Investigation of the electrochemical behaviour of metal electrodes in biological media

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University of Wollongong

Recommended Citation
Investigation of the Electrochemical Behaviour of Metal Electrodes in Biological Media

A thesis submitted in fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

Simon Edward Moulton, BTech. (Environmental Geochemistry), BSc. (Hons)

Department of Chemistry

2002
Declaration

I, Simon Edward Moulton, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The work described in this thesis has never been submitted for a higher degree at any other university or institution.

Simon Edward Moulton

2002
I dedicate this Thesis to my family

Louise, Aleida and Liam
Acknowledgements

I would like to sincerely thank my supervisors Prof Gordon Wallace and Dr Norm Barisci for giving me the opportunity to undertake my PhD in an extremely fascinating area of research. I would also like to thank them for their tireless efforts in assisting and guiding me through all aspects of my project.

This PhD would not have been possible if it were not for my Industry partner, Polartechnics Ltd. The warm welcome I received from all Polartechnics staff, the supply of materials and the invaluable discussions have helped me immensely. Their financial assistance to attend an international conference was greatly appreciated. Special thanks goes to Dr Rita Stella, Dr Warren Smith, Dr Richard Thomson and Mr Andrew Bath for helpful discussion, particularly on the operation of TruScan™ and the development of the SUS units.

I would like to thank Jason McArthur, Darren Saunders and Nick Andronicus from the Biological Sciences Department (UoW) for their assistance in performing the radiolabelling work. A big thanks also for their patience as they tried to teach an electrochemist the concepts of protein chemistry.

The spectroscopic work was made possible by the generosity and hospitality of Prof Jim McQuillan from the Chemistry Department at Otago University, Dunedin, New Zealand. Lee Bronkhurst of the Engineering Facility at UoW provided invaluable assistance in obtaining the AFM images presented in this thesis.
I would like to thank everyone at IPRI for their friendship and assistance during my PhD and for the 6 years I have been at IPRI. Especially Dr Michael Davey, Dr Rod Shephard, Dr Andy Minnet, Dr Peter Innis, Dr Chee On Too, Dr Toni Campbell and Prof Leon Kane Maguire. Thanks to Violeta Misoska for her help in acquiring the AFM images.

Thankyou to my whole family who helped me in various ways over the last 4 years. Especially Dad, Mum and Fred, Caroline and Simon, Jan and Tim. Thankyou from the bottom of my heart.

Finally to the most important people, who without, my PhD would have gone by the wayside years ago. Thank you Louise, Aleida and Liam for being there for me. Your love and unwavering support has helped me through the difficult times and kept me focussed.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>A</td>
<td>Amp</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Angular frequency</td>
</tr>
<tr>
<td>$i_a$</td>
<td>Anodic peak current</td>
</tr>
<tr>
<td>$E_a$</td>
<td>Anodic peak potential</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>Attenuated total reflection infrared spectroscopy</td>
</tr>
<tr>
<td>C</td>
<td>Capacitor</td>
</tr>
<tr>
<td>$i_c$</td>
<td>Cathodic peak current</td>
</tr>
<tr>
<td>$E_c$</td>
<td>Cathodic peak potential</td>
</tr>
<tr>
<td>$R_{ct}$</td>
<td>Charge transfer resistance</td>
</tr>
<tr>
<td>Q</td>
<td>Const phase element</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<td>Counts per second</td>
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<tr>
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<td>Curie</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>dps</td>
<td>Disintegrations per second</td>
</tr>
<tr>
<td>$C_{dl}$</td>
<td>Double layer capacitance</td>
</tr>
<tr>
<td>EEC</td>
<td>Electrical equivalent circuit</td>
</tr>
<tr>
<td>EIS</td>
<td>Electrical impedance spectroscopy</td>
</tr>
<tr>
<td>F</td>
<td>Farad</td>
</tr>
<tr>
<td>$V_{fb}$</td>
<td>Flat band potential</td>
</tr>
<tr>
<td>F</td>
<td>Frequency</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>$Z_{im}$</td>
<td>Imaginary impedance</td>
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<tr>
<td>Ig.G</td>
<td>Immunoglobulin G</td>
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<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Z</td>
<td>Impedance</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>Ω</td>
<td>Ohm</td>
</tr>
<tr>
<td>OCP</td>
<td>Open circuit Potential</td>
</tr>
<tr>
<td>R_{ol}</td>
<td>Oxide layer resistance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Z_R</td>
<td>Real impedance</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SUS</td>
<td>Single use sensor</td>
</tr>
<tr>
<td>R_s</td>
<td>Solution resistance</td>
</tr>
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<td>volt</td>
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Abstract

The development of an optoelectric device (TruScan™) for the detection of cervical cancer provides the framework for this thesis. TruScan™ stimulates the cervical tissue with low energy electrical pulses producing a tissue response. This response is recorded at the three electrode tip and the signal is interpreted for a tissue classification. The aim of this work was to measure the extent of protein adsorption onto metal electrodes under various conditions, and then investigate the effects this protein layer had on the electrochemical behaviour measured at the electrode/solution interface.

The adsorption trends of proteins, and the effect that adsorption has on electrochemical responses of metal electrodes has been studied using various experimental techniques. The effect of pH and electrode potential on adsorption trends has been investigated using the proteins human serum albumin (HSA) and human immunoglobulin G (Ig.G) at both gold (Au) and titanium dioxide (TiO₂) metal surfaces.

This work showed that protein adsorbs spontaneously at metal electrodes at neutral pH. The protein adsorption trends followed electrostatic interactions, with the greatest amount of adsorption occurring when the electrode and protein had opposite charges, and the least amount when they possessed the same charge. The extent of protein adsorption also appeared to be influenced by the waveform of the applied potential. Only a small percentage of the total adsorbed protein could be removed. This was attributed to the denaturing of the protein allowing it
to form multiple binding sites, which increased its binding strength. This result lead to the hypothesis of two types of bound protein, namely weakly and strongly bound. Spectroscopic studies showed that the presence of phosphate in the protein solution hinders adsorption, resulting in lower adsorbed amounts. Phosphate was also shown to partially displace preadsorbed protein from a TiO$_2$ surface. The fact that not all of the preadsorbed protein was displaced strengthened the hypothesis of two types of bound protein.

The effect of protein adsorption on the electrochemical behaviour of metal electrodes was studied using electrochemical impedance spectroscopy and cyclic voltammetry. Upon adsorption of protein at Au and TiO$_2$ the capacitance decreased, which was attributed to the formation of a proteinaceous layer on the electrode surface. This decrease in capacitance was accentuated when the electrode surface was treated in such a way that the surface area and roughness increased. The formation of a proteinaceous layer on the electrode surface had the effect of increasing the charge transfer resistance. The resistance to charge transfer was shown to increase as the amount of adsorbed protein increased due to the increase in proteinaceous layer thickness. The electrode/electrolyte interface, for both electrode materials, was successfully modelled using a modified Randles circuit.
Chapter 1 - Introduction
1.1 General Introduction

The purpose of this project was to achieve an understanding of the characteristics of metal electrodes operating in a proteinaceous environment which is used in detecting pre-cancer and cancer of the cervix. This was investigated by considering the factors influencing the transient electrochemical responses obtained at these electrodes in a biological test medium. Protein adsorption trends at metal electrodes under various conditions were determined and the effect that the adsorbed protein has on the electrochemical responses of the metal electrode in biological media investigated.

This work was motivated by the development of a new optoelectronic medical device, developed by Polartechnics Pty Ltd., for the detection of cervical cancer. The device is called TruScan™. The environment in which TruScan™ works means that interaction of the probe electrodes with proteinaceous material is inevitable.

1.2 TruScan™

TruScan™ is currently being developed by Polartechnics Pty Ltd (Sydney-Australia). Polartechnics has been developing TruScan™ for the last 14 years and the device in now nearing commercialisation in some countries [1]. TruScan™ is a computerised medical device designed to screen for cervical pre-cancer and cancer by instantaneously assessing the normality of tissue by measuring the response of the tissue to low energy light and electrical pulses.
In contrast to competing cervical pre-cancer and cancer screening methods, the technology utilised in TruScan™ is based on the physical characteristics of the cervical tissue, such as tissue capacitance, rather than its chemical or visible characteristics, such as visual examination or the response of the non-living tissue to a chemical reagent. With TruScan™ the tissue response to an optical and electrical stimulus is compared algorithmically in real time to that stored in a databank of cervical tissue types. Once a tissue type is matched a result is displayed to the clinical operator. Independent trials last year showed TruScan™ was up to 20% more accurate than the currently employed Pap smear for cervical cancer detection [2].

The conceptual initiation of such a device has its foundations in the pioneering work of Fricke and Morse, who in 1926 conducted a study involving the electrical capacitance measurements of breast tumors [3, 4]. This was followed in 1949 with a study of electrical parameters derived from measurements of cervical tissue by Langman and Burr, who reported a significant difference in cancerous and non-cancerous tissue [3, 5].

The TruScan™ device is shown below in Figure 1.1(a) and consists of two parts, a console and the TruScan™ probe. The console incorporates a control module, a digital signal processor, a power supply, a liquid crystal display screen, a keypad and the TruScan™ probe connector. The TruScan™ probe is about 170 mm in length and tapers down to the tip, which has a diameter of approximately 5 mm.
Situated at the tip are the optical and electrical electrodes which are shown in Figure 1.1(b).

![Image](image_url)

Figure 1.1  (a) TruScan™ device showing the console and probe unit. (b) Schematic of the probe tip showing the three electrodes (A, B, C) surrounding the light emitting and detecting fibres. (Source: http://www.polartechnics.com.au)

The sequence of tissue stimulation, tissue response, response signal detection and signal processing is shown in Figure 1.2. All six stages shown below constitute one cycle and during this cycle the light emitting fibres are activated. During each cycle the electrical measurements are stimulated by the delivery of 0.8V
electrical pulses of 260μs duration. Following removal of the applied electrical potential, the residual charge dissipates within the tissue with a decay constant dependant on the tissue capacitance, the electrode/tissue interface and electronic and ionic conductance [3].

**Figure 1.2** The TruScan™ tissue stimulation and processing. (Source: http://www.polartechnics.com.au)

The electrical pulses are delivered across varying combinations of the three electrodes. In each case one electrode acts as the working electrode whilst the other two are held as the counter electrode. Electrical pulse delivery and the corresponding relaxation curve measurements are continually cycled through all three electrode combinations. The use of three electrodes allows the detection of conditions of asymmetric charge imbalance between the electrodes resulting from poor contact between the tip and cervical tissue caused by operator error [3].
The early version of TruScan™ used gold as the electrode material and was a reusable system, which required thorough cleaning and disinfecting between each patient. This situation was not ideal as cross infection, although very unlikely, was still a possibility due the variation in effectiveness of cleaning from operator to operator. To alleviate this problem Polartechnics developed a single use sensor (SUS), which fits over the TruScan™ probe (Figure 1.3).

Figure 1.3  The single use sensor developed to fit over the TruScan™ probe handle. (Source: http://www.polartechnics.com.au)

The SUS tip contains the metal electrodes with connection being made with the probe by internal contacts. The three electrodes surrounded a clear window to allow the optical sensors to work. The development of the SUS also saw a change in electrode material from gold to the semiconductor, titanium dioxide (TiO₂).
1.3 Electrode Material

1.3.1 Gold

Gold electrodes can either be single crystal, such as Au (100), Au (110), Au (111) or polycrystalline which combine two or more of the single crystal types. The number in brackets refers to their lattice structure. Polycrystalline gold is one of the most commonly used noble metals in the manufacturing of electrodes. Its appeal lies in the very favorable electron-transfer kinetics and a large anodic potential range, however, the low hydrogen overpotential of this electrode limits the cathodic potential window [6]. In acid chloride media the anodic range of gold is severely limited by oxidation of the metal to complex chlorides [7].

The experimentally determined potential limits of gold electrodes in a variety of electrolytes at various pH regularly exceed the theoretical limit determined using equations (1.1) and (1.2). The variation occurs due to the fact that these equations do not take into account the electrode material and specific interactions between the electrode metal and background electrolyte [7].

\[
\text{Reduction of hydrogen ions: } E = -0.059pH \quad (1.1)
\]

\[
\text{Oxidation of water: } E = 1.23 - 0.059pH \quad (1.2)
\]

Surface oxidation of gold introduces complications. While the anodic limit of gold in 1.0M perchloric acid is given to be +1.5V (vs. SCE) [7] Bauman and Shain [8] point out the practical range extends to only +0.8V (vs. SCE) due to interferences by the gold oxide formation. Gold does not sorb hydrogen to any
appreciable extent [6] and after hydrogen evolution gold shows practically none of the hydrogen dissolution current associated with platinum [7].

Gold electrodes have been used extensively in many areas of research such as the development of biosensors [9-12], the study of redox proteins [13, 14] and the investigation of adsorption processes [15-17] to name a few.

1.3.2 Titanium Dioxide (TiO$_2$)

Titanium dioxide is a semiconductor and has been studied as an electrode in electrochemical systems for many years, particularly in its use as an electrode for the conversion of solar energy to electrical energy. Fujishima and Honda [18, 19] were the first to report the continuous conversion of light to current and a chemical reaction using a semiconductor electrode. TiO$_2$ has three naturally occurring crystal phases: rutile, anatase and brookhite. To date, all electrochemical work has been performed on rutile or anatase, or a mixture of both [20]. The electrochemical properties of polycrystalline and single crystal TiO$_2$ electrodes have been investigated by many researchers [21-23].

There are several techniques that can be employed to fabricate TiO$_2$ electrodes, namely, thermally oxidised titanium, sintered compressed powders, sol-gel methods from such material as Degussa P25, and electrochemical oxidation at either sweep potential or constant current or either solid titanium or a sputtered film of titanium.
Electrical properties of TiO$_2$ necessarily refer to the doped form due to the high resistivity of undoped TiO$_2$. TiO$_2$ can be doped by reduction or substitution creating donor levels near the conduction band, and hence the semiconductor is always n-type doped. The term n-type refers to the fact that electrical current is carried predominantly by the negative charge carriers in the conduction band. Doping by reduction can occur either by heating in a vacuum, hydrogen, argon, or a mixture of CO and CO$_2$ or by biasing the electrode negative of the flatband potential ($V_{fb}$). Flatband potential refers to the unique potential of a semiconductor for which the potential drop between the surface and the bulk of the electrode is zero.

The electrochemical properties of TiO$_2$ in aqueous solutions are characterised by charging currents, which are proportional to the space charge capacitance, which diminish for increasing positive potential. The magnitude of the current peaks are proportional to the sweep rate in a linear scan voltammogram, indicating that the redox process is confined to the TiO$_2$ surface and not dependent on mass transfer of a substance from the solution [20]. At a significantly positive potential the small faradaic current characteristic of a depletion layer abruptly rises, and oxygen evolves from the electrode [24-26]. Water reduction is omnipresent at potentials negative of the $V_{fb}$ and is often the dominant process and at potentials biased ca. 0.5V more negative than $V_{fb}$ the TiO$_2$ exhibits a number of altered properties, such as a changed photocurrent spectrum and an increase in the electrode capacitance [27, 28].
1.4 Proteins

The environment in which TruScan™ operates in is one of high biological activity. Surrounding the cervix is a protective layer of cervical mucus, which is a complex mixture, produced continually by the endocervical cells called goblet cells [29]. The major structural components of mucus are mucins – highly glycosylated proteins, the major mass of which are O-linked carbohydrates [30].

Proteins are a large group of nitrogenous compounds of high molecular weight that are essential constituents of all living organisms. The stunning diversity of the thousands of proteins found in nature arises from the intrinsic properties of only 20 commonly occurring amino acids [31]. These aminoacid units are joined together in a precise sequence when the protein is made on a ribosome. The chain is then folded, often into a very compact form [31].

The structure of a typical amino acid is shown below in Figure 1.4. Central to the structure is the tetrahedral alpha-carbon (Cα), which is covalently linked to both the amino group and the carboxyl group. Also bonded to this α-carbon is a hydrogen and a variable side chain (R). These side chain groups fill much of the space in the interior of a protein molecule and also protrude from the external surfaces of the protein where they determine many of the chemical and physical properties of the molecule [31]. It is the side chain that gives each amino acid a particular identity. Amino acids fall into two general classes, polar (hydrophilic) and non-polar (hydrophobic).
Chapter 1 - Introduction

The characteristic linkage in the protein polymer is the peptide (amide) linkage. The formation of a peptide linkage is shown below (1.3). The peptide backbone of a protein consists of a repeated sequence of -N-Cα-C-, where the N represents the amide nitrogen, the Cα is the α-carbon of the amino acid in the polymer chain, and the final C is the carboxyl carbon of the amino acid, which in turn is linked to the amide N of the next amino acid down the line.

\[
\begin{align*}
\text{H}_3\text{H} & \text{H} \text{C} \text{H} \text{C} \text{O} \text{N} \text{H} \text{C} \text{O} \text{O} \\
\text{R} & \text{R}
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{H} & \text{H} \text{C} \text{H} \text{C} \text{O} \text{N} \text{H} \text{C} \text{O} \text{O} \\
\text{R} & \text{R}
\end{align*}
\]

(1.3)

The chain formed by the polymerisation of amino acids, referred to as a polypeptide, constitutes the primary structure of proteins. A typical globular protein (e.g. albumin) contains all of the 20 amino acids. All proteins are made in the same manner but as the growing peptide chains peel off the ribosome, each of the thousands of different proteins in a living cell folds into its own tertiary structure [31]. The highly folded globular proteins vary considerably in the tightness of packing and the amount of internal water of hydration [32, 33]. During the folding process the amino acids, which contain non-polar side chains, are often packed together in the center of the protein with the polar amino acids...
occupying the exterior of the protein. This results in most proteins having a hydrophilic exterior, which shields the hydrophobic interior from the surrounding water.

Upon folding of the polypeptide chain a process called crosslinking occurs. This linking together of two different sections of the peptide chain is extremely important in living beings [34]. One of the most common of crosslinkages is the disulfide bridge, formed when two $-\text{SH}$ groups of the cysteine side chains are close together and are oxidised by $\text{O}_2$ or some other reagent (1.4). It is the degree of crosslinking that gives the protein its stability, and resistance to denaturation.

$$\begin{align*} \text{V} \quad \begin{array}{c} \text{—H}_2\text{C—} \\ \text{S—H} \end{array} + & \begin{array}{c} \text{—H—H} \\ \text{S—C—} \end{array} \rightarrow \begin{array}{c} \text{—H}_2\text{C—} \\ \text{S—S—} \end{array} \text{CH}_2^- \\ \text{O}_2 \end{align*}$$

(1.4)

Denaturing is the process where the protein undergoes an extreme conformational alteration caused by either heating or by treatment with strong acids and bases. Denaturation leads to protein unfolding to form a more random conformation, which in turn exposes the once shielded non-polar (hydrophobic) amino acids. Complete denaturing of a protein was once regarded as an irreversible process until Anfinsen showed that denatured ribonuclease could refold spontaneously [35].

Because proteins contain many acidic and basic amino acids, the properties of proteins are greatly influenced by pH [31]. At low pH, proteins are positively
charged due to the carboxylates, -S$^-$ and imidazole groups accepting protons to become neutral, resulting in an excess of positively charged acidic amino acids remaining. At high pH the protein is negatively charged due to the loss of protons. The point between the high and low pH where the protein charge is zero is called the isoelectric point (pI).

The two proteins used in this study are both globular proteins, and are Human Serum Albumin and human Immunoglobulin G. These proteins are described in more detail below.

1.4.1 Human Serum Albumin (HSA)

Human Serum Albumin (HSA) is a globular protein and, as the name suggests, is part of the albumin family of proteins. HSA has a molecular weight of approximately 66kD and has been described to have a shape of an oblate spheroid with dimensions of 38 x 150Å. Through the use of sophisticated computer graphics it is possible to show the structure of HSA using crystallographic data (Figure 1.5). The wide ribbons are used to show the β strands and the helical turns while narrower ribbons are used for bends and loops of the peptide chains. The arrowheads on the β strands indicate the direction from the N terminus to C terminus. No individual atmos are shown and all side chains are omitted.
Serum albumin is a long-standing acquaintance of the protein chemist who often selects this readily available peptide as a model for physical or chemical studies [36]. HSA has been characterised to be acidic, very soluble and have a high stability with an isoelectric point at pH = 4.6. Albumins' stability, or resistance to denaturing, has long been recognized and withstands heating to 60°C for 10hrs and retains its structure at pH = 3-11 [37, 38].

Albumins are characterised by a low content of tryptophan and methionine and a high content of cysteine, which results in a high degree of crosslinking, hence its relatively high stability. They also contain charged amino acids, aspartic and
glutamic acids, lysine, and arginine giving the molecule a net negative charge at neutral pH. HSA is not uniformly charged throughout its length, rather is has three domains each exhibiting different charges. The amino terminal of HSA is highly negative and the carboxyl terminal is nearly neutral.

Due to HSA being well characterised and a commonly available protein it was used in this work to validate the effectiveness of several experimental techniques in investigating the adsorption behavior of protein at metal electrode surfaces.

1.4.2 Immunoglobulin G (Ig.G)

Immunoglobulin G (Ig.G) is also a globular protein with a molecular weight of approximately 156kD, which falls into the glycoprotein family, and is one of the antibodies found in cervical mucus. A glycoprotein as described by Metzler [31] is a protein, including the ones secreted from living cells and many that are components of cell surfaces, which carry covalently attached oligosaccharides. Spiegelberg [39] described human immunoglobulins as a group of structurally and functionally similar glycoproteins that confer humoral immunity in man. In short, immunoglobulins are antibody proteins which are present in fluid (mucus) excreted from living cells.

The immunoglobulin (Ig) protein “backbone” consists of heavy and light chains, terms based on their relative size. There are five types of heavy chains, which distinguish the class of immunoglobulins Ig.M, Ig.G, Ig.D, Ig.A and Ig.E, and two types of light chains [40]. The shape of all Ig’s is that of a “Y” (Figure 1.6).
The protein consists of two heavy chains and two light chains connected by four disulphide (SS) bonds. The two adjacent disulphide bonds linking the two heavy chains are located in the so-called hinge position. The $F_{ab}$ region is where antigen attachment occurs while the constant $F_c$ region interacts with effector molecules such as complementary proteins and $F_c$ receptors [41].

![Figure 1.6 Schematic picture of human Ig.G showing the two ( ) light and ( ) heavy chains. The $F_{ab}$ and $F_c$ regions are also shown.](image)

Ig.G was the first immunoglobulin to be discovered back in 1939 by Tiselius and Kabal [42]. Within the Ig.G protein there have been four subclasses identified, labelled 1, 2, 3, and 4 by the World Heath Organisation (WHO) [43], based on their relative concentration in normal serum and their frequency of occurrence as myeloma proteins. Each subclass is referred to as a monoclonal antibody while an Ig.G that contains all four subclasses is referred to as a polyclonal antibody.

1.5 Proteins at Interfaces

A significant proportion of biological processes occur at interfaces. The predominant interface in biological systems is that between an aqueous solution
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and a cell membrane. Adsorption/desorption processes and surface reactions take place at this surface [44]. Interfacial electrochemical processes take place on both, electrode surfaces and, between redox systems on the two sides of a phase boundary. Given the fraction of biological processes which proceed at interfaces, it is desirable to gain an adequate understanding of basic interfacial science, including adsorption and interfacial electrochemical processes.

The study of the adsorption of proteins at solid surfaces has attracted a great deal of interest in the last few decades with notable reviews by Haynes and Norde [45-48], MacRitchie [49] and Andrade and Hlady [50]. Often, this interest originates from the importance of the interfacial behaviour of proteins in a variety of applications in medicine, biotechnology, diagnostics, and food technology [51]. For example, medical scientists, pathologists and biomedical engineers have determined that thrombosis development on cardiovascular implant materials is intimately related to protein adsorption processes involving fibrinogen, Factor 11 and Factor 12, high molecular weight kininogen, and possibly a number of other plasma proteins [52-54]. This undesirable medical effect is contrary to recent applications of controlled protein adsorption in the development of drug delivery systems [55].

An important result of these rich and diverse research efforts has been the steady accumulation of new experimental and theoretical strategies for studying protein adsorption [45]. Of primary interest is the kinetics of adsorption, adsorbed amount, conformation of adsorbed protein and activity of the adsorbed protein.
The measurement of the adsorption kinetics and adsorbed amounts of protein require high accuracy since the amount of adsorbed protein are usually very low [56]. Examples of techniques that can be used for the measurement of adsorbed amounts and conformational changes upon adsorption are shown in Table 1.1 accompanied with references of research articles published in each technique. It must be noted that to gain a clear understanding of the forces at work in protein adsorption it is essential that several techniques be employed, as one single technique will not reveal all of the factors involved.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Ads Amount</th>
<th>Conf Change</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioisotope labelling</td>
<td>Y</td>
<td>N</td>
<td>[57, 58]</td>
</tr>
<tr>
<td>Quartz crystal microbalance (QCM)</td>
<td>Y</td>
<td>N</td>
<td>[59-62]</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA),</td>
<td>Y</td>
<td>N</td>
<td>[58, 63, 64]</td>
</tr>
<tr>
<td>Total internal reflection fluorescence (TIRF)</td>
<td>Y</td>
<td>Y</td>
<td>[63-68]</td>
</tr>
<tr>
<td>Atomic force microscopy (AFM)</td>
<td>Y</td>
<td>N</td>
<td>[69-71]</td>
</tr>
<tr>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
<td>Y</td>
<td>Y</td>
<td>[72-74]</td>
</tr>
<tr>
<td>Attenuated total reflectance infrared (ATR-IR)</td>
<td>Y</td>
<td>Y</td>
<td>[75-79]</td>
</tr>
<tr>
<td>Ellipsometry</td>
<td>Y</td>
<td>N</td>
<td>[80-82]</td>
</tr>
<tr>
<td>Circular Dichroism (CD)</td>
<td>N</td>
<td>Y</td>
<td>[74, 83-85]</td>
</tr>
<tr>
<td>Dynamic Contact Angle</td>
<td>Y</td>
<td>N</td>
<td>[86-88]</td>
</tr>
<tr>
<td>Inverse Chromatography</td>
<td>Y</td>
<td>N</td>
<td>[89, 90]</td>
</tr>
</tbody>
</table>

Table 1.1  Techniques used to investigate protein adsorption behaviour. 
Ads Amount = Adsorbed Amount; Conf Change = Conformation Change.
The way in which the protein interacts with the solid surface is dependent on many factors, such as, protein net charge, the charge on the substrate, protein size and the occurrence of hydrophobic domains in the protein surface to mention a few. However there are few general rules as to the effects of surface charge, hydrophobicity, etc., on protein adsorption [56, 91-96].

1.5.1 Influence of Charge

In terms of their electrostatic properties, solid substrates can be categorized as (1) uncharged surfaces, (2) surfaces where the charge is determined by ions other than protons, (3) surfaces that contain strongly acidic or basic groups, which titrate at extreme pH values, or (4) surfaces with charged groups that protonate at moderate pH values [45]. Surfaces (1) and (2) are ideal for protein adsorption studies as the surface charge is not affected by the protein pH and therefore the coulomb interactions on protein adsorption are less ambiguous.

Protein adsorption at a charged surface involves the overlap of the electrical double layer at the solvated surface and the solvated protein surface [45]. It has already been mentioned that by varying the protein pH it is possible to vary the overall protein net charge. If these two surfaces have opposite charges then electrostatic attraction results or repulsion if the charges are the same. This was clearly demonstrated by Norde and coworkers [45, 46, 97] who presented isotherms showing the adsorption of several proteins at pH = 7.0 onto negatively and positively charged polystyrene beads. In general, the results showed that for those proteins carrying a negative charge maximum adsorption was observed on
the positively charge polystyrene and vice versa for the positively charged proteins. This suggests that electrostatic interactions are the driving force behind adsorption, however, some results in the above study did not agree with this trend indicating that while global electrostatic forces undoubtedly affect adsorption, they do not dominate them.

1.5.2 Hydrophobicity

As mentioned above protein surface hydrophobicity is influential in protein adsorption. With respect to hydrophobicity the state of the substrate and protein must be taken into account. Due to the complex nature of proteins it is not possible to simply label one protein hydrophobic and another hydrophilic. The true picture of a protein is one with regions of hydrophobicity and regions of hydrophilicity dispersed throughout the surface, this is also true for most solid surfaces.

Most proteins tend to adsorb more extensively at hydrophobic than hydrophilic surfaces [45, 98]. Malmsten [99] was able to show using protein-protein exchange experiments that more limited exchange occurred at hydrophobic surfaces than at hydrophilic surfaces, which suggests that, proteins tend to adsorb more strongly at hydrophobic surfaces. These effects are most likely the result of differences in conformation of the adsorbed protein at the respective surface.

Regnier [100] and Gorbunov [101] et al have provided an excellent review on the effect of protein hydrophobicity on adsorption using studies related to
hydrophobic interaction chromatography. van Oss [102] used contact angle measurements to show a strong correlation between protein surface hydrophobicity and the driving force for adsorption. Haynes and Norde suggested that an alternative approach to understanding the influence of protein surface hydrophobicity on adsorption behaviour was to correlate surface hydrophobicity with adsorbed amounts (isotherm plateau values - $\Gamma^{pl}$). When this was done for several similar sized proteins on negatively charged polystyrene (PS) the $\Gamma^{pl}$ increased with increasing surface hydrophobicity, suggesting that the driving force is related to the protein surface hydrophobicity. Unfortunately one protein ($\alpha$-lactalbumin) deviated greatly from the correlation, indicating that another factor governs its adsorption behavior on PS. $\alpha$-Lactalbumin has low structural stability compared to the other protein used in the study [45], therefore structural rearrangement may dominate its adsorption behavior.

1.5.3 Influence of Protein Structural Stability

The stability of proteins in an aqueous environment varies from protein to protein. Globular proteins in an aqueous environment for example can be denatured by a modest change in environment, such as an increase in temperature, change in solution pH or the introduction of a foreign surface or interface. Conformational stability is important for determining the state of adsorption of a protein, and notably the adsorbed layer structure and adsorption strength; however, the stability of a protein may also affect the extent of adsorption [96].
In the native state the protein conformation is quite restricted, and hence the conformational entropy is low. When proteins undergo denaturing upon adsorption, its conformation changes and frequently loses a fraction of their overall structure (e.g., the content of the α-helix and β-sheet decreases). Therefore, the adsorption process may be associated with a conformational entropy gain, which in principle can act as an adsorption driving force [45, 98]. Proteins with low internal stability such as α-lactalbumin, β-casein, hemoglobin and catalase generally tend to adsorb on all surfaces irrespective of the electrostatic interactions, owing to a gain in conformational entropy resulting from adsorption [103, 104]. Upon adsorption on hydrophobic surfaces, these proteins change their conformation to a great extent.

Vroman was one of the first to recognize that proteins denature at solid surfaces. He stated [105] that “those (globular proteins) which can open easily will do so when they see a hydrophobic surface, and will turn themselves inside out to paste their fatty hearts onto that surface”. Carrying on from Vroman’s earlier observation many researchers have studied the denaturing process of proteins upon adsorption. Norde [46] performed extensive thermodynamic studies on the adsorption of HSA and bovine pancreas ribonuclease on polystyrene surfaces to confirm Vroman’s hypothesis. Norde and Favier [106] were able to show, using transmission circular dichroism, that the extent of α-helix breakdown increases as the protein native structure stability decreased. Furthermore Buijs [107] et al used ATR-FTIR to show that the adsorbed amounts of Ig.G decreased as the β-
sheet content was reduced upon adsorption. This reduction in \( \beta \)-sheet content was attributed to a slow conformational change upon adsorption. However, not all proteins that adsorbed undergo conformation changes, which leads to the conclusion that while protein structural rearrangement contributes to protein adsorption, it is not the sole contributor.

It is clear that the driving force for protein adsorption is very complex involving contributions from hydrophobic interactions, electrostatic interactions between oppositely charged surfaces, proteins or protein domains and conformational change of the adsorbed protein to name a few.

Much of the work performed in the area of protein interfacial behaviour has been concerned with adsorption trends and the effect various conditions have on these trends. Due to the complex nature of even the simplest proteins, investigation into the mechanism of protein binding (i.e., binding site chemistry) has been difficult. A way in which many researchers overcome this difficulty is to look at the binding mechanism of simpler amino acids and some small peptides. The amino acid compositions and/or sequences of the peptide regions might be determinant for the adsorption behaviour of proteins on solid surfaces [56].

The adsorption of peptides on solid surfaces has been investigated in connection with the formation mechanism of various solid surfaces. The \textit{in-situ} infrared spectroscopic investigation of the adsorption of lysine and polylysine to TiO\(_2\) performed by Roddick-Lanzilotta and McQuillan [78] found that the carboxylate
group was involved in the peptide/TiO$_2$ interaction. Recently efforts have been made to screen peptides that have a high affinity to solid surfaces. The chemical structures of such peptides provide fundamental information on the adsorption mechanism of peptides and proteins. One way of doing this is to synthesise various peptides with different amino acid sequences to compare their adsorption affinity. Whaley et al found by doing this that the peptides with high adsorption affinity were found to contain serin- (I) and threonine-rich (II) regions in addition to the presence of asparagine (III) and glutamine (IV) residues.

\[
\begin{align*}
\text{(I)} & \quad \text{OH} \quad \text{H} \quad \text{R} \quad \text{C} - \text{OH} \\
\text{(II)} & \quad \text{H} \quad \text{R} \quad \text{C} - \text{CH}_3 \\
\text{(III)} & \quad \text{H} \quad \text{R} \quad \text{C} - \text{N} - \text{H} \\
\text{(IV)} & \quad \text{R} \quad \text{C} - \text{C} - \text{H} - \text{O} - \text{N} - \text{H} \\
\end{align*}
\]

\[ R = \begin{array}{c}
\text{OOC} \\
\text{+H}_3\text{N} \\
\text{C} - \text{H}
\end{array} \]

The adsorption of amino acids on metal surfaces has been investigated since the 1980s mainly for the fundamental understanding of the biocompatibility of artificial implant materials [56]. Liedberg et al [108] found using glycine that upon adsorption to a gold surface it was oriented with an $-\text{NH}_3^+$ group close to the surface and with a $-\text{COO}^-$ group further away. When adsorption of glycine occurred at a copper surface Uvdal et al [109] and Ihs et al [110] found that both the amino and carboxyl regions were orientated close to the metal surface. Imamura et al [111] and Roddick-Lanzilotta et al [79] studied the adsorption of amino acids on stainless steel and TiO$_2$ respectively. Their findings agreed and
showed that adsorption occurred by the interaction between two dissociated carboxyl groups and the surface metal ions.

While investigation of the adsorption mechanisms for amino acids and peptide goes a long way towards elucidation of protein adsorption mechanisms, the amount of published results is rather limited and further work is required in this area to aid in understanding and controlling adsorption of proteins.

1.5.4 Protein Adsorption and Electrochemistry

Protein adsorption and electrochemistry has long been associated with the development of biosensors. The most famous of these being the glucose biosensor developed from pioneering work by Clarke and Lyons some 40 years ago for the treatment of diabetes [112]. The deliberate entrapment of DNA, RNA and oligonucleotides has also been performed for the development of a polypyrrole based electrochemical biosensor [113]. In more recent times electrochemistry has been the technique of choice for many researchers investigating protein adsorption trends. Many studies have provided a valuable insight into the effects that protein adsorption has on electrochemical responses of various electrode material.

Electrochemical methods are frequently applied for analysis of inorganic substances such as electroactive metallic ions and low weight organic and organometallic compounds [95]. The sensitivity of these methods for a substance depends on the possibility of electron transfer between the electrode and the
substance. The possibility of electron transfer depends on both the material and potential of the electrode and on the character of the substance. Interpretation of electrochemical data from proteins is very complex due to the mobility of the electroactive group in a protein being hindered by its bonds to the surrounding organic framework. Electron transfer reactions may occur at an electrode in a protein solution if electroactive groups are present within the protein moiety and if these groups lie close enough to the electrode surface for a charge transfer [95].

Electrochemical methods reveal charge transfer processes occurring within a very narrow zone close to the electrode surface, the electrode/electrolyte interface. Experimentally, this interface region has been shown to behave similar to a capacitor, which implies a charge separation at the electrode/electrolyte interface. The most accepted model for this charge distribution is a double layer structure [114], which is described in Figure 1.7. This double layer structure suggests three ionic zones to be formed on the solution side as a compensation for the excess of charge, of either positive or negative sign, within the electrode. The layer of ions closest to the electrode surface is called the inner Helmholtz layer and contains water molecules and specifically adsorbed ions that are not strongly hydrated. The plane passing through the centers of these ions is called the inner Helmholtz plane (IHP). Fully solvated ions, referred to as nonspecifically adsorbed ions, are separated from the IHP with the locus of centers of the nearest solvated ions in the outer Helmholtz plane (OHP). The forces binding the ions in the IHP depends on the nature of the ions as well as the
potential of the electrode, while the ions in the OHP are attracted by the electric field from the ions in the IHP, and/or the charge on the electrode [95].

Proteins are long chain molecules, which carry ionic side groups, and in an aqueous solution, in general, will turn its polar residues towards its aqueous surrounding. These polar residues possess charge and dipole moments creating a double layer around the protein molecule. Electrons are able to be passed through the double layers of the protein and electrode. Kuznetsov et al [115] proposed a

Figure 1.7 Schematic of the electrical double layer at a metal/electrolyte interface with an adsorbed protein shown. Water molecules shown both individual, where the arrow symbolises the dipole moment and as a structured hydration layer. IHP and OHP are the inner and outer Helmholtz planes, respectively and $\overset{\circ}{S}$ represents the disulfide bond [95].
model where electron transfer between an electroactive group of a protein and a metal electrode calls for an overlap of the double layers at the protein molecule and electrode surface, respectively, and that the electroactive group is present in the overlap region. Therefore, based on this model, the compact double layer zone is the sensitive zone for electrode processes in voltammetry.

However, the interaction of so called non-electroactive proteins at interfaces can also be studied using electrochemical techniques. It is possible to study electron transfer properties of a protein solution that contain electroactive species such as potassium ferricyanide that are not attached to the protein molecule. It is also possible to apply a polarizing potential to an electrode in a non-electroactive protein solution to induce electron transfer through the formation of reduction processes, such as the reduction of dissolved oxygen or water.

Phillips *et al* [116] used electrochemical techniques, namely electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) to study the adsorption of yeast alcohol dehydrogenase at a platinum electrode. They found that the surface charge density and corresponding polarisation resistance, resulting from protein adsorption and its oxidation, respectively, are directly proportional to the amount of adsorbed protein, indicating that the adsorption at anodic potential is accompanied by the transfer of charge, that is, chemisorption through carboxylate groups on the protein. Omanovic *et al* [117] found, using electrochemical impedance spectroscopy, that the adsorption of bovine serum albumin on stainless steel increased the dissolution rate of the metal. The
adsorption of the amino acids, serin (Ser), tyrosine (Tyr) and histidine (His) was studied by Slojkowska et al [118] using EIS with the results indicating that adsorption of amino acids on the gold electrode decrease in order, Tyr > His > Ser.

Roscoe et al have used various electrochemical techniques, such as cyclic voltammetry and electrochemical impedance spectroscopy, to study the interfacial behavior of proteins such as β-lactalbumin [119-121], α-lactalbumin [122], ribonuclease [119, 120], lysozyme [119, 120], BSA [122], and hemoglobin [123] at a platinum surface. Oliva et al [124] used electrochemical impedance spectroscopy to study the interaction of HSA at a TiO₂ nanocrystalline electrode, while Jackson et al [125] studied the adsorption behavior of fibrinogen, a fibrous protein, at a commercially pure titanium surface.

The studies listed above were able to show trends in adsorbed amounts of each protein as well as the extent of protein denaturing upon adsorption, with their results agreeing very well with those obtained by others using non-electrochemical techniques. The above studies were also used to elucidate the changes in the electrochemical properties of the electrode due to adsorbed protein. In all cases it was found, that upon adsorption of protein, the electron transfer rates occurring between the electrode surface and electrolyte are severely hindered. This effect was also clearly demonstrated by the work performed by Guo et al [126] using cyclic voltammetry at a gold disc, platinum and glassy
carbon electrode in potassium ferricyanide before and after adsorption of serum albumin. They found that as protein was adsorbed, the formation of the inert protein layer caused a decrease in anodic peak current and an associated oxidation/reduction potential shift resulting in a decrease in the electron transfer in the redox reaction of Fe(CN)$_6^{4-}$/Fe(CN)$_6^{3-}$ ions at the electrode surface.

1.6 Aims of this Work

TruScan™ identifies cancerous tissue in the cervix by detecting variations in electrical signals, in particular capacitance, generated in cervical tissue as a result of external electrical stimulation. It is therefore critical to elucidate any effect biofouling (protein adsorption) has on the TruScan™ probe electrodes sensitivity in detecting these signal variations. The main aim of this work was to investigate the electrochemical behaviour of metal electrodes in biological solutions using a variety of experimental techniques. Two metal electrodes were used, namely, gold and titanium dioxide, while the proteins used were human serum albumin and immunoglobulin G.

This main aim was further divided into two areas with the first one being to monitor the adsorption trends of two proteins at metal electrodes under various experimental conditions. Protein adsorption trends were studied from buffered solutions of various pH using non-electrochemical techniques such as quartz crystal microbalance, radiolabelling and attenuated total internal infrared spectroscopy. The effect of electrode potential on the adsorption trends was also investigated.
The second area involved the investigation of the effects the adsorbed protein had on the electrochemical behaviour of the metal electrodes. Electrochemical responses were studied at various electrode potentials using the electrochemical techniques cyclic voltammetry and electrochemical impedance spectroscopy.

Since TruScan initially was manufactured using gold (Au) as the electrode material followed by the use of titanium dioxide (TiO₂), these two metals were used as the test electrode material for all of the work presented in this thesis. Two model proteins were used to foul the electrode surface, namely human serum albumin (HSA) and immunoglobulin G (Ig.G). These proteins were chosen since HSA is a protein which has been well characterised over the years and is a common protein used in these types of experiments. Ig.G was chosen as this antibody is found in cervical mucus which covers the cervix and is therefore expected to play a role in the fouling of TruScan™ during operation.
References


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Chapter 2

Experimental Techniques and Procedures
2.1 Quartz Crystal Microbalance

In 1957 Sauerbrey showed that the piezoelectric effect of a crystal could be used to monitor mass changes [1]. Sauerbrey for the first time was able to show a relationship between mass loading or removal and the associated change in the crystals' resonant frequency. The change in frequency of the crystal can be converted to mass by considering the Sauerbrey equation (2.1).

\[
\Delta m(ng) = \frac{\Delta f \times A}{2 \times 10^6 \times f_0^2}
\]  

(2.1)

The Sauerbrey equation is the most useful formula for QCM/EQCM where \( \Delta f \) is the measured frequency shift and \( \Delta m \) is the mass of deposited material (g). \( f_0 \) is the fundamental frequency of the crystal (Hz) and \( A \) is the electrode area (cm\(^2\)). For an AT-cut 10MHz crystal, the mass sensitivity per unit area is 0.226 Hz cm\(^2\) ng\(^{-1}\), thus a crystal having a projected area of 0.25cm\(^2\) should have an absolute mass sensitivity of 0.0565 Hz ng\(^{-1}\).

2.1.1 Experimental

The Quartz Crystal Microbalance (QCM) used was a commercially available ELCHEMA\textsuperscript{TM} EQCN 701-702 nanobalance and faraday cage, which was interfaced with an Apple Macintosh computer using a MacLab/4e/interface (AD Instruments). The gold quartz crystals consisted of a quartz wafer coated on both sides with 1000Å 99% purity gold with a total surface area of 0.196cm\(^2\) per side. The gold regions are connected using fine wire which are, in turn, connected to
the crystal contacts. A MacLab Micropump™ controlled by Chart v3.1.5 software was used to deliver the protein solution to the QCM cell.

The gold crystal electrode was attached to the QCM three-electrode cell, placed inside the faraday cage, and connected to the commercial QCM. The three electrode cell and holder were designed and built in the mechanical workshop at the University of Wollongong. The cell was filled with 5mL of pH = 7.0 PBS and allowed to equilibrate for a period of 30min, ensuring a steady baseline. At this point the Micropump was activated delivering a quantity of protein to the cell. Gold was the only electrode used for this work while HSA was the only protein used that had a pH = 7.0. The HSA concentration investigated was 100µg mL⁻¹. The adsorption behaviour of the HSA was investigated at various applied potentials.

2.2 Radiolabelling

Radiolabelling has long been an efficient and relatively easy way to study many biological systems. Iodine 125 (¹²⁵I) has a sixty day half-life making it ideal as a radio label for adsorption experiments. ¹²⁵I is a low energy gamma emitter and its radioactivity can easily be measured using a gamma counter. When used with proteins the iodine attaches adjacent to the hydroxyl on the free tyrosine sections following the reaction (2.2) mechanism.
Once the protein has successfully been labelled it is possible to calculate the amount of radiation per mass of protein being emitted in units of, $\mu$Ci $\mu$g$^{-1}$ (see below). The unit Ci is a Curie which is a measure used to describe the amount of radioactivity in a sample of material. This measure of radiation per mass of protein is called the specific activity of the labelled protein and is used to calculate the mass of adsorbed protein. This is done by measuring the radioactivity of the sample after protein adsorption ($\gamma$-counts per second - cps) and converting this value into Ci (see below). A high precision of the count rate can be attained by rigorous shielding and counting over long periods, making radiolabelling one of the most reliable methods for determining adsorption [2].

It is essential that the labelling process does not alter the chemical properties of the protein, since altering the chemical properties has a marked effect on the adsorption characteristics. This point was not lost on Ramsden [2] or Soderquist [3] who stated that the general disadvantage of the labelling procedure is that the properties of the protein, especially those relevant to transport and adhesion, are likely to be significantly altered by the addition of such groups. However, modern day techniques in protein labelling virtually eliminate structural and chemical changes brought about by the addition of the radiotracer [4].
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2.2.1 Experimental

All experiments took place in a controlled “hot” laboratory located in the Biological Sciences building at the University of Wollongong and were performed behind a lead impregnated Perspex shield set up in a fume hood. A stock solution of 500μg mL⁻¹ of protein was prepared using 0.2M PBS (pH = 7.0). Concentrated HCl and NaOH were added to the protein solution to prepare the pH = 3.0 and pH = 9.0 solutions. Each solution was made up to contain 0.15M NaCl.

\[ ^{125}I \text{ Labelling Protocol} \]

The standard protocol (adjusted to suit required concentrations) for attachment of \(^{125}\text{I}\) to proteins as set out by Pierce Ltd. is as follows,

1. Wash and dry three IODO-BEADS\textsuperscript{®} on filter paper using 100mM PBS. Place beads along with 500μL of 100mM PBS into an Eppendorf vial.

2. Add 2.0μL of \(^{125}\text{I}\) to Eppendorf and leave for 5 min.

3. Add 800μL of stock protein to Eppendorf and leave for 15 min.

4. Remove solution from Eppendorf to stop reaction and place in a second Eppendorf, discard IODO-BEADS\textsuperscript{®} in the appropriate manner for radioactive waste.

5. Condition a PD-10 gel filtration column by passing 5.0mL of 20mM PBS through it, discard PBS to waste.

6. Pass labelled protein through the PD-10 column and collect the fraction in an Eppendorf vial.
7. Pass 10 x 1.0ml portions of 20mM PBS through the column and collect each fraction in a separate Eppendorf vial.

8. Place 2.0μL of each fraction onto a separate piece of filter paper, place the filter paper into glass tubes and measure the radioactivity using a gamma counter.

9. Record the counts per minute (cpm) over a 10 min period. Convert the cpm to counts per second (cps) and plot these values for each fraction.

10. Calculate the specific activity of the labelled HSA (see below for details).

**Specific Activity Calculations**

The plot of cps versus fraction should exhibit two distinct peaks, the first one due to the labelled protein and the second due to the free $^{125}$I. Determine which fractions are responsible for the protein peak and add all the cps values together, this gives a total cps for all of the labelled protein used in those fractions. For example, if fraction 3, 4, 5 and 6 contributed to the protein peak then adding all the cps will give a total cps for 8.0μL of labelled protein (2.0μL of each fraction was placed onto the filter paper which was then measured to produce the cps – step 8). Once all the above data is gathered the following calculations can be done.

Total Protein (cps in μL) = β $(2.3)$

(μL in the above paragraph is 8.0μL)

Total Protein (cps mL$^{-1}$) = $\left( \frac{β}{μL} \right) \times 1000 = δ \quad (2.4)$
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Total Protein (cps \( \mu g^{-1} \)) = \frac{\delta}{ppm_{pr}} \quad (2.5)

Where \( ppm_{pr} \) is the concentration of the protein used in the labelling process in \( \mu g \ mL^{-1} \).

Strictly speaking gamma counters measure disintegrations per sec (dps) rather than cps, however the measured cps can be converted into dps using equation (2.6). Where \( \text{eff}_{\gamma-counter} \) is the efficiency of the gamma counter for the radiolabel of interest:

\[
dps = \frac{cps}{\text{eff}_{\gamma-counter}} \quad (2.6)
\]

Knowing that \( 3.7 \times 10^4 \) dps equals \( 1 \muCi \) (Ci = Curie) it is possible calculate the specific activity of the labelled HSA (2.7):

\[
\text{Specific activity} = \frac{dps}{3.7 \times 10^4} \ (\muCi \ \mu g^{-1}) \quad (2.7)
\]

Specific activity is a measure of the radiation per mass of labelled protein. Therefore, when an experimental measurement is made and the resulting cps are converted to \( \muCi \) then the amount of labelled protein present can be calculated using the specific activity. The specific activity of a labelled protein solution remains the same regardless of the amount of the labelled protein used for adsorption experiments.
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**Decay Correction**

$^{125}\text{I}$ has a half-life of 60 days, it is therefore necessary to correct for decay with time. When the $^{125}\text{I}$ is ordered the supplier provides a table presenting the rate of decay. Equation (2.8) is used to correct for decay, where DF is the decay factor provided by the supplier.

$$c_{\text{PS corrected}} = \frac{c_{\text{PS}}}{DF} \quad (2.8)$$

**Adsorption**

When the iodination of the protein was complete and the specific activity calculated, adsorption experiments were performed. Various amounts of labelled (hot) and unlabelled (cold) protein were combined to produce final concentrations of hot to cold protein of a 1:100ppm ratio.

The gold electrode used for this work was gold Mylar sheeting while the TiO$_2$ electrode was a Ti coated polycarbonate sheet, which had been electrochemically oxidised. The gold and TiO$_2$ samples had an area of 0.50cm$^2$. The gold samples were cleaned in methanol, then in water and dried in a 60ºC oven for 5min to remove any surface impurities. No pretreatment of the TiO$_2$ samples was performed. The samples were placed in the protein solution for various lengths of time, during which various external potentials were applied. The potentials were applied using a BAS CV-27 voltammograph. The adsorption at an applied potential was performed in a three electrode cell.

A 1480 Wizard “3” Automated Gamma Counter was used to measure the cps which operates at an efficiency for $^{125}\text{I}$ of 82%. Due to time constraints resulting
from the need to conduct the experiments and cps measurements in the same day for \(^{125}\text{I}\) decay reasons, each experiment was only performed in duplicate. The adsorbed amount results are presented as an average of the duplicate measurements. The error bars show the range of data as an indicator of the measurement error in the experiments.

Each gold and TiO\(_2\) sample was placed in an Eppendorf vial and 200\(\mu\)L of the 1:100 protein solution was added. After the desired exposure time the sample was removed and rinsed in 0.2M PBS followed by Milli-Q water, and then left to dry at room temperature. The sample was transferred to a scintillation tube for counting in the gamma counter for 10 min.

EChem v1.3.2 software was used to apply the pulse potential. For the pulsed potential and various pH experiments the above procedure of adsorption, counting and calculating adsorbed amount was also used. The pulsed experiments were only performed at pH = 7.0, with the potential being pulsed on for one second and off for one second between 0.0V and a potential \(E_1\) (Figure 2.1). The \(E_1\) potentials used were 700mV and -800mV. The adsorption time for both the pulsed and pH experiments was 30 min.
Figure 2.1  Square wave pulse routine used for adsorption.

To study the effect of cleaning to remove adsorbed protein, both a chemical and mechanical cleaning procedure was used. The adsorption and cps measurements outlined above were used for this work.

2.3 Electrochemical Impedance Spectroscopy (EIS)

EIS uses a range of low magnitude polarization voltages, much like linear polarization. However, EIS voltages cycle from peak anodic to cathodic magnitudes using a spectrum of alternating current (AC) voltage frequency. Resistance and capacitance values are obtained for each frequency, and these quantities can provide information on electrode processes such as charge transfer rates and diffusion. EIS polarisation voltage amplitudes typically range from 5 to 20mV and are usually centered around open circuit potential or a set DC voltage. Voltage frequencies used for EIS can range from 100kHz to several mHz.
A charged surface in contact with an electrolyte solution is expected to attract ions of opposite charge and repel ions of like charge. Two parallel layers of charge are formed – the charge on the surface itself and the layer of oppositely charged ions near the surface, together they form the electric double layer. In the Gouy-Stern model \([5, 6]\) the sorbent/solution boundary is set at a hypothetical surface, \(x = 0\), containing all sorbent surface charge. In a system containing no specifically adsorbed ions, there is an ion-free layer adjacent to the sorbent surface which extends to \(x = d\), where \(d\) is the distance of closest approach of a hydrated ion to the sorbent surface.

An electrical double layer can be described to have characteristics similar to a simple electrical circuit. This circuit is commonly referred to as the Randles circuit and is depicted in Figure 2.2. \(R_s\) is the electrolyte solution resistance (ohms), \(C\) is the double layer capacitor (farads) and \(R_{ct}\) is the charge transfer resistance (ohms).

\[
\begin{array}{c}
-R_s \quad C \\
R_{ct}
\end{array}
\]

Figure 2.2  Electrical circuit used to describe the double layer.

AC and DC current and voltage are vectors because they have both magnitude and direction. Consequently, impedance is also a vector because it is AC voltage divided by current. An impedance vector can be resolved into component vectors as shown in Figure 2.3.
Total impedance for a circuit like that shown above is:

\[
Z = R_1 + \frac{R_2}{1 + (\omega R_2 C)^2} + \frac{j\omega R_2^2 C}{1 + (\omega R_2 C)^2}
\]  

(2.9)

where:

- \(Z\) is total impedance in ohms
- \(R_1\) and \(R_2\) are resistors in circuit 1 (ohms) (\(R_s\) and \(R_{ct}\) respectively)
- \(C\) is the capacitor capacitance in Farads
- \(\omega = 2\pi\) (AC voltage frequency)
- \(j\) is the square root of \(-1\), an imaginary number.

The magnitude of the X-component in Figure 2.3 is equal to the second term in equation (2.9) and is referred to as the real impedance. The third term of equation (2.9) is equal to the Y-component magnitude and is referred to as the imaginary impedance due to the fact that this term is multiplied by the imaginary number, \(j\).

Equation (2.9) illustrates that each polarizing voltage frequency produces a different magnitude for phase angle, total impedance, and the component vectors.
Impedance data can be presented in various ways, with the most common graphs being, Nyquist, Bode Z and Bode phase angle. The Nyquist plot is formed by plotting the real impedance against the imaginary impedance. From the plot it is possible to calculate solution resistance as well as the charge transfer resistance. The shape of this plot also helps to elucidate the electrode process occurring at the particular dc polarisation voltage. The Bode Z and phase angle plots are formed by plotting the logarithm of frequency against the absolute impedance and phase angle, respectively. These graphs also aid in elucidating electrode processes. Another graph, which is not as commonly used, is the capacitance plot, where the real capacitance is plotted against the imaginary capacitance. From this plot the electrode total capacitance can be calculated.

2.3.1 Experimental

All impedance measurements were made in a three-electrode cell using a Princeton Applied Research BES Impedance system, utilizing a PAR 263 Potentiostat plus 5210 lock-in amplifier. The impedance spectra were recorded using PowerSine™ V. 2.0 software between 100kHz and 50mHz with an AC amplitude of 10mV.

Prior to every impedance measurement the gold electrode was polished on a 0.45μm alumina slurry for 3-5min, followed by sonication in Milli-Q water for 2min. No polishing was performed for the TiO₂ electrode. The gold electrode was roughened (Results Chapter 4: section 4.2.4.3) by polishing the gold disc electrode using a 800grit emery (wet and Dry paper) using Milli-Q water as the
lubricant. After roughening, the electrode was rinsed in methanol followed by Milli-Q water to remove any impurities. For the work performed at a roughened electrode surface the electrode For all experiments, except impedance measured at open circuit potential (OCP), the electrode was conditioned prior to performing the impedance measurement. This involved the EIS system measuring the OCP for 15s, followed by the application of this open circuit potential for 15s, finally the electrode was held at the potential which the impedance measurement was to be recorded at for 15s. The electrodes were conditioned in the media in which the impedance measurements were to be made.

Impedance spectra were recorded in 0.2M PBS before and after immersion into solutions of protein and PBS. For all experiments the concentration of the protein solution was 1000μg mL⁻¹ and for PBS solution it was 0.2M. The impedance measured before immersion is always referred to as the “Initial”, whilst the impedance measured after immersion into protein is always referred to as “Final Ig.G” or “Final HSA” respectively (depending on the protein). The Impedance recorded after immersion in 0.2M PBS is referred to as the “Final PBS” measurement. This “Final PBS” measurement was used as the blank when the protein adsorption experiments where completed.

Impedance was also measured at a single frequency, 1000Hz, with a measurement taken every 8s. The impedance measurements were performed immediately after the Au and TiO₂ electrodes were placed in the protein solution
and continued for 256 measurements. The double layer capacitance was calculated from the imaginary impedance using equation (2.10).

\[
C_{dl} = \frac{1}{2\pi f Z_{im}}
\]  

(2.10)

Due to the varying experimental conditions used, the measured OCP will differ from experiment to experiment within one data set. To avoid this variation it was necessary to measure the OCP manually and use this value for all subsequent experiments performed at OCP. This was done by connecting the working electrode to the impedance analyser then placing the electrode in a three electrode cell containing a solution of 0.2M PBS. Using the impedance analyser, the potential between the working and reference electrodes was measured and plotted against time until a steady value was reached. At that time the electrode potential was assumed to be at equilibrium and this value was used as the OCP.

The impedance data recorded under the various experimental conditions was modelled using Zview™ version 2.2 (Scribner – USA) EEC software. The impedance spectra were fitted to various models using initial starting resistance and capacitance values calculated from the impedance data. For each set of experimental conditions all three spectra (Initial PBS, Final PBS and Final protein) were modelled using the same circuit. In this way it was possible to investigate the changes to the circuit brought about by adsorption.
All impedance experiments were performed in duplicate. Where applicable the capacitance data are presented as an average of the duplicate measurements. The error bars show the range of data as an indicator of the measurement error in the experiments. Where impedance spectra are presented the Figure is representative of the trends observed.

2.4 Cyclic Voltammetry

Cyclic voltammetry is one of the most common voltammetric techniques used, where the potential at an electrode is scanned from one potential to another and back with the resulting current recorded. Recording a cyclic voltammogram in a solution of ferricyanide yields a typical response which exhibits clear redox peaks originating from the Fe(CN)$_6^{4-}$/Fe(CN)$_6^{3-}$ redox couple (Figure 2.4). The resulting current-potential curve, or voltammogram (CV), is in essence, the electrochemical equivalent of an adsorption spectrum obtained by the conventional spectroscopic techniques [7].

![Cyclic voltammogram for a gold disc electrode in the presence of 0.01M K$_3$Fe(CN)$_6$ containing 0.1M NaNO$_3$.](image)

**Figure 2.4** Cyclic voltammogram for a gold disc electrode in the presence of 0.01M K$_3$Fe(CN)$_6$ containing 0.1M NaNO$_3$. 

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The main parameters of interest of a cyclic voltammogram are: anodic (oxidation) peak current ($i_{pa}$); cathodic (reduction) peak current ($i_{pc}$); anodic peak potential ($E_{pa}$) and cathodic peak potential ($E_{pc}$).

### 2.4.1 Experimental

The following experimental procedure was performed for both HSA and Ig.G proteins. A three-electrode cell was used for the CV experiments. For the work performed with the gold electrode, protein solutions with a concentration of 100μg mL$^{-1}$ were prepared in 0.2M PBS, at pH = 3.0, 7.0 and 9.0. The CVs were recorded in a 0.01M potassium ferricyanide containing 0.1M sodium nitrate solution. The CV was recorded between 700mV and -400mV at a scan rate of 100mV s$^{-1}$, for four cycles.

The gold electrode was polished with 0.45μm alumina slurry using a polishing cloth. Once polished, the electrode was sonicated for 2min in Milli-Q water then placed into the ferricyanide solution and four CVs recorded. The fourth CV in each set acted as the reference which successive CVs were compared against. The electrode was polished again and placed in one of the protein solutions with an applied potential and for a given time. On completion of the adsorption, the electrode was removed from the protein solution and rinsed in PBS and then in Milli-Q water. Following the rinsing, the electrode was placed in the ferrocyanide solution and the CVs recorded. This procedure continued until all the applied potential, pH and adsorption time combinations were completed.
For the TiO$_2$ work, the CVs were recorded between 600mV and -400mV at a scan rate of 100mV s$^{-1}$, for four cycles. Only the protein Ig.G was used for this work and it was at pH = 7.0. No polishing of the electrode was performed. The TiO$_2$ electrode was placed in the ferricyanide solution and the CVs recorded. The electrode was then rinsed and transferred to the protein solution for 30min. After this immersion, another CV was recorded in the ferricyanide solution.

All CV experiments were performed in duplicate. The adsorption profiles are presented as an average of the duplicate measurements. The error bars show the range of data as an indicator of the measurement error in the experiments. Where cyclic voltammograms are presented the Figure is representative of the trends observed.

2.5 Attenuated Total Reflectance Infrared (ATR-IR) Spectroscopy

Spectroscopy using the total internal reflection technique can be done in the ultra-violet, visible or the infrared region of the spectrum with appropriate waveguides. The attenuated total internal reflection technique is almost exclusively used in the mid-infrared region where adsorptions due to molecular vibrations can be used to monitor proteins at interfaces. While the adsorptions at each reflection of each wave are small, we can increase the attenuation of the incident infrared radiation by multiple reflections along the length of the waveguide such as zinc selenide (ZnSe) crystals. The ATR crystal needs to be
chemically inert, to have a useful IR window, and to have a high refractive index [8].

If a beam of radiation strikes the back face of an internal reflection crystal at the correct angle total single internal reflection occurs. When this occurs a standing wave with the same frequency as in the crystal forms in the optically rarer medium (thin film). This electromagnetic field is called the evanescent wave and decays exponentially [9]. Figure 2.5 shows a schematic of a single internal reflection and decay of the evanescent wave.

![Figure 2.5](image)

**Figure 2.5**  *Schematic of single internal reflection and decay of the evanescent wave through a particle thin film immersed in a solution.*

It is the absorptions of this evanescent wave by the adsorbed surface species that provide the IR spectra. Due to the short wavelength of the evanescent wave in the thin film the ATR technique does not suffer the same problem as conventional IR associated with solvent IR absorptions in aqueous solutions. The use of submicrometer solid particles as suspension or deposited as thin films on ATR
crystals gives sufficiently high surface area to obtain good quality IR spectra of solid-solution interfacial species [8].

2.5.1 Experimental

Single internal reflection 45° ZnSe prisms in conjunction with a Prism Liquid Cell accessory (Harrick) were used in all ATR-IR experiments. The TiO₂ films were formed by vacuum evaporation of 100 μL aqueous suspension (10⁻² M) of titanium dioxide (Degussa, P25) onto the ZnSe prism. The ZnSe prisms were covered with a hemispherical glass solution cell sealed to the prism surface with an O-ring (Figure 2.6).

![Figure 2.6 Schematic of the ATR-IR cell set up used to measure the IR spectra.](image)

Solutions were pumped through the cell at a flow rate of ~1 mL min⁻¹ using a peristaltic pump. The tubing used was a Masterflex 96420 14 Tygon 3350 Silicon tube supplied by Norton, which has low protein binding.

All infrared spectra were recorded between 4000 and 700 cm⁻¹ over 64 scans at a resolution of 4 cm⁻¹ using a BioRad Digilab FTS60 spectrometer fitted with a DTGS detector and using WinIR software. Background spectra were initially
recorded by flowing the solution of interest, but without the protein component, through the cell. Subsequently the protein solution was flowed through the cell and absorbance spectra of the adsorbed species obtained from these two measurements. In each case, spectra were recorded 30min after the TiO$_2$ film was exposed to the solution. Unpublished work performed in our laboratories indicates that 30min adsorption results in equilibrium being reached between the adsorbing protein and the protein in solution.

Due to time constraints the ATR-IR work was not performed in duplicate. The results presented in Chapter 7 are of data obtained from single experiments.

2.6 Solutions and Reagents

All chemicals and reagents were used as received unless otherwise stated. Human Serum Albumin (HSA) and human polyclonal Immunoglobulin G (Ig.G) were obtained from Sigma Aldrich and were reagent grade isolated from pooled normal serum. Potassium ferricyanide, sodium chloride, sodium nitrate, disodium orthophosphate and sodium dihydrogen orthophosphate were all obtained from Sigma Aldrich. All solutions were prepared in Milli-Q water. The buffer used was phosphate buffer saline solution (PBS), which was prepared by combining 0.2M Na$_2$HPO$_4$ and 0.2M NaH$_2$PO$_4$. Enough NaCl was added to produce a final saline concentration of 0.15M. This buffer was prepared at pH = 7.0 and concentrated HCl or NaOH solutions were added to lower or raise the pH.
The $^{125}$I isotope was obtained from Amrad/Biotech. The IODO Beads and PD-10 Sefadex Gel Filtration columns used in the labelling process were purchased from Lab Supply and Amersham respectively. Nitric acid was obtained from BDH chemicals whilst sodium hydroxide and sodium dodecylsulphonate was obtained from Sigma Aldrich. The enzymatic detergent Medizyme was obtained from Whiteley Industries Pty. Ltd., NSW, Australia. The gold quartz crystals were purchased from International Crystal Manufacturing, Oklahoma, USA. The gold Mylar sheets were purchased from Courtaluds Performance Films (USA) while the Ti coated polycarbonate films were obtained from Polartechnics Pty Ltd.

Where a three-electrode cell was used the working electrode was either gold or TiO$_2$. The auxiliary and reference electrodes were a platinum mesh and Ag/AgCl (3M NaCl) electrode respectively. Figure 2.6 depicts the typical three electrode cell set up. Conducting foil was folded over one edge of the Au, Ti and TiO$_2$ samples and alligator clip were attached to the foil to ensure electrical contact with these samples.
Chapter 2 – Experimental Techniques and Procedures

Figure 2.7  Three electrode cell showing the working, auxiliary and reference electrodes connected to a potentiostat.

Contact angle measurements were made using the sessile drop contact angle technique in air. The contact angles were measured using a Rame-Hart Model 100-00-230 Contact Angle Goniometer (USA). Three 12μL drop of Milli-Q water (polar solvent) and diiodomethane (non-polar solvent) were placed at different regions of the gold substrate. The contact angle of both drop edges were measured for each drop (Figure 2.8). The contact angles for the polar solvent and non-polar solvent were averaged to determine the overall contact angle for a polar and non-polar solvent.

Figure 2.8  Schematic of the contact angle measurement for a polar and non-polar solvent where Θ₁ and Θ₂ are the two measured contact angles.
The roughness values reported for the AFM images were calculated using the NanoScope (USA) v2.1 software. The software calculates the total surface area of the scanned region based on the imaged topography. It then calculates the surface roughness by dividing the total surface area by the projected area of the scanned region.
Chapter 2 – Experimental Techniques and Procedures

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Chapter 3

Adsorption of Protein on Gold
3.1 Introduction

3.1.1 Radiolabelling

Radioactive isotopes are widely used in chemical and biological investigations. The study of metabolism was revolutionised by the introduction of isotopic tracers [1, 2]. Radiolabelling with Iodine 125 is the method of choice due to it being chemically much easier and the specific radioactivity is higher than other common isotopes such as $^3$H and $^{13}$C [3]. As far back as 1960 Yallow and Berson were successful in demonstrating for the first time the use of radioimmunoassay in detecting endogenous insulin in human blood [4]. Cornelius and Brash [5] used the radiolabelling technique to investigate the adsorption of glycoprotein on various substrates. The adsorption of two plasma proteins, HSA and fibrinogen, on TiO$_2$ was investigated by Liu et al [6] using the $^{125}$I radiolabelling method.

Radiolabelling has also been used extensively as a complementary technique with other proven techniques. An example of this is the use of radiolabelling by Westbrook [7] et al to complement the identification of proteome elements by MS (Q-TOF). Many authors [8-11] have compared adsorbed protein amounts obtained using non-radiolabelled techniques with those obtained by radiolabelling and found the results to be similar.

3.1.2 Quartz Crystal Microbalance

In 1957 Sauerbrey showed that the piezoelectric effect of a crystal could be used to monitor mass changes [12]. Sauerbrey for the first time was able to show a relationship between mass loading or removal and the associated change in the
crystals resonate frequency. From his work Sauerbrey was able to quantify his findings showing that frequency decreased linearly with mass uptake [12]. However, subsequent studies performed by Miller and Bolef [13] showed that this linear relationship had limitations relating to the elastic nature of the deposited films.

The modern day QCM is capable of extremely sensitive mass measurements. It oscillates in a mechanically resonant shear mode by application of an alternating, high frequency electric field using electrodes that are usually deposited on both sides of the crystal [14]. Up until 20 years ago it was thought that crystal oscillation in liquid was not possible due to excessive energy loss to the solution from viscous effects. At that time Konash and Bastiaans as well as Nomura demonstrated the use of the QCM in the liquid environment for the determination of mass changes at the crystal surface [14].

When using the QCM it is important to remember that other factors, besides mass loading or removal, affect the oscillation of a crystal. When a planar surface that is oscillating in the shear mode is immersed into any medium, a shear wave propagates normally away from the surface into the medium. Immersion has the effect of dampening the oscillating frequency \( f_0 \). Kanazawa and Gordon [15] showed that the \( f_0 \) of a crystal decreased by approximately 700Hz when transferred from vacuum to pure water at 20°C. A change in temperature of 5°C can change the \( f_0 \) by as much as 41Hz.
Jones and Meiure [16] showed, in 1981, the use of a QCM in an electrochemical context to determine trace metals plating onto the crystal metal surface. A QCM used in an electrochemical context is often referred to as an Electrochemical Quartz Crystal Microbalance (EQCM). When used as an in-situ technique for measuring mass changes, one of the electrodes is used simultaneously to provide for the alternating electric field that drives the oscillation of the crystal and as the working electrode in the electrochemical cell [14]. The EQCM experiment involves the measurement of mass change at the crystal as well as one or several electrochemical parameters such as potential, current or charge.

The QCM technique has been shown by several investigators to be a very sensitive tool to study protein adsorption kinetics in aqueous solutions with sensitivity in the ng cm\(^{-2}\) (sub-monolayer) regime [17-19]. Kößlinger et al [20] investigated the use of a QCM in the immunosensing of specific antibodies in human sera, while Minunni et al [20] employed a QCM to monitor, in real time, protein adsorption of and immunoreactions of immunoglobulin (h-\textit{lg} G). Måsson et al [21] studied the use of a QCM as a bioaffinity sensor for biotin based on the bioaffinity complex between avidin and immobilized desthiobiotin. Wang et al [22] used an EQCM as an investigative tool to study the electrochemical behaviour of riboflavin at gold electrodes.

The commercial QCM instrument used for this work has an internal frequency to mass conversion calibration constant of 0.226 Hz cm\(^{-2}\) ng\(^{-1}\) for AT-cut crystals of a base resonant frequency of 10MHz. Therefore it is not necessary to perform a
frequency to mass calibration commonly performed for other QCM systems. QCM/EQCM was used to investigate the adsorption behavior of human serum albumin (HSA) at a gold electrode under conditions of open circuit potential and applied potential.

3.1.3 Electrochemical Impedance Spectroscopy

Adsorption at metal surfaces can also be monitored using electrochemical impedance spectroscopy (EIS). Roscoe [8, 9, 23-26] et al have published extensively in the area of protein adsorption investigation using EIS for the food processing industry. EIS involves the application of a DC potential with an AC potential superimposed on top, typically with a value of 10mV. The frequency of this AC potential is varied usually in the range from kHz to mHz which allows for complete characterisation of the electrode surface.

The way in which EIS was employed for this work differs slightly from that described above. A fixed AC potential frequency was chosen which allows for the monitoring of the electrical double layer capacitance ($C_{dl}$). The $C_{dl}$ can be calculated from the imaginary impedance using equation (3.1). This technique has been used previously by Subramanian et al [27] to study the kinetics of adsorption of alkanethiols on gold and by Bernabeu et al [28] who back in 1998 used EIS to study the adsorption of albumin on a platinum rotating disc electrode.

$$C_{dl} = \frac{1}{2\pi f Z}$$  \hspace{1cm} (3.1)
3.2 Results and Discussion

Adsorption of protein was studied at the OCP as well as when the electrode was polarized. It is therefore pertinent to introduce the electrode characteristics at the potentials used. The gold disc electrode cyclic voltammogram (CV) shown in Figure 3.1 was recorded in a pH = 7.0 PBS solution before and after degassing the solution with N₂ gas. The region between 0.0V and ~0.7V is one of little electroactivity where no faradaic processes are occurring and is referred to as the double layer region. The only thing occurring in this region is the charging of the double layer. At potentials greater than 0.7V the sharp increase in anodic current is due to the formation of an oxide layer on the gold surface [29]. The reduction current between 0.0V and approximately -0.6V arises from the reduction of dissolved oxygen present in the aqueous electrolyte. This was confirmed by the decline in current observed when the CV was recorded after deoxygenating the PBS solution with N₂ gas. The sharp increase in reduction current at potentials more negative than ~0.6V is attributed to the reduction of water, present in the PBS solution, resulting in the formation of hydrogen gas.
Figure 3.1  Gold disc electrode cyclic voltammogram recorded in 0.2M PBS before and after degassing at a scan rate of 100mV s\(^{-1}\). The arrows show the direction of the scan.

Quartz crystal microbalance (QCM) and radiolabelling have been used to investigate the adsorption of protein at a gold electrode under various pH and electrode potential. The work performed using QCM was carried out at pH = 7.0 only, whilst the radiolabelling work was carried out at pH = 4.0 and 9.0 as well as pH = 7.0. The effect of applying a potential to the electrode during adsorption was investigated at each pH using both QCM and radiolabelling techniques. EIS was used to investigate the adsorption kinetics of IgG at various electrode potentials. The protein concentration used for the QCM and radiolabelling work was 100\(\mu\)g mL\(^{-1}\), whilst for EIS it was 1000\(\mu\)g mL\(^{-1}\).
3.2.1 Adsorption at Constant Potential

3.2.1.1 Adsorption of HSA

Adsorption of HSA was studied using two techniques, namely quartz crystal microbalance (QCM) and iodine 125 (I\textsuperscript{125}) radiolabelling. When HSA is adsorbed under OCP conditions the adsorption profile shows adsorption still occurring at 60min. However the rate at which adsorption is occurring is less than 5% per minute and at 60min the adsorption is considered to have reached a pseudo-plateau value. At 60min the electrode surface is considered to be saturated with adsorbed protein with a mass loading of approximately 60ng being observed (Figure 3.2). This mass loading is equivalent to an adsorbed amount of approximately 3.0mg m\textsuperscript{2}, which agrees with previously published work by Malmsten [30] (3.4 mg m\textsuperscript{2}).

![Figure 3.2](image_url)  
Figure 3.2 Typical time-dependant change in mass due to HSA adsorption on a Au-coated QCM crystal at OCP from a solution of HSA (100\mu g mL\textsuperscript{-1}) in 0.2M PBS, pH = 7.0.
Figure 3.3 shows the adsorption trend of HSA from the results obtained using the radiolabelling technique. Like the profile shown above, adsorption is still occurring after 60min, however the rate of adsorption is such that at 60min a pseudo-plateau value is reached. The adsorbed amounts recorded at OCP using the two techniques are comparable.

![Figure 3.3 Adsorption profile of gold Mylar sample in 100\mu g mL^{-1} HSA at pH = 7.0 during adsorption at OCP. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0\%.]

The effect of applied potential on adsorption of HSA was then investigated using EQCM (Figure 3.4). Adsorption is still occurring at 60min, although, like the OCP results the rate of increase is small. When adsorption occurs at 700mV the mass loading on the crystal after 60min is approximately 100ng, which corresponds to an adsorbed amount of \(~5.2\text{mg m}^{-2}\). At \(-800\text{mV}\), the mass loading was \(~40.0\text{ng}\) which corresponds to an adsorbed amount of \(~2.1\text{mg m}^{-2}\).
Chapter 3 – Adsorption of Protein on Gold

Figure 3.4  Typical time-dependant change in mass due to HSA adsorption on a Au-coated QCM crystal at 700mV and −800mV from a solution of HSA (100µg mL⁻¹) in 0.2M PBS, pH = 7.0.

The adsorption of HSA at an electrode potential of 700mV and −800mV monitored by radiolabelling is shown in Figure 3.5. The adsorption profiles are similar to those shown in Figure 3.4 with the adsorption trends being the same as those shown above, namely, greater adsorption at positive and OCP potential than when the electrode potential is biased negative.

Figure 3.5  Adsorption profiles of gold Mylar samples in 100µg mL⁻¹ HSA in 0.2M PBS at pH = 7.0. Adsorption occurred at an electrode potential of 700mV and −800mV. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0%.
At all three potentials (-800mV, OCP and 700mV) the adsorbed amounts calculated from the QCM experiments are slightly greater than those calculated from radiolabelling. When the change in frequency of the oscillating crystal is converted into mass it is assumed that the adsorbed species is of a rigid nature [12, 14], however proteins adsorb as soft flexible species [31, 32]. This may cause the recorded mass loading to be higher than expected for the frequency shift observed.

Another point to consider is that in QCM, the mass loading of the crystal may not be due to protein adsorption only. Proteins in solution are solvated as well as having ions from solution bound to the charged regions of the protein [33]. When proteins adsorb, these ions and water molecules are also adsorbed; which will contribute to the mass loading. Lee et al [34] showed that water adsorbs to gold at low relative humidity (ca. 10%) whilst Connor et al [35] and Michelmore et al [36] showed that phosphate ions adsorb strongly to metals. However, there was only a slight increase in mass during the 30min PBS equilibration period, which, over the duration of the experiment was taken to be negligible.

While both techniques proved valid in the study of protein adsorption and adsorption trends, it was the radiolabelling technique that provided better reproducibility of results for multiple experiments. It was therefore decided to continue the investigation of adsorption trends and amounts using only the radiolabelling technique.
3.2.1.2 $^{125}$I Radiolabelling

Figure 3.6 shows the adsorption profiles of Ig.G under various applied potential, HSA results have been included for comparison. The increase in the amount adsorbed with time can be equated to an increase in mass at the gold QCM crystal. It was observed that the application of 700mV to the electrode resulted in the greatest adsorbed amount for both proteins while the application of −800mV resulted in the least amount. HSA is a highly charged molecule ($z = -18$) [37] at physiological pH [38], therefore, adsorption at a positively charged surface will be favored over a negatively charged surface, as observed in Figures 3.4 and 3.5. Based on electrostatics, the result for Ig.G agrees with the HSA results, however the pi (discussed below) for Ig.G is not as defined as for the HSA protein. The exact pi of the polyclonal Ig.G antibody used for this work is unknown but it is considered to be between pH = 5 and pH = 8.0 [39], therefore at pH = 7.0 the overall charge on the Ig.G molecule is unknown although it can be assumed to be near neutral.

Adsorption at negative applied potentials shows that Coulombic interactions between the HSA or Ig.G and the gold surface do not dominate the adsorption process. Norde and co-workers [40] observed adsorption of negatively charged protein molecules at negatively charged surfaces. The overall adsorption process may be considered to be the result of various processes:

- Redistribution of charged groups,
- Van de Waals interactions between the protein and the sorbent, and
- Structural rearrangement in the adsorbing protein molecule [41].
Roscoe et al [42-44] as well as other workers [45, 46], using thermodynamic and FTIR studies, respectively, have shown this last point to be significant for “soft proteins” (HSA and Ig.G) [47] in the adsorption of proteins.

![Adsorption profiles of gold Mylar samples in 100µg mL⁻¹ HSA and Ig.G at pH = 7.0. Applied potentials during adsorption are indicated on the figure. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0% (HSA) and 5.5% (Ig.G).]

The HSA molecule has been described as a “stubby cigar-shaped albumin molecule” [48] with dimensions of 14 x 4 x 4nm [49]. While Ig.G is assigned a “Y” shape configuration with dimensions of 4.5 x 4.5 x 23.5nm [49]. Adsorption of proteins has been considered to be either horizontal or vertical adsorption.
(Figure 3.7) and many papers provide calculated data for both orientations of adsorbed proteins at complete surface coverage [37, 50, 51]. Using the dimensions of the HSA and Ig.G molecule, the adsorbed amount at complete electrode surface coverage for vertical and horizontally oriented adsorption is 6.85mg m$^{-2}$ and 1.96mg m$^{-2}$ for HSA and 12.02mg m$^{-2}$ and 2.30mg m$^{-2}$ for Ig.G.

![Figure 3.7 Orientation of protein adsorption at a solid surface. Horizontal (A) and vertical (B). The sizes represent the thickness of a monolayer adsorption for HSA and in brackets for Ig.G.](image)

Based on the calculated adsorbed amounts for each adsorption orientation the adsorbed amount at OCP in Figure 3.6 corresponds to approximately a monolayer of horizontally adsorbed HSA and Ig.G molecules. The result for HSA agrees with Norde et al [52] who proposed horizontal adsorption of HSA at hydrophobic titanium oxide. However, the Ig.G adsorption orientation assigned, based on adsorbed amounts, does not agree with Malmsten [49] who assigned vertical orientation based on ellipsometry results.

At 700mV the adsorbed amount increases to $\sim$4mg m$^{-2}$ and $\sim$4.3mg m$^{-2}$ for HSA and Ig.G, respectively. These amounts are greater than a monolayer of horizontally adsorbed protein but less than a vertical oriented layer. This suggests that under these conditions, the adsorbed layer consists of both vertical and
horizontally adsorbed protein molecules. Finally the amount adsorbed at an
electrode potential of $-800 \text{mV}$ is less than one horizontally oriented monolayer at
$\sim 1.2 \text{mg m}^{-2}$ for HSA while for Ig.G the adsorbed amount is equal to a monolayer
of horizontally adsorbed protein at $\sim 2.4 \text{mg m}^{-2}$. These results suggest that the
nature of the electrode potential has an effect on the orientation of the adsorbing
species, which in turn has an effect on the adsorbed amounts. This suggestion has
also been put forward by Slojkowska and Herbich [53] who studied adsorption of
amino acids on a polycrystalline gold electrode.

The adsorbed amount for HSA and Ig.G under OCP at a gold electrode at
pseudo-plateau saturation (ca. 60min) was found to be $\sim 2.0 \text{mg m}^{-2}$ and $\sim 2.5 \text{mg}
\text{m}^{-2}$, respectively. The HSA adsorbed amount is very similar to the value obtained
using QCM (Figure 3.2). Sessile drop contact angle measurements of the gold
Mylar substrate, were $64^\circ$ for the polar drop and $49^\circ$ for the non-polar drop,
indicating the surface to be more hydrophobic in nature than hydrophilic. Lensen
et al [54] and Malmsten [30] have studied the adsorption of HSA at hydrophobic
surfaces, with the results presented here agreeing with their findings. Malmsten
was able to calculate this adsorbed amount to equate to a mean optical film
thickness of $4 \pm 2 \text{nm}$, which corresponds to a monolayer of horizontally adsorbed
HSA molecules. In another study Malmsten [49] showed the adsorbed amount of
Ig.G at saturation to be $\sim 3.0 \text{ mg m}^{-2}$, which also agrees with the above findings.
Malmsten assigned a vertical adsorption orientation of the Ig.G molecule.
While the adsorbed layer has been considered to consist of either vertical or horizontally adsorbed protein molecules, it is obvious that is not the case. In most instances the adsorbed layer comprises of a mixture of both orientations, although there is evidence to suggest that one preferred orientation dominates at all times. To gain a better insight into the nature of the adsorbed protein the use of a complementary technique such as ellipsometry, which provides information on the adsorbed layer thickness, would be desirable. Ellipsometry was performed for this work, however due to the instrument being a fixed angle system and the substrate being gold which had a non-uniform morphology no meaningful results were obtained.

So far only the charge on the substrate has been investigated. It is well known that the charge on the protein has a significant effect on the adsorption processes [10, 51, 55] and one way to vary the charge on the protein is to vary the protein pH about its pi.

3.2.1.2.1 Effect of pH

Changing the pH of the protein had a dramatic effect on the adsorption characteristics at OCP (Figure 3.8). The pi of HSA is at pH = 4.6, therefore at pH = 3.0 the protein charge is positive and at pH = 7.0 and 10.0 it is negative. Unlike HSA the pi for Ig.G lies between pH = 6.0 and pH = 8.0, however by choosing pH = 3.0 and 10.0, which is outside this range, the charge on the Ig.G molecule will be varied in accordance with HSA. As well as changing the protein charge by varying the solution pH, the interfacial environment between the electrode
and electrolyte will also vary due to the excess of H\(^+\) and OH\(^-\) ions present at acidic and basic conditions respectively. This variation in the interfacial region will have an effect on the electrode processes occurring [56] as well as the formation of oxide layers [29]. Therefore the local environment of the protein and electrode surface must be considered when studying the effect of solution pH on adsorption, particularly when adsorption occurs at a polarised electrode.

**Figure 3.8** Adsorption of HSA and Ig.G (100\(\mu\)g mL\(^{-1}\)) in 0.2M PBS at various pH. Adsorption time was 30min at OCP. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0%.

At OCP the electrode possesses a positive charge (Chapter 4 – Section 4.2.3). Adsorption at pH = 7.0 and 10.0, where HSA possess a negative charge, appears to follow an electrostatic trend, namely greater adsorption than when the protein pH = 3.0 and possesses a positive charge. The electrostatic interactions occur between regions of the protein and electrode surface that possess opposite charge. Adsorption of Ig.G also appears to follow an electrostatic trend, namely greater adsorption at pH = 10 (protein negative charge) than at pH = 3.0 (positive charge).
protein charge). Adsorption at pH = 7.0 however shows greater adsorption than at pH = 10.0 which is opposite to the HSA adsorption results at the same pH. Several authors have observed a maximum in the amount of Ig.G adsorbed with pH, and indicate that it is due to the decrease in conformational stability of the Ig.Gs with increasing net charge on the molecule [39]. Galisteo-Gonzalez et al [57] showed that as the pH increased from pH = 7.0 to 10.0 the extent of Ig.G adsorption at a positively charged polystyrene surface decreased, which agrees with the above findings. While there are other processes occurring which influence adsorption, such as hydrophobic interactions, hydrogen bonding, and van der Waals interactions, it appears that under OCP experimental conditions electrostatic interactions dominate.

The fact that the adsorbed amounts recorded for HSA at pH = 7.0 and 10.0, where the protein’s charge is the same, are different, indicates that other factors are involved in the adsorption process. This is also true for Ig.G. As well as altering the protein’s charge in solution, varying the pH has also been shown to alter the level to which native-state proteins undergo structural alterations upon adsorption to solid surfaces [58]. Norde concluded from his work that structural rearranging upon adsorption to solid surface increase the further the protein pH is moved from its pI. The results presented in Figure 3.8 highlights the complex nature of protein adsorption at solid surfaces. Haynes and Norde [55] suggest that this complex dependence of adsorbed amounts on pH arises from increased lateral electrostatic repulsions between charged proteins on the surface preventing the formation of close packed protein layers. This, in turn, creates
competition between protein-protein and protein-surface electrostatic interactions. This explanation has also been backed up by experimental evidence from Shastri and Roe [59].

3.2.1.2.2 Protein Removal

Much of the published work in the area of adsorbed protein removal comes from the food industry, in particular the dairy industry [60-66]. The cleaning of processing equipment is important in maintaining the high quality and sterility of products. The cleaning and disinfection process is also crucial in avoiding cross contamination in the medical industry. Whilst ultraviolet treatment in conjunction with autoclaving are the preferred method for sterilisation [67] not much has been published in the area of protein removal in the medical industry.

To evaluate the effectiveness of a cleaning procedure used in clinical trials in conjunction with TruScan™ a range of chemically different media to remove bound protein was investigated. For this work, only the Ig.G protein was used due to this protein being present in cervical mucus, which contributes to the fouling of the TruScan™ probe tip.

The removal of adsorbed protein from the gold electrode was investigated. Based on previously published work, four cleaning solutions were chosen 8.0M HNO₃, 1.0M NaOH, 0.1M SDS (sodium dodecylsulphonate) and 0.6% v/v Medizyme solution. Timperley et al [68] reported the use of nitric acid in the two stage cleaning process used in the dairy industry, whilst Karlsson et al [61] reported
the use of sodium hydroxide and the surfactant SDS to remove β-lactoglobulin from stainless steel. The procedure used for the cleaning and disinfecting of TruScan™ involves the use of the 0.6% Medizyme solution (unpublished report).

The first treatment was a chemical cleaning process that involved immersing the protein pre-adsorbed electrodes in one of the cleaning solution for various lengths of time. In all cases the gold sample was immersed in a solution of 1:100 hot to cold protein solution for 30min at OCP, pH = 7.0. After this time the samples were γ-counted and the adsorbed amounts calculated. These samples were then treated for various lengths of time with the cleaning solutions mentioned above. After the cleaning process each sample was rinsed with Milli-Q water and allowed to dry at room temperature. The radioactivity of the samples was then measured and the amount of protein remaining was calculated.

The cleaning and disinfecting process used for TruScan™ also involved a mechanical cleaning step involving the use of a hard bristle brush, soaked in Medizyme. To investigate the effectiveness of the scrubbing procedure in removing bound protein a second cleaning treatment incorporating a mechanical step with the chemical treatment was used. After an initial soak in the cleaning solutions for 5min, each sample was scrubbed using the same solution for 5min. Following the scrubbing the samples were rinsed with Milli-Q water and left to dry at room temperature followed by measurement of radioactivity.
The adsorption of protein was monitored and then the effect of leaving the sample in each cleaning solution was investigated (Figure 3.9). The solution containing 1.0M NaOH was the most effective in removing the adsorbed protein, followed by nitric acid, SDS and finally the enzyme detergent Medizyme. There is no result shown for 1080min 1.0M NaOH since at this time the NaOH solution removed the gold film from the Mylar substrate.

After 90min of desorption the acid treatment had removed 30% of the bound protein, whilst the basic solution removed 42%. When SDS was used 25% of the total adsorbed protein was removed, which disagrees with results published in a review by Hidalgo-Alvarez and Galisteo-Gonzalez [39] which showed complete removal of adsorbed Ig.G from the copolymer polystyrene/polyacrylic acid by a solution of 1% SDS. Medizyme was only capable of removing only 17% of the adsorbed Ig.G. For all of the solutions used, except NaOH, further removal of the bound protein was negligible at longer soaking time (ca. 1080min).
Figure 3.9 Removal of adsorbed protein from gold samples after soaking in each cleaning solution. Adsorption time was 30min in 100μg mL⁻¹ IgG at pH = 7.0. The time in the figure refers to the immersion time in the cleaning solution. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.0%.

The use of a mechanical method to remove adsorbed protein is only slightly more effective than using a 30min chemical method (Figure 3.10). The chemical method of removal for 8.0M HNO₃ was able to desorb 9% of the adsorbed protein whilst the mechanical method was only slightly better at 14%. There is no mechanical method for 1.0M NaOH result due to the fact that the scrubbing procedure removed the gold film from the Mylar sample. For the SDS solution 38% of the adsorbed protein was removed chemically while the mechanical method was only 12% more effective. 20% removal of protein was observed for 0.6%v/v Medizyme when chemical removal was used and 29% when a mechanical method was used. Therefore, in all cases except NaOH, the addition of a mechanical process to remove adsorbed protein was only at best 12% more efficient.
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Figure 3.10 Effectiveness in removing adsorbed protein from gold samples adsorbed in 100μg mL⁻¹ IgG at pH = 7.0. Adsorption time was 30 minutes at OCP. Chemical cleaning results (30min) are included for comparison. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.5%.

Other researchers [28, 69] have proposed that the adsorbed protein layer contains two types of adsorbed protein, namely, reversibly (weakly) and irreversibly (strongly) bound protein. The strongly adsorbed proteins are those molecules that adsorbed in the initial stages of protein/surface contact. As adsorption time increases these earlier adsorbed proteins undergo structural rearrangement to become strongly attached to the substrate surface. As this process of structural rearrangement is taking place new protein molecules are adsorbing. It is these latter adsorbed proteins that do not have time to undergo structural rearrangement, that constitute the reversibly bound protein. This theory of reversibly and irreversibly adsorbed proteins may help explain why not all of the adsorbed protein is removed after soaking in the cleaning solutions for 1080min and after a harsh mechanical process was used.
3.2.2 Adsorption Profiles with Pulsed Potential Routine Applied

The effect of an applied potential waveform was investigated with respect to adsorbed amounts of HSA and Ig.G at pH = 7.0. As outlined in the experimental section a square wave pulse of 1.0s duration was applied to the electrode for 30min. The square wave involved pulsing the electrode from 0.0mV to either 700mV or -800mV for 1.0s then back to 0.0mV for 1.0s. Figure 3.11 presents the adsorption data for HSA and Ig.G.

![Graphs showing adsorption amounts](image)

**Figure 3.11** Amounts of protein adsorbed when the potential is pulsed during adsorption in 100μg mL⁻¹ HSA and Ig.G at pH = 7.0, adsorption time was 30min. The adsorption at constant potential results is included for comparison. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0%.
From the results outlined above more adsorption occurs when a positive potential is applied to the electrode and less adsorption occurs at negative potentials. When the electrode potential is pulsed between 0 and 700mV less adsorption of HSA occurs than when the potential is constant at 700mV. For Ig.G this trend is reversed. When the electrode potential is pulsed between 0 and −800mV the adsorbed amount is greater for both HSA and Ig.G compared to when the potential is kept constant at −800mV.

The adsorption trends observed for the pulse potential work could be explained by considering the amount of adsorbed protein when adsorption occurred at a constant potential. For the 0mV to 700mV results, when the potential is pulsed the electrode is at 700mV for only half of the 30min adsorption time, for the other half the electrode potential is 0mV, which is close to the OCP of the gold electrode. Figure 3.6 shows that more adsorption occurs at 700mV than at OCP, therefore it follows that more adsorption would be expected when the electrode is held at 700mV for 30min as opposed to when the electrode is held at 700mV for 15min and at 0mV for a further 15min.

The result obtained for Ig.G when the electrode potential was pulsed between 0mV and 700mV is opposite to what was observed for HSA. When the electrode was pulsed between 0mV and 700mV the amount of adsorbed Ig.G was greater than when the electrode was held constant at 700mV. This result may be explained by considering the electrode surface net charge at 0mV and 700mV, where the net positive charge at 700mV will be greater than at 0mV (see
Other workers have shown that the adsorption mechanism for Ig.G is relatively unstable and is easily affected by external influences, such as sorbent surface charge [70] and ionic strength [39, 71]. Therefore the rapid change in the magnitude of the surface charge may influence the adsorption mechanism of Ig.G which may account for the observed variation.

At an electrode potential of -800mV more adsorption for both HSA and Ig.G was observed when the potential was pulsed. As above, this result can be explained by the fact that more adsorption occurs at OCP than at -800mV for both HSA and Ig.G (Figure 3.6). When the electrode is held at -800mV for 30min, less adsorption will occur compared to when the electrode potential is held -800mV for 15min and 0mV for the remaining 15min.

Both HSA and Ig.G are “soft” proteins, which means they have low internal stability and generally tend to adsorb on all surfaces irrespective of electrostatic interactions [72]. Due to this low internal stability, these proteins upon adsorption on hydrophobic surfaces change their conformation to a great extent. A consequence of protein structure rearrangement (unfolding) is, that the once buried hydrophobic peptide moiety is now exposed. These hydrophobic peptides are now free to assemble and interact with the hydrophobic regions of the surface, resulting in a multi-point attachment with more interacting points. As adsorption time is increased, more of the “soft” protein can unfold, in turn creating more attachment sites. This protein unfolding and increased attachment has been used to help explain irreversible adsorption.
3.2.3 EIS Study - Protein Adsorption Kinetics

The adsorption kinetics of the protein Ig.G was investigated using the electrochemical impedance spectroscopy technique. Only Ig.G was used since this protein was the main protein of interest due to its presence in cervical mucus. To ensure that observable variations in the impedance spectra were obtained, the concentration of the protein used for this work was increased 10 fold, from 100μg mL⁻¹ to 1000μg mL⁻¹. Bernabeu et al [28, 73] have used similar protein concentration in their work using electrochemical impedance spectroscopy to study the adsorption of fibrinogen and albumin at conducting metal surfaces.

When the impedance was recorded at 1000Hz in a solution of 1000μg mL⁻¹ Ig.G (pH = 7.0) the change in C_{dl} could be monitored (Figure 3.12). There appears to be two regions where the rate at which the C_{dl} decay varies, one for the first 10min of adsorption and another from 10min to 30min adsorption. Bernabeu [28] associated these regions with various modes of protein adsorption, namely the formation of an irreversible layer during the first 10min of contact followed by the formation of a reversible layer at longer contact times. The shape of the capacitance decay appears to be the reciprocal of protein adsorption shown in Figure 3.6, where complete surface saturation occurs at approximately 60min. The plateau region in Figure 3.12 has been attributed to saturation of the electrode surface by the adsorbing protein [28]. The reason surface saturation occurs within 10min of adsorption, whereas in Figure 3.5 this saturation did not
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occur until 60min, may be due to increased protein concentration used in this work.

There was very little to no observable variation in the electrode $C_{dl}$ when the electrode was exposed to a solution of 0.2M PBS (pH = 7.0). This suggests that the variation observed in Figure 3.12, when the electrode is exposed to a solution of 1000μg mL$^{-1}$ prepared in 0.2M PBS, is due exclusively to the adsorption of Ig.G.

![Graph showing variation of $C_{dl}$ versus adsorption time.](image)

**Figure 3.12** Variation of $C_{dl}$ versus adsorption time. Impedance measured at a frequency of 1000Hz and a DC potential of 700mV in 1000μg mL$^{-1}$ Ig.G prepared in 0.2M PBS (pH = 7.0).

Since in all cases 98% of the total $C_{dl}$ change occurs in the first 10min, it appears that only the adsorption of the irreversible layer can be monitored using the double layer capacitance method. These results are in agreement with work performed by other researchers [74-76]. These workers concluded that the capacitance changes, recorded when protein adsorbed at an interface, are related
to the number of close contact points formed between the protein and the surface. Therefore, at adsorption time $t = 0\text{min}$ there are many available sites for the protein to bind to, and as result many contact points are formed. As the electrode surface becomes coated with protein, the amount of available sites for protein binding becomes less and the rate at which these contact points are formed decreases. The adsorption kinetics measured through the variation of the electrode capacitance with time can therefore be attributed to the adsorption of the irreversible layer of protein on the electrode surface.

By using Equation (3.2) and taking the saturation capacitance value as the capacitance of the fully covered electrode surfaces it is possible to calculate the surface coverage ($\Theta$) [27, 77], where $C_0$ is the bare electrode capacitance (capacitance at time $= 0$), $C_i$ is the capacitance at any time $t$ and $C_f$ is the capacitance of the fully covered surface.

$$\Theta_i = \frac{C_0 - C_i}{C_0 - C_f} \quad (3.2)$$

Figure 3.13 shows the adsorption kinetics of Ig.G at different applied potentials. There are at least two distinct surface coverage regions evident in the $\Theta - t$ plots at OCP and 700mV. During the first fast step ~80 – 85% of the final coverage is reached and the second slower step extends for the rest of the coverage. Full surface coverage is reached at both potentials by $10\text{min}$, but at OCP the capacitance decreases at longer adsorption times suggesting the removal of
bound Ig.G. The surface coverage profile for -800mV appears to only contain one region with the final coverage being reached after 30min of adsorption.

![Surface coverage variation due to adsorption of Ig.G onto gold. Adsorption occurred at the different applied potentials shown in 1000μg mL⁻¹ Ig.G/0.2M PBS.](image)

**Figure 3.13** Surface coverage variation due to adsorption of Ig.G onto gold. Adsorption occurred at the different applied potentials shown in 1000μg mL⁻¹ Ig.G/0.2M PBS.

For the 700mV and OCP data, complete coverage is reached after 10min of adsorption. Therefore, it is possible to calculate the adsorption rate constant ($k_m$) for this initial region. Two adsorption models were used for this study, namely the Langmuir and Diffusion Controlled Langmuir (DCL) models. The Langmuir model implies monomolecular reversible adsorption, while the DCL model implies that adsorption follows Langmuir kinetics that are influenced by diffusion processes occurring at the adsorption interface. The Langmuir model in which the surface coverage dependence on time of adsorption is expressed by equation (3.3), whilst equation (3.4) expresses the DCL model.

\[
\Theta_t = \left[1 - \exp\left(-k_m t\right)\right] \\
\Theta_t = \left[1 - \exp\left(-k_m \sqrt{t}\right)\right]
\]

(3.3)  
(3.4)
For rate constant analysis, only the first 10min of adsorption will be considered for OCP and 700mV, since after this time the surface coverage remains fairly constant at ~1.0. For adsorption at -800mV the whole 30min data will be modelled since complete surface coverage is not reached until then. Table 3.1 shows the calculated rate constant for both adsorption models and all three potentials. The rate constant was determined at five different adsorption times with standard deviation being calculated at 95% confidence.

Table 3.1  Rate constants for adsorption of IgG (1000μg mL⁻¹, pH = 7.0) on a gold disc electrode at different electrode potentials during adsorption using the DCL and Langmuir models. The 95% confidence interval is shown in brackets.

<table>
<thead>
<tr>
<th>Potential</th>
<th>Diffusion Controlled Langmuir $10^3 k_m$ (s⁻¹/²)</th>
<th>Langmuir $10^3 k_m$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>700mV</td>
<td>89.4 (±15.8)</td>
<td>7.06 (±0.7)</td>
</tr>
<tr>
<td>OCP (100mV)</td>
<td>76.8 (±13.5)</td>
<td>6.07 (±0.6)</td>
</tr>
<tr>
<td>-800mV</td>
<td>40.2 (±5.8)</td>
<td>3.2 (±0.5)</td>
</tr>
</tbody>
</table>

When surface coverage is plotted against the right hand side of equations 3.3 and 3.4 it is possible to determine which model predicts the adsorption kinetics. Adsorption at OCP follows the Langmuir model as can be seen from the plot in Figure 3.14.
When adsorption occurs at an applied potential the model that best describes the adsorption kinetics is the DCL model. The linear regression check for DCL adsorption model is shown in Figure 3.15 for adsorption at 700mV and -800mV. It must be noted that the Langmuir model could also be fitted to the 700mV data, however the DCL model gave a better fit. The variation in adsorption kinetics at 700mV and -800mV from adsorption at OCP may due to the application of a DC potential during adsorption. From Figure 3.1 is can be seen that when the electrode is biased to 700mV and -800mV faradaic processes begin to occur such as the formation of an oxide layer and the evolution of hydrogen gas respectively. These faradaic processes will have some bearing on the adsorption processes since the impedance measurement and adsorption are occurring simultaneously. It is therefore difficult to ascertain if the variation in kinetics is exclusively due to protein adsorption or other process occurring at the electrode interface.
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Figure 3.15  Linear regression plot to check the validity of DCL adsorption model for adsorption at 700mV and -800mV.

From the above results it is clear that the adsorption kinetics follows different mechanisms depending on the electrode potential during adsorption. Thus the adsorption process is under partial diffusion (DCL) when the electrode is biased away from OCP (700mV and -800mV) and follows Langmuir adsorption kinetics control at OCP. The consequence of the various adsorption kinetics can be seen in Figure 3.13 where the time it took to reach surface coverage equilibrium as greater at -800mV due to the influence of diffusion. While adsorption at 700mV was also fitted using the DCL model the effect on the adsorption profile in Figure 3.13 is harder to see, which may also account for the
fact that the Langmuir model could also be fitted to the 700mV data with limited success. This may be due to the formation of the oxide layer having only a marginal effect on adsorption kinetics. Under Langmuir adsorption kinetics Ig.G adsorption at Au reaches surface saturation relatively quickly.

3.3 Conclusions

At physiological pH, the amount of adsorbed protein was greatest when the electrode potential was biased positive followed by when the electrode was held at OCP. The least amount of adsorbed protein was observed when the potential was negative with respect to OCP. The surface coverage obtained suggests that the orientation of the adsorbed proteins was horizontal when the electrode potential was at OCP. When the electrode potential was positive the orientation of the adsorbed protein appeared to be a combination of both horizontal and vertical. When the electrode potential is negative the adsorbed amount of HSA was less than a monolayer of horizontally adsorbed protein, while for Ig.G it was approximately a monolayer of horizontally adsorbed protein. These results at physiological pH suggest that the electrode potential has an effect on the extent of adsorption, as well as the orientation of adsorption. The adsorption kinetics also varies depending on the electrode potential. When the electrode potential was at OCP the adsorption process followed the Langmuir type kinetics, however, when the electrode potential was biased positive or negative adsorption followed that defined as Diffusion Control Langmuir kinetics.
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When the charge on the protein is varied, by changing the protein pH, the adsorption characteristics varied considerably. In all cases the adsorption process appeared to follow the rule of electrostatics, namely, maximum adsorption was observed when the electrode surface and the protein had opposite charge and minimum adsorption was observed when the charge was the same.

Once the protein had adsorbed it was difficult to remove via chemical or mechanical cleaning means. Of all the solutions used for the chemical cleaning a NaOH solution removed the most, followed by the HNO₃ solution whilst the solutions containing the surfactants SDS and Medizyme removed the least. The use of mechanical means to remove the adsorbed protein proved to be only marginally better than the chemical method alone with, at best, approximately 50% of bound protein being removed. These results suggested that protein adsorbs in two modes, namely, reversible (weakly bound) and irreversible (strongly bond).

When the applied potential waveform routine was varied the amount of adsorbed protein also varied. When the electrode potential was held constant at 700mV the amount adsorbed was greater for HSA than when the electrode potential was pulsed between 700mV and 0mV. Conversely, when the electrode potential was held constant at −800mV the adsorbed amount was less than when the electrode potential was pulsed between −800mV and 0mV for both HSA and Ig.G.
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References


Chapter 4

Effect of Protein Adsorption on the Electrochemical Behaviour of Gold
4.1 Introduction

4.1.1 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) has been the main tool used to investigate processes occurring at a conducting solid/electrolyte interface, particularly in the food processing industry as well as the medical device industry in relation to biocompatibility. Roscoe et al [1-4] have used EIS extensively to study numerous conducting solid/electrolyte interfaces, while Xie et al [5] have used EIS in conjunction with QCM to investigate BSA adsorption at modified gold electrodes.

Electrochemical impedance spectroscopy was used to investigate the electrode/electrolyte interface and the processes that occur at a gold electrode surface in the presence of protein at ambient temperature. To ensure complete characterisation of the interface and surface processes, EIS measurements were made over several frequency decades, from 50 mHz up to 50 kHz.

Non-faradaic and faradaic processes were examined to investigate the electrical double layer (C_{di}) and electron transfer variations upon adsorption at the electrode surface. Impedance was also measured at a fixed AC voltage frequency since it has been demonstrated [6-8] that this experimental procedure also yields information on the C_{di} in real time.

The advantage of EIS over data measurements in the time domain (i.e., pulse or step response measurements) is that the measured dispersion data can be described analytically, using an electrical equivalent circuit [9] (EEC). The
elements of an EEC model represent the various processes involved in the transport of mass and charge. If the dispersion diagrams show distinct features that can easily be related to specific physical processes and thus to defined sub-circuits of the equivalent circuit model, analysis becomes quite simple. However, if the dispersions overlap in any way analysis becomes difficult and it is therefore necessary to use a more sophisticated method for analysis. Using Non Linear Least Squares Fit (NLLSF) techniques, all parameters in the equivalent circuit model are adjusted simultaneously, thus obtaining an optimum fit to the measured dispersion data [9].

4.1.2 Cyclic Voltammetry

Cyclic voltammetry (CV) was used to investigate the effect of adsorbed species at the electrode surface on electron transfer between the electrode and an electroactive species (ferricyanide) in solution. Ferricyanide provides one of the best-understood reversible redox couples (4.1), and for this reason it is often used for characterising the response of electrode substrates [10].

\[
\text{Fe(CN)}_6^{3-} \leftrightarrow \text{Fe(CN)}_6^{4-} + e^- \quad \text{(4.1)}
\]

When the electrode surface is modified or fouled by protein the resulting CV will vary.

Cyclic voltammetry has been used to study surface adsorbates such as self-assembled monolayers of thiols on gold [11] and lipids on platinum [12]. The interfacial behaviour of insulin at a platinum electrode [13], oxidation reactions of tryptophan and related dipeptides at a Pt electrode [14] and the effect of
surface activation on charge and mass transfer rates of an electroactive species at a modified carbon paste electrode have all been carried out using cyclic voltammetry. Guo et al [15] used CV to study the effect of an externally applied potential on the adsorption of serum albumin at a gold, platinum and glassy carbon electrode. They found that upon fouling of the electrode the separation between the oxidation and reduction peaks increased, while the oxidation current magnitude decreased. They associated these changes with the reduction of the electron transfer rate due to the formation of an inert protein layer at the electrode surface.

The concentration of protein used for this work was 1000 µg mL⁻¹, which is an order of magnitude increase in the concentration used for the adsorption work presented for the QCM and Radiolabelling (Chapter 3). The reason for this increase in concentration has previously been outlined in Chapter 3 - Section 3.2.3. Bernabeu et al [6, 16] have used similar protein concentration using electrochemical impedance spectroscopy to study the adsorption of fibrinogen and albumin at conducting metal surfaces.

4.2 Results and Discussion

4.2.1 Electrode Characterisation

To characterise the processes occurring at the gold disc electrode in PBS, cyclic voltammograms (CVs) were obtained (Figure 4.1). The CVs in Figure 4.1 are the same as those presented in Figure 3.1 and show that in the region between 0.0V and 0.7V no faradaic (electron transfer) processes occur and only double layer
charging processes are observed. The current observed between 0.0V and approximately -0.6V arises from the reduction of dissolved oxygen. This was confirmed by the decline in current observed when the CV is recorded in nitrogen purged PBS (Figure 4.1). The sharp increase in reduction current at potentials more negative than -0.6V is due to the reduction of the electrolyte producing H\textsubscript{2} gas.

![Gold disc electrode cyclic voltammogram recorded in 0.2M PBS (pH = 7.0) before and after purging the PBS solution with N\textsubscript{2} gas. Scan rate = 100mV s\textsuperscript{-1}. (The arrows show the direction of the scan).](image)

**Figure 4.1** Gold disc electrode cyclic voltammogram recorded in 0.2M PBS (pH = 7.0) before and after purging the PBS solution with N\textsubscript{2} gas. Scan rate = 100mV s\textsuperscript{-1}. (The arrows show the direction of the scan).

### 4.2.2 Open Circuit Potential (OCP) Measurement

For the impedance measurements to be performed at OCP it is necessary to determine the gold electrode OCP in 0.2M PBS (Figure 4.2). A steady state potential of 100mV was reached at approximately 120min, which was used as the OCP for all subsequent gold disc impedance measurements.
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Figure 4.2 Time dependence of the open circuit potential of the gold disc electrode in 0.2M PBS (pH = 7.0) vs. Ag/AgCl reference electrode.

4.2.3 Potential of Zero Charge

The capacitance plot as a function of electrode potential was obtained (Figure 4.3). The capacitance decreased with less negative potentials to around -250mV and began to increase again at approximately -200mV. A potential of zero charge (pzc) was interpreted to be -275mV. For potentials less negative than -275mV the electrode surface carries a positive charge and for potentials more negative than -275mV the electrode charge is negative. Bernabeu et al [16] used equation (4.2) to determine the charge (q) on a platinum and carbon electrode; where U is the potential used to measure the capacitance, U₀ is the pzc, Cₐ is the capacitance value at potential U and C₀ is the capacitance at pzc. The charge on the gold electrode at potentials above and below the electrode pzc is shown in Table 4.1.

\[ q = \frac{(U - U₀)(Cₐ + C₀)}{2} \]  \hspace{1cm} (4.2)
Table 4.1 Charge on the gold electrode calculated using equation 4.1. The capacitance was calculated from the impedance measurements performed in 0.004M PBS (pH = 7.0). The potential at which the impedance was measured is shown in the table.

<table>
<thead>
<tr>
<th>Potential (V)</th>
<th>Charge (μC cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.81</td>
</tr>
<tr>
<td>-0.1</td>
<td>1.61</td>
</tr>
<tr>
<td>-0.2</td>
<td>0.64</td>
</tr>
<tr>
<td>-0.3</td>
<td>-0.21</td>
</tr>
<tr>
<td>-0.4</td>
<td>-1.13</td>
</tr>
<tr>
<td>-0.5</td>
<td>-2.10</td>
</tr>
</tbody>
</table>

Figure 4.3 Capacitance vs. potential plot. Impedance was measured between 50kHz and 50mHz in 4.0x10⁻³M PBS solution at pH = 7.0. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.5%.

4.2.4 Non-Faradaic – Double Layer Effects

From the CVs shown in Figure 4.1 two of the electrode potentials at which protein adsorption was investigated in Chapter 3, namely 700mV and -800mV, are located in regions where faradaic processes are occurring. At 700mV surface oxides begin to form on the electrode surface [17] whilst at -800mV reduction
of dissolved oxygen and the evolution of hydrogen occurs [17]. These faradaic processes will alter the electrode surface causing misinterpretation of impedance spectra, with respect to spectra variation caused by protein adsorption. To avoid these complications in interpreting the impedance spectra it was decided to measure the impedance at intermediate potentials, such as 400mV, OCP (100mV) and −400mV. Whilst there are faradaic process occurring at −400mV, namely reduction of dissolved oxygen, this potential was chosen to allow for comparison with the titanium dioxide impedance work presented in Chapter 7.

4.2.4.1 Adsorption at OCP and Impedance at Various Potentials

The shape of the impedance spectra are dependent on the potential applied to the electrode during the impedance measurement (Figure 4.4). The impedance spectra show three distinct frequency dependent regions. In the high frequency region, the absolute impedance (Bode Z) is independent of the frequency with the phase angle value at or near zero degrees as represented in the Bode Phase plot. This is a typical response for resistive behaviour [2] and corresponds to a resistance of the phosphate solution between the working and reference electrode. In the medium frequency region a linear relationship can be observed between the absolute impedance and the frequency. This frequency region corresponds to the capacitive behaviour of the electrode/electrolyte interface [2]. The linear region extends further to lower frequencies for the impedance spectra recorded at positive potentials than it does for negative potentials.
Figure 4.4  Bode plots of bare gold disc electrode (Dia = 0.3cm) recorded in 0.2M PBS (pH= 7.0) solution. The potential applied during the impedance measurements are shown.

At the low frequency region the impedance spectra shape is different between the positive potential impedance and negative potential impedance. In this region the phase angle decreases for measurements at −400mV, which indicates an increase in the resistive behaviour of the electrode, relative to a capacitive one [2]. The absolute impedance (Bode Z) measured at negative potentials appears to become
independent of frequency, while the phase angle approaches zero again, corresponding to the polarisation resistance of the electrode.

From the CVs in Figure 4.1 it can be observed that the negative potential used in the impedance measurements are situated in the region of dissolved oxygen reduction whilst the OCP and positive potential are situated in the double layer region. The variation between the spectra recorded at negative and positive potentials is due to the presence of faradaic processes. The polarization feature (DC limit) at -400mV appears to be due to the presence of faradaic processes brought about by the reduction of dissolved oxygen.

Due to the absence of faradaic processes, except at -400mV, the impedance spectra are dominated by double layer variations. The capacitance of the electrode double layer can be calculated from the capacitance plot using the high to medium frequency range. The diameter of a circle fit to this region is the electrode capacitance in farads (F). The sensitivity of the EIS system is dependent on variables such as reproducibility in electrode polishing before each adsorption stage. It was found that the variation in electrode capacitance about the mean value for a bare electrode was approximately 10%. This was done by performing impedance measurements on the bare electrode after polishing a number of times. Therefore changes in electrode capacitance were not considered significant unless the variation was greater than 10%.
The capacitance values calculated from the capacitance plots are presented in Figure 4.5. The impedance was measured at the applied potential shown after exposure to solutions of 0.2M PBS, 1000µg mL\(^{-1}\) HSA and 1000µg mL\(^{-1}\) Ig.G at OCP (pH = 7.0). To accurately observe the effect of the adsorbing protein on the electrode capacitance, the changes due to interaction with PBS solution (Exposure to PBS) was subtracted from the change due to protein adsorption (Exposure to HSA or Ig.G depending on which protein used). This change is represented as a Δ% value in the figures and was obtained by dividing the change in capacitance due to protein adsorption (i.e., Exposure to Ig.G – Exposure to PBS) by the bare electrode value capacitance.

Upon adsorption of HSA and Ig.G the electrode capacitance decreased, with the greatest decrease being observed at the potentials 400mV and OCP. The work presented in Chapter 3 – Section 3.2.3 showed that the change in electrode capacitance could be related to the amount of adsorbed protein, which was also highlighted by other workers [18-20]. Therefore the potentials at which the greatest decrease in capacitance was observed also corresponded to the potentials at which the greatest amount of protein adsorbed.

From the adsorbed amounts reported in Chapter 3 for HSA and Ig.G it was possible to calculate the volume occupied by the mass of protein from the size of each protein and their maximum adsorbed amounts. At OCP the adsorbed Ig.G occupies twice the volume that HSA does, indicating a thicker protein layer since the electrode surface areas were the same for both proteins. This thicker Ig.G
layer explains why the decrease in the capacitance after adsorption of Ig.G is slightly greater than after adsorption of HSA.

Figure 4.5 Capacitance values for a gold disc electrode (Initial) before and after exposure to 0.2M PBS (exposed to PBS) and exposure to a 1000μg mL⁻¹ protein solution (exposure to HSA or Ig.G) for 30min (pH = 7.0) at OCP. Impedance was measured in 0.2M PBS (pH = 7.0) at the DC potential shown. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 2.5% (Initial), 5.5% (Exposed to PBS), 2.5% (Exposed to HSA) and 3.0% (Exposed to Ig.G).

A point of interest in Figure 4.5 is that the capacitance values, even after adsorption of protein, appear to be at a minimum between −400mV and OCP, which corresponds to the region of pzc shown in Figure 4.3. This trend is not surprising considering that capacitance and charge are related.
By comparing the Nyquist plot before and after exposure of the electrode to a protein solution it is possible to observe changes brought about by adsorption (Figure 4.6).

![Nyquist plot for a gold disc electrode after exposure to PBS (Final PBS) and Ig.G (Final Ig.G). Adsorption occurred at OCP with the PBS and Ig.G solution pH = 7.0. Impedance was measured at OCP and -400mV in 0.2M PBS (pH = 7.0).](image)

Figure 4.6 does not show the spectrum recorded for the bare electrode due to the fact that there was no difference between it and the spectrum recorded after the electrode was exposed to PBS for 30min (Final PBS). Since the Ig.G solution contains PBS it is appropriate that the spectra recorded after exposure of the electrode to Ig.G for 30min (Final Ig.G) be compared against the Final PBS
spectra to attain a clear understanding of the effects brought about by adsorption of Ig.G.

At OCP no faradaic processes are occurring and the impedance spectra should show a straight line, however, this is not the case since the system is not ideal. If the slight curve was allowed to continue (i.e., impedance measured to very low frequencies) a semicircle would appear resulting in a charge transfer resistance ($R_{ct}$) value at the point where the semicircle crosses the x-axis. This is easier to see for $-400\text{mV}$ were the semicircle is much smaller. The smaller semicircle of $-400\text{mV}$ is due to the faradaic processes occurring, (reduction of dissolved oxygen). At both OCP and $-400\text{mV}$ the adsorption of Ig.G causes the semicircle to enlarge which in turn shifts the $R_{ct}$ to larger values, this was also observed for impedance measured at $400\text{mV}$. This indicates that electrode fouling hinders the faradaic processes occurring at the electrode surface resulting in an increase in charge transfer resistance. These results were also observed when HSA adsorbed to the electrode, with the extent to which $R_{ct}$ increased after protein adsorption being almost identical for both proteins. These results were also observed when CVs were recorded in a solution containing the electroactive species, potassium ferricyanide, before and after exposure of the gold disc electrode to solutions of HSA and Ig.G (Section 4.2.5).
4.2.4.2 Impedance Measurements at Various Potentials

To investigate the effect of applying a potential during adsorption on the electrode capacitance, a new set of experiments were performed. For example, the 400mV results presented in Figure 4.7 were obtained by measuring the electrode impedance at 400mV after the electrode was exposed to solutions of PBS (Final PBS) and Ig.G (Final Ig.G) at 400mV for 30min. This work was performed using Ig.G only at solution pH = 7.0.

![Figure 4.7 Capacitance values for a gold disc electrode (Initial) before and after exposure to 0.2M PBS (Exposure to PBS) and after exposure to 1000μg mL\(^{-1}\) Ig.G (Exposure to Ig.G) (pH = 7.0) for 30min at the applied potentials listed. Impedance measured in 0.2M PBS (pH = 7.0) at the potential shown. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 2.5% (Initial), 5.5% (Exposed to PBS) and 2.5% (Exposed to Ig.G).]

The trends observed in Figure 4.7 agree with what was observed in Figure 4.5 when the electrode was exposed to a protein solution at OCP. The delta values suggest that when Ig.G adsorbed at a potential of 400mV and OCP the adsorption causes the capacitance to decrease to a greater extent than when absorption
occurs at $-400\text{mV}$. This again may be explained by considering that more Ig.G adsorbs at these potentials than at $-400\text{mV}$ (Chapter 3 Figure 3.3).

4.2.4.3 Effect of Electrode Surface Area

In this work the surface area (SA) of the gold disc electrode was varied, by roughening the electrode surface, to investigate the effect of adsorption on the double layer capacitance of the electrode. The terms “polished” and “treated” refer to the electrode before and after the electrode surface area was increased. Exposure to the 0.2M PBS and 1000$\mu$g mL$^{-1}$ Ig.G solutions was performed at an applied potential with the impedance measurement made at the same potential, as detailed in Section 4.2.4.1.2.

By plotting the capacitance against the log of the frequency (Figure 4.8) it is possible to ascertain the effect of modifying the electrode surface. As explained previously the double layer can be defined in terms of an electrical circuit (Figure 2.2). If the capacitor was ideal then the calculated capacitance would not vary with frequency, however from Figure 4.8 it can be seen that the value does vary suggesting that the capacitor does not behave in an ideal way. This was confirmed by the requirement of a constant phase element in the equivalent circuit model (Section 4.3.6).
Figure 4.8 Capacitance vs. the logarithm of frequency of a polished and treated gold disc electrode. Impedance was measured in 0.2 M PBS (pH = 7.0) at OCP.

The increase in capacitance values and variation in capacitance over the frequency range for the treated electrode is associated with the increased electrode surface area and roughness respectively. The non-ideal behavior has also increased for the treated (roughened) electrode.

When overlaying the impedance spectra for the polished electrode, before and after adsorption (Figure 4.9) it is apparent that, when the capacitive behaviour dominates, only a slight difference between the spectra can be noticed. However, when the electrode SA and roughness are increased a marked difference in the spectra is observed. As the capacitive behaviour predominates, a significant difference between the spectra can be noticed. Since the Ig.G solution contains PBS it is appropriate that the Final Ig.G spectra be compared against the Final PBS spectra to attain a clear understanding of the effects brought about by adsorption of Ig.G.
Figure 4.9 The Bode Z plot of the polished and treated gold disc electrode. Impedance was measured at OCP in 0.2M PBS (pH = 7.0). Spectra (x) Final PBS and (●) Final Ig.G. Exposure to PBS and Ig.G solutions occurred at OCP for 30min.

The capacitance values presented in Figure 4.10 were measured after the electrode was treated. When the bare electrode capacitance values in Figure 4.10 are compared with those shown in Figure 4.7, an increase is observed which is due to an increase in electrode SA and roughness. As the SA and roughness increased, adsorption of Ig.G resulted in a large decrease in capacitance compared to the polished electrode capacitance decrease. There appears to be very little variation in the change in capacitance after exposure to PBS between the treated and polished electrode, suggesting that upon increasing the SA and roughness of the electrode the effects of PBS interaction on the electrode capacitance are not accentuated as they are for Ig.G adsorption.
This accentuation of capacitance decrease after Ig.G adsorption could be due to the increase in SA and roughness increasing the amount of binding sites for Ig.G. The increase in Ig.G binding sites may arise due to the increased morphological SA due to the generation of valley and peaks bought about by surface roughening. If more area is open to adsorption then there must be more available binding sites for Ig.G to attach to. An increase in binding sites would have the effect of increasing the amount of adsorption occurring at the electrode surface. Arnebrant and others [18-20] have shown that as the amount of contact points between the protein and electrode surface increases the variation between a non-fouled electrode and fouled electrode capacitance also increases.

![Figure 4.10 Capacitance values for a treated gold disc electrode before (Initial) and after exposure to 0.2M PBS (Exposed to PBS) and after exposure to 1000μg mL⁻¹ Ig.G (Exposed to Ig.G) (pH = 7.0) for 30min at the applied potentials listed. Impedance measured in 0.2M PBS (pH = 7.0) at the potential shown. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 3.0% (Initial data), 6.0% (Exposed to PBS) and 3.5% (Exposed to Ig.G).](image)

**Figure 4.10** Capacitance values for a treated gold disc electrode before (Initial) and after exposure to 0.2M PBS (Exposed to PBS) and after exposure to 1000μg mL⁻¹ Ig.G (Exposed to Ig.G) (pH = 7.0) for 30min at the applied potentials listed. Impedance measured in 0.2M PBS (pH = 7.0) at the potential shown. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 3.0% (Initial data), 6.0% (Exposed to PBS) and 3.5% (Exposed to Ig.G).
This variation in the capacitance after adsorption of Ig.G agrees with the results presented in Figure 4.9 where for the treated electrode the variation in the spectra in the capacitive region after adsorption is greater than for the polished electrode. This work shows that the effects on the electrode capacitance upon adsorption of Ig.G were enhanced when the SA and roughness of the electrode was increased. This is possibly due to the increase in available binding sites for the Ig.G protein to establish close contact points upon adsorption.

### 4.2.4.4 Adsorption of Ig.G with Pulsed Potential Routine Applied

This work was performed using a Au single use sensor (SUS) electrodes (Figure 1.3) obtained from Polartechnics Ltd. Impedance measurements were performed before and after exposure to solutions of 0.2M PBS and 1000μg mL⁻¹ Ig.G, both at pH = 7.0, for 30min under pulse potential conditions. The TruScan™ was used to apply the pulses during the exposure. The same SUS electrode was used for the before exposure (Initial) and after exposure (Final) measurements, however, separate electrodes were used for the measurements performed in PBS and Ig.G. Impedance measurements were performed in 0.2M PBS (pH = 7.0) at OCP. Initial and Final capacitance values for each electrode were obtained and the variation in capacitance as a percentage change are reported. By comparing the percent change after exposure to PBS with the percent change after exposure to Ig.G it is possible to ascertain the extent of capacitance change due to Ig.G alone.

After exposure to PBS the capacitance increased by 36% whilst after adsorption in Ig.G the increase was only 14% (Figure 4.11). When the PBS effect is
removed from the Ig.G effect the net change in capacitance, due to Ig.G only, is a decrease of 22%. This decrease in capacitance due to adsorption of Ig.G agrees with the results presented above (Figure 4.7). TruScan applies a rapid 800mV pulse to one electrode (working electrode) whilst the other two act as the counter electrode. This pulse is applied to the next electrode whilst the other two act as the counter. This pulsing routine is cycled around all three electrodes at a rate of one pulse every 260\(\mu\)s (see Section 1.2). Since this system is a two electrode arrangement the 800mV is distributed between the working and counter electrodes. Therefore when the working electrode is pulsed at 400mV the counter electrode is poised at -400mV. This means that when the SUS electrode is pulsed during exposure to the PBS and Ig.G solutions the electrodes are held at positive and negative potentials. Therefore it is expected that after exposure under pulse potential conditions the change in capacitance due to Ig.G adsorption should be between that seen at positive and negative potential when adsorption occurs at constant potential. The net decrease is 22%, which is between that observed for adsorption at a constant positive (-35%) and negative (-21%) potential (see Figure 4.7).
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Figure 4.11 Comparison of capacitance values recorded (Initial) before and (Final) after exposure to 0.2M PBS and 1000µg mL⁻¹ Ig.G (pH = 7.0). Exposure occurred for 30min under TruScan™ pulsed potential conditions. Impedance was measured at OCP in 0.2M PBS (pH = 7.0). The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.25% (Initial) and 5.75% (Final).

The impedance was measured at OCP (100mV) and from the CV shown in Figure 4.12 it can be observed that this potential is located in the region dominated by double layer charging. At potentials below 0mV the cathodic current decreases rapidly due to reduction of dissolved oxygen, which was confirmed by the decrease in this cathodic current after purging the PBS solution with nitrogen.
Figure 4.12  Gold SUS electrode cyclic voltammogram recorded in 0.2M PBS (pH = 7.0) before and after purging the PBS solution with N₂ gas. Scan rate = 100 mV s⁻¹. (The arrows show the direction of the scan.) The asterisk indicates the potential at which the impedance spectra were obtained.

For a fully capacitive (ideal) system the Nyquist plot should be a straight line, associated with the capacitive nature of the electrode at this potential. However, the system is not ideal and some faradaic nature appears in the Nyquist plot, which makes it possible to calculate the charge transfer resistance (R_{ct}) of the electrode. This is achieved by extending the impedance plot to low frequencies to where the impedance plot crosses the x-axis, which corresponds to the R_{ct} value. By calculating the R_{ct} before and after exposure to a PBS and Ig.G solution it is possible to study the effect of Ig.G adsorption on the electron transfer processes occurring at the electrode surface (Figure 4.13).
Figure 4.13  Impedance data in the form of a Nyquist plot for the Au SUS electrode recorded (Initial) before and (Final) after exposure to solutions of 0.2 M PBS and 1000 μg mL⁻¹ Ig.G (pH = 7.0). Impedance was measured in 0.2M PBS (pH = 7.0) at OCP. Exposure occurred at a pulsed potential for 30min.

After exposure to PBS the R_{ct} appeared to decrease, however after exposure to Ig.G the R_{ct} increased indicating that adsorption of Ig.G blocks electron transfer processes occurring at the electrode surface. When the above experiment was repeated the changes observed after exposure to PBS and Ig.G are the same as that shown in Figure 4.13, suggesting that while the changes are not dramatic the trends are the same. These results have been confirmed by subsequent work, where similar adsorption experiments were performed in the presence of an electroactive species (section 4.25) and adsorption monitored by cyclic voltammetry.
4.2.4.5 Impedance at a Single Frequency

The electrochemical interface can be represented as an ideal Randles electrical circuit (Figure 2.2) composed of the electrolyte resistance ($R_s$) in series with the parallel combination of the double layer capacitance ($C_{dl}$) with charge transfer resistance ($R_{ct}$) [5, 8]. For the Randles circuit it is possible to write:

$$\frac{Z'}{Z''} = R_s + \frac{R_{ct}}{1 + \omega^2 R_{ct}^2 C_{dl}^2}$$  \hspace{1cm} (4.3)

And

$$\frac{Z'}{Z''} = \frac{R_{ct}^2 \omega}{1 + \omega^2 R_{ct}^2 C_{dl}^2}$$  \hspace{1cm} (4.4)

Where $Z'$ and $Z''$ are the real and imaginary components of impedance, $R_{ct}$ and $R_s$ are the charge transfer resistance and solution resistance, respectively, $C_{dl}$ is the double layer capacitance of the electrode/solution interface and $\omega = 2\pi f$ where $f$ is the frequency of the applied AC voltage. In a system where $\omega^2 R_{ct} C_{dl}^2 \gg 1$ it can be assumed $Z'' = (1/C_{dl}\omega)$ [8].

To determine the frequency to use, a plot of capacitance against log frequency is constructed using the Nyquist data recorded in PBS (Figure 4.14). The frequency is chosen from the pseudo-plateau region of the plot. A RC circuit generally describes this pseudo-plateau region, and the corresponding capacitance assimilated to the double layer capacitance, even if it is not really frequency independent [6].
Therefore, choosing a frequency in this region allows for the monitoring of the double layer capacitance. In this work, electrochemical impedance was thus measured at 1000Hz, since this frequency is located within the central section of the pseudo-plateau region.

![Capacitance as a function of the logarithm of frequency of a bare gold disc electrode. Impedance was recorded at OCP in 0.2M PBS (pH = 7.0).](image)

**Figure 4.14** Capacitance as a function of the logarithm of frequency of a bare gold disc electrode. Impedance was recorded at OCP in 0.2M PBS (pH = 7.0).

The potentials OCP, 400mV and -400mV were used with the results for -400mV and 400mV presented below in Figure 4.15. The capacitance profiles for both PBS and Ig.G adsorption are shown. At all potentials the extent of capacitance variation due to exposure to PBS was much less than the variation due to adsorption of Ig.G. All of the initial capacitance values (capacitance at time = 0min) were normalised to zero to be able to get a clear picture of the variation in capacitance over time at each potential.
When the electrode potential was biased to $-400 \text{mV}$ during exposure to 0.2M PBS and 1000μg mL$^{-1}$ of Ig.G the capacitance of the electrode increased. This is opposite to what was observed when the capacitance was calculated from the Capacitance plot (Figure 4.7). This increase in capacitance recorded at $-400 \text{mV}$ may be due to the faradaic process (reduction of dissolved oxygen) causing an increase in double layer charge which is great enough to compete against the adsorbing protein which causes the double layer to decrease as seen for the 400mV result (no faradaic processes occurring). This result suggests that this method of monitoring the $C_{dl}$ at a single impedance frequency is not valid when faradaic processes are occurring at the electrode/electrolyte interface.

When the $C_{dl}$ was monitored at electrode potentials of OCP and 400mV the capacitance decreased upon exposure to 0.2M PBS and 1000μg mL$^{-1}$. The electrode capacitance change after 30min adsorption for each duplicate
experiment was averaged with the results highlighted in Table 4.1. Once again the delta values represent the change in capacitance due to Ig.G adsorption (PBS effect removed) and the % presents this delta value as a percentage change in capacitance relative to the non-normalised initial (exposure time = 0min) capacitance value measured in Ig.G (4.5).

\[
\% = \frac{\Delta C}{Cap_{t=0}^{Ig_G}} \times 100\%
\]  

(4.5)

<table>
<thead>
<tr>
<th>Potential (mV)</th>
<th>-400</th>
<th>OCP</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta C (\mu\text{F cm}^{-2}))</td>
<td>+0.48 (±0.02)</td>
<td>-0.62 (±0.03)</td>
<td>-0.98 (±0.05)</td>
</tr>
<tr>
<td>%</td>
<td>+11.9</td>
<td>-14.3</td>
<td>-16.0</td>
</tr>
</tbody>
</table>

Table 4.1 Average change in capacitance after adsorption of Ig.G. Exposure time to the Ig.G solution was 30min. Impedance performed in Ig.G, pH = 7.0. The percentage value is the extent at which the capacitance varied from the non-normalised initial capacitance value measured in Ig.G (equation 4.5). The 5.5% error of the averages is shown in the brackets.

There appears to be two regions involved in the \(C_{dl}\) decay shown in Figure 4.15, one for the first 10min of adsorption and another for 10 to 30min adsorption. The shape of the capacitance decay appears to be the reciprocal of protein adsorption shown previously in Chapter 3 (Figure 3.3 and 3.4). Bernabeu et al [6] also observed this relationship for adsorption of BSA at a platinum electrode. His group associated these regions with various modes of protein adsorption, namely the formation of an irreversible layer during the first 10min of contact, followed by the formation of a reversible layer at longer contact times.
Since in all cases, except -400mV, 98% of the total $C_{dl}$ change occurs in the first 10min, it appears that only the adsorption of the irreversible layer can be monitored using the double layer capacitance method. These results are in agreement with work performed by others [18-20]. These workers concluded that the capacitance decrease, recorded when protein adsorbs at an interface, is related to the number of close contact points formed between the protein and the surface. Therefore, at adsorption time $t = 0$min there are many available sites for the protein to bind to, and as a result many contact points are formed. As the electrode surface becomes coated in protein, the amount of available sites for protein binding become less and the rate at which these contact points are formed decreases.

It is interesting to note that 98% of the $C_{dl}$ change occurs in the first 10min of adsorption whilst the radiolabelling work showed only ~50% of surface coverage of Ig.G at this time. This indicates that the Ig.G adsorbing after this time has little to no effect on the electrode capacitance variation. However, the adsorption results are of those using an Ig.G concentration of 100μg mL$^{-1}$ whilst this work is performed in 1000μg mL$^{-1}$. It may be possible that at this concentration an adsorption maximum may have been reach after 10min adsorption.
4.2.5 Faradaic – Electron Transfer Effects

4.2.5.1 Cyclic Voltammetry in K₃Fe(CN)₆

4.2.5.1.1 Adsorption at OCP

The results presented here were obtained using a protein concentration of 100µg mL⁻¹. This concentration allowed for observable and reproducible results to be recorded and enabled the comparison of this work with the adsorption results presented in Chapter 3. The proteins HSA and Ig.G were both used for this work to investigate the effect of protein size on the electron transfer blocking process. Figure 4.16 shows successive CVs in ferricyanide after adsorption in 100µg mL⁻¹ HSA or Ig.G (pH = 7.0) at OCP for various lengths of time. The anodic currents are shown as positive currents whereas the cathodic currents are negative. All CVs were scanned in the positive direction from OCP. For all CVs a blank in PBS was performed at all adsorption times, pH, and applied potential. In all sets this blank CV showed very little variation from the bare electrode CV indicating that the change in CV after contact with the protein/PBS solution is due solely to the protein.
Figure 4.16  Comparison of CVs recorded on a gold disc electrode after adsorption of HSA and Ig.G (100μg mL⁻¹, pH = 7.0) at OCP. CVs were recorded in a solution of 0.01M K₃Fe(CN)₆ containing 0.1M NaNO₃. The exposure times are listed in the figure. Scan rate = 100mV s⁻¹.

As the adsorption time increases the anodic and cathodic peaks decrease, accompanied by a positive and negative direction shift in the respective potentials. Table 4.2 shows the peak-to-peak separation as a function of adsorption time. As the adsorption time increases so too does the separation.
Table 4.2  Peak-to-peak separations for the anodic and cathodic potential as a function of adsorption time. Results were obtained from the CVs shown in Figure 4.16.

<table>
<thead>
<tr>
<th>Adsorption Time</th>
<th>HSA</th>
<th>Ig.G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Electrode</td>
<td>0.146</td>
<td>0.152</td>
</tr>
<tr>
<td>10s</td>
<td>0.179</td>
<td>0.203</td>
</tr>
<tr>
<td>30s</td>
<td>0.386</td>
<td>0.376</td>
</tr>
<tr>
<td>5min</td>
<td>0.546</td>
<td>0.514</td>
</tr>
</tbody>
</table>

At adsorption times greater than 10min the peak currents associated with the oxidation of the ferricyanide species was not distinguishable from the background charging current. The shape of the anodic and cathodic peak broadens as the adsorption time increases. The above changes in the CV are bought about by a decrease in the electron transfer rate constant [21] due to the electrode surface becoming coated with an inert protein layer. This layer acts as a barrier preventing the ferricyanide ions from approaching close to the electrode surface. This view is supported by the work performed by Anzani et al [22] and Willner et al [23]. Delahay and Trachtenberg also noted that coverage of the electrode by a foreign substance decreases the effective current density and increases the over-voltage [24].

Electron transfer in a microstructured film is a process involving the dynamics of several elementary events that overlap and cross-influence on one another. Therefore this film controls the kinetics of charge transport associated with the observed current. Two models have been proposed by other research groups [25, 26] to depict current responses displayed by surface-modified electrodes and are
referred to as the membrane and pinhole models. The latter model assumes the protein coating to be impervious except for small pinholes through which the electroactive species can diffuse, while the membrane model presents the coating as a uniform phase barrier with a certain permeability.

When the anodic peak current at \( t \) adsorption time (\( i_{a(t)} \)) is divided by the bare electrode current (\( i_{a(0)} \)) an anodic current profile can be constructed (Figure 4.17). For each anodic current the background charging current for each CV has been subtracted. The profiles show that as the adsorption time increases, the anodic peak current decreases. After 10min adsorption the reduction in anodic peak current appears to have plateaued off, at which time the electron transfer process is completely blocked. This is indicated by the \( i_{a(t)}/i_{a(0)} \) value being approximately 0.

![Figure 4.17](image.png)

**Figure 4.17** Anodic current profile for the gold disc electrode. Data obtained from CVs recorded in 0.01M \( \text{K}_3\text{Fe(CN)}_6 \) containing 0.1M \( \text{NaNO}_3 \) after exposure to solutions of 100\( \mu \)g mL\(^{-1}\) (---) HSA and (—) Ig G (pH = 7.0). Exposure occurred at OCP. Scan rate = 100mV s\(^{-1}\). The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.25% (HSA) and 5.75% (Ig.G).
The rate and extent of electron blocking for both HSA and Ig.G appear to be the same at all time over the 10min adsorption period. However, when the adsorbed amounts (adsorption at OCP) are compared for HSA and Ig.G at 10min (Chapter 3 - Figure 3.6) there appears to be approximately twice as much Ig.G adsorbed compared to HSA, although, due to their different molecular mass the number of adsorbed molecules are very similar. When the number of adsorbed molecules is converted into volume occupied it shows that the Ig.G molecules occupy approximately twice the volume of the HSA molecules. While the volume of the adsorbed HSA layer is smaller than that of the adsorbed Ig.G the extent of electron blocking is the same which suggests that the HSA protein layer is more effective in blocking the electron transfer process than Ig.G.

The appearance of the plateau region at 10min adsorption indicates that at longer adsorption times no further reduction in electron blocking occurs. From the radiolabelling and QCM results presented in Chapter 3, adsorption equilibrium under the same experimental conditions is not reached until approximately 60min. The fact that at adsorption times greater than 10min no further decrease in anodic current is observed implies that the continued growth of the protein layer does not influence the electron transfer process.

4.2.5.1.2 Effect of Applied Potential during Adsorption

In this work a potential is applied to the electrode during the adsorption of HSA and Ig.G from solutions of pH = 7.0. At this pH the proteins carry a negative charge. From the adsorption work presented in Chapter 3 it was found that by
applying a potential to the electrode during adsorption the amount of adsorbed protein varied.

The anodic current profiles presented in Figure 4.18 shows the effect of varying the applied potential during adsorption. Like the profiles shown in Figure 4.17, the adsorption profiles have reached a plateau by 10min adsorption. Figure 4.18 demonstrates clearly that the electron blocking nature of the protein film on a gold electrode varies under the influence of the electrode potential applied during adsorption.

The extent to which the $i_{a(t)}/i_{a(o)}$ value decrease indicates the level of protein layer formation. The degree of layer formation can be attributed to the amount of adsorbed protein. Therefore, the amount of electrode coverage increases as the $i_{a(t)}/i_{a(o)}$ value approaches zero. It can be seen in Figure 4.18 that for both proteins the extent of electron transfer blocking is approximately the same at 700mV and OCP after 10min adsorption, whilst for -800mV it is less. The -800mV result for both proteins show that after 10min of adsorption the $i_{a(t)}/i_{a(o)}$ value is approximately 0.2, suggesting not all of the electron transfer is blocked. These results indicate that for both proteins the amount of adsorbed protein is greatest when the electrode potential is 700mV followed by OCP with the least amount being adsorbed at -800mV. These results may be explained by considering electrostatic influences. Since both proteins carry a negative charge adsorption at positive surfaces (700mV and OCP) will be favoured over negative surfaces.
(-800mV). These results agree with the adsorbed amount results for HSA and Ig.G presented in Chapter 3 - Section 3.2.1.2.

![Graph](image)

**Figure 4.18** Anodic current profile for the gold disc electrode. Data obtained from CVs recorded in 0.01M K$_3$Fe(CN)$_6$ containing 0.1M NaNO$_3$ after exposure to solutions of 100μg mL$^{-1}$ (---) HSA and (---) Ig.G (pH = 7.0). Exposure occurred at the potentials shown. Scan rate = 100mV s$^{-1}$. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.25% (HSA) and 5.75% (Ig.G).

### 4.2.5.1.3 Effect of Solution pH

Figure 4.19 shows the effect of varying the pH during adsorption of HSA and Ig.G at OCP. From the pzc work (Section 4.2.3) the electrode carries a positive
charge at OCP. The profiles recorded for HSA and Ig.G when the solution pH = 7.0 and 10.0 showed that complete electron blocking occurred after approximately 10 min of adsorption. The rate at which the electron transfer rate decreases is the same at pH = 7.0 and 10.0 for both HSA and Ig.G. This is due to both proteins having the same charge at these pHs, namely a negative charge, and therefore the adsorption trends will be similar.

![Graph showing anodic current profile for gold disc electrode](image.png)

**Figure 4.19** Anodic current profile for the gold disc electrode. Data obtained from CVs recorded in 0.01 M K$_3$Fe(CN)$_6$ containing 0.1 M NaNO$_3$ after exposure to solutions of 100 µg mL$^{-1}$ (—) HSA and (—) Ig.G at various pH. Exposure occurred at OCP. Scan rate = 100 mV s$^{-1}$. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.25% (HSA) and 5.75% (Ig.G).
The profile for Ig.G adsorption from a solution pH = 3.0 shows that the rate and extent of electron transfer blocking is less than pH = 7.0 and 10.0 after 10 min adsorption. This is due to the fact that at pH = 3.0 the electrode and the protein at have the same charge (positive) and electrostatic repulsion hinders the adsorption process.

When adsorption of HSA occurs from a solution of pH = 3.0 the anodic peak current has completely disappeared after 30s adsorption, indicating complete blocking of the electron transfer process. Based on the discussion presented above, as the protein layer increases the $i_a(t)/i_a(o)$ value decreases, this trend appeared erroneous since the charge of the electrode at OCP is positive and at pH = 3.0 the charge on HSA is also positive. With both the electrode and the protein having the same charge it would be expected that due to electrostatics alone the amount of adsorbed HSA at pH = 3.0 would be less that at pH = 7.0 and 10.0 where HSA has a negative charge. If adsorption was less at pH = 3.0 then the extent at which the $i_a(t)/i_a(o)$ value decreases should also be less, clearly it is not.

The fact that complete electron blocking occurs after 30sec suggests that other factors are involved in the adsorption process of HSA at pH = 3.0. HSA has a relatively low native-state stability as inferred from its large conformational adaptability to changes in its environment [27] (i.e., unfolding). Hayes and Norde noted that since a globular protein’s folded conformation in aqueous solution are only marginally stable under the best of conditions they can usually be denatured...
by a modest change in environment, such as a change in pH [28]. Add to this the natural tendency for proteins to denature at solid surfaces then the reason for this complete blocking of the electron transfer by the adsorption of HSA after only 30 sec at pH = 3.0 is most likely due to denaturing of the protein. This denaturing of the protein causes it to unfold its secondary structure to occupy more volume, which in turn will block further electrode sites where ferricyanide can be oxidised.

4.2.5.2 Electrochemical Impedance Spectroscopy in K$_3$Fe(CN)$_6$

Prior to examining the effect of modification of the gold disc electrode with protein, it was necessary to confirm the CV behaviour of the Fe(CN)$_6^{4-}$/Fe(CN)$_6^{3-}$ species at the gold electrode. The gold disc cyclic voltammogram recorded in 0.01 M potassium ferricyanide containing 0.1 M NaN$_3$ is presented in Figure 4.20. The CV shows the characteristic reversible oxidation and reduction of the ferrocyanide/ferricyanide ion at +285 mV and +135 mV (vs. Ag/AgCl reference) respectively with a peak-to-peak separation of 150 mV.

The OCP of the gold disc electrode measured in 0.2 M PBS was 100 mV. Since EIS spectra are sensitive to the applied DC potential it was necessary to use this same OCP value (even though it will be different to the OCP in K$_3$Fe(CN)$_6$) to allow for comparisons to be made between spectra recorded in PBS and K$_3$Fe(CN)$_6$. 

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Figure 4.20 Cyclic voltammogram of a gold disc electrode recorded in a solution of 0.01M $K_3Fe(CN)_6$ containing 0.1M $NaN_3$. Potentials are vs. Ag/AgCl reference electrode. The arrows show the direction of the scan. Scan rate = 100mV s$^{-1}$. The asterisks indicate the potentials at which impedance spectra were obtained.

The shape of the impedance spectra recorded in $K_3Fe(CN)_6$ is quite different compared to those recorded in PBS (Figure 4.21). The semicircle present in Figure 4.21-$K_3Fe(CN)_6$, highlighted with a dashed line for ease of identification, is due to faradaic processes associated with the reduction of ferricyanide ions since OCP is situated at the point of ferricyanide reduction (see Figure 4.20).
When protein adsorbs at the electrode surface the impedance of the system increases (Figure 4.22). The increase in the semicircle represents an increase in the $R_{ct}$ (charge transfer resistance). This increase in resistance to electron flow agrees with the results presented above where the adsorption of protein decreased the rate of electron transfer. The increase in $R_{ct}$ after adsorption was also observed at $-400\text{mV}$, however it was difficult to observe for impedance recorded.
at 400mV due to this potential being situated in the double layer charging region of the CV where very little faradaic processes are occurring.

**Figure 4.22** Nyquist plot of impedance spectra of a gold disc electrode recorded in 0.2M PBS containing 0.01M $K_3Fe(CN)_6$ containing 0.1M NaNO$_3$ at OCP ($pH = 7.0$). The impedance spectra were recorded (Final PBS) after exposure to 0.2M PBS ($pH = 7.0$) and (Final Ig.G) after exposure to 1000μg mL$^{-1}$ Ig.G ($pH = 7.0$).

### 4.2.6 Electrical Equivalent Circuit (EEC) Modelling

EEC modelling, like mathematical modelling, involves fitting simulated circuits (equations) to the data to get an accurate fit between simulated results and experimentally obtained results. Just like mathematical modelling it is possible to fit any equation to any data set provided the equation is complex enough. Therefore it is crucial to know and understand the electrode surface characteristics and the processes occurring at the electrode surface so as to fit a simple and logical circuit.

Of all the circuits used in the modelling process the circuit shown in Figure 4.23 produced the best fit to experimental data. The circuit is a modified Randles
circuit, comprising of a resistor ($R_s$) in series with a constant phase element ($Q$) in parallel with a resistor ($R_{ct}$). Instead of pure capacitance, a constant phase element ($Q$) was introduced in the modelling procedure. Often a constant phase element is used in a model in place of a capacitor to compensate for non-homogeneity in the system. For example, a rough or porous surface can cause a double-layer capacitance to appear as a constant phase element. The use of $Q$ in the model shown in Figure 4.3 is due to a distribution of relaxation times as a result of inhomogeneities present at the microscopic level at the electrode/electrolyte interface [29].

![Figure 4.23](image)

**Figure 4.23** Electrical equivalent circuit used to model the experimental data. Where $R_s$ and $R_{ct}$ represents the solution and charge transfer resistance respectively and $Q$ is the constant phase element representing the double layer capacitance.

The electronic components $R_s$ and $R_{ct}$ represent the solution resistance between the working and reference, and the charge transfer resistance respectively. $Q$ is the capacitance of the electric double layer at the electrode/electrolyte interface and is represented by a constant phase element and its exponent $n$.

Fitting simulated data, using the circuit shown above, to experimental data obtained at a potential of $-400\text{mV}$ was difficult, particularly in the low frequency region of the spectra. The low frequency region of impedance spectra is characterised by slow electrode processes, such as diffusion across the double
layer [9]. At $-400\text{mV}$ perturbations are introduced at the electrode surface as a result of the reduction of dissolved oxygen. The diffusion of the dissolved oxygen across the double layer may be the cause for the difficulty in fitting the experimentally obtained data at $-400\text{mV}$. A Nyquist plot showing the experimental data and the simulated fit is presented in Figure 4.24.

![Figure 4.24](image)

**Figure 4.24** Nyquist plot of impedance data for a bare gold disc electrode recorded in 0.2M PBS at OCP ($pH = 7.0$). The symbol (●) represents the experimental data while (••••) is the simulated fit using the EEC shown above.

When IgG was adsorbed onto the electrode surface at OCP a significant increase in the $R_{ct}$ was observed (Table 4.3). The value of $Q$ also increased after adsorption, although not to the same extent while the exponent $n$ value remained at $-0.9$. This trend was also observed at 400mV and $-400\text{mV}$. The increase in charge transfer resistance is associated with the formation of an inert protein layer at the electrode surface. The use of a constant phase element was justified by the exponent $n$ values all being approximately 0.9, this is, neither capacitive ($n = 1.0$) nor resistive ($n = 0$).
Chapter 4 - Effect of Protein Adsorption on the Electrochemical Behaviour of Gold

Table 4.3  The values of the equivalent circuit elements obtained from the impedance measurements for a gold disc electrode recorded in 0.2M PBS at OCP (pH = 7.0).

<table>
<thead>
<tr>
<th></th>
<th>$R_{ct}$ (MΩ cm$^{-2}$)</th>
<th>$Q$ (μF cm$^{-2}$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial PBS</td>
<td>6.7</td>
<td>1.3</td>
<td>0.908</td>
</tr>
<tr>
<td>Final PBS</td>
<td>5.91</td>
<td>1.4</td>
<td>0.899</td>
</tr>
<tr>
<td>Final Ig.G</td>
<td>29.6</td>
<td>2.5</td>
<td>0.871</td>
</tr>
</tbody>
</table>

4.3 Conclusions

When protein adsorption occurs at the electrode a decrease in electrode capacitance is observed. This decrease in capacitance is attributed to the formation of a proteinaceous layer interacting with the electrical double layer at the electrode surface. Upon treatment of the electrode, which increased the electrode surface area and roughness, adsorption of Ig.G also resulted in a decrease in capacitance. The extent of capacitance decrease for the treated electrode was greater than for the polished electrode, which was attributed to the increased surface area and modification of the electrode surface due to the roughening procedure. The magnitude of the capacitance values measured increased after treatment, which was attributed to the increased surface area. This increase in surface area appeared to accentuate the decrease in capacitance observed after adsorption. Of the two proteins used, the greatest capacitance change occurred when Ig.G adsorbed at the electrode surface, which agrees with the protein adsorption results that indicated a greater adsorption of Ig.G.
When the electrode double layer capacitance was measured at a single frequency a decrease was observed at the potentials 400mV and OCP, while the -400mV profile showed an increase in capacitance. The decrease in capacitance was rapid within the first 10min of adsorption and slowed down as adsorption time increased. By approximately 10min of adsorption, the electrode capacitance had reached a steady value. This equilibrium value was attributed to the formation of an inert protein layer on the electrode surface. At times greater than 10min, adsorption was still occurring however it had no effect on the electrode capacitance. It was proposed that monitoring of the double layer capacitance in this manner only allows for monitoring effects associated with the formation of the irreversibly bound protein layer.

Using EIS and CV it was possible to study the effects of adsorption on the electron transfer processes at the electrode/solution interface. Under all experimental conditions the adsorption of protein resulted in an increase in the charge transfer resistance. This increase is attributed to the blocking of the electron transfer process by the formation of an inert protein layer at the electrode surface. When the charge on the electrode and protein was varied electrostatic interaction dominated the adsorption process. When the protein and electrode had similar charges the effects due to adsorption were reduced compared to when they had opposite charges. When the size of each protein is compared, it appears that HSA is more efficient at blocking the electron transfer process. The anodic current, associated with oxidation of the electroactive species, was indistinguishable from the background charging current after 10min

of adsorption, suggesting complete blocking of electron transfer. Since adsorption continues past 10min it was proposed that the continued formation of the inert protein layer has no further effect on the electron transfer processes. This result agrees with the $C_{dl}$ results obtained at a single frequency, where $C_{dl}$ levelled off after 10min of protein adsorption.

It was possible to model the electrode/protein interface with a modified Randles circuit using the EEC method. For this model to allow a best fit, it was necessary to use a CPE in the circuit, confirming the non-ideal nature of the electrode/protein interface.
Chapter 4 - Effect of Protein Adsorption on the Electrochemical Behaviour of Gold

References


24. P. Delahay and I. Trachtenberg, 2094 (1958)


Chapter 5

Adsorption of Ig.G on Titanium Dioxide
5.1 Introduction

Metallic titanium spontaneously generates an oxide surface layer when exposed to air or aqueous media [1]. This oxide layer plays a vital role in the biocompatibility of surgical implants since this oxide layer acts as a passive layer protecting the implant from corrosion [2]. Protein interaction with passive films such as titanium dioxide (TiO$_2$) is of major concern in the medical industry as they may have bearing on film breakdown and ultimately implant failure [2]. The interaction of TiO$_2$ with proteins in solution forms the basis of many studies into the biocompatibility of this material [3-5].

Topoglidis et al [6] studied the factors that affect protein adsorption on nanostructured titanium dioxide films and found protein immobilisation to be remarkably stable, which they attributed to secondary binding processes occurring as immobilisation time increased. In addition Giacomelli et al [1], using ellipsometry to study the adsorption of BSA on Ti/TiO$_2$ electrodes, found that adsorption occurs in a two step process. The first step is characterised as a fast attachment process governed by electrostatic interactions. The second step is much slower and involves rearrangement of the adsorbed protein molecules to find their more energetically favoured orientation with respect to the TiO$_2$ surface. Fukuzaki et al [7] studied the adsorption of BSA onto metal oxides using the Lowry-Folin [8] method.

Cervical mucus, the secreted membrane covering the cervix, is characterised by a high content of macromolecules called mucins. One of the functions of cervical
mucus is to act as a protective layer between the cervical tissue and the environment. To aid with this protection, mucins contain antibodies, one of which is immunoglobulin G (Ig.G). Therefore the protein Ig.G is more pertinent for this study with Chapters 5 and 6 being restricted to the investigation of Ig.G at pH = 7.0. By maintaining a solution pH = 7.0 it is possible to compare adsorption trends at TiO₂ with that for gold, and pH = 7.0 is close to physiological pH under which TruScan™ operates. The potentials used during adsorption and impedance measurements were −100mV (OCP), 400mV and −400mV. The reason for choosing 400mV and −400mV is due to the fact that TruScan™ applies a positive potential of 800mV during each pulse (see Chapter 1). TruScan™ works as a two-electrode system where one TiO₂ electrode is the working and the other two are the counter electrodes. Therefore, the 800mV pulse is distributed between the working electrode and the counter electrode combination, effectively only applying a pulse of 400mV to the working electrode. Whilst the working electrode is held at 400mV the counter electrode combination is held at −400mV, which is why adsorption under an applied potential of −400mV was also investigated.

The TiO₂ electrode used for this work was fabricated at IPRI by the electrochemical oxidation of titanium coated polycarbonate sheets (provided by Polartechnics Ltd.) in sulfuric acid. The electrochemical oxidation process involves cycling the potential from 0.0V to 1.6V and back to 0.0V at a scan rate of 100mV s⁻¹ in a solution of 1.0M H₂SO₄.
5.2 Results and Discussion

5.2.1 Adsorption at Constant Potential

The adsorption of Ig.G from a buffered solution (pH = 7.0) at TiO$_2$ under various applied potentials was investigated using $^{125}$I radiolabelling (Figure 5.1). Protein adsorption was greatest when the electrode potential was 400mV, it decreased at OCP (-100mV) and further decreased at -400mV. The pI of polyclonal Ig.G lies in the pH range of 5-8, and at pH = 7.0 the charge on the Ig.G molecule is likely to be close to neutral. Therefore electrostatic effects will play a role in determining the adsorption characteristics. This trend in the amount of protein adsorbed was observed for adsorption of HSA and Ig.G at the gold disc electrode (Chapter 3). Increased adsorption of Ig.G at positive potentials was also observed by Guo et al [9]. Adsorption appeared to still be occurring after 60min, however, like the gold results shown in Chapter 3 the rate of adsorption had reached a pseudo plateau value. The amount of Ig.G adsorbed after 60min was taken to be the saturation point.

![Figure 5.1 Adsorption profiles for TiO$_2$ samples in 100µg mL$^{-1}$ Ig.G at pH = 7.0. Applied potentials during adsorption are indicated on the figure. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0%.](image)

Figure 5.1 Adsorption profiles for TiO$_2$ samples in 100µg mL$^{-1}$ Ig.G at pH = 7.0. Applied potentials during adsorption are indicated on the figure. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0%.
The total amount of protein adsorbed after 60min at OCP for the TiO\textsubscript{2} electrode is greater than that recorded at the Au electrode, 4.57 and 3.32mg m\textsuperscript{-2} respectively. This increase in the amount of adsorbed Ig.G is illustrated by comparing the Au and TiO\textsubscript{2} adsorption isotherms recorded at OCP and pH = 7.0 (Figure 5.2).

**Figure 5.2** Adsorption isotherm for Ig.G measured for Au and TiO\textsubscript{2}. Adsorption occurred from an Ig.G solution of pH = 7.0 at OCP. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.75% (Au) and 8.25% (TiO\textsubscript{2}).

At surface saturation for both substrates (100 μg mL\textsuperscript{-1}) the adsorbed amount of Ig.G at TiO\textsubscript{2} is 34% greater than at Au. The AFM surface roughness calculations indicated the surface area of TiO\textsubscript{2} to be 9% greater than Au (shown below). This increased surface area however, does not account for the 34% increase in adsorbed Ig.G recorded at TiO\textsubscript{2}. These results suggest that Ig.G has a greater affinity for TiO\textsubscript{2} than Au. A well defined maximum adsorption plateau is observed at concentrations greater than 100μg mL\textsuperscript{-1}. There appears to be two
plateau regions in each isotherm, one at low concentrations and the other at high concentrations. The presence of a step or "kink" has been reported elsewhere for globular protein isotherms [10]. The presence of the step indicates that protein binding to the surface is bimodal. To explain the appearance of this kink Koutsoukos et al [11] proposed that orientation of the adsorbed protein varies with surface coverage, i.e. a side-on to end-on transition. Evidence for such an orientation transition has come from surface force measurements of albumin on various hydrophilic surfaces [10, 12].

This increase in adsorbed amount at the TiO₂ electrode is associated with the increased surface roughness compared to the Au electrode, which can be seen in the atomic force microscopy (AFM) images (Figure 5.3).
Chapter 5 - Adsorption of Ig.G on Titanium Dioxide

Figure 5.3  AFM image of (a) gold electrode and (b) TiO₂ electrode. AFM images were obtained using contact mode in air.

The roughness of the electrodes were calculated as described in Chapter 2 and were found to be 0.1% for Au 9.0% for TiO₂. The increase in roughness is attributed to the formation of the oxide layer upon electrochemical oxidation in 1.0M H₂SO₄. As the roughness increases so too does the surface area (SA) of the electrode. It has been shown that nanoporous TiO₂ has a SA typically much greater (by up to 1000 times) than its geometric area. It is this increased SA with
pores of similar dimensions to those of a protein, which allows high levels of protein adsorption without loss of protein structure and activity [6]. The adsorbed amounts shown in Figures 5.1 and 5.2 are reported per geometric SA. Since the surface roughness of the TiO$_2$ electrode is 90 times greater than that of the Au electrode it is expected, on this account alone, that more adsorption would occur at the TiO$_2$ surface when geometric areas are considered.

In Chapter 3 the dimensions of the Ig.G molecule was given to be 4.5 x 4.5 x 23.5nm with the calculated adsorbed amount at complete electrode surface coverage (monolayer) for vertical and horizontal orientation being 12.02mg m$^{-2}$ and 2.30mg m$^{-2}$, respectively. Since the adsorbed amounts at all electrode potentials shown in Figure 5.1 range from 3.26 at -400mV to 4.97mg m$^{-2}$ at 400mV, the orientation of the adsorbed protein probably comprises of a mixture of both vertical and horizontal.

5.2.2 Adsorption at Pulsed Potential

The effect of the applied potential waveform was investigated with respect to adsorbed amounts of Ig.G from a buffered solution at pH = 7.0 (Figure 5.4). As outlined in the experimental section a square wave pulse of 1.0s duration was applied to the electrode for 30min. For the pulsed potential work performed with the gold electrode (Chapter 3 – Section 3.2.2) the potential was pulsed between 0.0mV and either 700mV or -800mV. For this work the TiO$_2$ electrode was pulsed between OCP (-100mV) and either 400mV or -400mV. The reason for choosing OCP rather than 0.0mV is due to the fact that the TruScan™ pulse
Chapter 5 - Adsorption of Ig.G on Titanium Dioxide

Routine incorporates a pulse from OCP to 800mV. Therefore OCP was chosen to be able to compare these results with capacitance results presented in Chapter 6 – Section 6.2.4.2.2.

Figure 5.4 Amounts of protein adsorbed when the potential is pulsed during adsorption in 100μg mL⁻¹ Ig.G at pH = 7.0. Adsorption time was 30min. The adsorption at constant potential results is included for comparison. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0% (Constant) and 7.5% (Pulsed).

The delta values in Figure 5.4 represent the difference in the amount of adsorbed Ig.G between the constant potential and pulsed potential results. When the electrode was pulsed between OCP and 400mV during adsorption the amount of adsorbed Ig.G was reduced by 51% compared to when the electrode potential was held constant at 400mV during adsorption. This result can be explained by considering the amount of adsorbed Ig.G when the electrode potential is held constant during adsorption (Figure 5.1). The amount of adsorbed Ig.G was greater when adsorption occurred at 400mV than at OCP. When the electrode is pulsed between OCP and 400mV the electrode is biased at 400mV for half of the
30min adsorption period and at OCP for the other half. Therefore the amount of adsorbed Ig.G will be less under pulsed potential conditions than if the potential was held constant at 400mV for the whole 30 min adsorption period.

When the electrode potential was pulsed between OCP and -400mV during the adsorption, the amount of adsorbed Ig.G remained the same. This result was unexpected since, based on the reasoning presented above, pulsing the electrode between OCP and -400mV should have resulted in a greater amount of adsorbed Ig.G compared to maintaining a constant potential of -400mV during adsorption. This result may be explained by considering the change in electrode/protein interactions when pulsing occurs. When the electrode is pulsed to -400mV from OCP the total potential change is only 300mV. This change may not be enough to alter the electrode surface characteristics, which in turn may not affect the electrode/protein interactions. If the electrode/protein interactions do not vary, then the amount of adsorbed Ig.G would be similar to those recorded when the electrode potential was held constant at -400mV.

5.2.3 EIS Study - Protein Adsorption Kinetics

Similar to the work presented in Chapter 3 – Section 3.2.3, to ensure that observable variations in the impedance spectra were obtained, the concentration of the protein used for this work was increased 10 fold, from 100μg mL⁻¹ to 1000μg mL⁻¹. When the impedance was recorded at 1000Hz in a solution of 1000μg mL⁻¹ of Ig.G (pH = 7.0) the change in C_{dl} could be monitored.
All of the initial capacitance values (capacitance at time = 0min) were normalised to zero to be able to get a clear picture of the variation in capacitance over time at each potential. A typical response of change in capacitance over time, due to the interaction of Ig.G, is shown in Figure 5.5. The profile recorded when the electrode was biased to 400mV was similar to the profile recorded at OCP, however it was not included to aid in interpretation of the Figure 5.5 data. The profile recorded when adsorption occurred at an electrode potential of -400mV also had the same shape as that recorded at OCP and 400mV, however the time it took for the profile to level off was much longer (~20min).

**Figure 5.5**  Plot of the variation of $C_{dl}$ versus time as a consequence of exposure to Ig.G. Impedance was measured at OCP (-100mV) in 1000μg mL$^{-1}$ Ig.G (pH = 7.0). Impedance was measured at 1000Hz.

Similar to the gold disc electrode results there appears to be two regions involved in the $C_{dl}$ variation profile for exposure to Ig.G. When the electrode is biased at -400mV during adsorption the first region extends from 0min to approximately 20min but, when the electrode is biased at OCP (-100mV) and 400mV, the first region extends to approximately 10min. The second region for all profiles
extends for the remainder of the adsorption period. The difference in the time it took for the profiles to reach a plateau (OCP, 400mV = 10min; -400mV = 20min) maybe due to the fact that more protein adsorbs when the electrode potential during adsorption is biased at OCP (Figure 5.1), because the $C_{dl}$ value has been shown by others [13-15] to be related to the number of protein-surface contacts (i.e., amount of adsorbed protein). The initial region corresponds to irreversibly adsorbed protein while the second region accounts for reversibly adsorbed protein [13-15]. This suggestion of two types of adsorbed Ig.G has also been proposed in Chapter 7 - Section 7.2.2.

The presence of two regions in a TiO$_2$ electrode $C_{dl}$ variation has also been observed by Oliva et al [16] who proposed the results suggest that the adsorption is consistent with a two-consecutive step mechanism. The two consecutive reactions occurring at the interface can be considered as follows:

$$P_{sol} \xrightarrow{k_1} P_{ads1} \xrightarrow{k_2} P_{ads2}$$

Where $P_{sol}$ refers to the protein in the electrolyte solution and $P_{ads1}$, $P_{ads2}$ are the two different states of the adsorbed protein. The proposed mechanism shown above was also reported for platinum and glassy carbon rotating disc electrodes with bovine serum albumin (BSA) [17, 18]. A schematic representation of this process is shown in Figure 5.6.
In Chapter 3 – Section 3.2.3 it was demonstrated that it is possible to calculate the extent of surface coverage with protein using equation 5.1. Like the \( C_{dl} \) variation plot shown in Figure 5.5 the surface coverage profiles also show two regions of variation (Figure 5.7). The profile recorded when the electrode potential was 400mV is also included.

\[
\Theta_i = \frac{C_o - C_i}{C_o - C_f}
\]

(5.1)

Figure 5.6  Scheme of the TiO\textsubscript{2}/Ig.G system in 0.2M PBS showing the different steps from initial protein adsorption until equilibrium conditions are established.

Figure 5.7  Surface coverage variation due to adsorption of Ig.G. Adsorption occurred at the potentials shown in the figure on a TiO\textsubscript{2} electrode from a solution of 1000µg mL\textsuperscript{-1} Ig.G prepared in 0.2M PBS (pH = 7.0).
It is possible to calculate the adsorption rate constant \( (k_m) \) in the same ways as performed for the gold disc electrode in Chapter 3 – Section 3.2.3. The same two adsorption models used for the gold disc electrode are used here, namely, Langmuir and Diffusion Controlled Langmuir (DCL). The equation for each model is shown in (3.3) and (3.4) respectively.

The rate constant at each potential for each model is shown in Table 5.1. The rate constant was determined at five different adsorption times with the standard deviation being calculated at 95% confidence.

**Table 5.1** Rate constant for Ig.G adsorption on TiO₂ electrode. Adsorption occurred at the different applied potentials shown using the DCL and Langmuir models. The concentration of Ig.G was 1000µg mL⁻¹. The standard deviation at 95% confidence is shown in brackets.

<table>
<thead>
<tr>
<th>Applied Potential</th>
<th>Diffusion Controlled Langmuir ( 10^3 k_m (s^{-1/2}) )</th>
<th>Langmuir ( 10^3 k_m (s^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>400mV</td>
<td>83.1 (24.0)</td>
<td>6.3 (0.3)</td>
</tr>
<tr>
<td>OCP (-100mV)</td>
<td>36.5 (8.2)</td>
<td>2.8 (0.1)</td>
</tr>
<tr>
<td>-400mV</td>
<td>31.1 (4.7)</td>
<td>2.5 (0.3)</td>
</tr>
</tbody>
</table>

For both models and at all potentials, the rate constants calculated for adsorption at TiO₂ are less than those calculated for adsorption at Au. The smaller rate constants for TiO₂ indicate that the processes affecting the C₅l are slower [18] than for Au processes, which are evident by the longer time it took for C₅l to reach equilibrium (Figure 5.5).
These rate constants were substituted into equations (3.3) and (3.4) to determine which model predicts the adsorption kinetics. For the $C_{dl}$ profiles recorded when the electrode potential was $-400$ mV during exposure to the Ig.G solution only the data between 0 min and 20 min was used while only the data between 0 min and 10 min was used for the profile recorded when the electrode potential was OCP and 400 mV during exposure to the Ig.G solution. When the Langmuir model was applied to the data, a good fit was obtained at low to intermediate electrode coverage, however when the surface coverage approached 100% the fit deteriorated. Therefore, the DCL model was applied to the experimental data with a good fit resulting at all surface coverage except at low coverage were the data tends to pull the fit down. Despite this deviation at low coverage the DCL model gave a much better fit that the Langmuir model. The data recorded at OCP (-100 mV) is shown in Figure 5.8.

![Figure 5.8](image)

**Figure 5.8** Linear regression plot to check validity of the DCL model fitted to the $C_{dl}$ data obtained from impedance data recorded at 1000 Hz and at OCP (-100 mV). Impedance was recorded in a 1000 µg mL$^{-1}$ Ig.G solution at pH = 7.0.
From Table 5.1 it can be seen that the rate constant is larger as adsorption occurs when the electrode is polarized positive. This may be due to the applied potential creating a potential gradient within the oxide layer that in turn creates diffusion of charged species within the layer.

From the work performed by others [19-21] phosphate has been shown to interact with TiO₂. The results presented in Chapter 7 also showed that the presence of phosphate reduced the extent of Ig.G adsorption onto TiO₂. Therefore it must be noted that the above determination of adsorption kinetics has not considered the effect of the phosphate on Ig.G adsorption.

5.3 Conclusions

Adsorption of Ig.G at a TiO₂ electrode occurs spontaneously at pH = 7.0. The amount of adsorbed protein is increased upon application of a potential positive of the OCP and decreased upon application of a potential negative of OCP. The adsorption isotherms showed Ig.G to have a greater affinity for TiO₂ than Au when adsorption of Ig.G, prepared in 0.2M PBS (pH = 7.0), occurred at OCP. When the potential is pulsed between OCP and -400mV during the adsorption stage the adsorbed amount is similar to the amount recorded when the potential is held constant at -400mV. This was not the case when the potential was pulsed between OCP and 400mV. The adsorbed amount after pulsing the electrode was much less (89%) than when the potential was held constant during the adsorption stage.
It was possible to use EIS to monitor the $C_{dl}$ as adsorption occurs. From the data it was possible to determine the adsorption rate constant ($k_m$) for two adsorption models, then use these constant to determine which model best describes the adsorption kinetics. It is also possible to use this technique to investigate the effect of various applied potentials during adsorption on the rate of adsorption and hence the rate constant and adsorption model. The calculated rate constants for adsorption at TiO$_2$ are smaller than those calculated for Au, indicating that the processes affecting the $C_{dl}$ are slower for TiO$_2$. The model which best describes the adsorption kinetics at all electrode potentials used during exposure to the Ig.G solution was the Diffusion Controlled Langmuir model.
Chapter 5 - Adsorption of Ig.G on Titanium Dioxide

References


Effect of Ig.G Adsorption on the Electrochemical Behaviour of Titanium Dioxide
6.1 Introduction

An introduction to EIS has already been presented in Chapter 3. While there are many papers published regarding protein adsorption and electrochemical effects at metal electrodes [1-5], there is very little information regarding the effects of protein adsorption on semiconductor electrochemistry. A recent paper by Oliva et al [6] has gone some way to filling this void and provides a valuable insight into the semiconductor-protein interface. The work presented in this section involves the investigation of the electrochemical responses of a titanium dioxide (TiO$_2$) electrode upon adsorption of protein.

The TiO$_2$ electrodes used for this work were prepared and supplied by Polartechnics Ltd. The electrode has three kidney shaped electrodes on the tip (Chapter 1 - Figure 1.1). The electrode is of a three-layer construction, the first layer is the polycarbonate substrate that is sputtered with three kidney shaped gold contacts (second layer), on top of the gold is sputtered Ti (third layer), and the thickness of the Ti is approximately 50nm. The Ti covered electrode is electrochemically oxidised as detailed in Chapter 5 Introduction.

All impedance measurements, except for the single frequency work performed in the Ig.G solution and determination of the potential of zero charge, were carried out in 0.2M PBS at pH = 7.0. Since the TiO$_2$ electrode is a single use electrode it was not possible to measure impedance after exposure to PBS and Ig.G on the same electrode. Therefore, for each electrode an Initial impedance measurement was made before exposure and a Final one after exposure. For this reason, the
impedance and capacitance data had to be plotted in a slightly different way to that for the Au disc electrode. The changes in capacitance and impedance, due to adsorption of Ig.G and the interaction of phosphate from the PBS, were converted to percentages and were compared for separate electrodes for PBS and Ig.G adsorption.

When the TiO₂ electrodes are manufactured at Polartechnics Ltd. the capacitance is measured to determine the reproducibility in forming the electrode surface. The reproducibility in manufacturing the TiO₂ electrodes is approximately 95%. Therefore any variation in impedance or capacitance upon adsorption that was less than 5% was not considered significant, as this variation may be due to the variability in the electrode surface and not from the adsorbed layer. This was confirmed by multiple capacitance measurements made in our laboratory that showed a variation about the mean (0.85μF cm⁻²) of approximately 4%.

6.2 Results and Discussion

6.2.1 Electrochemical Characterisation

The CV for the formation of the TiO₂ film is presented in Figure 6.1. The large oxidation current is associated with the formation of the oxide layer. The nature of the oxide layer varies depending on the final oxidizing potential [7] and the TiO₂ film forms as a polycrystalline layer consisting of a mixture of rutile and anatase.
Chapter 6 - Effects of IgG Adsorption on the Electrochemical Behaviour of Titanium Dioxide

Figure 6.1  Cyclic voltammogram showing the oxidation of Ti in 1.0M H₂SO₄ to form TiO₂. Scan rate was 100mV s⁻¹ with the arrows showing the direction of the scan.

Atomic Force Microscopy (AFM) was used to image the Ti and TiO₂ films. A flat surface for the Ti sample in (a) and the rougher more porous TiO₂ sample in (b) are obvious (Figure 6.2). The surface defects observed in Figure 6.2(a) are the result of scratches that occurred during sample preparation. Apart from these defects the surface of the Ti sample is flat with very little roughness observed, whilst the TiO₂ image shows a much rougher surface due to the formation of the oxide layer. When the roughness of each sample was calculated the Ti value was 0.46% and the TiO₂ sample 9.07%, resulting in a surface roughness increase by a factor of 20.
To characterise the processes occurring at the TiO$_2$ electrode in PBS a CV was recorded in a solution of 0.2M PBS at pH = 7.0, (Figure 6.3). The region between 0.0V and 0.6V is one of little electroactivity where no faradaic processes are occurring and is referred to as the double layer region. The only thing occurring
in this region is the charging of the double layer. The reduction current between 0.0V and approximately -0.4V arises from the reduction of dissolved oxygen. This was confirmed by the decline in reduction current observed when the CV is recorded in deoxygenated PBS, (Figure 6.3).

![Graph showing cyclic voltammograms](image)

**Figure 6.3** TiO$_2$ electrode cyclic voltammogram recorded in 0.2M PBS (pH = 7.0), before and after deoxygenation at a scan rate of 100mV s$^{-1}$. (The arrows show the direction of the scan.) The asterisks show the region where the impedance potentials are located on the CV.

EIS measurements were performed on the Ti and TiO$_2$ samples to determine the electrochemical changes due to oxidation of the electrode. The capacitance and Bode Phase plots are presented in Figure 6.4 for the Ti and the TiO$_2$ samples.
Figure 6.4 Capacitance and Bode plots of the impedance data for the Ti and TiO$_2$ samples recorded in 0.2M PBS. Impedance was recorded at OCP (-100mV) in the frequency range of 50mHz-50kHz.

At high frequencies there appears to be no difference between the Ti and TiO$_2$ phase angle and absolute impedance. As the frequency decreases the TiO$_2$ absolute impedance starts to become independent of frequency and the phase angle approaches zero, indicating an increase in a resistive behaviour of the electrode, relative to a capacitive behaviour for the Ti sample. This response at low frequency is attributed to the formation of the oxide layer that has a higher resistance than the bare Ti surface. From the Capacitance plot (not shown) it can
be seen that after oxidation the electrode capacitance has increased which is also attributed to the formation of the porous oxide layer.

6.2.2 Open Circuit Potential (OCP) Measurement

For the impedance measurements to be performed at OCP it is necessary to determine the TiO$_2$ OCP in 0.2M PBS (Figure 6.5). A steady state potential of -100mV was reached at approximately 100min, which was used as the OCP for all subsequent TiO$_2$ impedance measurements. This value agrees reasonably well with the value obtained by Oliva *et al* [6] for a nanocrystalline Ti/TiO$_2$ electrode in 0.1M NaCl.

![Figure 6.5](image)

*Figure 6.5*  *Time dependence of the open circuit potential of the TiO$_2$ electrode measured in 0.2M PBS at pH = 7.0 vs. Ag/AgCl.*

6.2.3 Potential of Zero Charge

The capacitance plot as a function of electrode potential was obtained (Figure 6.6). When the impedance potential was varied from 200mV to -500mV the capacitance decreased to a minimum value at approximately -100mV at which point capacitance began to increase. The potential of zero charge was interpreted
to be $-100\text{mV}$, therefore, when potentials are more positive than $-100\text{mV}$ the electrode surface carries a positive charge and for potentials more negative than $-100\text{mV}$ the electrode charge is negative.

![Capacitance vs. potential plot](image)

*Figure 6.6* Capacitance vs. potential plot. Impedance was measured between 50kHz and $50\text{mHz}$ in $4.0\times10^{-3}\text{M}$ PBS solution at $\text{pH} = 7.0$. Potentials are vs. Ag/AgCl reference. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 7.3%.

Bernabeu *et al* [8] used equation (6.1) to determine the charge on a platinum and carbon electrode, where $U$ is the potential used to measure the capacitance, $U_0$ is the pzc, $C_d$ is the capacitance value at potential $U$ and $C_0$ is the capacitance at pzc. The charge ($q$) on the TiO$_2$ electrode at potentials above and below the electrode pzc is shown in Table 6.1.

$$q = \frac{(U - U_0)(C_d + C_0)}{2}$$

(6.1)
**Table 6.1** Charge on the TiO₂ electrode calculated using equation 6.1. The capacitance was calculated from the impedance measurements performed in 0.004M PBS (pH = 7.0). The potential at which the impedance was measured is shown in the table.

<table>
<thead>
<tr>
<th>Potential (V)</th>
<th>Charge (µF cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>1.36</td>
</tr>
<tr>
<td>0.1</td>
<td>0.86</td>
</tr>
<tr>
<td>0.0</td>
<td>0.40</td>
</tr>
<tr>
<td>-0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>-0.2</td>
<td>-0.42</td>
</tr>
<tr>
<td>-0.3</td>
<td>-0.95</td>
</tr>
<tr>
<td>-0.4</td>
<td>-1.82</td>
</tr>
</tbody>
</table>

6.2.4 Non-Faradaic - Double Layer Effects

6.2.4.1 Impedance and Adsorption at Constant Potential

In this work adsorption and impedance were performed at -400mV, OCP and 400mV (Figure 6.7). Like the impedance spectra obtained for the gold disc electrode the TiO₂ spectra vary depending on the potential applied during the impedance measurement.

All of the Bode Z plots have a similar shape revealing the high frequency region to be independent of the frequency with the Rs value similar at all potentials (~60ohms); once again all three phase angles are at or near zero. In the medium frequency region a linear relationship is observed between the absolute impedance and frequency, and the phase angle approaches 90°, corresponding to the capacitive behaviour of the electrode/electrolyte. In the low frequency region the OCP and 400mV phase angle do not decrease, while at -400mV it does. At
the same time the $-400\text{mV}$ absolute impedance appears to become independent of frequency, associated with reaching the electrode DC limit.

![Bode plots of TiO$_2$ electrode recorded in 0.2M PBS (pH = 7.0). The potential applied during the impedance measurement are shown. The frequency range was 50mHz-50kHz.](image)

**Figure 6.7** Bode plots of TiO$_2$ electrode recorded in 0.2M PBS (pH = 7.0). The potential applied during the impedance measurement are shown. The frequency range was 50mHz-50kHz.

The differences in the impedance spectra recorded at OCP, 400mV and $-400\text{mV}$ can be explained by looking at the TiO$_2$ CV recorded in 0.2M PBS (Figure 6.3). The potential of 400mV is situated in the double layer region of the CV whilst OCP (-100mV) is situated at the start of the region associated with the reduction of dissolved oxygen. The $-400\text{mV}$ potential on the other hand is situated well within the region of the CV where reduction of dissolved oxygen is occurring. The CV recorded in the deoxygenated PBS solution confirmed the region
corresponding to the reduction of dissolved oxygen. The differences observed in the impedance spectra are due to the reduction process occurring at the electrode surface.

The capacitance was measured from the circle fit applied to the capacitance plot (Figure 6.8). At all potentials the capacitance increased after exposure to a solution of 1000μg mL⁻¹ Ig.G prepared in 0.2M PBS, with the delta values indicating the increase with respect to the Initial capacitance value.

Figure 6.8 Capacitance values for a TiO₂ electrode (Initial) before and (Final) after exposure to solutions of 1000μg mL⁻¹ Ig.G prepared in 0.2M PBS (pH = 7.0). Impedance measured in 0.2M PBS at the DC potential shown. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 6.5% (PBS) and 5.5% (Ig.G).
This increase in electrode capacitance observed in Figure 6.8 agrees with results published elsewhere [7], where an increase in capacitance occurs when the electrode is biased from the $V_{fb}$ (flatband potential) at a given pH. Finklea also points out that it is possible to dope TiO$_2$ electrochemically at room temperature by biasing the electrode negative of the flatband potential and the increased doping accounts for the capacitance change.

The extent of capacitance increase after Ig.G adsorption was less than after exposure to the PBS solution. When the delta value due to PBS interaction is subtracted from the delta value due to Ig.G interaction net effect due to Ig.G adsorption is a decrease in the TiO$_2$ electrode capacitance. The extent at which the capacitance decreased was 10%, 5% and 4% for the values measured at an electrode potential of $-400$ mV, OCP and $400$ mV respectively. While the last two percentage values are within the 10% significance range the decrease trend agrees with the results presented previously for the Au electrode (Figure 4.5).

The largest decrease in capacitance was recorded when the potential was $-400$ mV, which is located in the reduction region of the CV (Figure 6.3). The reduction process could be the reason the results recorded after exposure to the PBS solution at $-400$ mV showed a larger increase than the other two potentials.

By comparing the Nyquist plot before and after exposure of the TiO$_2$ electrode to solutions of PBS and Ig.G it is possible to observe the changes brought about by adsorption (Figure 6.9).
Figure 6.9  The Nyquist plot for a TiO$_2$ electrode (Initial) before and (Final) after exposure to solutions of 0.2M PBS and 1000μg mL$^{-1}$ Ig.G (pH = 7.0). Adsorption and Impedance was performed at OCP in PBS (pH = 7.0).

As adsorption occurred at the electrode surface the semi-circle feature became smaller indicating a reduction in the R$_{ct}$. This reduction in R$_{ct}$ after adsorption is the opposite of what was observed for the Au disc electrode, however, it was observed when adsorption occurred at the Au SUS electrode (Chapter 4 – Section 4.2.4.2). This decrease in R$_{ct}$ could be due to modification of the TiO$_2$ surface during protein adsorption. The surface modification appears to activate the surface and be associated with either doping or further oxidation of TiO$_2$, since the electrode is polycrystalline comprising of a mixture of rutile and anatase forms of TiO$_2$. Anodic oxidation results of TiO$_2$ are consistent with direct injection of electrons into the TiO$_2$ conduction band [9]. This electron injection
has the effect of further doping of the electrode [7]. While this anodic oxidation doping process does not change the composition of the polycrystalline TiO$_2$ sample it may account for the "activation" observed. Polartechnics Pty Ltd. have observed that the TiO$_2$ electrode dehydrates during storage, suggesting that hydration of the electrode during adsorption may also contribute to the activation process. This surface modification process was also observed in the work performed in potassium ferricyanide (Section 6.2.5).

It is clear that after exposure to the solution of PBS the $R_{ct}$ value decreased more than after Ig.G adsorption. This agrees with what was observed for the Au disc electrode where an increase in $R_{ct}$ was observed after Ig.G adsorption but not after PBS. If the activation of the TiO$_2$ electrode was the same after exposure to the PBS and Ig.G solutions then the decrease in $R_{ct}$ will be the same, however, if adsorption of Ig.G causes an increase in $R_{ct}$ during exposure then the final $R_{ct}$ value will be less than PBS. The same results were observed at OCP and 400mV, however the extent of the $R_{ct}$ decrease was less.

6.2.4.2 Impedance at OCP

6.2.4.2.1 Adsorption at Constant Potential

To investigate the effect of varying the potential at which adsorption occurred, the impedance was measured in 0.2M PBS at OCP before and after adsorption at an electrode potential of 400mV and $-400$mV (Figure 6.10). The results presented in Figure 6.8 and 6.10 show a significant variation in the electrode capacitance after exposure to the 0.2M PBS solution suggesting adsorption of
phosphate to the TiO$_2$ electrode surface. Phosphate has been shown in Chapter 7 to bind to TiO$_2$, which has also been reported elsewhere [1, 10, 11].

Figure 6.10 Capacitance values measured for a TiO$_2$ electrode (Initial) before and (Final) after exposure to 0.2M PBS and 1000µg mL$^{-1}$ Ig.G (pH = 7.0) for 30min. Impedance measured in 0.2M PBS (pH = 7.0) at OCP. The potentials shown indicate the potential applied during adsorption. The delta values represent the increase in capacitance from the Initial to Final. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 7% (PBS) and 5.0% (Ig.G).

When the effect of Ig.G adsorption (ΔIg.G − ΔPBS) is investigated, the results after adsorption at 400mV show a net decrease (Δ36% - Δ54% = -18%) in
capacitance. When adsorption of Ig.G occurs at an electrode potential of \(-400\text{mV}\) the net effect is an increase in capacitance \((\Delta 73\% - \Delta 24\% = 49\%)\). This increase in capacitance may be due to processes occurring at the electrode during adsorption due to the application of \(-400\text{mV}\).

The effects of adsorption on the Nyquist plot were investigated (Figure 6.11). Once again the semi-circle feature of the Nyquist plot is smaller after adsorption of phosphate and Ig.G. The changes shown below were also observed for the adsorption of PBS.

\[\text{Figure 6.11 The Nyquist plot for a TiO}_2\text{ electrode before (Initial) and after (Final) adsorption of Ig.G at 400mV and -400mV. Impedance was measured at OCP in 0.2M PBS.}\]
The Initial and Final $R_{ct}$ values and the associated change in $R_{ct}$ ($\Delta R_{ct}$) are shown in Table 6.2. Similar to the results presented in Section 6.2.4.1 the $\Delta R_{ct}$ at both potentials is less after adsorption of Ig.G than it is for phosphate. There is a greater difference between the $\Delta R_{ct}$ values (Ig.G and PBS) recorded when adsorption occurred at $-400\text{mV}$ than at $400\text{mV}$, however, the difference between the $\Delta R_{ct}$ value within the $-400\text{mV}$ data set (Ig.G versus PBS) is very slight. It was observed from the adsorption work presented in Chapter 5 that less protein adsorbed when the electrode potential was $-400\text{mV}$ as opposed to $400\text{mV}$. It has been proposed that the change in $R_{ct}$ is a result of adsorption, where greater adsorption resulted in a larger $\Delta R_{ct}$ value. Therefore, in this case this larger $\Delta R_{ct}$ value recorded when adsorption occurred at $-400\text{mV}$ must be due to factors other than protein adsorption. One possible explanation may be that the application of $-400\text{mV}$ to the TiO$_2$ electrode during adsorption of Ig.G and phosphate changes the electrochemical nature of the electrode surface due to the associated electrochemical processes occurring at this potential. This may also account for the increase in capacitance observed at $-400\text{mV}$ in Figure 6.10.

Table 6.2 $R_{ct}$ values (Initial) before and (Final) after exposure to 0.2M PBS and 1000$\mu$g mL$^{-1}$ Ig.G ($pH = 7.0$). Adsorption occurred at 400mV and $-400\text{mV}$. The $R_{ct}$ values were calculated from the Nyquist plot recorded at OCP in 0.2M PBS ($pH = 7.0$).

<table>
<thead>
<tr>
<th></th>
<th>400mV</th>
<th>-400mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>Ig.G</td>
</tr>
<tr>
<td>Initial $R_{ct}$ (M$\Omega$)</td>
<td>9.32</td>
<td>6.07</td>
</tr>
<tr>
<td>Final $R_{ct}$ (M$\Omega$)</td>
<td>5.52</td>
<td>3.84</td>
</tr>
<tr>
<td>$\Delta R_{ct}$</td>
<td>-41%</td>
<td>-37%</td>
</tr>
</tbody>
</table>
6.2.4.2.2 Adsorption of Ig.G with Pulsed Potential Routine Applied

To investigate the effect of the applied potential waveform during adsorption, impedance of the TiO$_2$ electrode was measured before and after exposure to solutions of 1000µg mL$^{-1}$ Ig.G and 0.2M PBS (pH = 7.0) under pulsed potential conditions at pH = 7.0 (Figure 6.12).

The capacitance values increased upon adsorption, with the result recorded after exposure to PBS showing the greatest increase. Since the Ig.G solution was prepared in PBS and the capacitance value after exposure to Ig.G is less than after exposure to PBS this suggests that the adsorption of Ig.G has the effect of decreasing the electrode capacitance. This result agrees with what was observed when adsorption occurred at a constant potential.

![Figure 6.12](image_url)  

**Figure 6.12** Capacitance values measured for a TiO$_2$ electrode (Initial) before and (Final) after exposure to solutions of 1000µg mL$^{-1}$ Ig.G and 0.2M PBS (pH = 7.0) at a pulse potential. Polartechnics TruScan™ system was used to apply the pulses during adsorption. Impedance measured in 0.2M PBS at OCP. The delta values represent the increase in capacitance from the Initial to Final in percentage. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 7% (PBS) and 6.3% (Ig.G).
Figure 6.13 compares the capacitance values measured before and after exposure to solutions of PBS and Ig.G at constant and pulsed potential conditions. The amount of adsorbed Ig.G presented in Chapter 5 showed that when the electrode potential was held constant at 400mV, more Ig.G adsorbed compared to when the potential was pulsed during the adsorption stage (Figure 5.4). It has been established that upon adsorption of Ig.G at a constant potential the electrode capacitance decreases (Figure 6.12). Therefore, since less Ig.G adsorbs at pulsed potential it is expected that the net decrease in capacitance would be less when the electrode potential is pulsed during adsorption.

When the delta value recorded after exposure to PBS is subtracted from the delta value recorded after adsorption of Ig.G ($\Delta_{Ig\cdot G}17\% - \Delta_{PBS}33\%$), under pulsed potential conditions, the net decrease is 16%. This is less than the net decrease observed when adsorption occurred at a constant potential of 400mV ($\Delta_{Ig\cdot G}36\% - \Delta_{PBS}54\%$), which was 19%.
Figure 6.13 Capacitance values measured for a TiO$_2$ electrode (Initial) before and (Final) after 30min adsorption of PBS and Ig.G. Impedance measured in 0.2M PBS at OCP. The delta value represents the percentage increase in capacitance from the Initial to Final measurement. The error bars were calculated as described in Chapter 2 and show a measurement error of approximately 5.0% (PBS) and 5.5% (Ig.G).

When adsorption occurred at a potential of $-400\text{mV}$ there appeared to be no difference in the adsorbed amount when the potential was constantly applied or pulsed during adsorption (Chapter 5 - Figure 5.4). Since there was no variation in the adsorbed amounts, and since the capacitance increase is associated with the amount of adsorbed Ig.G, it was expected that there would be no variation in capacitance after adsorption of Ig.G at a constant potential of $-400\text{mV}$ and under
pulsed potential conditions. Clearly there is a difference shown in Figure 6.13, which may also be attributed to the variation of the electrode surface due to the application of \(-400\text{mV}\) and the associated electrochemical processes during adsorption.

The changes in the various impedance plots upon adsorption at a pulsed potential are very similar to those stated for adsorption at constant potential (Figure 6.9). The Nyquist plots for the TiO\(_2\) electrode before and after adsorption at a pulsed potential are shown below in Figure 6.14.

Figure 6.14  The Nyquist plot for a TiO\(_2\) electrode before (Initial) and after (Final) exposure to solutions of 0.2M PBS and 1000\(\mu\text{g mL}^{-1}\) Ig.G (pH = 7.0) under pulse potential conditions. Impedance was measured at OCP in PBS (pH = 7.0).
PowerSine™ calculates the $R_{ct}$ value by applying a circle fit to the Nyquist plot data with the diameter of the circle fit being the $R_{ct}$ value in ohms. Due to the capacitive nature of the Nyquist plots shown in Figure 6.14 the PowerSine™ software was unable to apply a circle fit to the whole data set. Therefore, it was not possible to obtain $R_{ct}$ values for Initial and Final spectra recorded before and after adsorption. It is possible to see however, that if the spectra were allowed to continue to much lower frequencies and pass through the x-axis, the $R_{ct}$ value would decrease upon adsorption of phosphate and Ig.G. It is clear that after exposure to the solution of PBS the $R_{ct}$ value decreased more than after Ig.G adsorption. This agrees with what was observed in Figure 6.9 and for the Au disc electrode where an increase in $R_{ct}$ was observed after Ig.G adsorption but not after PBS. If the activation of the TiO$_2$ electrode was the same after exposure to the PBS and Ig.G solutions then the decrease in $R_{ct}$ will be the same, however, if adsorption of Ig.G causes an increase in $R_{ct}$ during exposure then the final $R_{ct}$ value will be less than PBS.

6.2.4.3 Impedance at Single Frequency

In Chapter 4 - Section 4.2.4.3 it was stated that it is possible to represent the electrochemical interface using a Randles electrical circuit (Figure 2.2). It is possible to apply equations 4.3 and 4.4 to a Randles circuit and use the equation (6.2) to calculate the $C_{dl}$.

$$Z'' = \left( \frac{1}{\frac{C_{dl}}{\omega}} \right)$$  \hspace{1cm} (6.2)
To determine the frequency to use, a plot of capacitance against log frequency was constructed using the Nyquist data recorded in 0.2M PBS (Figure 6.15). The frequency to be used was determined from the pseudo-plateau region of the Capacitance versus log \( f \) plot, which extends from approximately log 1 to log 4.5 (Figure 6.15). Impedance was measured at a constant frequency of 1000Hz corresponding with log 3 that is situated in the pseudo-plateau region.

![Capacitance as a function of the logarithm of frequency of a TiO\textsubscript{2} electrode. Impedance was recorded at OCP in 0.2M PBS (pH = 7.0).](image)

The impedance potentials used were OCP (-100mV), 400mV and -400mV. The initial (time = 0min) \( C_{dl} \) value recorded at each potential was different, so to be able to compare the \( C_{dl} \) profile trends it was necessary to normalise all the initial \( C_{dl} \) values to zero. The \( C_{dl} \) profile for impedance measured at OCP and -400mV (Figure 6.16) shows that capacitance increases for both phosphate and Ig.G adsorption, which agrees with the work presented in Section 6.2.4.1.
Figure 6.16  Profile of $C_{dl}$ variation when impedance was measured at a single frequency (1000Hz) in 0.2M PBS and 1000µg mL⁻¹ Ig.G (pH = 7.0). Impedance was measured at −400mV and OCP.

For the profiles recorded at OCP the capacitance increase is similar in both PBS and Ig.G solutions until 10min, when at longer times the capacitance values for PBS become greater. When impedance was measured at 400mV the trend was the same as that shown for impedance measured at OCP, however the $C_{dl}$ profile is not shown to avoid bunching the data making interpretation difficult.

The trend observed when impedance was measured at −400mV is opposite to those for OCP and 400mV. At all times the capacitance value measured in the Ig.G solution was greater than when measured in 0.2M PBS. As the adsorption time increased, the $C_{dl}$ value measured in the Ig.G solution levelled off and started to converge with the $C_{dl}$ profile recorded in 0.2M PBS.

Similar to the Au disc single frequency results, there appears to be two regions involved in the $C_{dl}$ variation. The first region extends from 0min to
approximately 15min for the profile recorded at OCP in the Ig.G solution while it extends from 0min to approximately 25min for the profile recorded at \(-400\text{mV}\). The shape of the capacitance decay is the same as the protein adsorption profile shown previously in Chapter 5 (Figure 5.1). Bernabeu et al [4] associated these regions with various modes of protein adsorption, namely the formation of an irreversible layer during the first stage of contact followed by the formation of a reversible layer at longer contact times.

When the impedance was measured at OCP, 400mV and \(-400\text{mV}\) the \(C_{dl}\) profiles levelled off within 25min of adsorption. Like the results presented for the Au electrode in Chapter 5, further adsorption of protein at times greater than the time it took for the \(C_{dl}\) value to level off has no effect on the electrode \(C_{dl}\). However, the adsorption results are for those using an Ig.G concentration of 100\(\mu\)g mL\(^{-1}\) and this work is performed in 1000\(\mu\)g mL\(^{-1}\). It may be possible that at this concentration an adsorption maximum may have been reached by 25min of Ig.G adsorption.

The final \(C_{dl}\) values from Figure 6.16 after exposure for 30min are listed in Table 6.3. The Ig.G solution contains 0.2M PBS, therefore, to determine the effect of Ig.G adsorption of the electrode \(C_{dl}\) the effect due to interaction of PBS must be removed from the data recorded in the Ig.G solution. The change in \(C_{dl}\) due to Ig.G only was calculated using the original (non-normalised data) and is expressed as \(\Delta C\) in Table 6.3. The \(\Delta C\) value is the percentage change between
the non-normalised $C_{dl}$ value recorded after 30 min exposure and the non-normalised initial (time = 0 min) $C_{dl}$ value.

Table 6.3 Change in capacitance over 30 min. Impedance performed in solutions of 1000 µg mL$^{-1}$ Ig.G and 0.2 M PBS (pH = 7.0), at a fixed frequency of 1000 Hz. $\Delta C$ represents the difference in capacitance value between impedance measured in PBS and Ig.G.

<table>
<thead>
<tr>
<th>Potential (mV)</th>
<th>-400</th>
<th>OCP (-100)</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>Ig.G</td>
<td>PBS</td>
</tr>
<tr>
<td>Final $C_{dl}$ (µF cm$^{-2}$)</td>
<td>0.208</td>
<td>0.218</td>
<td>0.101</td>
</tr>
<tr>
<td>$\Delta C$</td>
<td>+10.7%</td>
<td>4000</td>
<td>-33.3%</td>
</tr>
</tbody>
</table>

For the impedance measured at OCP and 400 mV the $C_{dl}$ adsorption of Ig.G resulted in a decrease in $C_{dl}$ whilst at and applied potential of -400 mV the $C_{dl}$ increased. This decrease in $C_{dl}$ agrees with the results presented in Section 6.2.4.1. Similar to the results presented in Chapter 4 – Section 4.2.4.3 the increase in capacitance when the impedance was measured at -400 mV indicates that the single frequency impedance method is not suitable for $C_{dl}$ monitoring at potentials where faradaic processes are occurring.

6.2.5 Faradaic – Electron Transfer Effects

6.2.5.1 Cyclic Voltammetry in $K_3Fe(CN)_6$

CVs were recorded in 0.01 M $K_3Fe(CN)_6$ containing 0.1 M NaNO$_3$ before and after exposure to Ig.G and PBS solutions. All of the Initial CVs recorded prior to exposure to the solutions showed no or only a very slight redox couple associated with the oxidation and reduction of the ferricyanide ions. However, after adsorption the redox couple was much more pronounced (Figure 6.17). An
explanation for this is that during the adsorption process the electrode oxide surface is modified (activated) in some way.

This suggestion of a modification of the electrode has been made in Section 6.2.4.1 and is supported by the OCP measurement (Figure 6.5). Initially the potential increased, followed by a slow decrease to where it reaches its OCP after approximately 100min. The Au electrode took only 10min to reach its OCP. This longer time to reach the OCP may be due to the modifications occurring at the electrode surface.

A cyclic voltammogram of the “activated” TiO₂ electrode, showing defined redox couple of ferricyanide, is presented in Figure 6.18. The redox peaks appear at approximately the same potential as they do for the Au disc electrode, however
the anodic and cathodic currents are less for the TiO$_2$ electrode possibly due to the semi-conductor nature.

**Figure 6.18** Cyclic voltammogram obtained using a TiO$_2$ electrode in 0.01M $K_3Fe(CN)_6$ containing 0.1M NaNO$_3$. The scan rate used was 100mV s$^{-1}$, with the arrows showing the direction of the scan. The asterisks indicate the region of the CV where the potentials at which impedance was measured are located.

### 6.2.5.2 Impedance in $K_3Fe(CN)_6$

Impedance measurements were performed at OCP, 400mV and -400mV in 0.01M $K_3Fe(CN)_6$ containing 0.1M NaNO$_3$ before and after exposure to solutions of 1000µg mL$^{-1}$ Ig.G and 0.2M PBS (pH = 7.0). The electrode was biased at 400mV and -400mV during the 30min adsorption period. The shape of the impedance spectra recorded in potassium ferricyanide was different when compared to those spectra recorded in 0.2M PBS (Figure 6.19). The main difference was the spectra recorded in potassium ferricyanide show more of a faradaic appearance due to the presence of electroactive species. The variation between impedance measured in 0.2M PBS and 0.01M $K_3Fe(CN)_6$ shown below
also occurred at the other two potentials (400mV and -400mV) however the variation was not as pronounced for the spectra recorded at 400mV.

![Nyquist plot of impedance spectra recorded at OCP in 0.2M PBS and 0.01M K₃Fe(CN)₆ containing 0.1M NaNO₃. The impedance was recorded prior to adsorption.](image)

**Figure 6.19** The Nyquist plot of impedance spectra recorded at OCP in 0.2M PBS and 0.01M K₃Fe(CN)₆ containing 0.1M NaNO₃. The impedance was recorded prior to adsorption.

After exposure to the solutions of PBS and Ig.G the Rₜₐ value decreased (Figure 6.20) indicating a decrease in the charge transfer resistance. This result has been observed previously in Figures 6.9, 6.11 and 6.13, and it is clear that after adsorption of phosphate from the PBS solution the Rₜₐ value decreased more than after Ig.G adsorption. If the activation of the TiO₂ electrode was the same during exposure to the PBS and Ig.G solutions then the decrease in Rₜₐ will be the same, however, if adsorption of Ig.G causes an increase in Rₜₐ during adsorption then the final Rₜₐ value will be less than that observed after exposure to 0.2M PBS.

These results were also observed when impedance was measured at 400mV and -400mV.
Figure 6.20 The Nyquist plot for a TiO₂ electrode (Initial) before and (Final) after exposure to solutions of 1000μg mL⁻¹ Ig.G and 0.2M PBS (pH = 7.0). Impedance was measured at OCP in 0.01M K₃Fe(CN)₆ containing 0.1M NaNO₃.

6.2.6 Electrical Equivalent Circuit (EEC) Modelling

The EEC that best fitted the impedance data recorded at OCP and 400mV is shown in Figure 6.21. The circuit is a modified Randles circuit and is similar to the circuit used to model the Au disc electrode/electrolyte interface. The circuit comprises a solution resistor (Rₛ) in series with a constant phase element (Q₆l - double layer capacitance) in parallel with a charge transfer resistor (Rₖt) and constant phase element (Qₖl - TiO₂ oxide layer capacitance) in series. Instead of pure capacitance, a constant phase element (Q) was introduced in the modelling procedure. This is due to a distribution of relaxation times as a result of inhomogeneities present on the microscopic level under the oxide phase and at
the oxide/electrolyte interface [12]. This may result from the contributions from static disorder due to porosity [13].

\[
\begin{array}{c}
\text{Rs} \\
\text{Rct} \\
\text{Qdi} \\
\end{array}
\]

**Figure 6.21** Electrical equivalent circuit used to model the experimental impedance data recorded at OCP and 400mV. Rs and Rct represents the solution and charge transfer resistance respectively. Qdi and Qol are the constant phase elements that represent the double layer and oxide layer capacitance.

The electronic component Qol was required to model the low frequency region of the impedance spectra, which is dominated by the slow diffusion processes occurring at the electrode/electrolyte interface, and therefore Qol has been used to define the oxide layer capacitance. Like the Au disc results, the use of a constant phase element was justified by the value of the Q exponent \( n \) being ~0.9. An impedance spectra showing the experimental data and the simulated fit is presented in Figure 6.22.

**Figure 6.22** Nyquist plot for a TiO\(_2\) electrode recorded in PBS at OCP before adsorption. The symbol (•) represents the experimental data while (-----) is the simulated fit using the EEC shown above (Figure 6.21).

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The circuit in Figure 6.21 was not successful in modelling the impedance spectra recorded at -400mV. Therefore the modified circuit shown in Figure 6.23 was used to model this data and is comprised of the same circuit shown above with a resistance element added in parallel with the oxide layer CPE. Roscoe and Omanovic [3, 12] have used this EEC to model a stainless steel electrode in PBS before and after protein adsorption, whilst a similar circuit was proposed by Protsailo et al [14] to model an Au (211) electrode covered in a SAM of various length thiols. They proposed that the EEC was capable of describing the impedance data in the presence of a slow electron transfer process.

\[ R_s - Q_{dl} - \begin{array}{c} R_{ct} \\ Q_{oi} \\ R_{ol} \end{array} \]

**Figure 6.23** Electrical equivalent circuit used to model the experimental impedance data recorded at -400mV. $R_s$, $R_{ct}$ and $R_{oi}$ represents the solution, charge transfer and oxide layer resistance respectively. $Q_{dl}$ and $Q_{oi}$ are the constant phase elements that represent the double layer and oxide layer capacitance.

It can be observed from the CV shown in Figure 6.4 that -400mV is situated in the reduction region, and therefore, when the impedance is measured at this potential, faradaic process are occurring due to the reduction process. The $R_{oi}$ therefore accounts for the resistance of the faradaic charge through the oxide layer, whilst the $R_{ct}$ accounts for the resistance of the faradaic charge between the oxide layer and the electrolyte solution. The experimental data and simulated fit using the EEC are presented in Figure 6.24.
6.3 Conclusions

The electrode capacitance increased upon interaction with Ig.G and PBS, however, when the capacitance increase due to exposure to PBS was subtracted from the capacitance increase due to Ig.G adsorption the net effect was a decrease in capacitance. These capacitance results recorded for the TiO₂ electrode were very similar to the Au results. This net decrease in capacitance is associated with a film of Ig.G forming at the electrode surface which influences double layer characteristics. When exposure of the electrode to PBS and Ig.G occurred using a pulsed potential waveform, the net decrease in capacitance due to Ig.G was less. This was attributed to the lower amount of adsorbed Ig.G measured under pulsed potential conditions, which was confirmed in Chapter 5.

When the electrode capacitance was monitored at a single frequency the variation in capacitance was dependant on the electrode potential. When the electrode
potential was OCP and 400mV the capacitance increased upon adsorption, with the adsorption of phosphate producing the greatest increase. When the effect due to Ig.G adsorption was compared, a net decrease was observed when the impedance and adsorption occurred at an electrode potential of OCP and 400mV. At an electrode potential of –400mV the capacitance increased upon adsorption of phosphate and Ig.G, with this increase being greater for Ig.G. The effect of Ig.G adsorption, when the impedance and adsorption occurred at –400mV, was a net increase. It was concluded that for the single frequency impedance method it was not possible to accurately monitor the electrode capacitance at potentials where faradaic processes occur.

Upon adsorption of Ig.G and phosphate, the charge transfer resistance decreased; which was attributed to activation of the electrode during the adsorption stage. When the extent of $R_{ct}$ decrease was compared after adsorption of Ig.G and phosphate, it appears that the adsorption of Ig.G in fact increases the $R_{ct}$, which agrees with the work presented for the Au electrode. When impedance was measured at OCP and adsorption occurred at an electrode potential of 400mV or –400mV, the extent of $\Delta R_{ct}$ variation was greater when the potential was –400mV, which coincided with large changes in the Nyquist plots. These changes in the Nyquist plots could not be explained on the basis of adsorption and therefore it was concluded that the application of –400mV to the TiO$_2$ electrode modified its surface to some extent.
It was necessary to use two equivalent circuits to model the impedance data, one for impedance recorded at OCP and positive potentials and another for impedance recorded at negative potentials. The circuit used to model the OCP and positive data was a modified Randles circuit, whilst the circuit used for negative impedance data incorporated an extra RQ component. The RQ component accounts for the diffusion processes occurring at the negative potential. Like the Au electrode, for these models to provide a best fit, it was necessary to use a CPE in the circuit, confirming the non-ideal nature of the electrode/protein interface.
References


Chapter 7

ATR-IR Spectroscopic Studies of Ig.G Adsorption onto Titanium Dioxide
7.1 Introduction

The study of protein layers at interfaces has previously been conducted using various techniques such as NMR, circular dichroism, and time-resolved fluorescence. However these techniques have some limitation and disadvantages. Alternatively, spectra of adsorbed protein layers can accurately be obtained by using the high sensitivity of the Fourier transform infrared (FTIR) method in combination with attenuated total reflectance (ATR) [1].

Infrared (IR) spectroscopy of thin particle films, deposited on an attenuated total reflection (ATR) crystal (Figure 7.1) and immersed in solution, has a high potential for investigating the chemistry of solid-solution interfaces [2]. In principle, the ATR-IR approach can be applied to any solid that can be formed into a particle film and remain intact when submerged in the relevant solution. Fourier transform attenuated total reflectance infrared (ATR-IR) spectroscopy was first developed by Fahrenfort [3]. Shortly after Fahrenfort, Brash and Lyman [4] used ATR-IR to study the adsorption of plasma proteins in solution to uncharged hydrophobic polymer surfaces [5]. Work performed by research groups such as Anderson [6], Sperline [7], Hug and Sulzberger [8], Connor, Dobson and McQuillan [9], as well as Buijs, Norde and Lichtenbelt [1] has led to the development of ATR-IR spectroscopy as a tool to investigate various solid-aqueous environments.
This Chapter presents the results of the investigation of the adsorption of Ig.G at a sol-gel TiO₂ film deposited on a ZnSe crystal. The effect of phosphate and protein pH on adsorption characteristics was also studied.

7.2 Results and Discussion

7.2.1 Effect of Phosphate on Ig.G Spectra

To study the effect of phosphate on the IR spectra of adsorbed Ig.G two 200µg mL⁻¹ Ig.G solutions were prepared, one in 0.2M PBS containing 0.15M NaCl and another in 0.15M NaCl only. Each solution was flowed over fresh TiO₂ thin films for 30min and the spectra were recorded. The two spectra were compared to examine the effect of phosphate on adsorption.

The Ig.G spectrum presented in Figure 7.2 is that of the Ig.G prepared in NaCl (no phosphate buffer) and shows several well defined peaks as well as several broad peaks. The main peaks of interest appear between 1000 and 1800 cm⁻¹ and are associated with the adsorbed protein.
Figure 7.2 IR spectra of species adsorbed from 200 µg mL⁻¹ IgG, pH = 7.0, prepared in NaCl only on TiO₂, with (a) whole spectra, (b) region of amide and phosphate absorbance bands. The background spectrum is from a solution of 0.15 M NaCl (pH = 7.0).

The sharp peak at ~1636 cm⁻¹ is attributed to the amide I vibration and consists mainly of the C=O stretching vibrations of the peptide bond groups [1, 10] with minor contribution from N-H stretching vibration [11]. Structural analyses of proteins are obtained from the amide I region since the frequency of this vibration depends on the nature of the hydrogen bonding in which the C=O group is involved.
The amide II vibration is observed at ~1545 cm\(^{-1}\) and is largely due to the N-H in plane deformation, with a smaller contribution from the C-N and C-C stretching coordinates \([12, 13]\). The bands at ~1450 and ~1400 cm\(^{-1}\) are associated with the C-H stretch from the CH\(_2\) side chains within the protein and the C-O symmetric carboxylate ion stretches respectively. The broad peak centered at ~1240 cm\(^{-1}\) is associated with the amide III region, whilst the broad absorbance band at ~1075 cm\(^{-1}\) is due to the P-O stretch vibrations of the phosphate ions. The observation of the phosphate band is attributed to the presence of trace amounts of phosphate in the supplied Ig.G. The peak at ~3267 cm\(^{-1}\) is observed superimposed on the negative O-H stretch band of water displaced from the TiO\(_2\) surface by adsorption of bulky Ig.G molecules \([12]\). The peak at ~2900 cm\(^{-1}\) is due to the C-H stretch of the adsorbed protein. The negative peak at ~2350 cm\(^{-1}\) is due to a change in the CO\(_2\) content of the spectrometer as a result of purging dry air through the cell chamber between sample and background spectra.

The amide I band and amide II band have been used to follow cell growth \([10]\), therefore it is possible to use these bands to monitor the extent of Ig.G adsorption at the TiO\(_2\) surface. However, only the amide II peak was used to monitor the extent of Ig.G adsorption, since water adsorbs in the region of the amide I peak, and can result in misleading absorbances. While the amide II absorbance can be used to monitor adsorption it is not possible to obtain quantitative data on the amount of protein adsorbed. Giacomelli \([14]\) \textit{et al} used the amide II absorbance band to compare,
quantitatively, the extent of bovine serum albumin and Ig.G adsorption onto various silica surfaces.

The spectrum obtained after exposure of TiO₂ to Ig.G containing PBS solution is shown in Figure 7.3. The obvious difference between this spectrum and the one shown in Figure 7.2 is in the absorbance. The presence of the phosphate severely reduces the absorbance in the protein band region, and hence the amount of adsorbed protein. Serro et al. [15] also found using the reflectometry and dynamic contact-angle techniques that the presence of phosphate ions reduced the amount of adsorbed bovine serum albumin (BSA) at a titanium dioxide (TiO₂) surface. Serro suggested the reason for the lower adsorbed amounts of BSA in the presence of phosphate was the adsorbing phosphate increasing the TiO₂ surface hydrophilicity. It is widely accepted that the affinity between proteins and solid surfaces increases with the increasing hydrophobic nature of the solid surface [16].

In the Ig.G/PBS solution the concentration of PBS is 0.2M. Connor et al. [17] have shown using ATR-IR that phosphate binds strongly to TiO₂ as a bidentate ligand and that saturated coverage is reached at a phosphate concentration less than 0.01M. Phosphate adsorption at TiO₂ has also been detected by XPS [18] and radiotracer experiments [19]. Since the phosphate binds to the surface of TiO₂ the Ig.G has to compete for adsorption sites reducing the extent of protein adsorption.
Figure 7.3  IR spectrum of amide and phosphate species adsorbed on TiO$_2$ from of 200 $\mu$g mL$^{-1}$ Ig.G, pH = 7.0, prepared in 0.2M PBS. The background spectrum is from a solution of 0.2 M PBS (pH = 7.0).

It is clear that the amide II absorbance in Figure 7.2(b) (no phosphate) is more intense than the amide II absorbance presented in Figure 7.3 (phosphate present), due to more protein present at the TiO$_2$ surface. The adsorption time and conditions were the same for both spectra and indicates that in the presence of phosphate the extent of protein adsorption is reduced. When the amide II absorbance is plotted against adsorption time, an adsorption profile of Ig.G prepared in PBS can be compared against that for the Ig.G prepared in NaCl solution. Figure 7.4 clearly shows the lower amide II absorbance for the TiO$_2$ film exposed to Ig.G prepared in PBS at all adsorption times.
Figure 7.4 Comparison of the adsorption profiles for Ig.G (200µg mL⁻¹ - pH = 7.0) prepared in 0.15M NaCl (0.15M NaCl) and 0.2M PBS (0.2M PBS) at a TiO₂ film. The absorbance values are that of the amide II peak (1545 cm⁻¹). The background spectrum for the 0.2 M PBS profile is from a solution of 0.2 M PBS (pH = 7.0) whilst the background spectrum for the 0.15 M NaCl profile is from a solution of 0.15 M NaCl (pH = 7.0).

The adsorption vs. time profile presented in Figure 7.4 shows the increase in absorbance of the amide II peak (1545 cm⁻¹) associated with protein adsorbing to the TiO₂ surface. After 1 hour of adsorption the absorbance was slightly increasing, indicating surface saturation has not been reached. However, after 30min of adsorption the change in amide II absorbance was less than 5% every 15min.

7.2.2 Phosphate Induced Ig.G Desorption

To investigate the effect of phosphate on preadsorbed Ig.G, solutions of varying phosphate concentration were washed over a TiO₂ film preadsorbed with Ig.G. A 200µg mL⁻¹ solution of Ig.G prepared in NaCl and was flowed over the TiO₂ film
for 30min with the spectra recorded upon completion. The amide II (1545cm⁻¹) and the phosphate (1080cm⁻¹) peak absorbance were recorded. After 30min adsorption of Ig.G, the PBS solutions (0.0002M, 0.002M, 0.02M and 0.2M) were introduced into the cell starting with the lowest concentration. Each PBS solution was flowed through the cell and an IR spectrum was recorded after 5min flow. The 5min of washing by each of the phosphate solutions was sufficient to establish the threshold phosphate concentration producing significant displacement of the adsorbed protein. After the spectra were recorded the PBS solution was changed to the higher concentration. The change in amide II (1545cm⁻¹) and phosphate (1080cm⁻¹) absorbance were recorded from each spectrum.

The phosphate absorbance band at 1080cm⁻¹ was also monitored. The change in the amide II and phosphate absorbance from those initially measured after preadsorption of Ig.G for 30min are presented in Figure 7.5
Figure 7.5  Plot of absorbance during PBS wash of (a) adsorbed protein amide II at 1540 cm\(^{-1}\) and (b) adsorbed phosphate at 1080 cm\(^{-1}\). Absorbance at \(t = 0\) min represents the surface saturation from 200\(\mu\)g mL\(^{-1}\) Ig.G solution. Washes for 5 min with PBS solutions (1) 0.0002 M, (2) 0.002 M, (3) 0.02 M, (4) 0.2 M PBS and for (5) 15min with 0.2 M PBS solution. The background spectrum is from a solution of 0.15 M NaCl (pH = 7.0).

In Figure 7.5, after the first PBS (0.0002M) wash, the amide II absorbance remained relatively constant whilst the phosphate peak slightly increased. It seems possible that the adsorbed protein, after 30min adsorption at 200\(\mu\)g mL\(^{-1}\), may not be able to tie up all of the sites to which phosphate would otherwise bind. This was confirmed by the fact when Ig.G adsorption occurs at pH = 7.0 for 30 min the amide II absorbance has not leveled off indicating surface saturation had not been reached (see Figure 7.4).

As the PBS concentration increased to 0.002M, the amide II absorbance decreased sharply indicating a reduction in the amount of Ig.G at the TiO\(_2\) surface. As the
amide II peak decreased the phosphate peak increased indicative of accumulation of phosphate ions at the TiO₂ surface. Adsorption of phosphate ions at pH = 7.0, where the TiO₂ film is marginally negatively charged, has also been observed by Connor [17] et al. The amide II absorbance continues to decrease during the third wash (#3, 0.02M – 15 min total wash time) while an increase in phosphate was observed. The increased phosphate absorbance at #4 is probably due mainly to the increasing solution contribution to the absorbance, since saturated coverage is expected at phosphate concentrations in excess of 0.01M [17]. However, the amide II absorbance decreases during wash #4 indicating displacement of Ig.G by phosphate ions, suggesting phosphate ions are accumulating at the TiO₂ surface, which also contribute to the phosphate absorbance. The phosphate absorbance at wash #5 is due to the solution contribution since no further reduction in the amide II absorbance is observed, suggesting no displacement of the bound Ig.G by phosphate ions. The above result is clear evidence of protein displacement by phosphate in a substitution type exchange at the TiO₂ surface, possibly influenced by the negative charge of the adsorbing phosphate.

The presence of amide II band after wash #5 shows that not all of the adsorbed Ig.G is removed and suggests two types of adsorbed Ig.G, weakly and strongly bound. This suggestion of weakly and strongly bound protein has also been proposed by Hidalgo-Alvarez and Galisteo-Gonzalez [5] based on work performed by other researchers [20, 21]. The exchange of Ig.G with phosphate may only occur at sites
of weakly bound Ig.G while the strongly bound Ig.G remains at the TiO$_2$ surface, even at phosphate concentration greater than those for surface saturation.

### 7.2.3 pH Studies

The isoelectric (pl) point for a polyclonal Ig.G is a range of pH, namely between pH = 6 and 8 [22]. At pH = 7 the exact charge on the Ig.G molecule is not known. Dobson et al [23] using the STIRS technique showed that the point of zero charge for an amorphous TiO$_2$ film is at pH = 5.0 while for P25 it is at pH = 6.0 [24]. This suggests that as the pH decreased from 10.0 to 4.0 the charge on the TiO$_2$ film becomes more positive.

Adsorption from 200$\mu$g mL$^{-1}$ Ig.G/NaCl solutions (pH = 4.0 or 10.0) and subsequent PBS wash at the same pH as used for the adsorption was investigated (Figure 7.6). At pH = 4.0 and 10.0 the amide II absorbance after 30 min adsorption of Ig.G (time = 0 min) is less than that observed at pH = 7.0, which is attributed to lower amounts of adsorbed protein at the TiO$_2$ surface. At pH = 4.0 and 10.0 the TiO$_2$ film and Ig.G molecule posses the same charge, that being positive at pH = 4.0 and negative at pH = 10.0. Lower amounts of adsorbed protein have been reported by other researches when the substrate and protein possess the same charge [25-27].

Displacement of adsorbed Ig.G by adsorbed phosphate occurred when solutions of pH 10.0 or 7.0 where used. With a solutions of pH = 4.0 little protein displacement was observed. In the pH = 4.0, 7.0 and 10.0 profiles the absorbance of the phosphate
band increases with increasing PBS concentration associated with the coordination of phosphate to unoccupied TiO₂ sites.

The lack of displacement of adsorbed Ig.G occurring at pH = 4.0 suggests that the Ig.G molecule has a stronger affinity to TiO₂ at this pH than at pH = 7.0 and 10.0, possibly due to partial unfolding (denaturing) of the Ig.G molecule at low pH. There have been reports [26-28] suggesting that upon adsorption proteins undergo some structural rearrangement due to entropic factors. This structural rearrangement could possibly expose the interior of the protein to the TiO₂ film effectively increasing the number of available binding sites of the Ig.G molecule to the TiO₂ film resulting in a stronger adsorption. This would lead to the conclusion that the degree of unfolding of a protein is greater in more acidic conditions. The possible unfolding of Ig.G when the protein pH is acidic (pH = 4.0) was also observed for adsorption at a gold electrode (Chapter 4, Section 4.2.5.1.3).
Figure 7.6 Plot of absorbance during PBS wash for (a) amide II and (b) phosphate at pH = 4, 7, and 10. Absorbance at t = 0 min represents the surface saturation from 200 µg mL⁻¹ Ig.G solution. PBS wash with (1) 0.0002M, (2) 0.002M, (3) 0.02M and (4) 0.2M. The background spectrum is from a solution of 0.15 M NaCl at the various pH indicated in each graph.
7.3 Conclusions

It is clear from the results that the presence of phosphate in the Ig.G solution reduces the extent of Ig.G adsorption at TiO₂ films. The phosphate is able to displace adsorbed Ig.G at relatively low phosphate concentrations, however, not all of the adsorbed Ig.G can be displaced, even at high phosphate concentrations. Adsorption kinetics showed the presence of Ig.G weakly and strongly bound to TiO₂. The displacement of adsorbed Ig.G by phosphate did not occur at pH = 4.0, possibly due to stronger binding arising from partial structural rearrangement of Ig.G at the TiO₂ surface. This is the first time that ATR-IR spectroscopy has been used to monitor the effect of phosphate on the adsorption and displacement of Ig.G on a TiO₂ film at various pH.
References

Chapter 7 - ATR-IR Spectroscopic Studies of Ig G Adsorption onto Titanium Dioxide


Chapter 8

Conclusions
Using a variety of experimental techniques it was possible to monitor the adsorption of proteins at metal electrodes and to study the effect this had on the electrochemical responses of the metal electrodes.

Adsorption trends were successfully studied using the iodine 125 radiolabelling technique. These results revealed that the adsorption of proteins at metal electrodes is not a straightforward process; rather, it is a complex system with numerous factors combining together to affect adsorption. The results also showed that the equilibrium between the protein in solution and the electrode surface can easily be shifted by subtle changes in either the protein pH or the charge on the electrode surface. The use of two proteins enabled the study of protein size and structure to be considered with respect to adsorption, and the results indicated that the larger the protein the greater the adsorbed mass.

Whilst the adsorption process of proteins is sensitive to changes in the system, the desorption results indicated that once the protein had adsorbed it was resistant to removal. The desorption results suggest that while some of the adsorbed protein is removed there still remains bound protein even after the harshest cleaning process was applied. These results indicate the possibility of two types of adsorbed protein, (i) weakly and (ii) strongly bound. This hypothesis was further strengthened from the results presented in Chapter 7. In this Chapter it was also reported that the presence of phosphate, from the PBS, severely reduced the extent of Ig.G adsorption. In the absence of phosphate the amount of Ig.G adsorption was increased by a factor of 10. Not only did phosphate suppress Ig.G
adsorption it was capable of removing pre-adsorbed Ig.G from a TiO$_2$ film simply by washing with PBS, however, phosphate was not able to remove all of the bound Ig.G.

EIS proved to be a very powerful technique for the investigation of the effect of adsorbed protein on the electrochemical behaviour of the metal electrodes. By combining EIS with the CV technique it was possible to obtain a clear understanding of the changes taking place at the electrode surface upon fouling with adsorbed protein. Similar to the adsorption trends, the trends in the change of electrochemical behaviour were dependent on the electrode potential and to a lesser extent on the protein size and structure.

For both electrode material, Au and TiO$_2$, adsorption of protein caused a decrease in electrode capacitance and was associated with the formation of a protein layer on the electrode surface. The extent of capacitance decrease was dependent on the extent of adsorption (adsorbed amounts). This decrease was enhanced upon increasing the Au electrode surface area by increasing the surface roughness. When impedance was measured at a single frequency it was possible to monitor the change in C$_{dl}$ due to protein adsorption. However, this technique had its limitations when the potential used to measure impedance created faradaic processes at the electrode/electrolyte interface.

The electron transfer properties of both Au and TiO$_2$ electrodes were affected by the formation of the protein layer. For the Au electrode, adsorption of protein
increased the charge transfer resistance indicating a reduction in the electron transfer capability of the electrode surface. The behaviour of the TiO$_2$ electrode varied somewhat to that of the Au, where upon exposure to PBS and protein the charge transfer resistance decreased. When the decrease associated with phosphate adsorption was compared to the decrease associated with Ig.G adsorption, it appeared that the adsorption of Ig.G resulted in a net increase in the charge transfers resistance. This was attributed to activation of the TiO$_2$ electrode during adsorption.

The consequence of these findings in relation to the operation of TruScan™ are two fold. It has been shown that adsorption of protein occurs instantaneously when the electrode surface comes in contact with proteinaceous solutions. It was also shown that the amount of adsorbed protein is dependant of the local environment pH and the potential at the electrode surface is biased. Since TruScan™ operates in an environment where local pH can vary and the system employs the pulsing of an applied potential to the electrode surface it must be concluded that TruScan™ does encounter biofouling during the screening of the cervix.

The biofouling of electrode surfaces has been shown to influence the electrochemical behaviour of the electrode in such ways that the measured electrode capacitance decreases. The fact that capacitance is one of the main parameters used to detect changes in the cervical tissue this finding has consequences in the further development of the TruScan™ technology.
It has been shown that the adsorption characteristics of two proteins vary depending on the electrode surface composition, surface morphology, protein pH and the electrode surface charge. The nature of protein adsorption is a dynamic one and as a result a variety of experimental techniques is required to gain a detailed understanding of the processes involved.

To help elucidate these processes further, future work might involve a step backwards to investigate the adsorption of smaller simpler amino acids. The use of other techniques such as ellipsometry, electrochemical surface enhanced resonance Raman scattering (EC SERRS), scanning tunnelling and atomic force microscopy (also in the electrochemical mode) (STM and AFM/ECAFM) would complement the work presented here.

Ellipsometry may provide valuable information on the thickness of the adsorbed protein layer, which provides additional information to the radiolabelling results. This would enable the researcher to gain a better insight into the nature of the adsorbed protein. EC SERRS would provide a means of being able to obtain spectroscopic information whilst performing various electrochemical procedures to the sample. The advantage of this technique is that it is performed in-situ and data is acquired in real time, thus allowing spectroscopic studies of the electron transfer reactions of proteins on electrode surfaces. This technique may provide information on possible protein rearrangement at the electrode surface to accommodate a change in the charge on the electrode surface.
STM and AFM, while prone to complications due to image artefacts due to probe-induced sample movement and image vibration, may provide a means of performing structural studies of proteins and amino acids. Similar to EC SERRS, the ECAFM technique may provide a means to image the protein or amino acid and study any conformational changes due to varying the charge on the electrode surface.