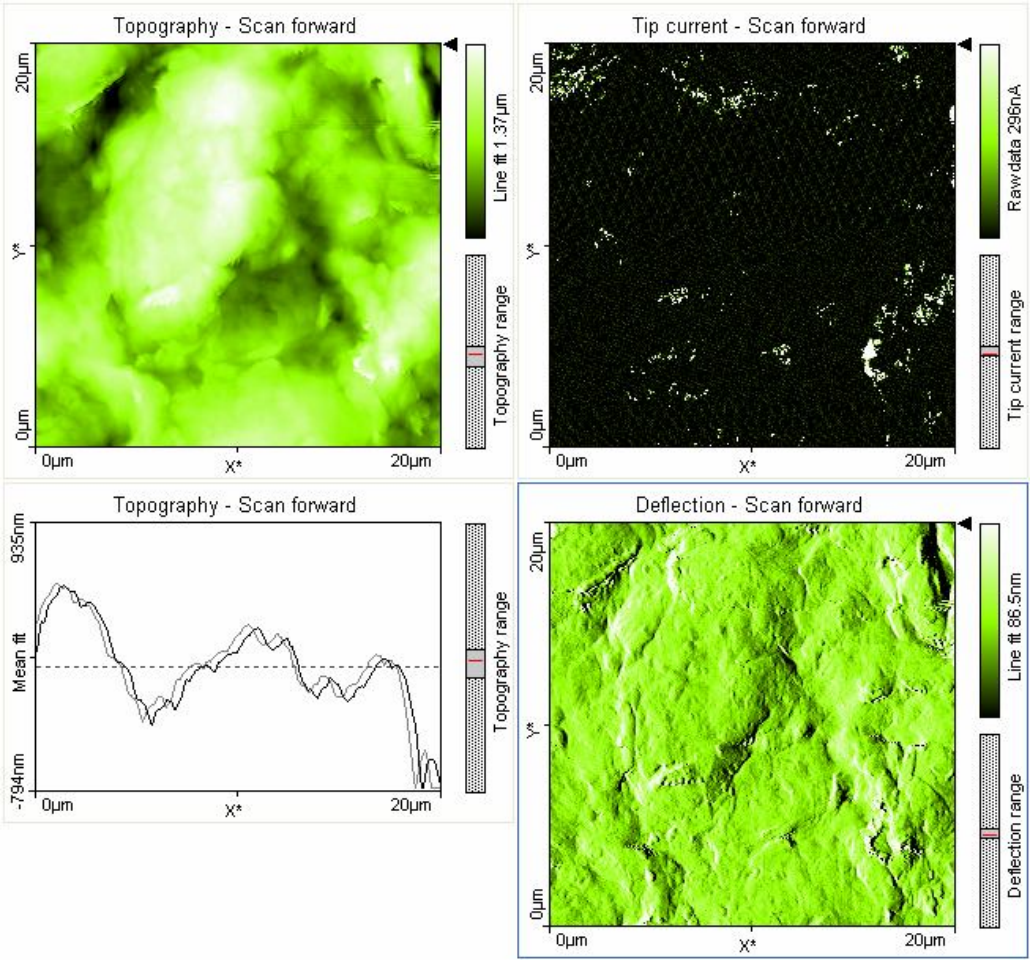

Figure 6.12: Non-lasered polycarbonate electrode

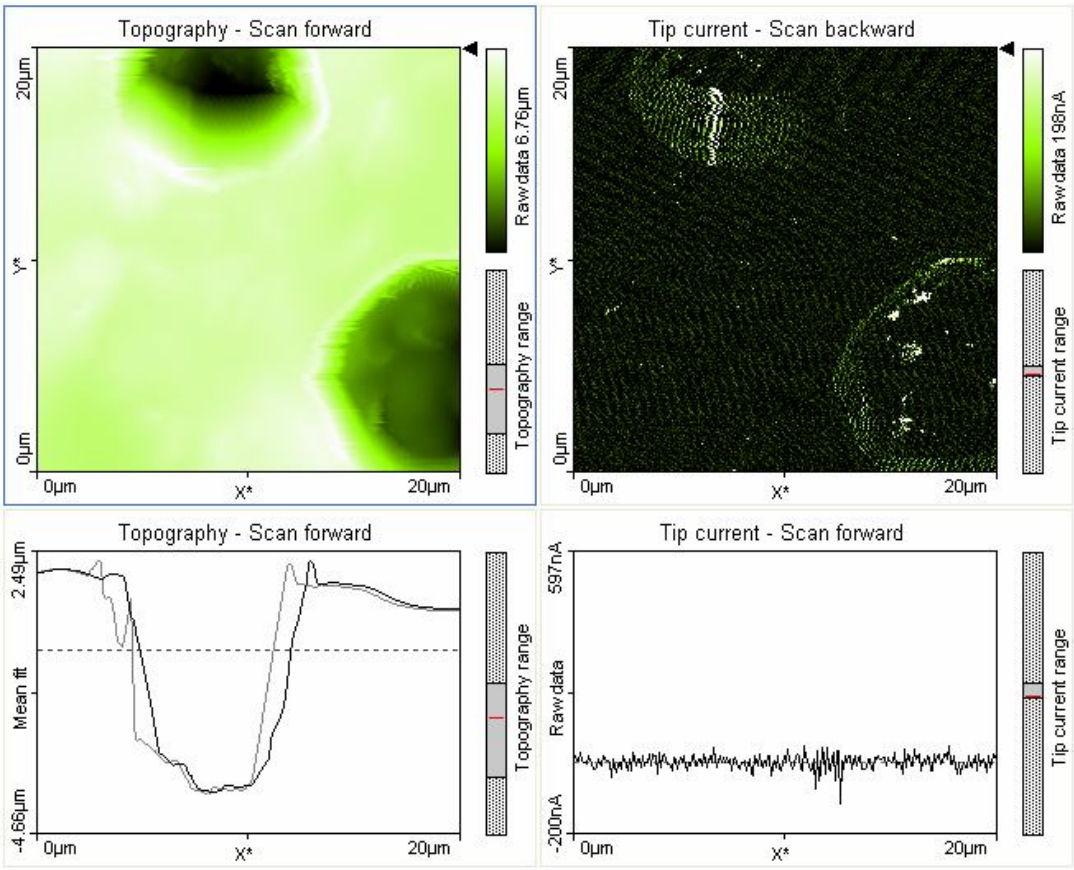

Figure 6.13: Laser-etched carbon composite electrode

The electrochemical properties of the in-house films were examined through comparing the voltammetric and potentiometric response to sulphite. Cyclic voltammograms detailing the response of the unmodified and laser etched films towards increasing sulphite (90 µM additions, pH 7) are compared in **Figure 6.14**. There is essentially no response to sulphite from the unmodified film whereas the laser etched film provides a significant oxidation peak process at +0.5V which responds linearly to sulphite. The background current is markedly greater for the laser etched film and could be expected given the fracturing of the surface and increased electrode area.

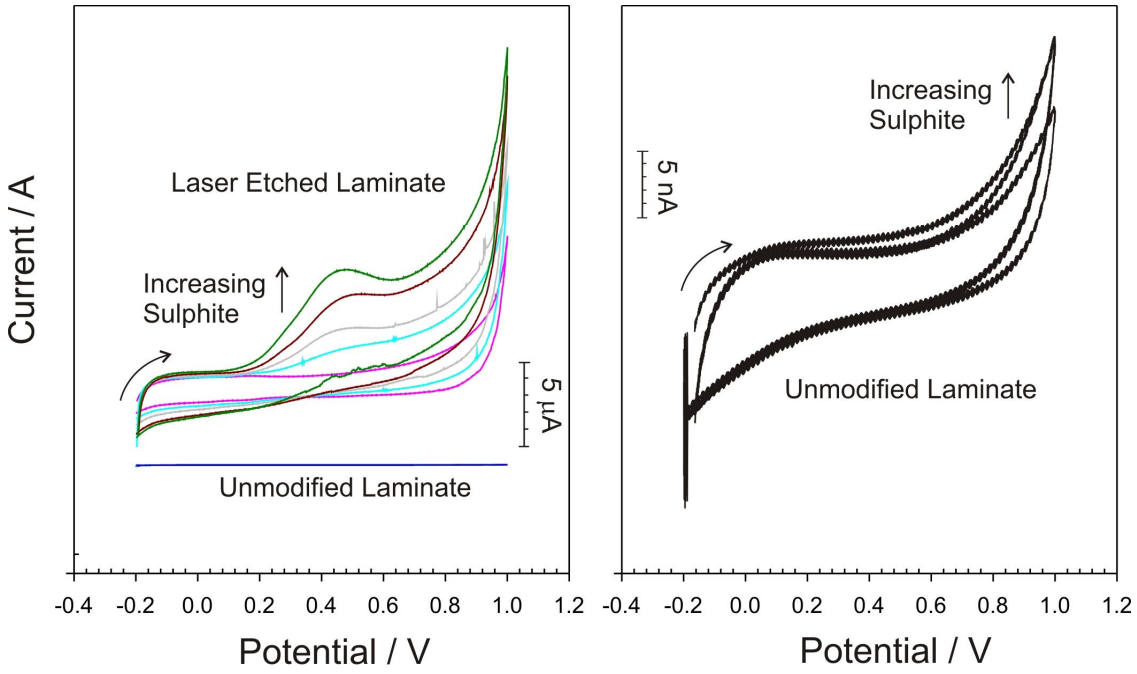

Figure 6.14 Cyclic voltammograms detailing the response of the unmodified and laser etched laminate electrodes towards increasing sulphite.

The response to naphthoquinone was also assessed. Cyclic voltammograms detailing the response of the composite electrodes to 0.5 mM naphthoquinone (pH 7) are shown in **Figure 6.15**. It can be seen that again there is essentially no response to the quinone from the unmodified film in comparison to the laser etched laminate.

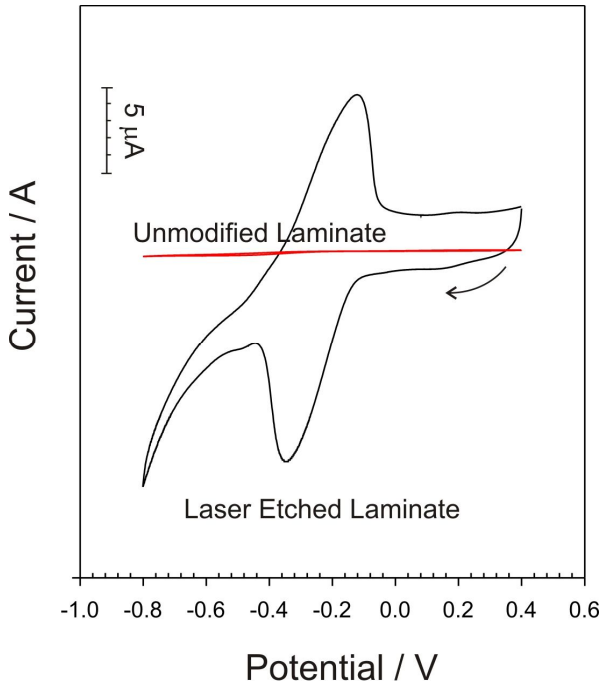

Figure 6.15. Cyclic voltammograms detailing the response of the un-modified and laser etched laminate films towards 0.5 mM naphthoquinone (pH 7).

The potentiometric responses towards sulphite were then assessed in an analogous procedure to that used for the commercial polycarbonate film. The responses towards the addition of 50 µM sulphite are compared in **Figure 6.16**. In contrast to the voltammetric systems – both electrodes were found to respond to sulphite and in almost equal measure. The laser modified electrode provided a qualitatively better signal – in that the response was equivalent to the profile observed previously with the commercial system. The unmodified film provided a similar change in potential though the signal was not as stable as that obtained with the laser etched composite.

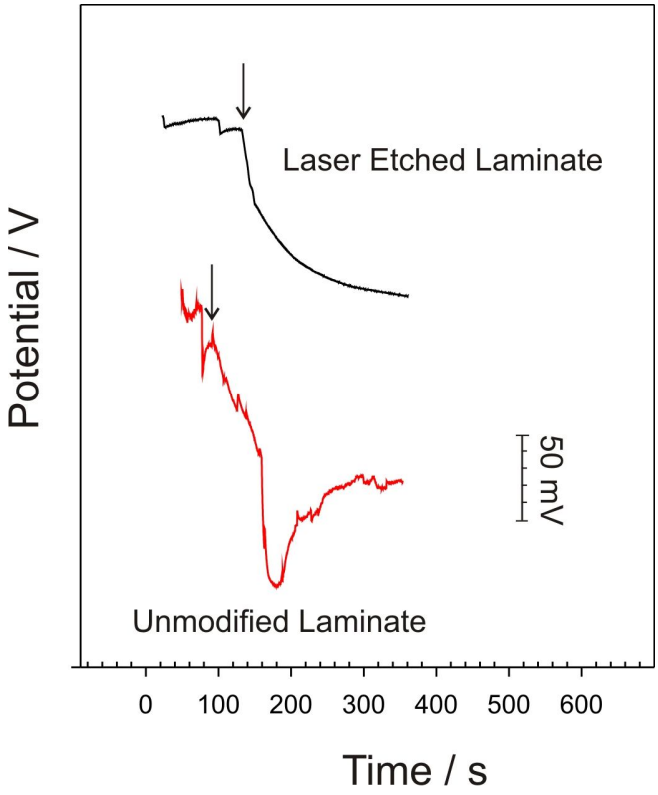

Figure 6.16 Chronopotentiometric traces detailing the response of the un-modified and laser etched films towards 50 μM sulphite

While the use of the adhoc casting procedure would not be advisable for the production of voltammetric sensors, it could be envisaged that through improvements to the casting procedure, the potentiometric response could be improved and would provide a viable possibility of preparing prototype sensors.

6. 8 Conclusion

Carbon is an extremely versatile material for the construction of electrodes – whether for industrial or analytical purposes. The work presented here has highlighted a new route through which carbon "Film" electrodes can be speedily constructed and has demonstrated the applicability of the CPE film as a viable sensing substrate. The use of the polyethylene and polycarbonate films can provide responses that are sufficiently responsive for potentiometric measurements yet can be produced and maintained for a fraction of the cost of conventional glassy carbon electrodes. A new approach to the measurement of sulphite has also been outlined and the analytical performance optimised to provide a selective and sensitive response to the anion. The overall approach advocated here is generic, eminently transferable and could form the foundation of studies involving the adaptation and, indeed, exploitation of carbon loaded films, tailored with specific electrical and/or chemical functionalities, to other potentiometric sensing applications.

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Chapter 7

Conclusions and Areas for Further Development

The ability of sulphydryl thiols to scavenge free radicals and prevent oxidative damage to cellular machinery is well known but it is only recently that their concentration could be used as a versatile measure of the physiological well being. Although not a specific biomarker the ability to monitor their concentration could be useful in a number of clinical contexts. Oxidative stress accompanies many disease processes and is an integral part of the pathophysiology of inflammation – the depletion of the anti-oxidant thiols could therefore be used as a gauge as to the severity of the underlying condition but also of the patients response to the treatment. The main problem preventing the adoption of such diagnostics is the lack of a suitable technology that can allow their concentration to be measured rapidly and accurately.

The laboratory based analysis of thiols is beset with a number of procedural difficulties and it is therefore no surprise that there are no point of care devices presently available. Given the problems of sample degradation then it is likely that the delays associated with taking the sample, referring it to an appropriate lab and then waiting for the return of the analysis results would effectively negate the diagnostic advantage that would originally been achieved had the results been immediately available. Moreover, the possibility of sample degradation through reaction with oxygen and losses resulting from the sample preparation compound the problems further.

The work presented here sought to investigate possible solutions to the problems of both sample preparation, response and possibility of delivering the analytical technology in a form that could be used at the patients bedside or, ultimately by the patient themselves. A novel approach was taken in which the thiols are selectively

labeled and measured without recourse to any conventional laboratory equipment which would be expected when attempting to perform standard thiol procedures. The rapid derivatisation of the thiol and the sensitivity of the resulting signal provides an analytical range that is clinically relevant and free from the interferences that prove problematic for most electroanalytical techniques. The underlying technology is based on a potentiometric methodology and as such represents a more realistic commercial opportunity that should enable the technology to be more accessible to the general clinical biochemistry laboratory. The accuracy of the approach was demonstrated through assessing the plasma thiol concentration of a number of volunteers and the results compared with the conventional spectroscopic technique.

An important aspect, although supplementary to the main goals, was the adaptation of the detection methodology to enable speciation – differentiation between low and high molecular weight thiols. Two novel approaches have been documented that lay the foundations for this work to be further exploited. The development of a tablet system for the selective detection of cysteine was briefly investigated and, although it was set aside in favour of pursuing the electrochemical system, showed considerable promise. The development of the centrifugal filter with integral potentiometric sensor was more flexible and clinically relevant and represents a strategy that has many advantages over the existing approaches yet still retains instrumental simplicity and, importantly, provides a clinically relevant service in terms of allowing the quantification of albumin.

The quinone system advocated here represents only a small selection of the possible indicators that are available and there is little doubt that through careful selection

of the quinone derivative/composition that greater sensitivity and selectivity could be achieved. A problem with attempting to pursue such investigations is in the production of the sensor. In the present work, the disposable sensors used for clinical evaluations were commercially prepared. It has to be appreciated that not all researchers will have access to such technology and, as such, an alternative method of preparing prototype sensors was investigated. This involved the use of polymer composites – which could either be purchased in bulk as the film or through in-house preparation. Sulphite was used as the target analyte as it shares many of the physiological anti-oxidant characteristics of the amino acid thiols previously investigated but has received far less attention. The composite approach was found to provide a quick, flexible and reliable means of quickly prototyping sensor designs with responses that were as good as – or better than the commercial equivalents.

There is much to be done to demonstrate the true clinical efficacy of the thiol and sulphite measurement systems investigated in the present work but the foundations have been laid. One area that has considerable promise but which time did not allow for was the development of the instrumental aspect of the measurement. As previously mentioned – the potentiometric methodology requires measurement instrumentation that is relatively simple. One approach which was initiated but not completed and hence the absence of any corresponding chapter was the transfer of the screen printed system to a portable measurement device. The use of a Personal Digital Assistant fitted with an Analoguesto Digital convertor card can allow the basic potentiometric measurements to be recorded – quite easily by a nurse or GP at a clinic consultation. The basic system is shown below in

Figure 7.1 along with the typical response obtained detailed in **Figure 7.2**. It can be seen that the potentiometric profile is similar to those obtained in **Chapter 5**.

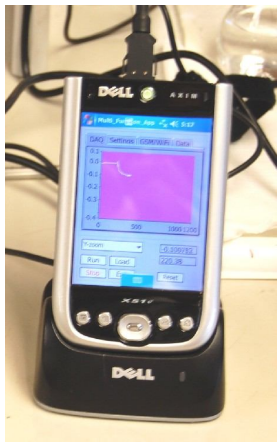

Figure 7.1 Dell Axim personal digital assistant fitted with a CC2 analogue to digital card that allows potential-time recording.

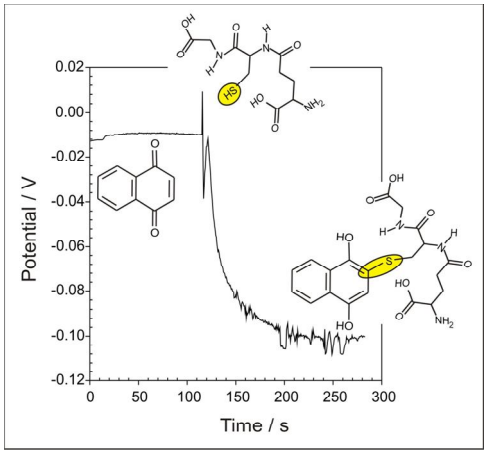

Figure 7.2 Chronopotentiometric response of a screen printed electrode containing 0.5mM naphthoquinone (pH 7) towards 0.1mM glutathione recorded using the Dell PDA fitted with a CC2 AD card. (Transferred from the data shown in the device screen in Figure 7.1)

The preliminary data presented here clearly demonstrate that the system is portable and transferable and provides an ideal platform for the further development of the work initiated in the present project.