

THE APPLICATION OF ANTIBODIES IN OPTICAL AND ELECTROCHEMICAL TRANSDUCTION PROCESSES

by

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A Thesis Submitted for the Degree of Doctor of Philosophy

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABBREVIATIONS, CONSTANTS AND SYMBOLS

A	Electrode area
Ab	Antibody
Ag	Antigen
AbAg	Antibody-antigen complex
Ag/AgCl	Silver/silver chloride
AFM	Atomic force microscopy
AP	Alkaline phosphatase
BCA	Bicinchoninic acid assay
Bpy	2,2'-bipyridyl
BSA	Bovine serum albumin
CDR	Complementarity determining region
CT	Charge transfer
CNT	Carbon nanotube
CV	Cyclic voltammetry
CVD	Chemical vapour deposition
Dcbpy	2,2'-bipyridine-4,4'-dicarboxylic acid
DCC	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DNS	5-dimethylaminonaphthalene-1-sulfonamido
DOS	Density of states
$E_{1/2}$	Half wave potential
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbant assay
<i>E. coli</i>	Escherichia coli
E_{em}	Emission energy
ΔE_p	Peak potential separation
Fc	Crystalline Fragment
Fab	Antigen binding fragment

F	Faraday's constant
F _v	Variable fragment
GOD	Glucose oxidase
GMP	Guanosine monophosphate
H	Heavy chain
HAT	1,5,5,8,9,12-hexaazatriphenylene
HiPCO	High pressure CO disproportionation process
HRP	Horseradish peroxidase
HSA	Human serum albumin
HupR	DNA binding protein of Rhodobacter capsulatus
I _{pa}	Anodic peak current
I _{pc}	Cathodic peak current
k ^o	Heterogeneous electron transfer rate constant
K _a	Association equilibrium constant
k _a	Association rate constant
k _d	Dissociation rate constant
k _r	Rate of radiative decay
k _{nr}	Rate of non-radiative decay
k _q	Quenching rate constant
LC	Ligand centered
L	Light chain
LMCT	Ligand-to-metal charge transfer state
LOD	Limit of detection
Mb	Myoglobin
MC	Metal centered
MLC	Metal ligand complexes
MLCT	Metal-to-ligand charge transfer state
MWNT	Multiwalled carbon nanotube
n	Number of electrons
N _A	Avogadro constant
NADH	β-Nicotinamide adenine dinucleotide

NHS	<i>N</i> -hydroxysuccinimide
NHSS	<i>N</i> -hydroxysulphosuccinimide
NMR	Nuclear magnetic resonance
opd	o-phenylenediamine
PANI	Polyaniline
PEO	Polyethylene oxide
PBS	Phosphate buffered saline
Phen	1,10-phenanthroline
PLL	Poly(L-lysine)
Q	Charge passed in electrolysis
QCM	Quartz crystal microbalance
R	Universal gas constant
rpm	Rotations per minute
$\Delta S_{\text{rc}}^{\circ}$	Reaction entropy
SAM	Self-assembled monolayer
ScAb	single chain antibody fragments
SCE	Standard calomel electrode
ScFv	Single chain fragment variable
SEM	Scanning electron microscopy
SWNT	Single walled carbon nanotube
s-SWNT	Shortened single walled nanotube
P	Polarisation
pI	Isoelectric point
pKa	pH of deprotonation
pKa*	pH of excited state deprotonation
p2p	1,2-bis(4-pyridyl)ethane
R	Steady state anisotropy
R_0	Förster distance
RBM	Radial breathing mode
TAP	1,4,5,8-tetra-aza-phenanthrene
t	Time

T	Temperature
T_c	Cytotoxic T cells
T_H	Helper T cells
TEM	Transmission electron microscopy
T_s	Suppressor T cells
(w/v)	weight per volume
V	Volt
V_H	Variable heavy chain
V_L	Variable light chain
(v/v)	volume per volume
α	Transfer coefficient
Γ	surface coverage
ϵ_r	Dielectric constant
ν	Scan rate
θ	Rotational correlation time
λ	Spin orbit coupling constant

ABSTRACT

Chapter 1 relates the background information of the structure of antibodies, the nature of their interaction with antigen and their production as polyclonal antibodies. Examples of antibodies which have been produced against luminescent molecules are given and it is illustrated how the specific interaction of antibodies with luminescent complexes can be used to gain information on the structure, function and rotational dynamics of antibodies. The principle features of immunoassays are outlined with a focus on amperometric immunosensing including some areas of active research within amperometric immunosensing. This serves as a general introduction to Chapter 4; the development of an amperometric immunosensor based on single wall carbon nanotubes.

Chapter 2 begins with a review of the fundamental chemical, photochemical and electrochemical properties of Ru(II) and Os(II) polypyridyl complexes. It is shown how their photophysical properties can be modulated by the interaction of the complexes with biomolecules such as proteins, nucleic acids and antibodies. The synthesis and characterisation of [Os(bpy)₂dcbpy] and some related Os(II) and Ru(II) complexes is described. The production and characterisation of a [Os(bpy)₂dcbpy]-thyroglobulin conjugate which was used as the immunogen is described, as well as the purification and characterisation of the resulting polyclonal antibody. Competition ELISA served to confirm the cross-reactivity of the antibody with the Os and Ru complexes synthesised.

Chapter 3 describes the effect of antibody binding on the spectrochemical properties of the complexes. Changes in the emission spectra and lifetimes were examined. Association constants were derived from emission titrations. The extent that the antibody binding site protects the complexes from excited state deactivation via interaction with solvent was investigated. The possibility of energy transfer from [Ru(bpy)₂dcbpy] to [Os(bpy)₂dcbpy] when both were bound to the same antibody was investigated, as were the effects of antibody binding to a self-assembled layer of [Os(bpy)₂(p2p)₂]²⁺.

Chapter 4 begins with an introduction to the structure and properties of CNTs and outlines their application thus far in biosensing. The assembly of oxidatively shortened SWNTs onto Nafion/iron oxide coated pyrolytic graphite electrodes is described and characterised by both AFM and resonance Raman spectroscopy. The immunosensing

strategy investigated involved the adsorption of anti-biotin antibody to the carbon nanotube surface. The presence of HRP-labelled biotin was determined via the reduction of hydrogen peroxide in the presence of the soluble mediator hydroquinone. A short investigation is also presented on the ability of HRP-modified SWNT forest electrodes to detect H_2O_2 produced by a mutant catalase negative *E. coli* bacteria which was co-immobilised with the HRP.

Recommendations for future work arising from this thesis are given in Chapter 5.

CHAPTER 1

The immunoreaction and its application in amperometric immunosensing: a literature review

1.1 Introduction

This literature review offers a broad outline of the principles involved in the reactions between antibodies and antigens including the production of polyclonal antibodies, antibody structure and binding interactions. An overview will be given on the information which has been obtained from the specific association of antibodies to luminescent molecules including an insight into the nature of the antibody's active site and the hydrodynamic behaviour of immunoglobulins and their fragments. The principle features of immunoassays are outlined with a focus on amperometric immunosensing.

1.2. Antibody structure and function

Antibodies (immunoglobulins) are serum proteins that provide an important part of the body's defense against infection. They achieve this by binding to foreign molecules called antigens. The high degree of specificity and strength of binding (affinity), which is inherent in the antibody-antigen interaction, is fundamental to the success of antibodies as a defense mechanism in vertebrates. It is also the basis of many diagnostic tests in biological, medical and environmental science.

The basic immunoglobulin 'unit' consists of two heavy (H) polypeptide chains linked together by disulphide bonds and each linked to one of an identical pair of light (L) chains (Figure 1.1). The H and L chains have a molecular mass of 50-80 kDa and 23 kDa, respectively. There are two intra-chain disulphide bonds within each L chain and four within each H chain. Their effect is to cause the chains to form into loops (domains) of approximately equal size (Eales, 2003).

The antigen binding site is located at the NH₂-terminal end of each H-L pair, also known as the Fab region (fragment antigen binding). The COOH-terminal portions or Fc region (fragment crystallizable) is complementary to ligands or receptors in certain tissues on immune cell membranes.

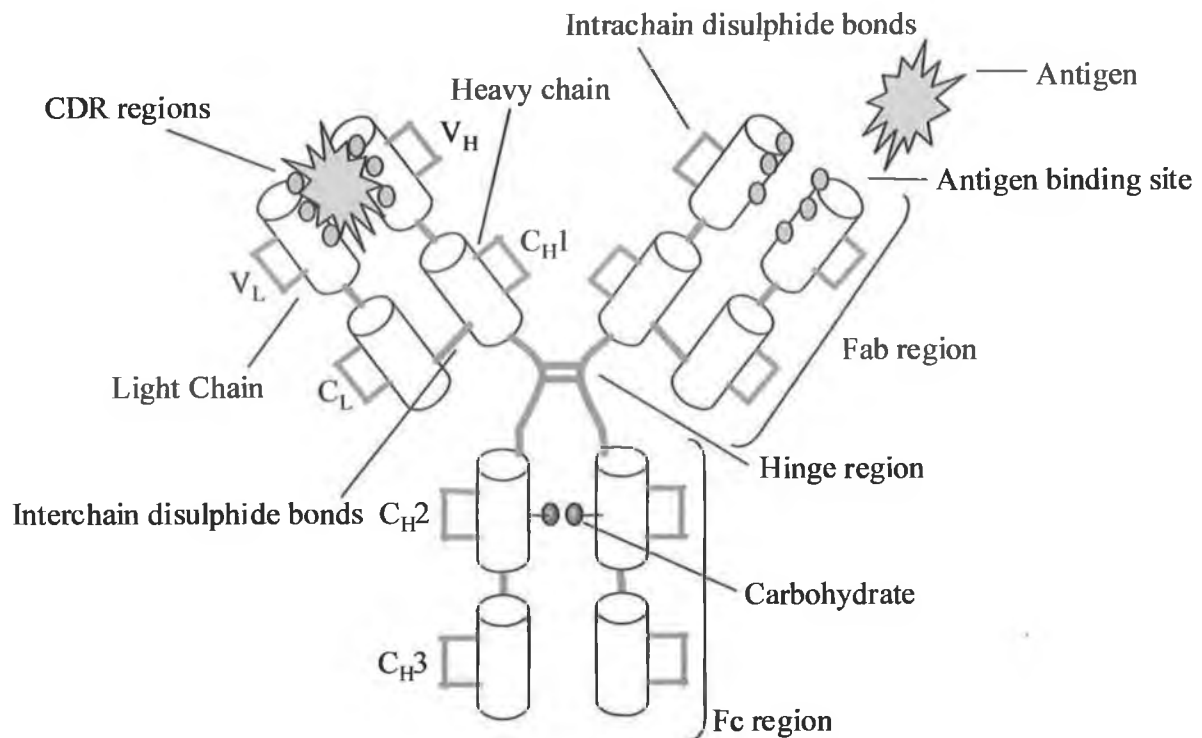


Figure 1.1. Structure of the IgG immunoglobulin molecule.

Both H and L chains can be divided into constant and variable regions. The domains are thus designated V_H, V_L, C_L, C_H1, C_H2 etc. Each antigen binding site is composed of hypervariable loops which form complementarity determining regions (CDRs) in the variable domains of a pair of H and L chains. There are three hypervariable clusters in the V_H domain, each comprising 10 to 15 amino acids. The V_L domain contains three smaller clusters.

The dimensions of the antigen binding site are approximately 15-20 Å long, 6-15 Å wide, and 6-12 Å deep (Mayforth, 1993). As a reference an IgG molecule is approximately 150 Å in diameter. The antigenic determinants are approximately 5-30 Å long. The exact shape of the antigen binding site is extremely variable and depends on the precise sequence of amino acids in the hypervariable loops and framework regions (FRs) which flank them. In some antibodies, the antigen binding site resembles a narrow groove that forms a pocket for the antigen. In others, the binding site is more planar, resembling an open basin. Small antigens can be almost entirely buried within the binding site.

The hinge region is a short amino acid sequence situated between C_H1 and C_H2. This contributes to the great flexibility of the molecule by allowing the distance between the two antigen binding sites to vary and considerable rotation of the Fab regions to occur relative to one another (Davey, 1989).

Five classes of antibody have been characterised on the basis of differences in the constant region of the heavy chains (Inchley, 1981). Two of the classes (IgM and IgA) exist as polymers of the basic structure, in association with additional polypeptide chains. The distinct H chains are named μ , γ , α , δ and ϵ after the molecule (IgM, G, A, D, E) in which they are respectively found. IgG makes up approx. 80% of the total amount of immunoglobulins in human serum. Some classes of immunoglobulins are further divided into subclasses also on the basis of H chain structure and functional differences (e.g. IgG1-IgG4). The antibody light chains exist in only two types, κ or λ , also depending on variability in the amino acid sequences in the constant region. Their relative incidence varies from species to species and from one immunoglobulin class to another.

1.3 The humoral immune response and generation of antibodies

The main function of the immune system is the identification and elimination of foreign or malignant cells, viruses and macromolecules from the body. The immune response can be broadly divided into two categories; non-adaptive and adaptive (Eales, 2003). The non-adaptive or innate immune system responds non-specifically to foreign molecules using cells such as phagocytes and natural-killer cells. The adaptive immune response is highly specific and possesses immunological memory. It can be further divided into the humoral and cell-mediated immune response.

The humoral immune response involves the generation of antibodies as a tool for antigen recognition and immobilisation. The main effector cells of this task are lymphocytes (white blood cells). Lymphocytes occur as two major types; B cells and T cells. Each T cell has an individual antigen receptor (TCR) specialised for one particular ligand (Paul, 1991). They recognise antigens only when they are presented on the surface of an antigen presenting cell (APC) such as a macrophage in association with a major

histocompatibility complex (MHC). TCRs are similar to immunoglobulins; both have hypervariable CDRs to allow for individual specificity towards an antigen.

T cells can be divided into helper T cells (T_H), cytotoxic (cell-killing) T cells (T_C) or suppressor T cells (T_S). Once activated, T_H cells are responsible for the release of cytokines that play a crucial role in the amplification of the immune response. T_H cells are made up of two distinct populations, namely T_{H1} and T_{H2} , that differ in their cytokine secretion characteristics. T_{H1} cells are primarily responsible for the activation of cytotoxic T-cells via interleukin 2 (IL-2) and interferon γ (IFN- γ), whilst T_{H2} cells are responsible for the activation of B cells by secreting IL-4 and IL-5.

The general mechanism of the humoral response is illustrated in Figure 1.2. When an antigen is introduced into the body, it is phagocytosed by macrophages and B cells. B cells are genetically programmed to express a specific antibody molecule on their surface. In contrast to T cells they can recognise and bind to an antigen directly from solution. Macrophages and B cells digest the antigen and present short segments of the antigen molecules on their cell surface using MHC Class II. The combination of an antigen presenting cell and MHC Class II proteins is recognised by determinant-specific T_H cells. Binding of the T_H to the antigen/MHC complex provokes the release of IL-1 by certain APCs including macrophages. Antigen-stimulated T_H cells secrete IL-2 and make IL-2 receptors. Subsequently, binding of IL-2 to its receptors causes the T_H cells to proliferate. IL-2 is also an inducer of the proliferation of B cells. Activated T_{H2} cells secrete IL-4 and IL-5, which act on B cells to induce activation and differentiation into antibody-forming cells (plasma cells) and memory B cells. Memory B cells remain dormant and form the basis of the secondary response, the intensified mechanism that is provoked by a repeat challenge by the same antigen.

Two processes accompany the differentiation of B cells (Harlow and Lane, 1988). The first is somatic mutation of the variable regions of the L and H chains. The second is the process of class switching. Somatic mutations produce a large pool of B cells with antibodies having different affinities for the antigen. Some mutations yield antibodies with higher affinities than the original B cell clone. These cells are selected preferentially to proliferate, leading to maturation of the antibody affinity during the immune response. Class switching allows the same variable region to be expressed with different constant

regions. During the maturation of a typical response, the IgM antibodies that are characteristic of the early response are replaced by IgG antibodies.

On antigen binding the Fc region of the antibody activates complement facilitating phagocytosis and phagocytes also bind through their antibody receptors. When antibody secretion is no longer required, the system responds to falling antigen concentration by a number of mechanisms which curtail the production of new lymphocytes, and induce apoptosis in many of those which already exist. This involves T_S cells.

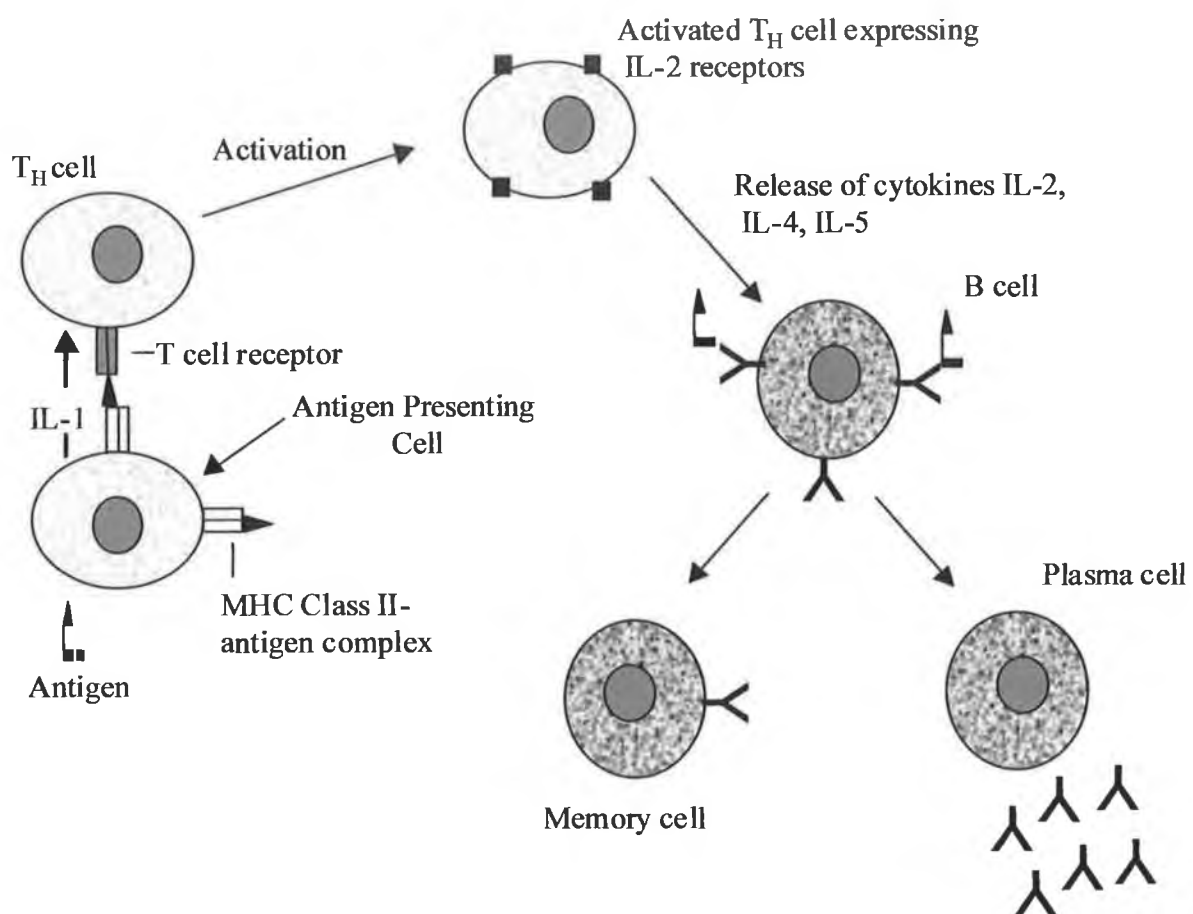


Figure 1.2. The interaction between an antigen presenting T cell and B cell leading to T cell and B cell activation, with the consequential production of memory and immunoglobulin-secreting plasma cells (from Coico, 2003).

1.4 The production of polyclonal antibodies

Polyclonal antibody production is a very practical means of obtaining large quantities of antibody. They can be synthesised relatively quickly and cheaply. Polyclonal antibodies consist of a mixture of antibodies since they are made by many different B lymphocytes. The antibodies will vary with regard to the exact antigen site to which they bind (epitope) and binding affinity. However, not only will polyclonal antibodies lack uniform characteristics within one immunisation but also in immunisations of different host animals and different types of host animals. This is in contrast to monoclonal antibodies which are obtained by fusing individual antibody-forming cells with a myeloma B cell to produce a constantly dividing clone dedicated to making one antibody type of defined specificity and affinity. The ability of polyclonal antibodies to bind to many antigenic determinants is an advantage in certain situations such as signal-amplification applications, where an increase in sensitivity can be achieved compared to the use of a labelled monoclonal antibody.

Antibodies can be produced against any molecule capable of eliciting an immune response in a host animal. A molecule is defined as being immunogenic if it elicits a complete immune response in the host. A certain molecular size and complexity is necessary. Species with molecular weights greater than 10,000 Da are almost invariably immunogenic. Immunogens of this size are required since the lymphocyte mechanism outlined above appears to be triggered by the need for a number of receptors on the B cell to be bound simultaneously (Dent and Aslam, 1998). Thymus-independent antigens are those with repeating determinants which cross-link many surface receptors providing a persistent signal to the B cell and allow for direct activation of B cells. The majority of antigens, however, are unable to activate B cells without the assistance of T lymphocytes, which are specific for determinants of different specificity on the antigen. This explains the need for haptens to be coupled to large molecular weight proteins. Haptens consist of a single non-repeating determinant. This does not allow for T cell cooperation. The 'carrier' determinants of the protein provide for T cell cooperation leading to a strong B cell stimulation and production of immunoglobulin.

Although a number of species have been used for polyclonal antibody production such as mice, sheep or goats, New Zealand white rabbits are widely used. Rabbits are easy to keep and handle for immunisation and bleeding purposes and reach immune maturity at 12 weeks (Law, 1996).

Adjuvants are non-specific stimulators of the immune response and are commonly used to enhance the humoral response to weak immunogens, such as the hapten-protein conjugates. These alter the physiological state of the water-soluble immunogens resulting in stable oil-in-water suspensions that form deposits *in vivo* providing a long-lived reservoir of antigen. This results in the prolonged persistence of the immunogen in tissues and a continuous stimulation of the immune system. Freund's complete adjuvant (FCA) is commonly used for the first administration of immunogen. This contains heat-killed *Mycobacterium tuberculosis*, which expand the T cell population and lipopolysaccharides which are a specific stimulant of B cell proliferation. FCA also leads to the local formulation of granulomata, which are rich in macrophages. Freund's incomplete adjuvant (FIA) (no mycobacterium) is used for subsequent booster injections, due to its reduced pathogenic effects.

The general immunisation schedule for the production of polyclonal antibodies involves immunising at 3-4 week intervals. A primary response is caused by the first exposure to a given immunogen and results in the production of mainly IgM antibodies, as mentioned previously, after a relatively long lag period. After a lapse of time, another exposure to the immunogen produces a quite different, stronger secondary response. This is characterised by a shorter lag period, the production of predominantly IgG antibodies and, after a peak has been reached, a slower decrease. Antibodies of the secondary response usually have a higher affinity, which may increase even more with time or with subsequent antigenic challenge (Coico, 2003). It has been argued that to obtain a maximum secondary response, boosting should not be carried out until the antiserum titre has decreased to a low level, or at least plateaued (Law, 1996). If sufficient time is not allowed between injections and a high circulating amount of antibody remains, the newly injected antigen will be rapidly cleared. Frequently, a compromise is made when choosing the time period before boosting and the number and frequency of booster injections to allow completion of the immunisation program within a reasonable time

frame. A standard immunisation schedule would typically consist of three booster injections given 21 days apart, the first booster performed 21 days after the initial immunisation.

1.5 The nature of antibody-antigen interactions

Several different forces are involved in the binding of an antigen to an antibody and not all of these come into play simultaneously (Davies, 1994). All of the bonds formed are weak and noncovalent. The primary attraction is ionic, between oppositely charged sites in the epitope and antigenic binding sites, operating over distances of over 100 Å. Weaker Van der Waals forces may also be involved in the primary reaction, where electrostatic interactions arise between fluctuating dipoles in one atom and dipoles induced by it in another. These initial bonding reactions represent only a small proportion of the total bond energy. As the epitope draws closer to the binding site, water may be effectively excluded, permitting the formation of hydrogen bonds between adjacent amino, hydroxyl and carboxyl groups at distances of between 1.5 to 5 Å. Van der Waals forces also become increasingly important in this secondary stage as the two components draw together. Eventually, direct protein-protein interactions occur which further strengthen the bond by exclusion of water molecules. A lack of complementarity (steric factor) between antigen and antibody binding sites is important among the repulsive forces and prevents a close approach of the two molecules necessary for water elimination and for the weak, attractive forces to be effective.

It is difficult to predict the optimum conditions for antigen-antibody interactions since the forces holding the molecules together are quite heterogeneous (Law, 1995). Electrostatic interactions are sensitive to changes in pH and ionic strength. Hydrogen bonding is essentially an exothermic reaction and thus is favoured by lower temperatures. Interactions in which these bonds dominate are therefore more stable at lower temperatures. Chaotropic ions (SCN^- , I^- , Br^- and Cl^-) distort, particularly at higher concentrations, the three-dimensional structure, leading to disruption of the antibody-antigen interacting structures (Tijssen, 1985). Organic solvents may also disrupt

interactions and organic acids of low surface tension disrupt Van der Waals bonds and dissociate antibody-antigen complexes.

1.6 Kinetics of antibody-antigen interactions

Affinity is a measure of the strength with which a monovalent antigen binds to one of the antibody binding sites at equilibrium. For the reaction;



at equilibrium the association constant (K_A) is given by;

$$K_A = [\text{AgAb}]/[\text{Ag}][\text{Ab}] = k_1/k_2 \quad (1.2)$$

where $[\text{Ag}]$ is the concentration of the antigen, $[\text{Ab}]$ is the concentration of the antibody and $[\text{AgAb}]$ is the concentration of the antibody-antigen complex. k_1 and k_2 are the rate constants for the forward (attractive forces) and back reactions (repulsive forces), respectively. Equation 1.2 can only be applied when the antigen possesses one epitope for binding and when the antibody has a single binding site that recognises one epitope of the antigen with one affinity. Values for K_A in the region of 10^6 to 10^{12} M^{-1} are commonly encountered, although for a sensitive and robust immunoassay, affinity constants of at least 10^{10} are probably necessary (Davies, 1994). The molar concentration of antigen necessary to bind half the antibody concentration can be taken as a measure of the affinity of the interaction. The lower the concentration of antigen required to reach half maximum occupancy of the antibody, the higher the affinity of the antibody for the antigen. An antibody with high affinity must have $k_1 \gg k_2$. k_2 can vary from around 10^{-4} for high affinity antibodies to 10^3 for low affinity antibodies (Tijssen, 1985). The half-life of antibody-antigen reactions varies between a few seconds and a fraction of a second depending on the affinity. This results in a continuous dissociation-association process.

As mentioned previously, the binding of an antigen can be severely influenced by pH, ionic strength, temperature and non-polar solvents. Thus, only under precise

conditions can absolute figures for K_A be generated. Furthermore, as polyclonal sera will contain several species of antibody, each with different binding characteristics for a given antigen. Accurate and meaningful affinity figures can therefore only be generated for monoclonal preparations.

The term “avidity” is used to describe the overall affinity arising from the binding of a heterogeneous mixture of antibodies to a multivalent antigen (Law, 1996). Multivalent antibody-antigen interactions often exhibit extreme stability and reduced dissociation rates as a result of the multimeric interactions (e.g. IgM), although the individual dissociation rates would resemble the univalent interaction.

1.7 Luminescent molecules as haptens

Antibodies to luminescent molecules have provided a sensitive means to determine the nature of the antibody's active site, the binding interactions involved and the hydrodynamic behaviour of immunoglobulins and their fragments. They have also provided a convenient method to study the changes in antibody affinities during the immune response and under varying immunisation conditions. Antibody binding to fluorescent molecules may result in either fluorescent enhancement or quenching. Examples of haptens which undergo fluorescent enhancement are 5-dimethylaminonaphthalene-1-sulfonamido (DNS) (Watt *et al.*, 1977) and 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) (Iwaki *et al.*, 1993). Examples of haptens which undergo fluorescent quenching on antibody binding are fluorescein (Watt *et al.*, 1977). and anthracycline antibiotics (Chien *et al.*, 1975).

Fluorescein is a polyaromatic, highly fluorescent dye. It is a relatively simple site-filling ligand that accommodates only a finite number of protein-ligand interactions. The binding of fluorescein to anti-fluorescein has proven to be an excellent model system for studying the biochemical basis of molecular recognition and as a result a large amount of physico-chemical and structural information is available for these antibodies. The spectral properties of fluorescein allow for sensitive measurements relating to the antibody binding site to be made. Antibody-bound fluorescein exhibits a red-shifted absorption spectrum (Watt *et al.*, 1977). This red shift can vary between 1-30 nm and is a

unique spectral property of each antibody active site. A more useful phenomenon is that the high quantum yield of fluorescein (0.92) is reduced by 90% or more when bound to the anti-fluorescein antibody. This is accompanied by a slight shift in maximum emission wavelength from 512 to 517 nm.

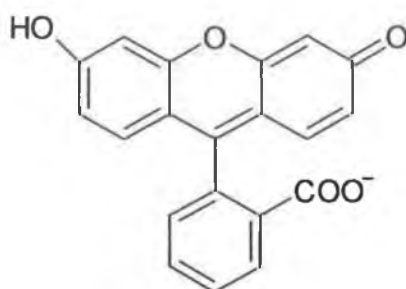


Figure 1.3. Structure of fluorescein.

Initial studies suggested that the quenching of fluorescein was due to a complex mechanism involving both static and dynamic components by tryptophan and tyrosine amino acids in the antibody binding site (Watt *et al.*, 1977). Another publication came to a similar conclusion on the quenching mechanism of the anthracycline antibiotic adriamycin when antibody-bound. The authors suggested that the decrease of the fluorescence of the aromatic compound may result from the formation of non-fluorescent charge transfer complexes or π -complexes between the aromatic residues and the anthracycline ring (Chien *et al.*, 1975).

The three-dimensional structure of the Fab fragment complexed with fluorescein has been determined based on x-ray diffraction studies and the active site contact residues were defined (Herron *et al.*, 1989). This confirmed that fluorescein binds tightly in an aromatic slot and participates in a network of electrostatic interactions. The active site contact residues were defined as L27^{His}, L32^{Tyr}, L34^{Arg}, L91^{Ser}, L96^{Trp} and H33^{Trp}. Site-specific mutagenesis of single-chain antibodies (scFv) has been used to define the relative roles of the fluorescein-amino acid contact residues in the variable domains as assessed by hapten fluorescence quenching and spectral shifts in the absorption spectra (Denzin, *et al.*, 1993). ScFvs involve only the variable immunoglobulin domains, V_L and V_H associated by a polypeptide linker between the carboxyl terminus of V_L and the amino

terminus of V_H. When the six contact residues in turn were changed to Ala, reduced binding affinities and quenching maxima were observed for each mutant. Ala was chosen as it should not impose constraints on surrounding amino acids. The scFv^{TyrL32^{Ala}}, Ser^{L91^{Ala}} and Trp^{H33^{Ala}} mutants exhibited the greatest change with binding affinities ~ 1,000-fold lower and greatly reduced fluorescence quenching values of 3.7%, 20.6% and 4.9%, respectively.

These studies emphasised the importance of aromatic amino acids as contact residues in the binding site. Comparison of crystallographic analyses of antibody active sites has shown that tryptophan and tyrosine residues are two of the most common contact residues. Mian *et al.* (1991) have suggested that these two amino acids may produce a binding site that behaves as a template responsible for general binding and that fine tuning of the binding site results from the addition of other antigen contact residues.

Iwaki *et al.* (1993) produced a monoclonal antibody which was specific for 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) and cross-reactive with 9-(dicyanovinyl)julolidine (DCVJ). The quantum yields of these fluorophores are dependent not on the dielectric constant of the medium but rather on the rotational relaxation of the molecules themselves. They are characterised by a charge-transfer-excited singlet state which can rapidly deactivate through internal rotation about the donor-acceptor bond. This results in a predominantly radiative decay pathway in highly constrained environments, and a large increase in the fluorescent quantum yield is observed. The CCVJ or DCVJ binding to IgG and Fab was accompanied by a significant increase in fluorescence quantum yield, suggesting that antibody binding decreased the nonradiative internal rotation about the donor-acceptor bond of the fluorophores. Scatchard plots determined that the association equilibrium constant (K_a) of CCVJ to the protein A-purified IgG was $6.8 \times 10^7 \text{ M}^{-1}$ and to the Fab component was $5.4 \times 10^7 \text{ M}^{-1}$. The K_a of DCVJ to IgG and Fab were 9.3×10^5 and $7.4 \times 10^5 \text{ M}^{-1}$, respectively. The quantum yield of CCVJ increased from 0.0014 to 0.062 for both IgG and Fab binding. Similar increases in lifetime of CCVJ were also observed for both. Free CCVJ had a lifetime of 62 ps, while IgG-bound had a value of 388 ps and the Fab-bound, 383 ps. This suggested that the nonradiative rate constants of CCVJ bound to antibody molecules were not dependent on the molecular size of the bound molecules but instead reflect the local

rotational motions of the fluorophore in the pocket of the antigen binding sites. The rates of the local rotational motions of CCVJ in the antigen combining sites can be approximated by the nonradiative rate constants. They were estimated to be 2.42 and 2.46 ns⁻¹ for IgG-bound and Fab-bound CCVJ, respectively.

The binding of fluorescent molecules to their specific antibody has been used not only to probe the nature of the antibody binding pocket and antibody antigen interactions but also to probe the hydrodynamic behaviour of the immunoglobulins. Segmental flexibility is an important property of immunoglobulins. Changes in flexibility can significantly affect immunological functions.

Tan *et al.* (1990) have characterised genetically-engineered chimaeric human IgG3 and IgG4 anti-DNS antibodies. These IgG subclasses vary in hinge region amino acid compositions. (DNS is another common fluorophore used to probe antibody/antigen interactions. Antibodies specific for DNS have resulted in a 25 to 30-fold increase in quantum yield when bound to ϵ -DNS-lysine. This increase is accompanied by a shift in the emission maximum from 556 to 500 nm and a red shift in the absorption band from 330 to 340 nm (Watt *et al.*, 1977)). The fluorescence emission anisotropy kinetics of DNS-lysine bound to these engineered antibodies was measured to determine the effect of hinge region structures on Fab fragment flexibility. IgG4 have hinge regions consisting of 12-15 amino acids while IgG3 have an extended hinge region consisting of 62 amino acids. The mean rotational correlation time of DNS-lysine bound IgG3 was determined as 52 ± 4 ns while bound IgG4 was 93 ± 7 ns indicating a greater rigidity. This confirmed the correlation between upper hinge length and the segmental flexibility of the antibody molecule.

Oi *et al.* (1984) investigated a correlation between segmental flexibility and effector function of antibodies. Mouse monoclonal anti-dansyl antibodies with the same antigen combining sites but different heavy chain constant regions were generated. The extent of segmental flexibility was determined by measuring their fluorescence polarisation kinetics. The mean rotational correlation times of the Fab fragments of mouse IgG subclasses IgG1, IgG2a and IgG2b were nearly identical at approx. 28 ns. However, the values for the intact antibodies were variable. IgG1 was the most rigid (81 ns), IgG2a was intermediate (60 ns) and IgG2b was the most flexible (47 ns). This was

found to correlate with the capacity to fix complement, IgG2b being able to fix the most complement and IgG1 the least.

Lim and Herron (1995) and Lim *et al.* (1995) have investigated the conformational perturbations which occurred within an anti-fluorescein (4-4-20) Fab upon fluorescein binding. Molecular dynamic simulations were performed with and without fluorescein. Anisotropy measurements were performed to validate the predictions. Differences observed in the simulations with and without the antibody included structural rearrangements of key contact residues and significant differences in the degree of hydration of the antigen-binding site, and a more acute elbow bend angle and less correlated motions of amino acid residues in the unbound form. Steady state and dynamic anisotropy measurements were performed on the unbound Fab and a complex of the Fab with a non-fluorescent analog of fluorescein (fluoresceinamine). Both had 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (AEDANS), covalently attached to the C-terminus of the Fab as a fluorescence polarisation probe. Two rotational components were observed for the Fab which included a longer correlation time that corresponded to the global rotation of the Fab and a shorter correlation time that was attributed to local and/or segmental motion of the constant domain (C_L - C_H1) dimer. Dynamic anisotropy measurements indicated that the magnitude of the shorter correlation time increases by more than 50% upon antigen binding, suggesting the conformation of the Fab is more rigid in the bound form. The authors concluded that these results suggest that an induced fit mechanism may be involved in the binding of fluorescein.

Voss *et al.* (2001) have used the fluorescein/anti-fluorescein system to study affinity variations in subpopulations of polyclonal antibodies. The spectral properties of bound fluorescein in the subpopulations was also characterised. The Fab fragments from 10 different hyperimmune rabbit IgG populations were separated into subpopulations based on specific dissociation times of 0.1 day, 1 day, 10 days and 100 days from the adsorbent. This resolution is based on the principle that variations in affinity among antibodies is dependent on differences in the rate of ligand dissociation. Fab fragments were chosen since they represent single antibody active sites thereby eliminating considerations of multivalency. The affinities measured showed a correlation between Fab affinity and the time of elution from the adsorbent. Direct correlation was also

observed between increased R value (the moles of ligand bound per mole of Fab fragment) and the length of time of dissociation of Fab fragments. R was 0.63 after 0.1 days of incubation and 0.93 after 100.

Fab dissociation profiles based on protein concentration did not conform to Gaussian distributions challenging a frequent assumption of heterogeneous populations of antibodies. Most patterns were asymmetric being skewed toward early or late elution subfractions. The absorbance of fluorescein bound to various Fab preparations segregated into two groupings; either a maximum absorbance of 505-507 or 518-520 nm. The latter group has only been observed with hyperimmune rabbit anti-fluorescein antibodies. The red shift to either spectral grouping was independent of time of elution. Isoelectric focusing studies indicated that the number of clones that populated the 100 days fraction was a finite number. Thus, the authors concluded that the fractionation procedure developed indicated that the polyclonal population was subfractionated by affinity with a tendency toward relatively homogeneous fractions.

1.8 Immunoassay design

The antibody-antigen interaction can be used to identify, separate and quantify the substances to which they bind. This has led to the development of immunoassays where the antibody is used as a qualitative and quantitative tool. Immunoassays have found use in diagnostic and quantitative applications in a broad range of areas. They are highly sensitive, selective, resistant to interferences and relatively cost-effective (McCormack *et al.*, 1998).

The assays rely on the use of labels to detect the immunological interaction. For example, fluorescent, chemiluminescent and enzyme labels. Use of the once popular radioisotopic labels is now increasingly avoided due to safety reasons. Enzyme labels are used most frequently. These have the advantage of signal amplification due to the catalytic effects of the enzyme leading to high sensitivity. Examples include horseradish peroxidase (HRP), alkaline phosphatase (AP) and β -galactosidase. HRP is particularly popular due to its high turnover rate, low molecular weight and cost.

Immunoassays can be categorised as homogeneous or heterogeneous. Heterogeneous assays are those in which free and bound label are separated before detection. In a homogeneous assay, this step is not required. Immunoassays can be further divided into competitive and non-competitive. In the competitive method, competition occurs between the labelled antigen and sample antigen for the immobilised antibody. A slight variation on this is when the antigen is immobilised and competition occurs between the analyte and a fixed amount of labelled antibody. The signal produced in both of these methods is inversely proportional to the concentration of antigen in the standard or test solution.

Non-competitive assays are based on a sandwich format, where the analyte is sandwiched between a primary, surface-bound capture antibody and a secondary, labelled detector antibody. The response is then directly related to the amount of bound antigen. This configuration while very sensitive is restricted by the fact that the analyte must have multiple antibody binding sites and so small molecules are excluded.

1.9 Immunosensors

Immunosensors are solid-state affinity ligand-based biosensor devices in which the immunochemical reaction is coupled to a transducer (Luppa *et al.*, 2001). Transduction elements include electrochemical (potentiometric, amperometric conductometric, capacitive or impedimetric), optical or transducers sensitive to changes in mass or thermal properties. Most commonly employed are electrochemical and optical methodologies. An advantage of electrochemical detection of the label is that it can be used in turbid media, unlike optical detection (D'Orazio, 2003). Solid supports for amperometric immunosensors can be made of larger area to increase sensitivity, and the support is not required to be optically transparent.

1.10 Amperometric immunosensors

An amperometric immunosensor based on single wall carbon nanotubes is developed in Chapter 4. Amperometric immunosensors measure the current generated when an

electroactive species is either oxidised or reduced at an antibody- (or antigen) coated electrode to which an analyte binds specifically. The electrode is held at a constant potential. This requires the use of electrochemically-active labels such as enzymes which are themselves redox active or catalyse the production of an electrochemically detectable product. Common enzymes are HRP and AP. The current generated is directly related to the immunological binding event. The electrode sensing element is usually constructed of platinum, gold or various forms of carbon. Screen-printed electrodes are also frequently used since they are low cost, mass produced and disposable. There are a number of established immobilisation procedures, which are used to attach the antibodies or antigen to the solid phase. These include physical adsorption (Lei *et al.*, 2003), use of cross-linking reagents (Lu *et al.*, 1997), covalent attachment (Valet *et al.*, 2000) or entrapment with a gel, polymer or membrane.

Amperometric immunosensors commonly employ mediators. These are small redox molecules with high heterogeneous electron transfer rates which shuttle electrons between the enzyme and electrode surface thereby overcoming the slow electron heterogeneous transfer rate of enzymes. In the original amperometric sensor of Clark and Lyons (1962), oxygen acted as a diffusional electron transfer mediator between the enzyme redox site and the electrode surface. Later devices used small redox molecules such as potassium ferrocyanide or hydroquinone in place of oxygen. The avoidance of oxygen or pH dependencies means that the biosensor can operate at lower potentials, thereby reducing potential interferences from other redox active species.

In the last few years non-diffusional mediators have been used such as redox and conductive polymers. Redox polymers include osmium complexes such as $[\text{Os}(\text{bpy})_2(\text{PVP})_{10}\text{Cl}]\text{Cl}$ where bpy is 2,2'-bipyridyl and PVP is poly-4-vinylpyridine (Lu *et al.*, 1997) while examples of conductive polymers are polyaniline or polypyrrole (Sadik, 1999). The advantage of non-diffusional mediators is that both antibody-bound and unbound enzyme labelled analyte can be present but only the bound species is responsible for the generation of a catalytic signal. This has led to the development of a single step assay in which no separation step is required (Grennan *et al.*, 2003).

Amperometric immunosensors can achieve very low limits of detection. A recent example of this was the detection of simazine at a concentration of 0.1 ng L^{-1} (Zeravik *et*

al., 2003). This mediator-free immunosensor was based on the channeling of H_2O_2 produced by glucose oxidase (GOD) labelled simazine which was bound to the anti-simazine co-immobilised with a recombinant HRP on the gold electrode. The recombinant HRP had a histidine tag which was shown to enable very efficient direct electron transfer between the enzyme and the gold electrode. This was attributed to stronger adsorption and more optimal space adaptation of the engineered HRP. In the presence of glucose, H_2O_2 formed by the antibody-GOD conjugate was reduced electrochemically by the HRP-modified electrode.

The development of recombinant antibodies has been much anticipated in the field of amperometric immunosensing and immunosensing in general. These are produced using recombinant DNA technology. They offer several potential advantages including rapidity of production, an infinite supply at low cost and the use of animals is bypassed (Spinks, 2000). Antibodies with novel properties may be generated. For example, specificity, affinity and stability could be altered or enzyme labels could be produced as fusion proteins, thus linking specific antigen recognition with a direct measurable readout. Benhar *et al.* (2001) were the first to use single chain antibodies (scFvs) in amperometric immunosensing for the detection of lactose, *Listeria monocytogenes* and the enzyme MtKatG. Recently, Grennan *et al.* (2003) applied the use of single-chain antibody fragments (scAb) to achieve sensitive detection of atrazine. ScAb contain interdomain disulphide bonds between V_L and V_H . A separation-free amperometric immunosensor was described utilising a screen-printed carbon paste electrode onto which a polyaniline/polyvinyl sulphonate film was potentiodynamically applied onto which the scAb were electrostatically immobilised and a flow injection competitive immunoassay performed. An inhibition competition format resulted in a limit of detection of $0.1 \mu\text{g ml}^{-1}$ of atrazine.

Amperometric immunosensing based on paramagnetic microbeads as a mobile solid phase is another area of active research. The paramagnetic beads provide an easy means of separating the primary antibody from the reaction chamber with a magnetic field. This allows small volumes for incubations and detection. This has resulted in reduced assay time and low detection limits. Paramagnetic bead-based immunoassays can also be incorporated into automated microfluidic systems (Ronkainen-Matsuno *et al.*,

2002). A recent paper compared a microbead-based immunoassay in a microdrop using amperometric detection with a rotating disk electrode (RDE), a microelectrode and an interdigitated array electrode (IDA) (Thomas *et al.*, 2003). An enzyme-labelled sandwich immunoassay with mouse IgG as the model analyte was used to demonstrate the three techniques. Once the immunocomplex was formed on the microbead, the beads were transferred to a microdrop where the enzyme, either AP or β -galactosidase generated 4-aminophenol (PAP) which was then oxidised at the electrode surface. The detection limits were 50 ng ml⁻¹ for the glassy carbon RDE, 40 ng ml⁻¹ for the carbon fibre microelectrode (11 ± 2 μ m) and 26 ng ml⁻¹ for the platinum IDA electrode.

Immunosensors require antibodies with high affinity constants (10^{10} M⁻¹) for adequate analytical sensitivity (Hock, 1997). This makes regeneration of the antibody-immobilised surface difficult to realise. Acidic or alkaline solutions can be used to separate the antigen and antibody but this is potentially harmful to the binding ability and may lead to diminished lifetime of the immobilised antibodies (Luppa *et al.*, 2001). Other formats involve the complete removal of the immobilised antibody after each analysis, i.e. an alkaline solution removed antibodies adsorbed to a nano gold monolayer (Lei *et al.*, 2003). Most immunosensors reported are limited to single-use devices. Antibody engineering techniques may improve the chemical stability of antibodies resulting in the development of continuous use immunosensors.

1.11 SUMMARY

The antibody-antigen interaction has potential for high selectivity and affinity. Antibodies can be produced against any molecule capable of eliciting an immune response in a host animal. Polyclonal antibodies are produced as a mixed population with varying affinity and specificity. Several different noncovalent forces are involved in the binding of an antigen to an antibody. Complementarity between the antigen and antibody binding site is an important factor in the strength of these forces. Antibodies to luminescent molecules have provided a sensitive means to determine the nature of the antibody's active site, the binding interactions involved and the hydrodynamic behaviour of immunoglobulins and their fragments. Immunoassays and immunosensors harness the

antibody-antigen interaction to detect and quantify a large range of analytes. The evolution of electrochemical immunoassays has resulted in significant improvements in assay times and detection limits. Further improvements are expected due to the expansion of antibody production by recombinant techniques.

1.12 THESIS AIMS

The aim of this work was to explore the application of antibodies in optical and electrochemical transduction processes. The first part of the thesis outlines the production of a polyclonal antibody specific for Os(II) and Ru(II) polypyridyl complexes and attempts to investigate the interaction of the antibodies with the complexes. This included the effect antibody binding has on the spectrochemical properties of several complexes, on the electrochemical properties of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ and the potential to exploit energy transfer processes.

The second part explores the use of SWNT forests as a platform for amperometric immunosensing. Carbon nanotubes (CNTs) are new and exciting members of the carbon family offering high conductivity, large surface area and mechanical strength, properties which may be of benefit in the development of biosensors.

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CHAPTER 2

**The production and characterisation of a
polyclonal antibody for osmium(II) and
ruthenium (II) polypyridyl complexes**

2.1 INTRODUCTION

Os(II) and Ru(II) polypyridyl complexes exhibit interesting and useful photophysical and electrochemical properties. Such properties have been shown to be applicable in exploring the structure and rotational dynamics of biological molecules. This Chapter reports the production for the first time of an antibody raised against an Os(II) complex ($[\text{Os}(\text{bpy})_2(\text{dcbpy})]$, where bpy is 2,2'-bipyridyl and dcbpy is 2,2'-bipyridine-4,4'-dicarboxylic acid). The synthesis and characterisation of this complex and some related Os(II) and Ru(II) complexes is outlined, as well as the production and characterisation of a $[\text{Os}(\text{bpy})_2(\text{dcbpy})]$ -thyroglobulin immunogen and its associated bovine serum albumin conjugate. The conjugates were characterised using high performance size exclusion chromatography, UV-Vis and emission spectroscopy and lifetime measurements.

Following immunisation, the resulting rabbit polyclonal antibodies were purified by ammonium sulphate precipitation and protein A chromatography and characterised by traditional immunological methods. The cross-reactivities of $[\text{Os}(\text{bpy})_2(\text{dcbpy})]$, $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]$, $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ (where p2p is 1,2-bis(4-pyridyl)ethane) towards the antibody were examined using competition ELISA.

2.1.1 Ruthenium(II) and osmium(II) polypyridyls as probes of biomolecules

It is appropriate to begin this Chapter with a review of the properties of the Ru(II) and Os(II) polypyridyl complexes relevant to this thesis, including the fundamental chemical, photochemical and electrochemical properties. It is also illustrated how their photophysical properties can be modulated by the interaction of the complexes with other species, including a whole range of biomolecules such as nucleic acids, antibodies and other proteins.

2.1.1.1 The structure of ruthenium and osmium (II) polypyridyl complexes

Ru(II) and Os(II) are d^6 transition metals. They are involved in the formation of octahedral co-ordinatively saturated complexes of the type $[\text{M}(\text{LL})_3]^{2+}$, where M is either Os or Ru and the ligands (L) are bidentate polypyridyls. These ligands are usually

colourless molecules possessing σ -donor orbitals localised on N atoms and π^* -donor and π^* acceptor orbitals more or less delocalised on aromatic rings. Both $[\text{Ru}(\text{LL})_3]^{2+}$ and $[\text{Os}(\text{LL})_3]^{2+}$ exhibit D_3 symmetry. X-ray crystallography has shown that in $[\text{M}(\text{bpy})_3]^{2+}$ the metal-to-ligand bond lengths are short, indicating significant back-bonding between the metal and the π^* orbital of bpy (Balzani *et al.*, 1988). The greater extension of the metal d orbitals in Os enhances the metal-to-ligand back-bonding. The metal-ligand bond lengths for analogous complexes of Os and Ru are very similar due to the lanthanide contraction. This minimises the differences in steric effects and solvation (Kober *et al.*, 1988).

The choice of ligands in a series of complexes of the same metal ion can control the orbital nature of the lowest excited state and hence, the energy, lifetime, quantum yield, redox properties and chemical stability of the complexes can also be controlled. This allows a complex to be 'tuned' for a particular application.

2.1.1.2 Excited states of Ru(II) and Os(II)

The first act of any photochemical and photophysical process is the absorption of a photon by a complex. The excited state that is formed in this way is a high energy, unstable species which must undergo some type of deactivation. Excited state deactivation can occur via photochemical reaction, luminescence, radiationless deactivation or a quenching process (Balzani *et al.*, 1988).

There are three types of excited state; metal centered (MC) d-d states, ligand centered (LC) π - π^* states and charge transfer (CT) states. A charge transfer state is obtained either by promoting a d electron to a π^* antibonding orbital (MLCT) or promoting an electron from a π bonding orbital to an unfilled d orbital (LMCT). α -diimine ligands are easily reduced and thus involve only MLCT transitions (Demas and DeGraff, 1991).

All these excited states may have singlet or triplet multiplicity, although spin-orbit coupling causes singlet-triplet mixing in the MC and MLCT excited states. Inter-system crossing occurs with almost unit efficiency (Kober *et al.*, 1983). Os has a larger spin-orbit coupling constant (λ), which leads to more extensive mixing of excited states of different

spin multiplicities (Kober *et al.*, 1988). The high energy excited states of transition metal complexes undergo fast radiationless deactivation. Thus, only the lowest excited state and the upper states that can be populated on the basis of the Boltzmann equilibrium law may play a role in the luminescence emission and in bimolecular processes.

Over the years various lines of experimental evidence have been accumulated, leading towards a localised orbital picture for the MLCT excited state of $[M(bpy)_3]^{2+}$, i.e. the excited state formulated as $[M^{III}(bpy)_2(bpy^-)]^{2+}$. Evidence in favour of the localised model comes from the properties of the excited state species and from the chemical and electrochemical properties of the reduction products.

The energy of the MC excited state $(d\pi)^5(d\sigma^*)^1$ depends on the crystal field splitting energy of both the ligands and the metal (Kalyanasundaram, 1992). The MC excited state is strongly distorted with respect to the ground state geometry (involving promotion of a largely non-bonding electron to an anti-bonding σ orbital). Hence it undergoes very fast radiationless decay, which includes ligand substitutions. MC excited states can also be thermally and photochemically activated, whereby the excited electron initially in π^* is transferred to a $d\sigma^*$ orbital.

LC excited states are largely localised on the organic ligands and are spectroscopically very similar to those of the free ligand yielding high energy excited states. The energy of the CT state depends on the redox properties of the metal and ligands. The harder it is to oxidise the metal, the higher the energy of the MLCT state (Demas and DeGraff, 1991). LC and MLCT excited states are usually not strongly displaced compared to the ground state geometry. Thus, when the lowest excited state is LC or MLCT it does not undergo fast radiationless decay to the ground state and luminescence can usually be observed (Balzani *et al.*, 1988). However, at high temperature, thermally activated radiationless deactivation via upper lying MC excited states can occur. The 3MLCT is actually a cluster of closely spaced 3MLCT levels. At higher temperatures all of these excited states are populated and contribute to the excited state decay so that the excited state manifold may be considered as an average state and on emission, results in a broad band (Kober *et al.*, 1984, Lacky *et al.*, 1980).

In comparison to their Os analogues, Ru(II) polypyridyl complexes possess a higher energy MLCT since Ru is harder to oxidise than Os (Figure 2.1). The lower

energy MLCT state of Os analogues yields lower lifetimes and quantum yields, i.e., in aqueous deaerated solution the lifetime of $[\text{Ru}(\text{bpy})_3]^{2+}$ has been determined as 600 ns while that of $[\text{Os}(\text{bpy})_3]^{2+}$ was determined as 19 ns (Creutz *et al.*, 1980). The lower lifetime and quantum yield is explained by the energy gap law which states that the rate of non-radiative decay (k_{nr}) increases as the lowest excited state energy becomes closer to the ground state (Caspar *et al.*, 1982). The photochemical stability of polypyridine complexes of Os is of a direct consequence of the larger splitting energy value of 10 Dq. This is approx. 30% higher in the third transition series compared to the second so that low-lying d-d states occur at higher energies and are well removed from the emitting MLCT state. Ru complexes are more susceptible to radiationless decay at higher temperatures via activation of the MC state.

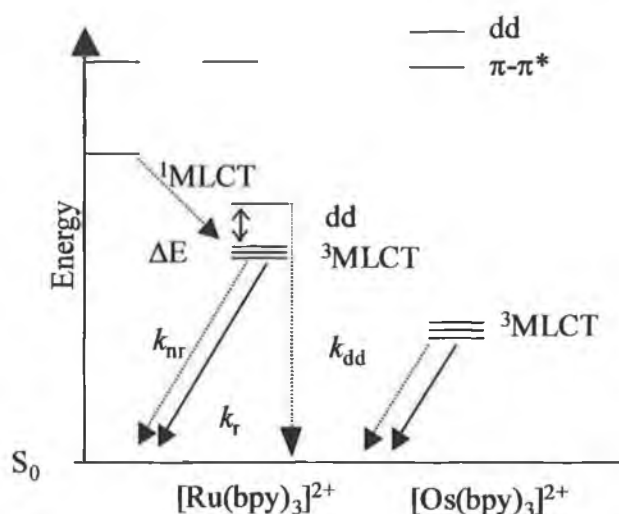


Figure 2.1. Diagram showing the relative positions of the d-d, π - π^* and $^3\text{MLCT}$ as a function of metal. S_0 is the ground state and k_r and k_{nr} are the radiative and non-radiative decay rates, respectively (from Demas and DeGraff, 1991).

2.1.1.3 Emission lifetime

2.1.1.3.1 Solvent dependence on emission energies and lifetime

Knowledge of the effect of solvent on the properties of Ru(II) and Os(II) polypyridyl complexes is important when studying the effects of an antibody binding to these complexes. As was discussed previously in section 1.5, the close approach of an antibody and antigen on binding, results in effective water elimination from the binding cleft, thereby altering the nature of the solvation of the antibody bound complex.

The solvent effects on the emission energies and lifetimes (τ) of Os(II) and Ru(II) have been investigated. Caspar *et al.* (1983) determined values for the radiative rate constant (k_r) and the non-radiative rate constant (k_{nr}) in a series of solvents from a combination of lifetime and quantum yield experiments. The k_r was shown to be only slightly solvent-dependent. No apparent systematic variation with emission energies (E_{em}) was found. However, the non-radiative rate constant (k_{nr}) and the emission energy were strongly solvent-dependent. This is in quantitative agreement with the predictions of the energy gap law for radiationless transitions. The energy gap law states that the radiationless decay constants increase as the emission maxima shift to lower energies (Caspar, 1982). Plots of $\ln k_{nr}$ vs. E_{em} of a series of polar organic solvents for $[\text{Os}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{bpy})_3]^{2+}$ had identical slopes within experimental error. The temperature-dependent thermal population of the metal centered d-d state is also solvent-dependent since both the preexponential (k) and exponential (ΔE) components of the following equation vary with solvent:

$$1/\tau(T) = (k_r + k_{nr}) + k \exp(-\Delta E/RT) \quad (2.1)$$

where T is the temperature, ΔE is the energy gap between the d-d state and emitting level, and k is the Arrhenius preexponential factor for thermal activation of the d-d state. The observed phenomena can be understood on the basis that the energy level of the MLCT state, which possesses a static dipole moment, is sensitive to the polarity change of the solution medium (Sun and Hoffman, 1993).

The k_{nr} values for Os and Ru complexes in hydroxylic solvents (solvents containing an OH group) are much higher than the trend observed in polar organic solvents, with H₂O having the most dramatic effect (Caspar *et al.*, 1983). Indeed, it has been shown that the k_{nr} of [Os(bpy)₃]²⁺ and [Os(phen)₃]²⁺ (where phen is 1,10-phenanthroline) in H₂O are large compared with those in D₂O despite the same emission energy (Masuda *et al.*, 1998). Hauenstein *et al.* (1983) reported a linear increase of the τ^{-1} of [Ru(bpy)₃]²⁺ on the mole fraction of H₂O in H₂O-D₂O mixtures. The linear dependence of the reciprocal lifetime (τ^{-1}) on the mole fraction of H₂O indicated that a H₂O-specific quenching process was present.

The existence of several hydrating water molecules, which penetrate between the bulky phen or bpy ligands of [Ru(bpy)₃]²⁺ and [Ru(phen)₃]²⁺ have been reported (Yokoyama *et al.*, 1997). This was determined by X-ray diffraction structural analysis of the complexes. The measurements of aqueous solutions of both complexes detected oxygen atoms of one or two H₂O molecules around 3.5-3.6 Å from the Ru²⁺. These were referred to as 'included H₂O molecules'. A specific interaction between the π electrons of the ligands and the included H₂O molecule was suggested. Ten or eleven H₂O molecules in the outer region of the complex were tightly hydrogen bonded to the adjacent H₂O molecules and to the included ones. It has also been reported that the overtones of the vibrationally excited states of H₂O lie close to the ³MLCT state of [Os(bpy)₃]²⁺ and are expected to act as energy accepting levels (Masuda *et al.*, 1998). Energy transfer via dipole-dipole interaction between the transition of the Os(II) complex (³MLCT → ground state) and that of the included H₂O (vibrational ground state → excited state) will increase the k_{nr} .

2.1.1.3.2 Bimolecular quenching of the excited state

The excited states of ML₃²⁺ are sufficiently long-lived to undergo bimolecular collisions with other molecules present in solution. Three processes must be considered as possible quenching pathways for the polypyridine excited states: Equations 2.2 and 2.3 below, involve, respectively, oxidation and reduction of the *ML₃²⁺ excited state, while in

equation 2.4 electronic excitation is transferred by an energy-transfer process to Q (the quencher).



The three quenching processes may proceed in parallel so that the observed quenching rate constant (k_q) may contain contributions from all three. The electron transfer quenching reactions are a function of the oxidation-reduction potentials of the reactants, while energy transfer processes depend on the spectral overlap considerations (Creutz *et al.*, 1980).

Oxygen is an effective quencher of excited states. For example the quenching constant of oxygen for $*[Ru(bpy)_3]^{2+}$ has been estimated as $3.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (Seddon and Seddon, 1984). The primary quenching path is energy transfer to form singlet O_2 .

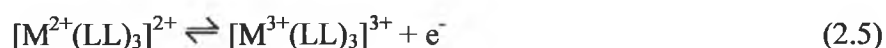
Following the quenching reactions, rapid, back electron transfer reactions can occur between either the M(III) or the ligand reduced M(I) complexes and the reduced or oxidised forms of the quencher (Creutz *et al.*, 1980).

2.1.1.4 Electrochemical properties

The redox properties of Os and Ru polypyridyl complexes depend on the π -accepting and σ -donating abilities of the ligands and the oxidation potential of the metal (which is also affected by electron donor and acceptor properties of the ligands). Os(II) and Ru(II) complexes undergo one metal centered oxidation and two or three ligand based reduction processes of reversible one-electron transfer. The oxidation peak potentials of the Os(II) complexes are found to be approx. 0.4 V more negative than those for the corresponding Ru(II) complexes (Matsumura-Inque *et al.*, 1986).

The effect of the ligand on the redox potential is due to the changing electron density at the metal centre. The π -accepting and σ -donating abilities of the ligand interact synergistically through π back-bonding. The weaker σ -donor capacity of the ligand gives rise to a greater effective nuclear charge on the metal centre and this results in stabilisation of the metal d orbitals. Further stabilisation of the metal centre can then occur by back-bonding of the $d\pi$ orbitals with the π^* levels of the coordinating ligands. Thus, the effect of ligands on the electrode potential can be explained in terms of intramolecular electron flow through the metal-ligand bond.

The oxidation process of Ru(II) and Os(II) bipyridine complexes corresponds to the removal of an electron from the t_{2g} orbital of predominant d character. This results in the formation of a genuine M(III) complex which is inert to ligand substitution:



The reduction process involves addition of electrons one by one to π^* orbitals predominantly of ligand character. The reduced form, keeping its low spin $4d_6$ configuration, is usually quite inert and the reduction process is reversible:



Correlations have been shown to exist between redox potentials and optical properties. This occurs as the π^* orbital involved in the first electrochemical reduction is the same as that involved in the MLCT absorption and emission process (Kalyanasundaram, 1992).

2.2.1.5 Self-assembled Os (II) and Ru (II) polypyridyl monolayers

The polyclonal antibodies produced in this thesis were capable of binding to $[Os(bpy)_2(p2p)_2]^{2+}$ where p2p is 1,2-bis-(4-pyridyl)ethane. In section 3.3.6 the effect of antibody binding to self-assembled $[Os(bpy)_2(p2p)_2]^{2+}$ on platinum microelectrodes is investigated.

Self-assembled and spontaneously adsorbed monolayers of Ru(II) or Os(II) polypyridyl complexes offer a facile means of controlling the chemical composition and physical structure of a surface. Self-assembled monolayers of these model systems have been used to probe fundamental properties such as electron transfer distance, molecular structure, and solvent dynamics on heterogeneous electron transfer dynamics (Forster and Faulkner, 1994a,b).

Self-assembled monolayers involve strong binding of a surface-active functional group to a surface. These are distinct from spontaneously adsorbed monolayers in that self-assembled monolayers also involve stabilising lateral interactions between the adsorbates. Investigations have focused primarily on self-assembled complexes of Os rather than Ru since their lower oxidation potential reduces interference from the effects of electrode oxidation. Many of the complexes studied involve adsorption through a pendant pyridine or thiol group. Types of surfaces used for these complexes involve mainly platinum and gold.

While chemisorption of pyridine is responsible for stable monolayer assembly, a publication by Campbell and Anson (1996) stressed the importance of hydrophobic interactions of ligands with the electrode surface and with each other. The mechanism of adsorption of $[\text{Os}(\text{bpy})_2\text{LCI}]^+$ on platinum, gold and graphite electrodes, where L was either 1,2-bis(4-pyridyl)ethane, 4-phenylpyridine, 4-(1-n-butylpentyl)pyridine or pyridine was investigated (Figure 2.2). Strong adsorption of all four complexes occurred on graphite with the exception of where L was pyridine on gold. This showed that adsorption was not restricted to platinum nor was the pendant pyridine group necessary for strong adsorption and emphasised the importance of hydrophobic interactions. It was suggested that the stability of the adsorbed complexes may also be due to intermolecular π - π interactions between aromatic rings of adjacent complexes.

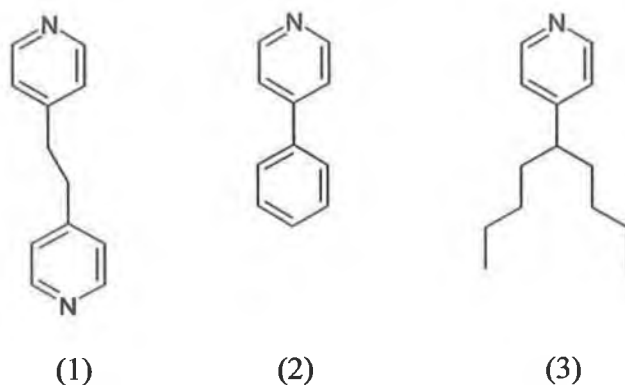


Figure 2.2. Ligands capable of strong adsorption on gold and pyrolytic graphite; (1) 2-bis(4-pyridyl)ethane, (2) 4-phenylpyridine, (3) 4-(1-n-butylpentyl)pyridine.

Self-assembled complexes of Os and Ru polypyridyls act as probes of their local microenvironment. The half wave potential is dependent on the monolayer surface coverage, the concentration and type of electrolyte and the solvent. Solvent molecules screen charge by intercalating between headgroups (Acevedo and Abruña, 1991). This decreases the repulsion between the adsorbed molecules making oxidation easier as the dielectric constant (ϵ_r) increases. The formation of ion pairs between anionic electrolytes and the charged headgroups serves to neutralise the monolayer, minimising repulsion. This is more effective in solvents of low ϵ_r . The variation of the half wave potential with coverage is also due to repulsion effects as a shift to more positive potentials occurs at higher coverage.

Forster and Faulkner (1994a) have investigated the electrochemical response of a series of $[\text{Os}(\text{bpy})_2\text{Cl}(\text{pNp})]^+$ monolayers self-assembled on platinum, where pNp is either 4,4-bipyridyl, 1,2-bis(4-pyridyl)-ethane or 4,4-trimethylenedipyridine. The effect of solvent, electrolyte, temperature and electron transfer distance was examined. The interfacial capacitance as a function of potential was determined. It was found that the interfacial capacitance becomes larger as the film becomes increasingly oxidised. This is the result of the movement of charge compensating counterions and solvent into the monolayer upon oxidation. Interfacial capacitance also depended on the nature of the electrolyte. Hydrophilic anions gave a higher interfacial capacitance than lipophilic anions suggesting the influence of the anion on ion pairing and solvent penetration. The

extent of ion-pairing was found to be solvent-dependent. In tetrahydrofuran, oxidation of the redox centers causes association of an extra anion, while two extra anions are bound to the oxidised centers in aqueous media. The reaction entropy ΔS_{rc}° quantifies the entropy difference between the reduced and oxidised forms of the redox couple and is expected to be dominated by differences in solvent and counterion ordering. A correlation was observed between the experimentally determined ΔS_{rc}° and that predicted by the Born dielectric continuum model. ΔS_{rc}° was found to be considerably larger in low dielectric media. The heterogeneous rate constant was found to be approximately independent of the supporting electrolyte concentration over the range 0.1 to 1 M suggesting that ion pairing is an equilibrium reaction which may either precede or follow electron transfer. A higher rate constant for reduction than oxidation for a given overpotential was obtained which suggested a through space tunneling mechanism. Subsequently, Forster and Faulkner (1994b) reported on the effect of solvent on electron transfer. The electron transfer rate was found to be approximately proportional to τ_1^{-1} where τ_1 is the longitudinal relaxation of the solvent. This suggested that the electron transfer process was sensitive to the ability of the solvent to relax dynamically to accommodate the new charge placed on the redox centre.

2.1.1.6 Interaction of Os(II) and Ru(II) with biomolecules

2.1.1.6.1 Interaction with proteins

Transition metal-ligand complexes (MLC) are becoming an increasingly important class of fluorophores for biological applications owing to their favourable properties of long lifetimes, adequate quantum yields, and high thermal, chemical and photochemical stability. These properties have contributed to the use of Ru(II) and Os(II) polypyridyls as probes of structural conformation and hydrodynamics of proteins. Certain MLCs also display high anisotropy when excited with polarised light. This property has allowed the use of Ru complexes in particular as anisotropy probes in fluorescence polarisation immunoassays for the detection of high molecular weight antigens such as proteins (Dürkop *et al.*, 2002).

The conjugation of a series of proteins to $[\text{Ru}(\text{L-L})_2(\text{NCSphen})]^{2+}$, $[\text{Ru}(\text{L-L})_2(\text{NH}_2\text{phen})]^{2+}$ and $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ has been reported (Ryan *et al.*, 1992). L-L is either 2,2'-bipyridine or 1,10-phenanthraline, NCSphen is 5-isothiocyanato-1,10-phenanthroline, NH_2phen is 5-amino-1,10-phenanthroline and dcbpy is 4,4'-dicarboxy-2,2'-bipyridine. The proteins used in the study were poly(L-lysine) (PLL), albumin and immunoglobulin G. The absorption and emission maxima of the labelled proteins were not found to be substantially altered upon conjugation but the emission lifetime was markedly affected.

The luminescence decay of the ruthenium complexes when conjugated could no longer be fitted to a single-exponential function, indicating that there must be more than one emitting site. This behaviour was found for both bovine serum albumin (BSA) and PLL conjugates of $[\text{Ru}(\text{L-L})_2(\text{NCSphen})]^{2+}$ or $[\text{Ru}(\text{L-L})_2(\text{NCSphen})]^{2+}$. The decay data in most cases could be fitted well to a double exponential function. In degassed solution, analysis of the data from BSA and PLL conjugates revealed one decay which was substantially longer than that of the free label and another which was significantly shorter. The short and long lifetime component of the conjugates each accounted for approx. 50%. The longer lifetime component was attributed to the more hydrophobic environment provided by the protein. This would result in a reduction of deactivation due to the interaction with water. It was concluded that the shorter lifetime component might arise due to electron transfer between the excited state and certain amino acid moieties. The quenching effects of oxygen were stronger in the PLL over the BSA conjugates. It was proposed that this was due to the more open tertiary structure of PLL.

Walsh *et al.* (1996) demonstrated the ability of ruthenium polypyridyl probes to monitor structural changes in biomolecules. In this report, PLL conjugates of $[\text{Ru}(\text{bpy})_2(\text{NCSphen})]^{2+}$ were prepared. The structure of PLL changes from a random coiled structure to an α -helix at a pH value of about 10. This changeover was, however, not observed at higher temperatures. The conformational change could be observed by monitoring the emission decay of the conjugates at increasing pH values. At lower pH both components of the biexponential decay exhibited longer lifetimes. This is most likely due to protection from quenching by oxygen in the sheltered environment of the polypeptide when in the random coil conformation. This corresponds to the behaviour in

deaerated solutions where the decay is quite uniform over the whole pH range as no oxygen quenching occurs. In aerated samples at 50°C, no lifetime drop was observed at any pH. This correlates well with the fact that at 50°C, PLL does not undergo a structural transition with pH but rather adopts a non-random type β -sheet conformation somewhat similar to the helical form. It was concluded that the results indicated the potential of ruthenium polypyridyl complexes as luminescent probes of the conformational structure of proteins.

Terpetschnig *et al.* (1995a) have used Os and Ru polypyridyl complexes to investigate the rotational motion of proteins based on their ability to display polarised emission when excited with vertically polarised light. The steady state anisotropy (r) of a labelled macromolecule is given by:

$$r = \frac{r_0}{1 + \tau / \theta} \quad (2.7)$$

where r_0 is the value observed in the absence of rotational diffusion and θ is the rotational correlation time. When the relatively small fluorescent complex becomes bound to a large protein molecule a marked decrease in rotational diffusion occurs with a corresponding increase in anisotropy. The values for polarisation and anisotropy can be interchanged using:

$$r = \frac{2P}{3 - P} \quad (2.8)$$

Information on the rotational motion is available over a time range extending to about three times the fluorescence lifetime, after which there is too little signal for accurate anisotropy measurements. The lifetimes of typical fluorophores range from 1 to 10 ns. This implies that they are limited to the measurement of short rotational correlation times of low molecular weight antigens. In contrast MLC displays emission from charge transfer states with decay times ranging from 100 to 4000 ns. As an example, a fluorophore with a lifetime of 400 ns could, in theory allow the analysis of biological systems with molecular weights up to 1×10^6 Da.

Terpetschnig *et al.* (1995b) reported on the use of Ru bis(2,2'-bipyridine)(2,2'-bipyridine-4,4'-dicarboxylic acid), $[\text{Ru}(\text{bpy})_2\text{dcbpy}]\text{PF}_6$ as a suitable anisotropy probe. The coupling reagents di-cyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were used to covalently attach the proteins to the complex through an amide linkage. Over a range of proteins it was found that the anisotropy decay increased as the molecular weight of the labelled protein increased. This led to the conclusion that the anisotropy decay of the Ru-labelled proteins was sensitive to the size and/or shape of the proteins.

A later publication provided a good example on the usefulness of this complex for probing high molecular weight protein hydrodynamics (Kang *et al.*, 2002). $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ was conjugated to IgG and IgM. The best fits of the anisotropy decay data were obtained using a biexponential model. The authors attributed the long rotational correlation time with the overall rotational diffusions of immunoglobulins and the short rotational correlation times with the local motion of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ within the antibodies. The long correlation times increased substantially with an increase in glycerol concentration, whereas the short correlation times increased much less with increasing glycerol content. The ratio of the long correlation times of IgM to IgG was 6.2. This is very close to the ratio of 6 for their molecular weights. This clearly indicated that this complex is a good probe for measuring high-molecular weight protein hydrodynamics.

This group also investigated the Os analogue of this complex as an anisotropy probe (Terpetschnig *et al.*, 1996). Typical of many Os polypyridyl complexes, this is characterised by low quantum yields and short decay times. However, its long wavelength absorption allowed for excitation with wavelengths up to 720 nm. This has an advantage since background fluorescence was highly reduced at longer wavelengths beyond 650 nm. The steady-state anisotropy of Os-labelled human serum albumin (HSA) in the presence of increasing amounts of monoclonal or polyclonal anti-HSA antibodies was examined. In both cases the anisotropy increased at least 60% in the presence of an eight-fold excess of anti-HSA.

Further studies showed that the anisotropy of the free Os complex decayed very rapidly and was essentially zero after 2 ns. When the complex was bound to HSA or antibody, the anisotropy decayed much more slowly and remained non-zero for as long as

40 ns. The anisotropy of Os-labelled HSA in the presence of antibody did not decay to zero at long times. This was due to the poor resolution of the long correlation time of the antibody-bound complex by this short lifetime probe. The authors concluded that an immunoassay could be based on a probe such as this which does not directly measure the rotational correlation time of the aggregates but simply reports on their presence.

There have been two previously published reports of the production of antibodies capable of binding Ru(II) polypyridyl complexes. The first concerned the elicitation of a polyclonal antibody via immunisation using a tris (2,2'-bipyridine)ruthenium(II)-methyl viologen hapten $[\text{Ru}(\text{mv}^{2+}\text{-bpy})(\text{bpy})_2]^{4+}$ (Figure 2.3) (Shreder *et al.*, 1995).

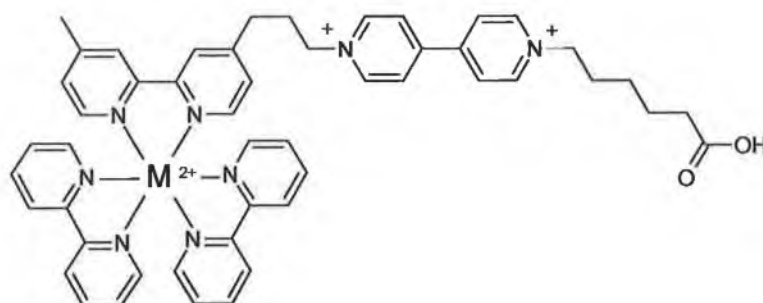


Figure 2.3. Structure of the hapten $[\text{M}(\text{mv}^{2+}\text{-bpy})(\text{bpy})_2]^{4+}$, where *M* is Ru or Co.

On binding of $[\text{Ru}(\text{bpy})_3]^{2+}$ to the polyclonal antibody a 3.3-fold increase in the emission was observed with a blue shift in the emission spectrum. A lower limit of the association constant for binding was assigned from this data as $5 \times 10^8 \text{ M}^{-1}$. It was found that the lifetime of $[\text{Ru}(\text{bpy})_3]^{2+}$ increased from 380 ns to 1300 ns when bound to antibody in air-saturated buffer. The lifetime of the bound species increased to 1500 ns in N_2 -saturated buffer. The antibody-bound $[\text{Ru}(\text{bpy})_3]^{2+}$ was found to have an increased protection from quenching by oxygen and methyl viologen.

In a proceeding paper by the same group a monoclonal antibody (AC1106) was produced on immunisation with the cobalt analogue of the hapten in Figure 2.3 (Shreder *et al.*, 1996). The kinetically inert cobalt(III) metal centre was chosen based on its reported stability to the conditions of immunisation. In addition, it was also taken into account that the dimensions of $[\text{Co}(\text{bpy})_3]^{3+}$ roughly approximate those of $[\text{Ru}(\text{bpy})_3]^{2+}$. The resulting antibody was found to be crossreactive for methylated derivatives of

$[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ in the order of $[\text{Ru}(\text{dmbpy})_3]^{2+} > [\text{Ru}(\text{dmbpy})(\text{bpy})_2]^{2+} > [\text{Ru}(\text{dmphen})_3]^{2+} > [\text{Ru}(\text{bpy})_3]^{2+} \gg [\text{Ru}(\text{phen})_3]^{2+}$ where dmbpy is 4,4'-dimethyl-2,2'-bipyridine and dmphen is 4,7'-dimethyl-1,10'-phenanthroline. It can therefore be seen that the immunological process that produced AC1106 gave particular emphasis to the methyl-substituted ligand of the haptens. Through a combination of steady state and time-resolved luminescence techniques and ELISA studies, the affinity constants of AC1106 for these metal complexes were found to range between 2×10^8 and $1 \times 10^3 \text{ M}^{-1}$.

A maximum 2.6-fold increase in emission was found on addition of $[\text{Ru}(\text{dmbpy})_3]^{2+}$. This was accompanied by a three-fold increase in the lifetime. The excited state lifetime for the AC1106- $[\text{Ru}(\text{bpy})_3]^{2+}$ was found to be 920 and 1200 ns in air and N_2 -saturated solution, respectively. These were shorter than those obtained previously for polyclonal antibody-bound $[\text{Ru}(\text{bpy})_3]^{2+}$ and shows the influence on the nature of the antibody-binding pocket.

The excited state lifetimes of free and antibody-bound $[\text{Ru}(\text{dmbpy})_3]^{2+}$ were measured in both N_2 -saturated H_2O -PBS and D_2O -PBS. The values obtained indicated that 72% of the antibody-bound $[\text{Ru}(\text{dmbpy})_3]^{2+}$ was inaccessible to excited state deactivation via interaction with solvent.

No evidence for simultaneous binding of methyl viologen and $[\text{Ru}(\text{dmbpy})(\text{bpy})_2]^{2+}$ inside the binding pocket of AC1106 could be found. Rather, the addition of methyl viologen was found to result in the displacement of AC1106-bound $[\text{Ru}(\text{dmbpy})(\text{bpy})_2]^{2+}$ from the antibody-binding site.

2.1.1.6.2 Interaction with nucleic acids

Parallels exist between the noncovalent binding of Ru or Os polypyridyl complexes to antibodies or nucleic acids. The trend is towards a higher emission intensity and longer lifetime on nucleic acid or antibody binding. While the specific nature of antibody-antigen interactions is well noted, the structure of the complex is also important for effective nucleic acid binding. In both cases, similar techniques are used to investigate the effects and modes of interaction.

Polypyridyl complexes of Ru(II) and Os(II) (particularly Ru(II)) have been extensively used as sensitive non-covalent probes of the polymer structure of DNA. The interaction of DNA with a metal ligand complex (MLC) has applications for the design of site- or conformation-specific probes for recognition of the biopolymer structure. They have also resulted in the development of selective DNA cleaving reagents. Their chiral nature enables enantioselective DNA binding.

DNA has a number of sites in which a molecule might bind. These include binding between two base pairs (intercalation), surface binding in the minor or major groove or non-specific electrostatic interaction on the outside of the helix.

More than any other compound, complexes with the dipyrido [3,2-a:2',3'-c] phenazine (dppz) ligand have attracted intense scrutiny because of their environment-sensitive photophysics. This ligand has an extended planar aromatic surface area which allows for tight binding to DNA via intercalative stacking. $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ (Figure 2.4) displays no detectable background emission in water but shows moderate luminescence in the presence of DNA ($\phi = 0.02$). It has been demonstrated that the excited state complex can be quenched by proton transfer (Turro *et al.*, 1995). However, recent time-resolved studies on fast timescales have indicated that more subtle processes involving two close-lying excited states may be responsible (Olofsson *et al.*, 2002, Onfelt *et al.*, 2000). The ability of $[\text{Os}(\text{phen})_2\text{dppz}]^{2+}$ to function as a DNA light switch has also been investigated (Holmlin *et al.*, 1995). No luminescence was detected from this complex in aqueous solution. In the presence of DNA the quantum yield was 0.0001. The ability of this complex to probe intercalative binding events on time scales below 10 ns while emitting at wavelengths in the red was illustrated. Other ruthenium complexes investigated incorporate ligands such as 2,2'-bipyridine, 1,10-phenanthroline (phen), 4,7-diphenanthroline (DIP) and 9,10-phenanthrenequinonediimine (phi). Based on the measurements of spectroscopic properties and binding isotherms, the intercalating ability appears to increase over the series $\text{bpy} \ll \text{phen} \leq \text{DIP} \ll \text{phi}$. These complexes bind to DNA with relatively low affinity. It has been estimated from equilibrium dialysis that $[\text{Ru}(\text{phen})_2(\text{phi})]^{2+}$ has a binding constant of approx. $4.6 \times 10^4 \text{ M}^{-1}$ (Pyle *et al.*, 1989). In contrast $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ has a binding constant of approx. 10^8 M^{-1} (Hiort *et al.*, 1993).

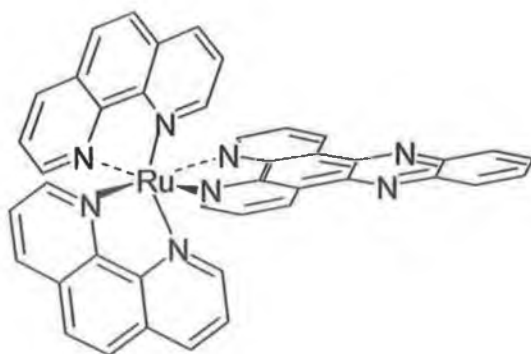


Figure 2.4. Structure of the complex $[Ru(phen)_2dppz]^{2+}$.

The increase in luminescence of DNA-bound complexes reflects the efficient protection of the excited state complex from radiationless deactivation due to interaction with water (Hiort *et al.*, 1993). It has also been attributed to the decrease in mobility of the complex when sandwiched into the helix. Protection from quenchers such as oxygen may also be a factor, or an increase in the energy gap between the emitting state and the other states involved in deactivation (Barton *et al.*, 1984). UV-Vis spectra in the presence of DNA show hypochromicity of the charge transfer bands and a red shift in the $\pi-\pi^*$ transition. This is typical in stacking interactions with base pairs.

Enantiomeric selectivity can be explained in terms of unfavourable steric interactions between the non-intercalated ligands of the Λ (left-handed) isomer and a right-handed DNA phosphate backbone. In contrast the Δ (right-handed) isomer fits easily in a right-handed helical groove (Barton *et al.*, 1984).

It has been reported that on binding of $[Ru(phen)_2dppz]^{2+}$ to DNA the relative quantum yield of the Δ isomer is some 6-10 times higher than that of the bound Λ isomer. Lifetime studies reveal a biexponential decay for each of the enantiomers of $[Ru(phen)_2dppz]^{2+}$ upon binding to DNA. The long lifetime component may originate from sequences of closely bound intercalator, while the shorter lifetime species may represent more or less isolated intercalating complexes. The lifetime of each component and their relative abundance was also found to depend on the type of DNA, the base sequence and the binding ratio (Hiort *et al.*, 1993).

Photoreactions of Ru(II) and Os(II) serve as an example of the effects that the tuning of the ligands can have on the interaction with the biological molecule. It has been demonstrated that photoinduced electron transfer from a nucleobase to the excited complex can occur when the complex is a strong oxidant in the excited state and thus can abstract electrons from donors. Strong oxidants are complexes with strong π -accepting ligands such as TAP (1,4,5,8-tetra-aza-phenanthrene) or HAT (1,5,5,8,9,12-hexaazatriphenylene) (Figure 2.5). The complex must contain at least two strong π -accepting ligands for it to be an efficient oxidant (Moucheron *et al.*, 1997).

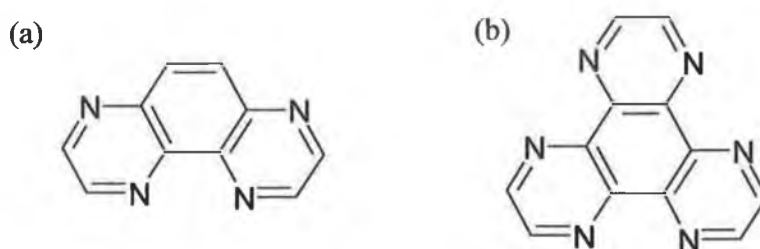


Figure 2.5. Structure of (a) TAP (b) HAT

In Ru(II) complexes photoreactions can occur from ^3MC or $^3\text{MLCT}$ excited states. Activation of the ^3MC state can lead to substitution of a ligand by a nucleobase and another monodentate ligand. The redox processes of photo-induced electron transfer from base to $^3\text{MLCT}$ can lead to DNA cleavage or the formation of an adduct of the complex to DNA (without ligand loss). The relative contributions of redox and dechelation-substitution reactions also depend on the concentration and electron potential of the electron donor. Although photosensitised cleavage has been reported for bpy and phen Ru(II) complexes, the efficiency is much lower than observed in strongly oxidising complexes.

The emission of $[\text{Ru}(\text{TAP})_3]^{2+}$ is quenched by calf thymus DNA, poly[d(G-C)] (Kelly *et al.*, 1987) and guanosine monophosphate (GMP) (Lecomte *et al.*, 1992). The reduced lifetime and emission may be attributed to reductive quenching by the guanine base. Flash photolysis experiments have demonstrated that the radical cation of guanine, formed by electron transfer to the excited complex, plays a key role in DNA strand cleavage. It has been reported that photoadduct formation is initiated by the coupling of

radicals formed from the electron transfer from guanine to the excited complex. This is followed by re-aromatisation of the TAP heterocycle. This results in the linkage of guanine by N2 to the α -position of a non-chelating nitrogen of the TAP ligand without destruction of the complex (Jacquet *et al.*, 1995).

Thermal activation from the $^3\text{MLCT}$ to the ^3MC state is not observed in Os(II) complexes. Therefore, photoreaction occurs exclusively from the $^3\text{MLCT}$ state. This is an advantage over Ru(II) complexes in certain applications. $[\text{Os}(\text{TAP})_3]^{2+}$ has been synthesised and characterised (Content *et al.*, 1997). Its lifetime is longer than that of the analogous Ru(II) complex since there is no radiationless deactivation via the ^3MC state. Laser flash photolysis experiments have shown that electron transfer takes place from guanosine monophosphate (GMP) to excited $[\text{Os}(\text{TAP})_3]^{2+}$, with the formation of the characteristic monoreduced complex.

2.2 MATERIALS AND INSTRUMENTATION

2.2.1 Materials

Ruthenium(III) chloride (11808) and potassium hexachloroosmate (12177) were purchased from Johnson Matthey (Dublin, Ireland). Nunc Maxisorp ELISA plates (439454) were purchased from Bioscience, (Dublin, Ireland). Centricon protein concentrators YM-10 (4205) were purchased from Millipore. All other materials and reagents were purchased from Sigma-Aldrich, (Dublin, Ireland). The buffers used were (a) 0.01 M phosphate buffered saline pH 7.4, 0.137 M NaCl, 0.027 M KCl. (b) 0.1 M bicarbonate buffer, pH 8.3, (c) 0.05 M phosphate buffer pH 6.8.

2.2.2 Instrumentation

2.2.2.1 Absorption and emission measurements

Emission spectra were recorded using a Perkin-Elmer LS50B luminescence spectrophotometer, which was equipped with a red sensitive Hamamatsu R298 PMT

detector and interfaced with an Elonex PC 466 employing Perkin-Elmer Fl Winlab software. Emission spectra were uncorrected for photomultiplier response. Emission and excitation slit widths were 15 nm. Absorption measurements were carried out on a Varian Cary 50 scan UV-Vis spectrometer. ELISA plate absorbances were measured on a Titretek Twinreader Plus plate reader.

2.2.2.2 Electrochemistry

Cyclic voltammetry was performed using a CHI 1000 instrument and a conventional three-electrode cell. Potentials were measured against a sodium chloride saturated silver/silver chloride (Ag/AgCl) reference electrode. The auxiliary electrode was a platinum wire. All solutions were degassed with argon. The electrolyte was lithium perchlorate (0.1 M).

2.2.2.3 NMR spectroscopy

^1H NMR spectra were obtained in d_6DMSO and recorded on a Bruker AC400 (400 MHz) NMR spectrometer.

2.2.2.4 High performance liquid chromatography (HPLC)

The purity of the complexes was verified using an analytical cation exchange column with a Waters HPLC system. The mobile phase used was (a) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20) containing 0.08 M LiClO_4 or (b) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20) containing 0.1 M LiClO_4 adjusted to pH 1.8 with HClO_4 . The flow rate was 2.5 ml min^{-1} .

2.2.2.5 Elemental analysis

Elemental analysis on C, H and N was carried out by the Microanalytical Laboratory at University College Dublin using an Exador analytical CE440.

2.2.2.6 Lifetime Measurements

Luminescence lifetime measurements were obtained using an Edinburgh Analytical Instrument time-correlated single-photon counting apparatus (TCSPC). This consisted of two model J-y A monochromators (emission and excitation), a single photon photomultiplier detection system model 5300, and a F900 nanosecond flashlamp (N₂ filled at 1.1 or 0.3 atm. pressure, 40 Hz) interfaced with a personal computer via a Norland MCA card. Data correction and manipulation were carried out using EAI F900 software version 5.1.3. All lifetime measurements were carried out in 0.01 M PBS, pH 7.4.

2.2.2.6.1 Lifetime decay data analysis

The lifetime of a monoexponential decay can be calculated according to:

$$\Phi(t) = A\exp(-kt) \quad (2.9)$$

where Φ is the emission intensity, A is the preexponential factor contributing to the signal at time zero and k is the decay rate constant. τ is the lifetime of the emissive state corresponding to the time taken for Φ to decrease to 1/eth of its initial value. The preexponential factor is a function of the spectral response of the detector, the concentration, emission and absorption characteristics of each emitting component, the spectral transmission properties of the filters and the spectral distribution of the exciting light.

A departure from first order kinetics is observed when a sample contains species with different lifetimes, which emit simultaneously and independently. In a biexponential decay the lifetimes of the species can be determined from:

$$\Phi(t) = [A_1\exp(-k_1t) + A_2\exp(-k_2t)] \quad (2.10)$$

To determine whether the decay behaviour is best suited to a single or multi exponential fit, and to judge the goodness of that fit, statistical analysis is performed. The standard statistical procedure is the use of the χ^2 test. A fit is not acceptable if the χ^2 value is over 1.2. (Birch and Imhof, 1991). To visualise the fit, a graph of percentage errors versus time is also plotted:

$$\% \text{ error} = [(F(t_i) - D(t_i)) / D(t_i)] \times 100 \quad (2.11)$$

where $D(t_i)$ is the calculated best fit and $F(t_i)$ is the observed decay data. This should yield a randomly distributed plot. A monoexponential fit of a multiexponential decay is indicated by a cosine wave distribution. A good fit should show an error of less than $\pm 5\%$.

2.2.2.7 Size exclusion chromatography

The HPLC system was a Beckman System Gold comprising a 166 detector and a 118 solvent module. The column used was a BioSep-SEC-S3000 size exclusion column. This had exclusion limits between 5-700 kDa. The mobile phase used was 0.05 M phosphate buffer, pH 6.8. UV-VIS detection at 215 or 290 nm and 440 nm as indicated. Flow rates of 1 ml min^{-1} were applied.

2.3 METHODS

2.3.1 Synthesis of $[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$

$[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ was synthesised following a procedure previously described with minor modifications (Kober, 1988). K_2OsCl_6 (300 mg, 0.62 mmol) and 2,2'-dipyridyl (203 mg, 1.3 mmol) were added to 3 ml of deaerated ethylene glycol and heated under vigorous reflux for one hour. On cooling, it was added to 5 ml of a saturated sodium dithionite solution and was allowed to stir overnight. This was to ensure complete

reduction of $[\text{Os}(\text{bpy})_2\text{Cl}_2]\text{Cl}$ to $[\text{Os}(\text{bpy})_2\text{Cl}_2]$. The resulting black precipitate was filtered and washed with water and diethyl ether. The yield was 61%.

2.3.2 Synthesis of $[\text{Os}(\text{bpy})_2\text{dcbpy}]\text{Cl}_2$

$\text{Os}(\text{bpy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (110 mg, 0.19 mmol) and 2,2'-bipyridine-4,4'-dicarboxylic acid (56 mg, 0.23 mmol) were refluxed together in 50 ml of a 1:1 ethanol/water solution until the solution was green in colour. Excess solvent was removed by rotary evaporation and the solution was allowed to cool. To recover as a chloride salt, sulphuric acid was added followed by 4 ml of saturated sodium chloride. This was allowed to stand on ice and finally filtered and dried under vacuum filtration while washing with diethyl ether. The yield was 65%.

2.3.3 Synthesis of $[\text{Ru}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$

This was carried out according to Sullivan *et al.* (1978). RuCl_3 (2.31 g, 1.1 mmol), bipyridine (3.44 g, 2.2 mmol) and lithium chloride (1.5 g, 3.5 mmol) were dissolved in 50 ml of deaerated DMF and set to reflux under nitrogen for 6 hours. The reaction mixture was stirred continuously during reflux. The purple solution was brought to 250 ml with acetone and cooled at -20°C overnight. The black crystalline product was filtered and washed with water and diethyl ether. The yield was 45%.

2.3.4 Synthesis of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]\text{Cl}_2$

This synthesis was carried out according to Bard *et al.* (1986). A minor modification was made in that the complex was precipitated as a chloride salt. After reflux in the 4:1 methanol:water solution and removal of excess solvent, a saturated aqueous solution of sodium chloride was added. After the solution was left to stand the complex precipitated. It was then filtered and washed with diethyl ether and dried under vacuum. The yield was 59%.

2.3.5 Synthesis of $[\text{Os}(\text{bpy})_2(\text{p2p})_2](\text{PF}_6)_2$

$[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ (200 mg, 0.35 mmol) was refluxed in 20 ml of deaerated ethylene glycol for 10 minutes to ensure complete dissolution. An excess of the p2p ligand was added (257 mg, 1.4 mmol) with 30 ml of deaerated deionised water. This was allowed to reflux for several days until the solution turned green in colour. The progress of the reaction was followed by cyclic voltammetry. The complex was precipitated by dropwise addition to a saturated aqueous solution of ammonium hexafluorophosphate (NH_4PF_6) (5 ml). The complex was then filtered and washed with water and diethyl ether. Yield 72%.

2.3.6 pK_a and pK_a^* measurements

Sample measurements were carried out in Britton-Robinson buffer (0.04 M boric acid, 0.04 M acetic acid and 0.04 M phosphoric acid). The pH of the solutions were adjusted using conc. H_2SO_4 or conc. NaOH . Luminescence titrations were carried out using an appropriate isosbestic point as the excitation wavelength. The inflection points were determined using Origin software.

2.3.7 Conjugation procedure

Conversion of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ to its active succinimide ester and conjugation to thyroglobulin or BSA was carried out according to Terpetschnig *et al.* (1996) with minor modifications. $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ (26 mg, 0.035 mmol) and a 2 mole excess of *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were dissolved in 1 ml of anhydrous DMF. The solution was allowed to stir for 5 hours. On succinimide ester formation the solution turned red in colour. It was then centrifuged for 2 min at 10,000 rpm and the supernatant was removed from the dicyclohexylurea precipitate. BSA or thyroglobulin (20 mg) was dissolved in 1 ml of 0.1 M bicarbonate buffer, pH 8.3. (A buffer of this pH is used to favour the NH_2 form of lysine groups, which react well with active esters over the unreactive NH_3^+ form.) The active ester solution was added slowly in small aliquots, the total volume added being equivalent to the desired molar excess of

the ester over the protein. The initial substitution ratios for [Os(bpy)₂dcbpy]-thyroglobulin were 100, 200 and 400:1. BSA conjugates were made with an initial molar excess in the range between 10 and 35. The remaining ester solution could be kept active by storing at -15°C. The conjugation reaction was allowed to proceed overnight while stirring slowly. The solution was again centrifuged for 2 min at 10,000 rpm. Unbound Os complex was removed by the use of gel filtration chromatography on Sephadex G-25 and exhaustive dialysis in 0.01 M PBS, pH 7.4.

2.3.8 Estimation of the conjugation ratio

The conjugation ratio is the molar ratio of complex to conjugated protein. The amount of complex present was determined by its absorption at λ max (485 nm) where the extinction coefficient is 11,500 (Terpetschnig *et al.*, 1996). No allowance was made for a change in the extinction coefficient upon binding to the biomolecules. The protein concentration was determined using a bicinchoninic acid assay (BCA) assay. Standard protein solutions with concentrations from 0-2 mg ml⁻¹ were prepared in PBS. 10 μ l of protein sample or standard was placed in wells of a 96 well microtitre plate. 190 μ l of BCA working reagent (50 parts reagent A to 1 part reagent B) was added. The plate was incubated at 37 °C for 30 min after gentle mixing. The absorbance of the wells was determined at 562 nm. A standard curve of known protein concentration versus absorbance was constructed from which unknown sample protein concentrations were determined.

2.3.9 Immunisation schedule

Conjugates to [Os(bpy)₂dcbpy] were prepared according to section 2.3.8. A New Zealand White rabbit was injected subcutaneously with 1 ml of a 1 mg ml⁻¹ solution of the hapten-thyroglobulin conjugate in PBS emulsified 1:1 with Freund's complete adjuvant. Three booster injections were performed 21 days apart with a 1 ml solution of the conjugate emulsified 1:1 with Freund's incomplete adjuvant. A test bleed was taken 12 days after the second boost. The final bleed was taken 10 days after the last

immunisation. The rabbit was housed and immunised at Bioresources, Trinity College, Dublin.

2.3.10 Preparation of rabbit serum

A 20 ml blood sample was collected and allowed to clot for 2 hours at room temperature. It was then left to stand overnight at 4 °C, thus allowing the clot to tighten. It was then centrifuged at 4,000 rpm for 20 min and the serum was removed from the red blood cells.

2.3.11 Antibody purification

Purification of polyclonal antibody from rabbit serum was initially carried out by precipitation with saturated ammonium sulphate. This was followed by affinity purification using a protein A chromatography column.

2.3.11.1 Saturated ammonium sulphate precipitation

A saturated ammonium sulphate solution was prepared by dissolving 50 g of ammonium sulphate in 50 ml of distilled water at 50°C. The solution was allowed to cool and then stirred overnight at 4 °C. The pH was adjusted to 7.2 by addition of NH_4OH . Measurement of the pH was taken on diluted aliquots to avoid salt errors and junction potentials obtained in the direct measurement of concentrated salt solutions. The cold saturated ammonium sulphate solution was added to an equal volume of rabbit serum on ice with stirring. The mixture was allowed to stir at room temperature for 30 min, followed by centrifugation at 3,000 rpm for 20 min. The supernatant was discarded and the pellet dissolved in 10 ml of 45% (v/v) saturated ammonium sulphate/PBS solution and centrifuged as before. The last step was repeated and finally the pellet was dissolved in 5 ml PBS and dialysed in 5 l of PBS overnight to remove any residual ammonium sulphate.

2.3.11.2 Protein A affinity chromatography

Protein A affinity purification was carried out using the Pure-1A antibody purification kit (Sigma). A volume of ammonium sulphate-purified serum containing 20 mg of protein as determined by a BCA assay was loaded onto the column. Non-specific protein was eluted by washing the column with binding buffer. The desalting cartridge was then attached and the elution buffer applied to remove the affinity captured antibody.

2.3.12 Determination of purified antibody concentration

The antibody concentration was determined spectroscopically as mg ml⁻¹ of IgG using the calculation (Delves, 1995):

$$A_{280} \times 0.7 \times \text{the dilution of the antibody measured}$$

where A_{280} is the absorbance at 280 nm.

2.3.13 Non-competitive ELISA for determination of polyclonal antibody titre in serum

100 µl of a solution of 100 µg ml⁻¹ of [Os(bpy)₂dc bpy]-BSA prepared in PBS was coated onto a Nunc Maxisorp ELISA plate and incubated overnight at 4 °C. Plates were washed three times with PBS containing 0.05% (v/v) Tween-20 and three times with PBS. The plates were then blocked with 150 µl of 2% (w/v) non-fat milk powder for 1 hour at 37 °C. Serial dilutions of polyclonal antibody in PBS were prepared in the range between 1/500 to 1/1,024,000. These were added in triplicate to the wells and allowed to bind at 37 °C for 1 hour. After washing, a 1 in 10,000 dilution of horseradish peroxidase-labelled goat anti-rabbit antibody in PBS was incubated on the plate for 1 hour at 37 °C. Plates were again washed and 100 µl of substrate was added to each well. The substrate used was Sigma Fast o-phenylenediamine (opd) provided in tablet form, and dissolved in the required volume of deionised water immediately before use. The chromogenic substrate

was left to develop at 37 °C for 30 min. The reaction was stopped on addition of 50 µl of 2 M H₂SO₄. The absorbance was read at 492 nm using a Titretrek plate reader.

The absorbance readings obtained at each antibody dilution (A) was divided by the absorbance reading determined in the presence of zero antibody (A₀), to give a normalised reading. This allows for comparison of ELISAs performed on different plates.

2.3.14 Competitive ELISA to determine cross-reactivity of antibody towards Os(II) and Ru(II) polypyridyl complexes

2.3.14.1 Determination of antibody working dilution and optimal conjugate loading density

Serial dilutions of BSA-hapten conjugate were prepared in the concentration range from 50 µg ml⁻¹ to 0.5 µg ml⁻¹ in PBS. A row of each concentration was coated on an ELISA plate, which was developed as per section 2.3.14. The optimal conjugate loading density was defined as that coating density that gave the widest linear working range over the greatest range of antibody dilutions used. The optimal dilution of purified antibody was determined as the dilution taken from the mid-point of the linear portion of the sigmoidal curve obtained.

2.3.14.2 Competitive ELISA

An ELISA plate was coated with the optimal conjugate loading density and blocked as described in section 2.3.14. Serial dilutions of competing antigen of a suitable range were prepared. 200 µl of each antigen concentration was incubated with 200 µl of purified antibody at 2 x working dilution, for 1 hour at room temperature. 100 µl of this solution was transferred in triplicate to the previously coated and blocked ELISA plate. The plate was then developed as described in section 2.3.14 (Figure 2.6).



Figure 2.6. Schematic of the principle of (a) direct ELISA, as used for the measurement of antibody titre and (b) competition ELISA to determine the cross-reactivity of the antibody (from Marco et al., 1995). Competition for antibody binding exists between free antigen and the antigen-protein conjugate which is adsorbed to the plate. Binding of the antibody to the conjugate can be detected upon addition of a species-specific secondary enzyme-labelled antibody. Addition of a substrate in the presence of the enzyme forms a coloured product.

2.4 RESULTS AND DISCUSSION

2.4.1 Characterisation of the synthesised complexes

The complexes were characterised using UV-Vis spectroscopy, cyclic voltammetry, ^1H NMR, CHN content and cationic high performance chromatography. The pK_a and pK_a^* of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ were determined.

2.4.1.1 Cation exchange HPLC

The retention times of $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ and $[\text{Ru}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ (Table 2.1) were very similar. The fast elution of these complexes is due to their net neutral charge. The pH of the mobile phase used in the chromatography of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ was approx. 1.8. This was to avoid adsorption of these complexes to the column. The pK_a values of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ were determined to be 2.7 and < 1 (section 2.4.2) and those of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ have been reported as 2.9 and 1.7. Therefore at pH 1.8 the complexes are expected to have a net +1 charge. This accounts for their slightly longer elution time (1.65 min and 1.55 min for $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$, respectively) in comparison to $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ which had an elution time of 1.44 min in this mobile phase. $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ had a retention time of approx. two minutes longer due to its net +2 charge. One peak was observed for all complexes indicating high purity except in the case of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ where an extra peak corresponding to its precursor $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ was observed (Figure 2.7). However, this accounted for only approx. 5% of the complex.

Complex	Retention time (min)
^(a) $[\text{Os}(\text{bpy})_2\text{Cl}_2]$	1.66
^(a) $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$	1.57
^(b) $[\text{Os}(\text{bpy})_2\text{Cl}_2]$	1.44
^(b) $[\text{Os}(\text{bpy})_2\text{dcbpy}]^+$	1.65
^(b) $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^+$	1.55
^(b) $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$	3.55

Table 2.1. Retention times for the osmium(II) and ruthenium(II) polypyridyl complexes. The mobile phase was either (a) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20) containing 0.08 M LiClO_4 or (b) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20) containing 0.1 M LiClO_4 adjusted to pH 1.8 with HClO_4 . The flow rate was 2.5 ml min^{-1} .

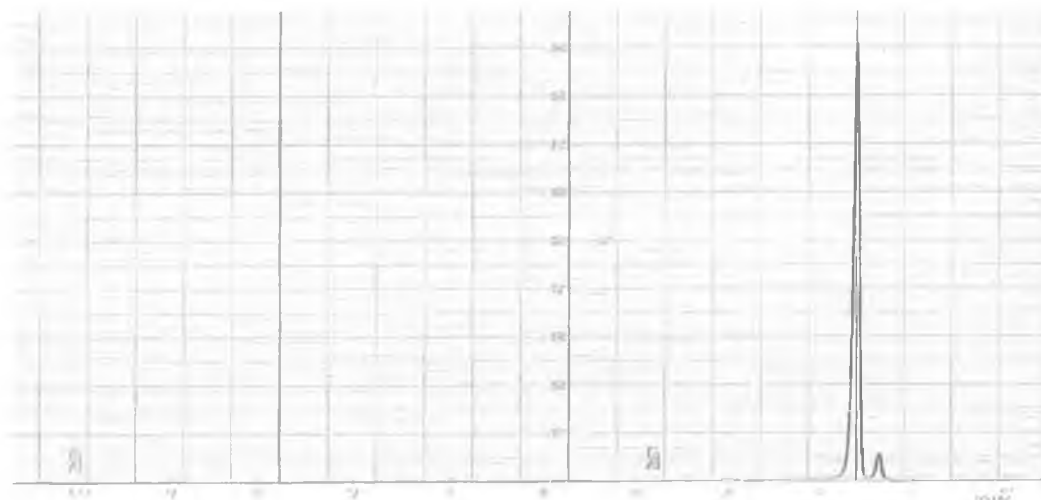


Figure 2.7. Cationic HPLC chromatogram of $[\text{Os}(\text{bpy})_2\text{dcbpy}]^+$. The mobile phase was $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20) containing 0.1 M LiClO_4 . Adjusted to pH 1.8 with HClO_4 . The flow rate was 2.5 ml min^{-1} .

2.4.1.2 UV-Vis spectroscopy

Figure 2.8 shows the UV-Visible absorbance spectra of $[\text{Ru}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ and $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$. The sharp absorption band observed at approximately 290 nm arises from a bpy based $\pi\text{-}\pi^*$ transition. In the visible region, broad bands exist between 350 and 560 nm. These bands are associated with metal-to-ligand charge transfer (MLCT). Broadness is also due to the effect of spin orbit coupling which is larger for Os(II) over the corresponding Ru(II) complexes.

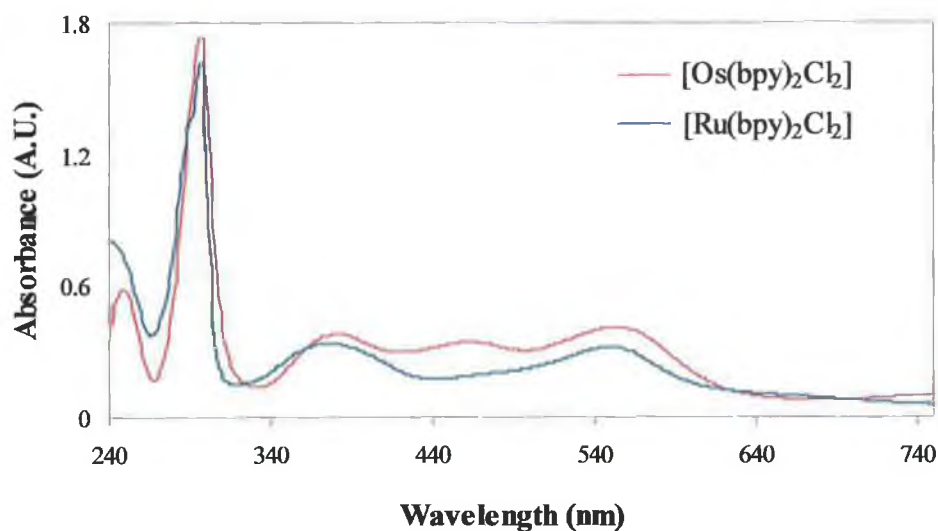


Figure 2.8. Absorption spectra of $[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ and $[\text{Ru}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ in acetonitrile.

UV-Vis absorption spectra of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ are shown in Figure 2.9. The MLCT bands were higher in energy relative to those of $[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$. This reflects the greater crystal field strength of the 2,2'-bipyridine-4,4'-dicarboxylic acid and 1,2-bis(4-pyridyl)ethane ligands. The absorption spectra of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ (Figure 2.10) is very similar to that of $[\text{Ru}(\text{bpy})_3]^{2+}$ having an intense MLCT at 452 nm. As expected, the MLCT bands were higher in energy than in its Os analogue. Os(II) complexes are also characterised by a weaker set of transitions appearing at lower energies in the spectra and extending to 750 nm. These have also been assigned as MLCT transitions but their low intensity and uniform appearance signify terminal excited states that distinguish them from the intense set (Pankuch *et al.*, 1980).

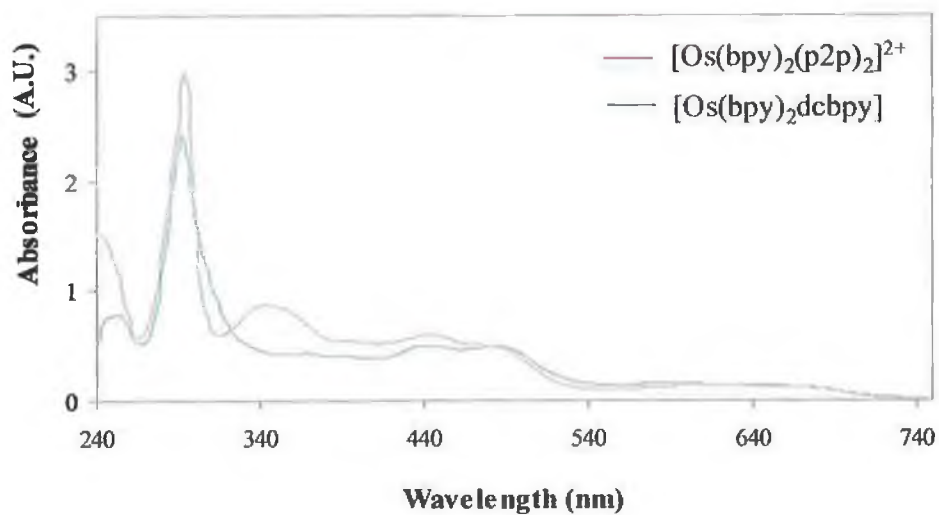


Figure 2.9. Absorption spectra of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Os}(\text{bpy})_2(\text{p2p})]^{2+}$ in acetonitrile.

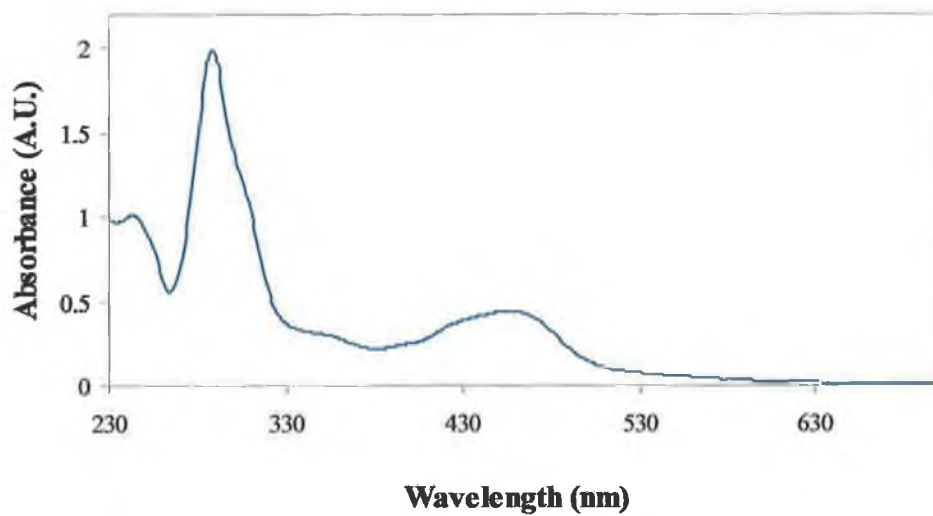


Figure 2.10. Absorption spectra of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ in acetonitrile.

2.4.1.3 Electrochemistry

The cyclic voltammograms for the Os(II) and Ru(II) complexes displayed a single metal-based redox couple in the potential range scanned (Table 2.2). Relative to Ru(II) complexes, polypyridyl complexes of Os(II) are more easily oxidised. The oxidation peak potentials of the Os(II) complexes were therefore more negative than those for the corresponding Ru(II) complexes. The oxidation potentials are strongly dependent on the σ -donor or π -acceptor properties of the ligands. This accounts for the positive shift in the redox potential observed when the electronegative chloride ions of $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ or $[\text{Ru}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ were replaced by 2,2'-bipyridine-4,4'-dicarboxylic acid or 1,2-bis(4-pyridyl)ethane. A cyclic voltammogram of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in acetonitrile is shown in Figure 2.11.

Complex	$E_{1/2}$ (V)
$[\text{Os}(\text{bpy})_2\text{Cl}_2]$	0.05
$[\text{Os}(\text{bpy})_2\text{dcbpy}]$	0.85
$[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$	0.62
$[\text{Ru}(\text{bpy})_2\text{Cl}_2]$	0.38
$[\text{Ru}(\text{bpy})_2\text{dcbpy}]$	1.26

Table 2.2 Half wave potentials vs. Ag/AgCl measured in acetonitrile with 0.1 M LiClO₄ as electrolyte.

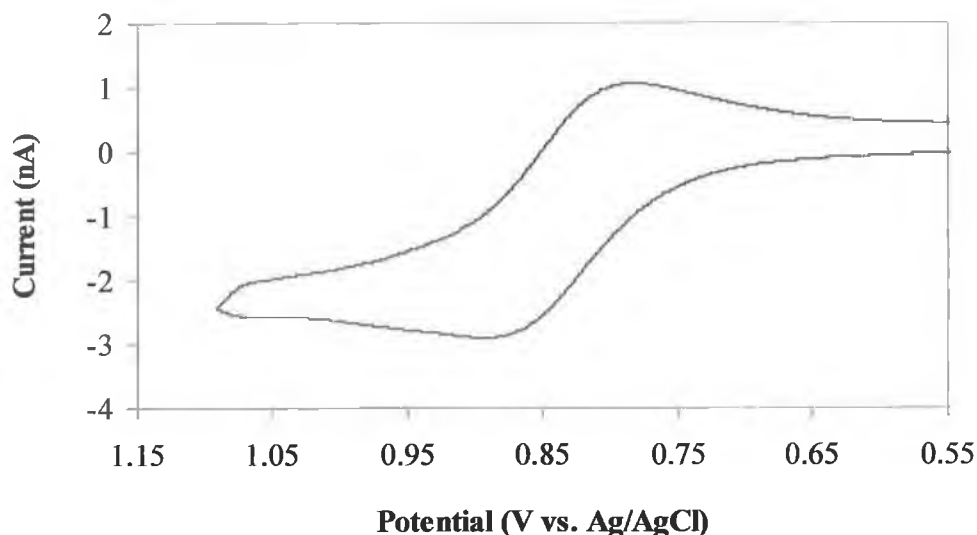


Figure 2.11. Cyclic voltammogram of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in acetonitrile with 0.1 M LiClO_4 as electrolyte. The scan rate was 0.5 Vs^{-1} and a $25 \mu\text{m}$ diameter platinum microelectrode was used with a Ag/AgCl reference electrode. The cathodic current is positive and anodic current negative.

2.4.1.4 ^1H NMR

The ^1H NMR in d_6DMSO spectra can be seen in the appendix (Figure 1-3). The ^1H NMR spectra of $[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ and $[\text{Ru}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ had eight peaks, each integrating for two protons. This corresponded to the eight protons of each dipyrindyl. Four doublets and four triplet peaks were observed. The doublets were attributable to the H3, H3', H6 and H6' protons and the triplets to the H4, H4', H5, H5' protons. The doublet at above 9.5 ppm, which was the H6 proton, was shifted downfield. This was due to the proton hanging over the pyridine ring on an adjacent bpy ligand resulting in a deshielding effect on the proton and the subsequent downfield shift. All the peaks in the $[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ spectrum were sharp and did not show broadening, indicating that there was no Os(III) present.

$[\text{Os}(\text{bpy})_2\text{Cl}_2] \cdot 2\text{H}_2\text{O}$ ^1H NMR; 6.8 (2H, t, H5), 7.23 (2H, d, H6), 7.28 (2H, t, H4), 7.56 (2H, t, H5'), 7.61 (2H, t, H4') 8.39 (2H, d, H3), 8.59 (2H, d, H3'), 9.61 (2H, d, H6').

[Ru(bpy)₂Cl₂].2H₂O ¹H NMR; 7.1 (2H, t, H5), 7.52 (2H, d, H6), 7.68 (2H, t, H4), 7.77 (2H, t, H5'), 8.07 (2H, t, H4'), 8.5 (2H, d, H3) 8.66 (2H, d, H3') 9.96 (2H, d, H6').

The integration of the ¹H NMR of the [Os(bpy)₂dc bpy] and [Ru(bpy)₂dc bpy] spectra revealed the expected 22 protons corresponding to the eight protons on each dipyriddy and six ring protons of the dc bpy ligand.

[Os(bpy)₂dc bpy] ¹H NMR; 7.43, 7.5 (4H, 2t, 5,5'H bpy), 7.61, 7.64 (4H, 2d, 6,6'H bpy), 7.72, 7.87 (4H, 5,5', 6,6'H dc bpy), 8.0 (4H, t, 4,4'H bpy), 8.88 (4H, t, 3,3' H bpy), 9.2 (2H, s, 3,3' dc bpy).

[Ru(bpy)₂dc bpy] ¹H NMR; 7.39, (4H, 2t, 5,5'H bpy), 7.66, (4H, t, 6,6'H bpy), 7.77, 7.9 (4H, 5,5', 6,6'H dc bpy), 8.0 (4H, 2t, 4,4'H bpy), 8.56 (4H, dd, 3,3' H bpy), 8.97 (2H, s, 3,3' dc bpy).

The integration of the ¹H NMR of the [Os(bpy)₂(p2p)₂](PF₆)₂ spectrum revealed the expected 32 aromatic protons.

[Os(bpy)₂(p2p)₂]²⁺ ¹H NMR; 7.18 (6H, m), 7.35 (4H, m), 7.79 (6H, m) 7.9 (2H, t), 8.1 (4H, d), 8.44 (4H,d), 8.57 (2H,d), 8.65 (2H,d), 8.78 (2H,d)

[Ru(bpy)₃](PF₆)₂ was kindly provided by Adrian Guckian. The integration of the ¹H NMR of the spectrum can be interpreted in terms of four coupled spins in each of six equivalent pyridine rings – each peak integrated to one.

[Ru(bpy)₃]²⁺ ¹H NMR; 7.3 (t, 5H bpy), 7.6 (d, 3H bpy), 7.8(t,4H bpy), 8.5 (d, 6H bpy)

2.4.1.5 Elemental analysis (CHN)

[Ru(bpy)₂dcbpy]

Calculated for [Ru(bpy)₂dcbpy]Cl₂·12H₂O, C, 40.76, H, 5.14, N, 8.92. Found C, 38.0, H, 3.48, N, 6.82.

[Os(bpy)₂dcbpy]

Calculated for [Os(bpy)₂dcbpy]Cl₂·4H₂O, C, 43.29, H, 3.64, N, 9.47. Found C, 42.16, H, 3.18, N, 8.95.

[Os(bpy)₂(p2p)₂]

Calculated for [Os(bpy)₂(p2p)₂](PF₆)₂·3H₂O; C, 43.49, H, 3.82, N, 9.22. Found C, 43.24, H, 3.22, N, 8.95.

The CHN results for [Os(bpy)₂dcbpy] and [Os(bpy)₂(p2p)₂] showed a satisfactory fit to the expected results. Errors in the CHN analysis of [Ru(bpy)₂dcbpy] are due to the addition of H₂SO₄, which was added after synthesis to precipitate the complex in its protonated form. This leads to difficulty in assigning the anion of the complex, which may be a mixture of Cl₂ and SO₄. This is also true of [Os(bpy)₂dcbpy]. Future mention of these complexes will therefore not include reference to the nature of the anions.

2.4.2 Acid-base properties of [Os(bpy)₂dcbpy]

The value of the pK_a of [Os(bpy)₂dcbpy] was determined from the point of inflection of the curve obtained by plotting absorbance at a suitable wavelength versus the pH in Britton-Robinson buffer. At pH 7.4 the absorption spectrum of [Os(bpy)₂dcbpy] consisted of bands at 290, 365, 439 and 489 nm with a broad tail extending to 760 nm. As the pH was decreased the bands at 439 and 489 nm lost intensity and became broadened and red-shifted. Further lowering the pH to less than 2.4 resulted in increasing the intensity of these bands which were shifted to 460 and 500 nm, respectively (Figure 2.12). The spectral changes were completely reversible. Above pH 1.5 two isosbestic

points were observed at 450 and 496 nm. Below this pH the spectra slightly traced off the isosbestic points. One inflection point was present between pH 1.7 and 8.5 at pH 2.7. It is likely that this inflection point represents the equilibrium between the deprotonated form and the monoprotonated form and indicates that the other equilibrium (between monoprotonated and deprotonated forms) is lower than pH 1 (Figure 2.13). Therefore pK_{a2} was assigned as 2.7 and pK_{a1} as < 1 . $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ therefore has a neutral charge at pH 7.4, which was the pH at which the measurements were taken in this work to investigate the effects of antibody binding. The pK_a values reported here were in contrast to the higher pK_a values of 2.3 and 5.5 reported by Ranatunga *et al.*, (2001). The pK_a of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ is well documented. Xie *et al.* (1999) reported a pK_{a2} of 2.9 and pK_{a1} of 1.7. These are in close agreement with the values reported by Nazeeruddin *et al.* (1989) of 2.85 and 1.75. The lower pK_a values of the Os complex are to be expected in accordance with its greater s-donor and backbonding effects.

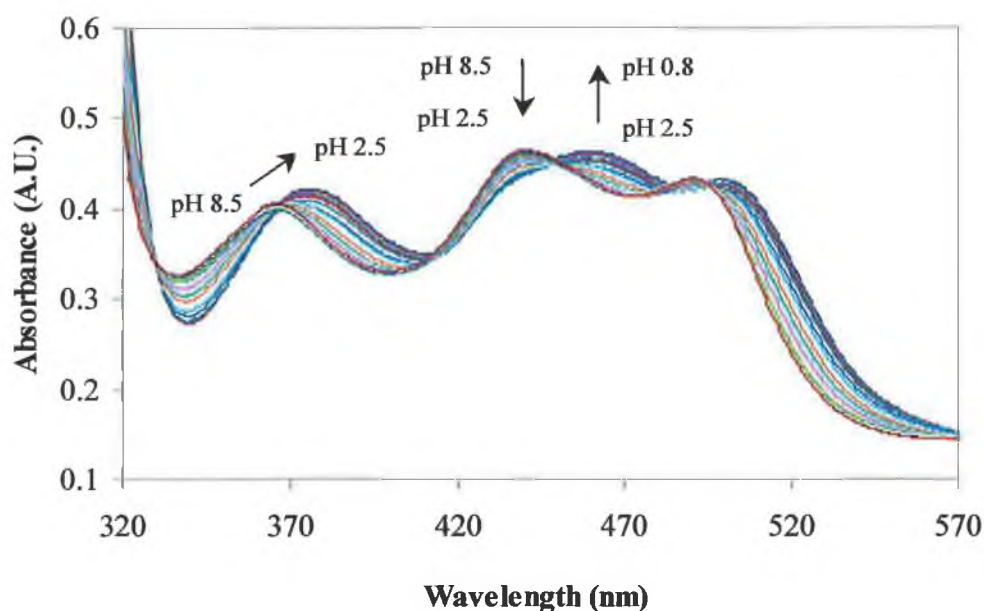


Figure 2.12. Ground state pK_a titration of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in Britton-Robinson buffer.

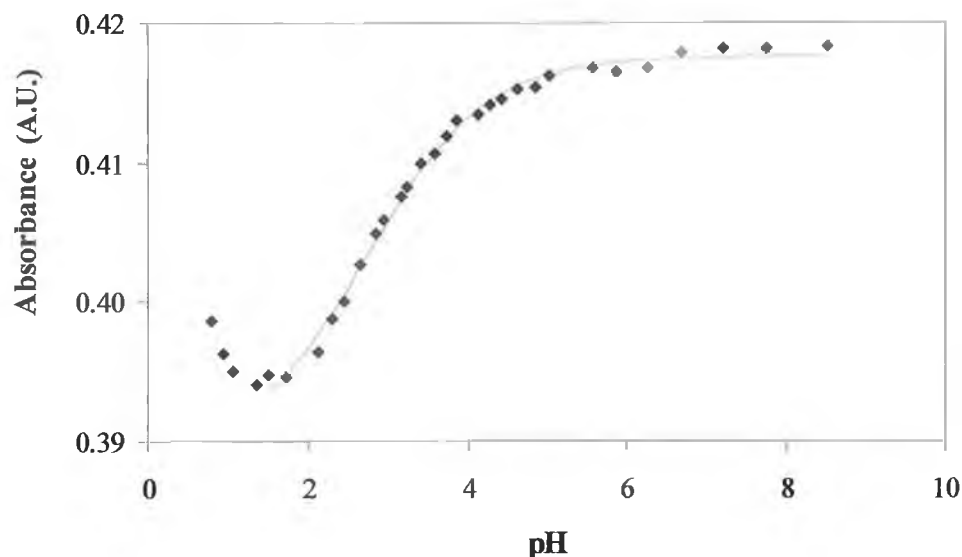


Figure 2.13. Plot of absorbance of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in Britton-Robinson buffer at 442 nm versus pH with fitted curve.

Figure 2.14 shows the changes which occur to the emission spectra of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ with pH. The emission was pH independent above pH 6. A large decrease in emission occurred between pH 5 and 2 with a red shift of 30 nm from 774 nm to 804 nm. One inflection point (pK_a^*) was observed in this region and was determined as 3.6 (Figure 2.15). In principle, to obtain the excited state pK_a (pK_a^*), the inflection point derived from the emission titration curve needs to be corrected for the differences in the excited state lifetime of the unprotonated (τ_b) and protonated forms (τ_a) according to equation 2.12. When the lifetime of the protonated species cannot be measured as in the case here, the Förster equation (equation 2.13) can be used to estimate the pK_a^* . This equation relates pK_a^* to the ground state pK_a and the emission maxima (in wavenumbers) of the protonated (ν_a) and deprotonated (ν_b) species at 298 K (Nazeeruddin *et al.*, 1989).

$$\text{pK}_a^* = \text{pK}_a^{*'} + \log (\tau_a/\tau_b) \quad (2.12)$$

$$\text{pK}_a^* = \text{pK}_a + 0.00209(\nu_b - \nu_a) \quad (2.13)$$

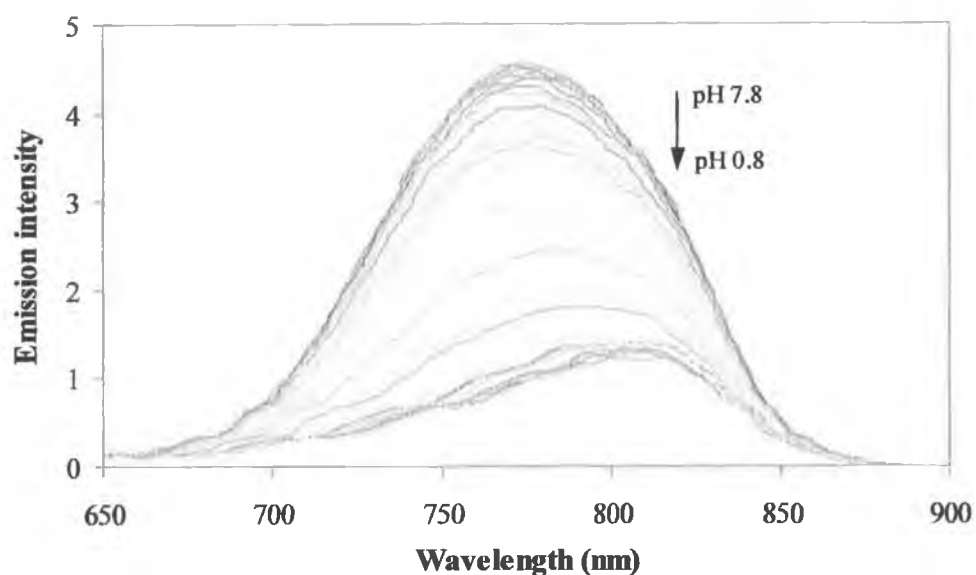


Figure 2.14. Excited state pK_a titration of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in Britton-Robinson buffer, excited at 450 nm (isosbestic point).

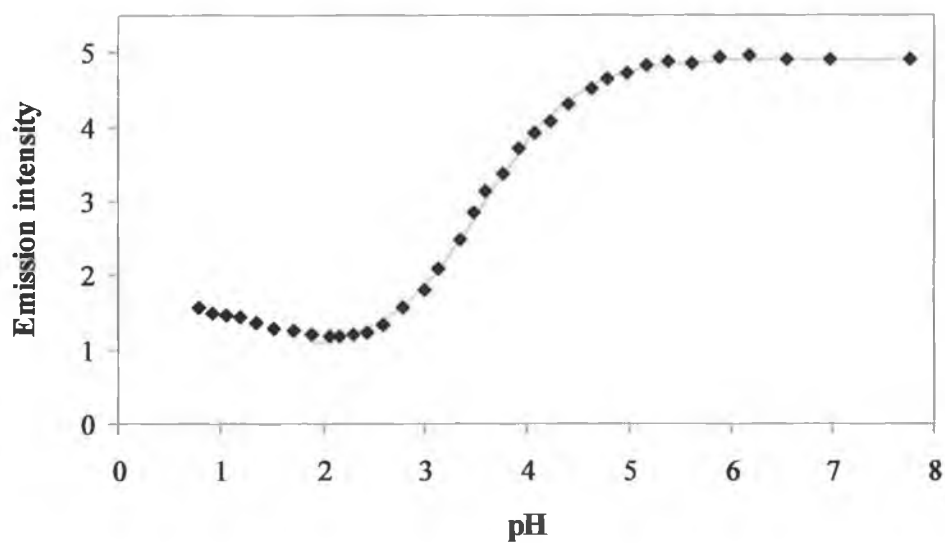


Figure 2.15. Plot of emission intensity of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ when excited at 450 nm versus pH with fitted curve.

Use of the Förster equation resulted in a pK_{a2}^* of 3.7. Again, it is likely that this represents the equilibrium between the deprotonated and monoprotonated form and

indicates that the pK_{a1}^* is well below pH 2. The larger value obtained for pK_{a2}^* when compared to the ground state value of 2.7 indicates that the deprotonated complex is a stronger base in the excited state than in the ground state. This suggests that the ligand electron density is significantly higher in the excited state than in the ground state and is evidence that the excited electron is localised on the dcbpy ligand. The pK_{a2}^* value reported here is also lower than that reported for its Ru analogue. Shimidzu *et al.* (1985) reported a pK_{a2}^* value of 4.1 ± 0.2 with $pK_{a1}^* < 0.2$.

2.4.3 Conjugation of [Os(bpy)₂(dcbpy)] to BSA and thyroglobulin using DCC and NHS

The cross-linking reagents DCC and NHS were used to conjugate amine groups of thyroglobulin and BSA to the carboxylate groups of [Os(bpy)₂dcbpy] (Figure 2.16). The central carbon atom of carbodiimides is rendered electrophilic by the electron-withdrawing effect of the adjacent nitrogens, and will therefore react with nucleophiles such as water and carboxylic acids. This results in an unstable *o*-acylisourea with which *N*-hydroxysuccinimide (NHS) reacts to form an NHS ester. This hydrolyses very slowly when compared to its rate of reaction with primary amino groups. The formation of the succinimide ester was therefore carried out in anhydrous conditions to avoid the reaction of water with DCC and also to avoid water reacting with the intermediate *o*-acylisourea. This side reaction results in the formation of dicyclohexylurea (DCU) and the regeneration of the carboxylate complex. *O*-acylisourea may also react with another carboxylate group to form a symmetrical anhydride. However, this is a frequent mechanism en route to the creation of an amide bond with an amine especially under anhydrous conditions (Dent and Aslam, 1998). A two step procedure was employed so that the DCC and NHS could react with [Os(bpy)₂dcbpy] first and not with any carboxylate groups present on the protein.

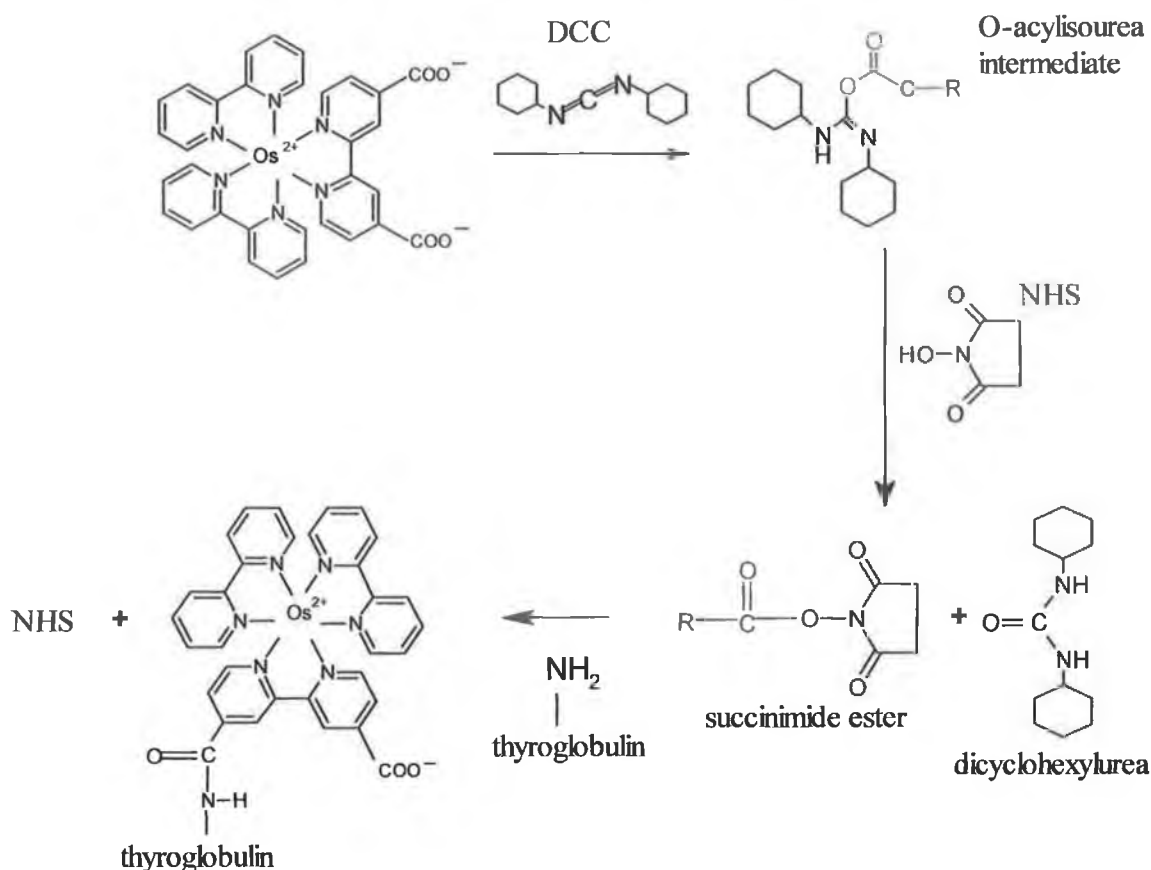


Figure 2.16. Conjugation of $[\text{Os}(\text{bpy})_2\text{dcbpy}] (\text{RCOO})$ with an amine group of thyroglobulin via a *N*-hydroxysuccinimide derivative. Carbodiimides react with carboxylic acids to form highly reactive *o*-acylisourea. The stability of this intermediate is improved when it is esterified with NHS. The resulting succinimide ester can react with a primary amine group of a protein and so becomes covalently attached via an amide bond.

2.4.4 Conjugate purification

The thyroglobulin and BSA conjugates were purified by gel filtration chromatography on Sephadex G-25 followed by exhaustive dialysis in PBS. Gel filtration chromatography provides a convenient initial assessment of the success of conjugation. Two coloured bands eluted from the column, the first being the higher molecular weight conjugate. The

second band was the unbound complex, its lower molecular weight allowing it to diffuse through the pores of the beads thereby slowing its progression through the column.

The conjugation procedure was also performed without the addition of DCC and NHS. This control served to show that non-covalent attachment of the osmium complex to the proteins did not take place. In this case, only one coloured band eluted from the gel filtration column and the osmium complex completely diffused from the dialysis membrane.

2.4.5 Conjugate characterisation

The conjugation ratios of the conjugates were determined. Other characterisation methods included size exclusion chromatography, UV-VIS absorption, luminescence and lifetime analysis.

2.4.5.1 Estimation of the conjugation ratio

As previously outlined, conjugation to a large molecular weight protein is necessary for immunisation as complexes of molecular weight less than 10,000 Da are not ordinarily antigenic. Thyroglobulin was chosen as the carrier protein as it is a large multisubunit protein with a molecular weight of 660,000 Da which is commonly used along with BSA and keyhole limpet hemocyanin (KLH) to successfully stimulate an immune response.

It is important to quantitatively determine the incorporation ratio of hapten-to-protein as it has been argued that it can greatly influence the IgG response. For example, it has been reported that very high incorporation levels of BSA resulted in a poor IgG response with between 5:1 and 19:1 giving good responses (Erlanger, 1980). Very high incorporation ratios are also known to produce antibodies of the IgM type with low binding affinity to the antigen (Malaitsev *et al.*, 1993). Since thyroglobulin has a molecular weight ten-times that of BSA, the substitution ratio should be approx. 10 times greater to maintain the same packing density (Law, 1996).

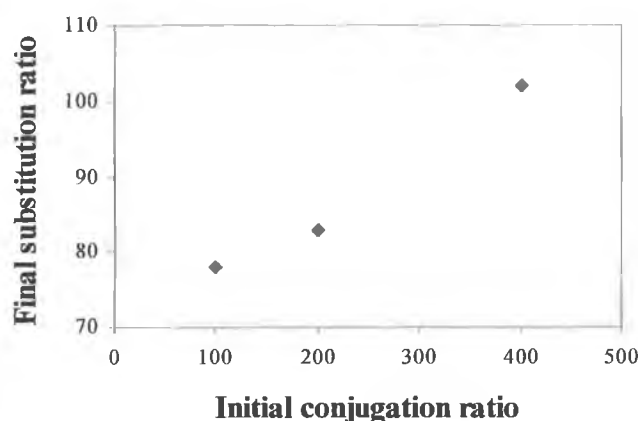


Figure 2.17. Substitution ratios obtained for [Os(bpy)₂dcbpy]-thyroglobulin conjugates. The protein concentration was determined using a BCA assay and complex from absorbance at 485 nm.

Figure 2.17 shows the substitution ratios for the [Os(bpy)₂dcbpy]-thyroglobulin conjugates. The 400:1 conjugation ratio of [Os(bpy)₂dcbpy]-to-thyroglobulin gave the highest substitution ratio of 102:1. This high ratio caused the conjugate to precipitate soon after conjugation. Although particulate materials are generally very immunogenic, problems arise with characterisation and accurate determination of complex and protein concentration. Therefore this conjugate was not characterised further. The 200:1 conjugate, which remained in solution and gave the next highest substitution ratio of 83:1 was chosen for immunisation purposes.

BSA conjugates were prepared for the purposes of antibody characterisation. Preparation of a protein hapten conjugate is a convenient way to immobilise the hapten to the ELISA plate where it binds primarily through hydrophobic interactions. The titre and cross-reactivity of the antibody can then be determined as outlined in Figure 2.6. For this purpose the use of conjugates employing different carrier proteins than those used for the initial immunisation is necessary to ensure that the antibody is binding to the hapten only and not the protein. The incorporation ratio of the plate conjugate is an important factor when controlling assay sensitivity. High incorporation ratios may lead to the distance between adjacent hapten molecules being similar to the distance between the two binding sites of the antibody. This may lead to bivalent antibody binding. Bivalent antibody binding is reported to have affinity constants which are 100 to 1000 times greater than

corresponding monovalent binding. Therefore, if the substitution ratio is high a poor sensitivity will result since the analyte will be unable to compete effectively with the hapten-protein conjugate for the antibody (Law, 1996).

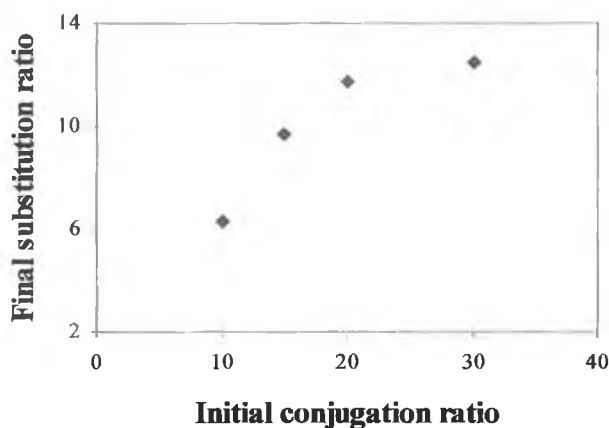


Figure 2.18. Substitution ratios obtained for [Os(bpy)₂dcbpy]-BSA conjugates.

Figure 2.18 shows the substitution ratio of the [Os(bpy)₂dcbpy]-BSA conjugates. It can be seen that the final substitution ratio had begun to level off at approx. 13:1. Although BSA has 57 lysine residues, there may only be 15 which are available for binding due to the complex structure of the folded protein (Hermanson, 1996). This may account for the comparable maximum final substitution of 13:1 obtained here. The [Os(bpy)₂dcbpy]-BSA conjugate with a final ratio of 13:1 was used as the plate conjugate in the ELISA.

2.4.5.2 Size exclusion chromatography

High performance size exclusion chromatography (gel permeation chromatography) was performed to confirm that conjugation had taken place and to ensure that all unbound complex had been removed by gel filtration and dialysis. Removal of non-covalently bound hapten is important as this may be adsorbed to the protein in a way which presents an epitope very different to that of chemically conjugated material. The net result of injecting an immunogen containing both covalently linked and adsorbed hapten could be

the increased generation of a mixed population of antibodies of differing specificity (Law, 1996).

High performance size exclusion chromatography is a separation technique based on the application of pressure to force molecules in solution through a chromatographic column filled with porous beads. The larger polymer molecules tend not to enter the pores of the beads and so pass through the column relatively quickly, whereas the smaller polymer molecules tend to diffuse through the pore structure of the beads and so take longer to pass through the column. Thyroglobulin and BSA have molecular masses of 670,000 and 67,000 Da, respectively and so should elute before the free osmium complex.

Absorption at 215 nm was used to detect the elution of protein. At this wavelength the peptide bond absorbs quite strongly. The aromatic amino acids also absorb such as tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine. Absorption at 440 nm confirms the presence of osmium in the elution peak. Therefore, simultaneous absorption at 215 nm and 440 nm at the shorter protein elution time would confirm successful conjugation.

The chromatogram of the free osmium complex revealed a large main peak at 12.6 min and a small second peak with the shorter elution time of 11.2 min which also absorbed at 440 nm and 215 nm (Figure 2.19). This smaller peak may be the 5% $[\text{Os}(\text{bpy})_2\text{Cl}_2]$ impurity that was detected in the cationic chromatography, although on a size exclusion column this would be expected to elute after the slightly heavier $[\text{Os}(\text{bpy})_2\text{dcbpy}]$.

Figure 2.20 shows the chromatogram of a control in which BSA spiked with osmium complex was injected onto the column. As expected, this showed two separate peaks at 215 nm characteristic of protein and osmium. This confirms that electrostatic interactions between protein and osmium did not take place between unconjugated species.

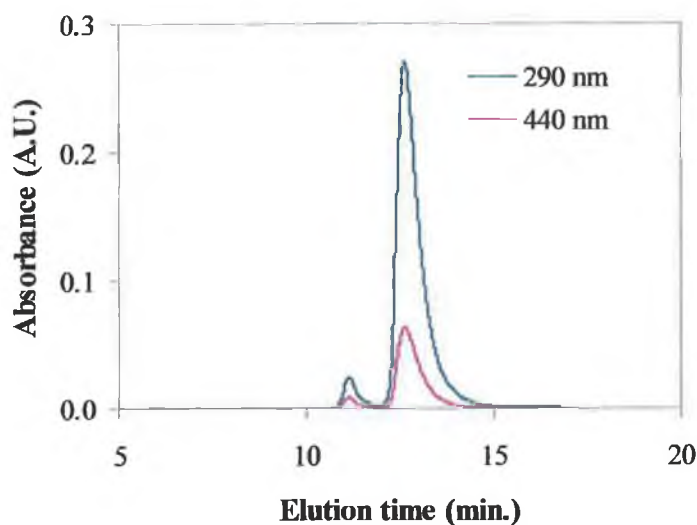


Figure 2.19. Size exclusion chromatogram of $[Os(bpy)_2dcbpy]$. The mobile phase was 0.05 M phosphate buffer, pH 6.8. The flow rate was 1 ml min^{-1} .

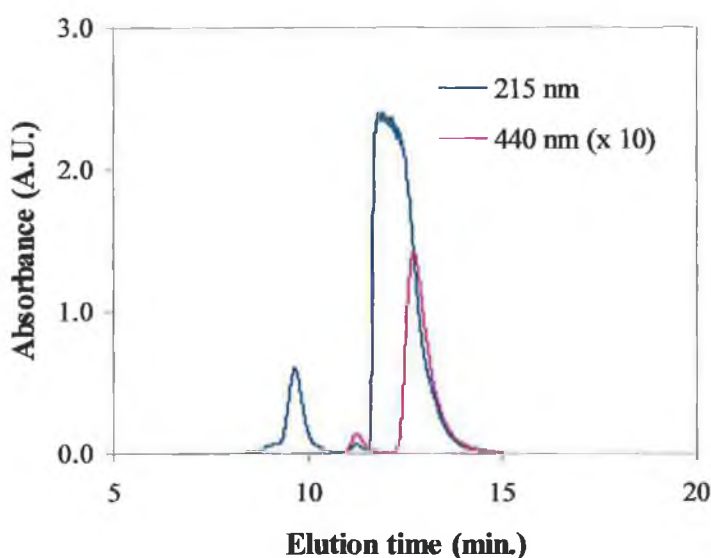


Figure 2.20. Chromatogram of BSA spiked with unbound $[Os(bpy)_2dcbpy]$.

The absence of a peak at 12.6 min in the chromatograms of the conjugates (Figures 2.21 and 2.22) indicated that free osmium complex was not present showing the effectiveness of gel filtration and dialysis in the removal of all non-conjugated complex.

As outlined in Table 2.3 the elution time of the free osmium complex was 12.6 min, while the larger molecular weight proteins eluted before 10 min. The conjugated

osmium complex eluted in the same time region or earlier than the protein band and showed absorption at 440 as well as 215 nm. This confirmed successful conjugation.

Protein/complex/conjugate	Initial conjugation ratio	Final substitution ratio	Elution time, mins
[Os(bpy) ₂ dc bpy]			12.6
BSA			9.6
Thyroglobulin			7.8
[Os(bpy) ₂ dc bpy]-thyroglobulin	100	78	7.8
[Os(bpy) ₂ dc bpy]-thyroglobulin	200	83	5.7-7.8
[Os(bpy) ₂ dc bpy]-BSA	30	13	9.7

Table 2.3. Elution times of free Os(II) complex, proteins and conjugated proteins using high performance size exclusion chromatography. Mobile phase was 0.05 M phosphate buffer pH 6.8. The flow rate was 1 ml min⁻¹.

It would be expected that the conjugated proteins would show a shorter retention time than free proteins due to their increased molecular weight. This did not occur in the case of conjugated BSA (ratio 13:1). However, conjugated thyroglobulin (ratio 83:1) showed two main peaks one at the original time of 7.8 min and one at a shorter time of 5.7 min (Figure 2.22). Adamczyk *et al.* (1994) have characterised BSA-hapten conjugates using matrix-assisted laser desorption ionisation mass spectrometry (MALDI) to reveal different populations of hapten incorporation within the same BSA conjugate. The authors discussed that one explanation for distinct populations could be a phenomenon of folding/unfolding of BSA during conjugation. Different populations of hapten incorporation could be a possible explanation for the multiple peaks in the chromatogram of the thyroglobulin conjugate especially since these peaks indicate a molecular weight greater than unconjugated thyroglobulin.

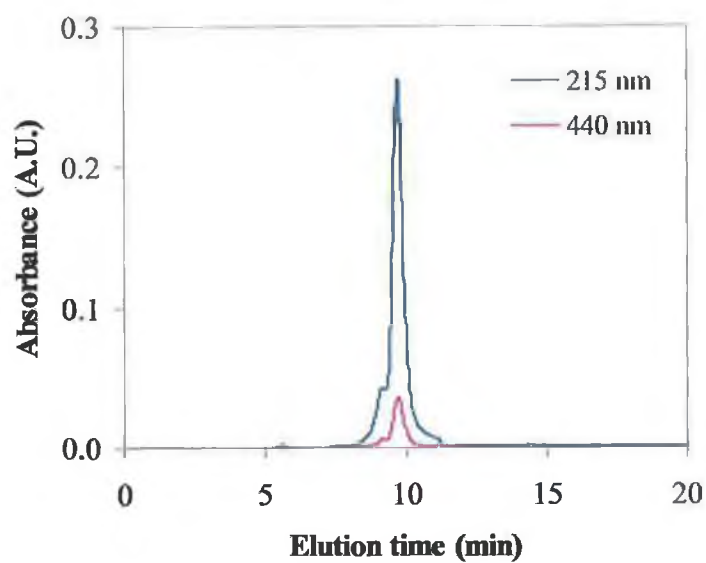


Figure 2.21. Chromatogram of covalently bound $[Os(bpy)_2dcbpy]$ -BSA (ratio 13:1).

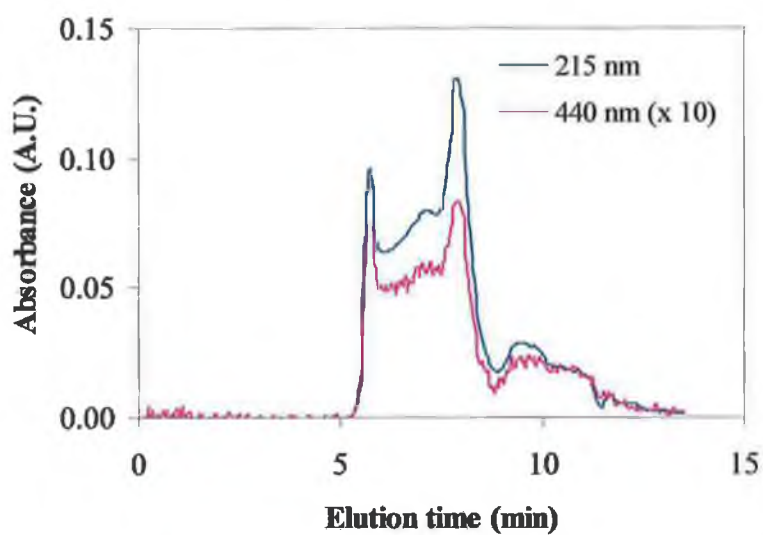


Figure 2.22. Chromatogram of $[Os(bpy)_2dcbpy]$ -thyroglobulin conjugate (ratio 83:1).

2.4.5.3 UV-Vis and emission spectra

The pK_a values for the two carboxylate groups of the 4,4'-dcbpy ligand of [Os(bpy)₂dcbpy] have been determined, with a pK_{a1} of 2.7 and pK_{a2} < 1 (section 2.3.1.5). The UV-Vis and emission measurements of the protein conjugates in Figure 2.22 and 2.23 were carried out in 0.01 M PBS, pH 7.4, so the unbound carboxylate groups of the conjugate were deprotonated. It is unlikely that complexes were conjugated through both carboxylate groups due to steric hindrance. Any of the carboxylate groups of the complex which have converted to esters and have not covalently bound to protein, will tend to revert to carboxylate groups as the half life of hydrolysis of N-hydroxysuccinimide esters is approx. 10 min at pH 8.6 and 4-5 hours at pH 7 (Wong, 1991). At this pH there is a net positive charge on the proteins, as the pI of thyroglobulin is 4.7 and that of BSA is 5.1 (Hermanson, 1996).

Slight changes were observed in the absorption spectra of the conjugated osmium complex in comparison to the free complex (Figure 2.23). The ³MLCT bands at 440 and 485 nm broadened and experienced a slight red shift of 5 nm upon conjugation. It is interesting to note that the changes on conjugation are intermediate to the changes observed in unconjugated [Os(bpy)₂dcbpy] on going from pH 7.4 to 1.7. This is also similar to the changes observed on conjugation and protonation of [Ru(bpy)₂dcbpy] (Terpetschnig *et al.*, 1995).

As can be seen in Table 2.4 and Figure 2.24, when [Os(bpy)₂dcbpy] was conjugated to BSA and thyroglobulin, a shift to lower energy and lower intensity emission occurred. This correlates with the slight red shift of the MLCT band in the UV-Vis absorption spectra. The emission maxima did not seem to depend significantly on the value of the conjugation ratio, indicating that there was little interaction between the complexes. There was also little difference between the conjugated thyroglobulin and BSA emission spectra with a red shift of approx. 20 nm and similar decreases in intensity.

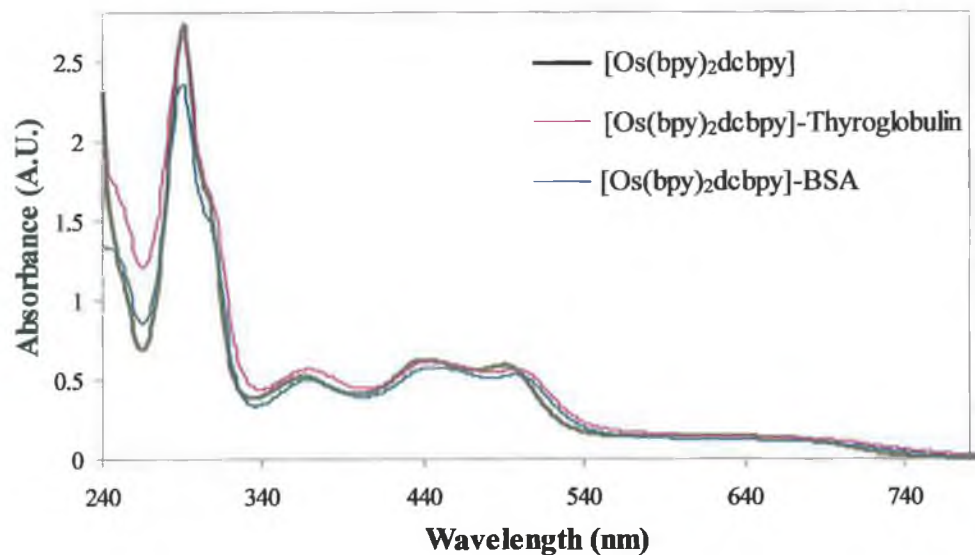


Figure 2.23. Absorption spectra of free $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and conjugated to thyroglobulin and BSA in 0.01 M PBS pH 7.4.

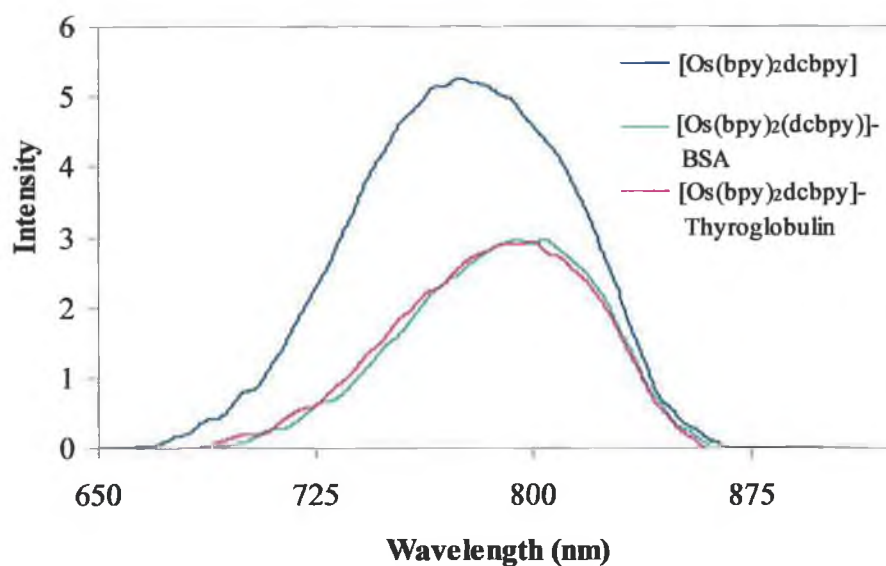


Figure 2.24. Emission spectra of free $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in 0.01 M PBS, pH 7.4, and after conjugation to BSA and thyroglobulin. The absorbance of all solutions were normalised at 440 nm before measurements were taken.

Complex/conjugate	Substitution ratio	Emission (nm)
[Os(bpy) ₂ dcbpy]		776
[Os(bpy) ₂ dcbpy]-thyroglobulin	83	796
[Os(bpy) ₂ dcbpy]-BSA	13	800

Table 2.4. Emission maxima of [Os(bpy)₂dcbpy] and when conjugated to BSA and thyroglobulin in 0.01 M PBS, pH 7.4.

Changes in the wavelength of maximum emission have also been reported for protein conjugates of Ru (II). However, whether a red or blue shift is observed depends on the ligands present in the complex. As mentioned earlier, Ryan *et al.* (1992) reported on the conjugation of [Ru(bpy)₂(NH₂phen)]²⁺, [Ru(phen)₂(NH₂phen)]²⁺, [Ru(bpy)₂(NCSphen)]²⁺ and [Ru(bpy)₂(NCSphen)]²⁺ to a series of proteins. Minimal shifts (within ± 6 nm) in emission were observed except for the conjugates of [Ru(bpy)₂(dcbpy)], which underwent a red shift of 18 nm. Szmecinski *et al.* (1998) found a 17 nm blue shift on conjugation of [Ru(dpp)₂(dcbpy)] to HSA (dpp is 4,7-diphenyl-1,10-phenanthroline). This was reduced to a blue shift of 8 nm on conjugation of [Ru(dpp)₂(mcbpy)]⁺ where mcbpy is 4-methyl, 4'-carboxylic acid-2,2'-bipyridine.

2.4.5.4 Lifetime measurements

The lifetime values obtained for [Os(bpy)₂dcbpy] and its thyroglobulin and BSA conjugates in 0.01 M PBS pH 7.4 are shown in Table 2.5. The lifetimes of all conjugates were best fit to a monexponential decay. The unbound [Os(bpy)₂dcbpy] resulted in a lifetime of 20.2 ns. This compares well to the 19.2 ns value reported in the literature at the same pH in aqueous solution. (Terpetschnig *et al.*, 1996). The [Os(bpy)₂dcbpy]-thyroglobulin conjugate (78:1) resulted in a lifetime of 21.4 ns, and [Os(bpy)₂dcbpy]-BSA (13:1) gave a lifetime 18.7 ns. The lifetime of the thyroglobulin conjugate (83:1) decreased to 17.6 ns (Figure 2.25). Terpetschnig *et al.* (1996) also found that the lifetime

of this complex when conjugated to HSA was only slightly affected, decreasing from 19.2 ns to 17.7 ns.

Complex\Conjugate	Substitution ratio	Lifetime (ns)
[Os(bpy) ₂ dcbpy]		20.2
[Os(bpy) ₂ dcbpy]-thyroglobulin	78	21.4
[Os(bpy) ₂ dcbpy]-thyroglobulin	83	17.6
[Os(bpy) ₂ dcbpy]-BSA	13	18.7

Table 2.5. Lifetime of free and conjugated [Os(bpy)₂dcbpy] measured in 0.01M PBS, pH 7.4. The average rsd was $\pm 3\%$, $n=3$.

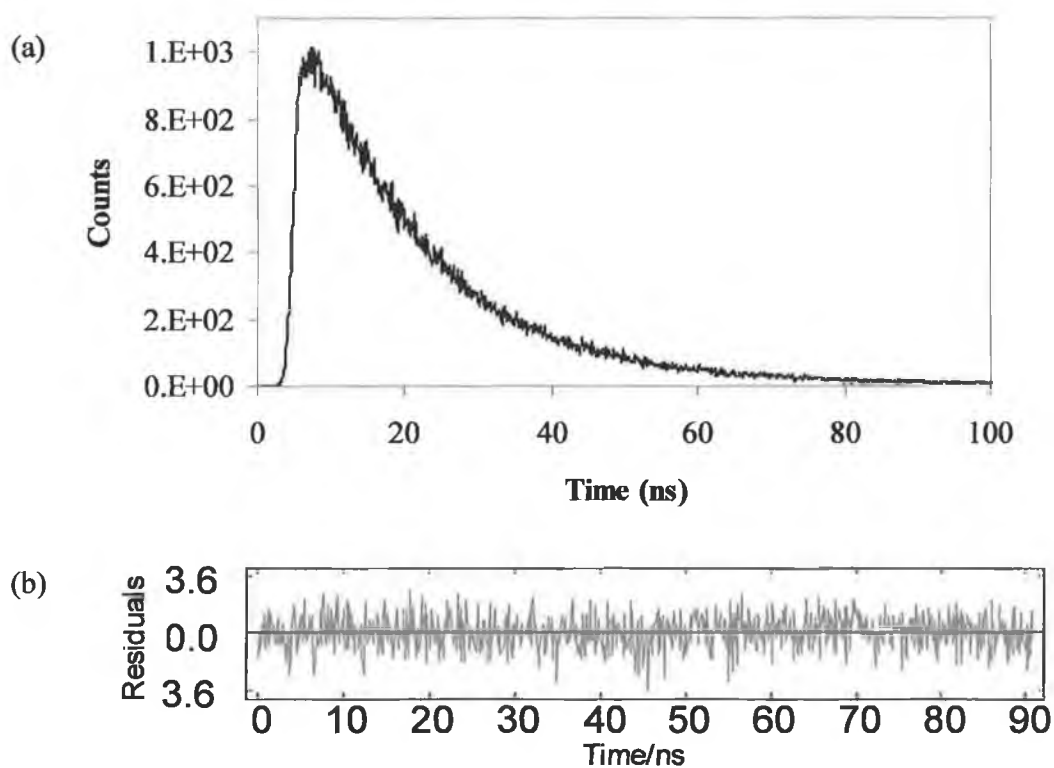


Figure 2.25. (a) Lifetime decay profile of [Os(bpy)₂dcbpy]-thyroglobulin (conjugation ratio 83:1) measured in aerated 0.01 M PBS, pH 7.4; (b) Residual analysis for the monoexponential decay.

2.4.6 Purification of immunoglobulins

Polyclonal sera are usually extremely stable. This is traditionally assumed to be due to the large number of disulphide bonds which are present on immunoglobulins (Campbell, 1984). This stability allows them to maintain biological activity following exposure to high salt concentrations or extremes of pH for short periods of time, and storage for relatively long periods at -20°C . The immunoglobulin classes differ from each other and from other serum proteins by their solubility in aqueous solution, molecular size, electrostatic density and isoelectric point. Therefore, these properties can be used to separate them from serum proteins or to fractionate them if necessary. The affinity of certain proteins, i.e. Protein A and Protein G, for immunoglobulins has also been utilised in their purification (Hudson and Hay, 1989).

Ammonium sulphate precipitation is a widely used technique for the preparation of a crude immunoglobulin fraction from whole serum. It is based on the relative solubility of proteins in salt solutions. The hydration of a large number of salt ions requires a considerable part of the solvent, thus lowering the solvation of the proteins and, consequently, inducing protein-protein interactions leading to precipitation (Tijssen, 1985). The precipitation reaction was carried out at pH 7.2 as this is the isoelectric point (pI) of IgG and so the pH at which they are least soluble. The salt concentration at which precipitation occurs is different for each protein. Most immunoglobulins precipitate free from the main contaminating protein (albumin) at 40% saturation with ammonium sulphate (Campbell, 1984).

The most common reagent to be utilised in antibody purification is Protein A. Protein A is a 42 kDa cell wall component of several strains of *Staphylococcus aureus* and it binds specifically to the Fc region of immunoglobulin molecules. The protein consists of four high affinity binding sites ($K_A = 10^8 \text{ M}^{-1}$), and is able to maintain activity after repeated exposure to extremes of pH rendering it suitable for routine purification procedures (Hermanson, 1996). Protein A can be easily coupled to a Sepharose column. Following application of the crude antibody preparation to such a column and subsequent washing to remove non-specifically bound protein, IgG antibody can be eluted on exposure to a low pH solution.

Other purification methods commonly employed include Protein G chromatography or affinity chromatography with antigen, gel filtration or ion-exchange chromatography.

2.4.7 Determination of titre of antibody raised against [Os(bpy)₂dcbpy]

The titre of an antibody refers to the highest dilution (lowest concentration) at which the antibody gives a signal above background in a given assay system. Several factors determine the titre of an antibody, but primarily it reflects both the concentration and functional affinity of the specific antibodies present in the preparation. It is also affected by the valency of the antibody and subclass distribution of the antibody response. The titre is highly dependent upon the assay system employed, and therefore the value obtained is only relevant for the assay system in which it was tested. The titre of the test serum (day 55) and the affinity purified antibody from the final bleed (day 83) were comparable and were determined to be greater than 1/64,000 (Figure 2.26). Since the titre of day 55 and day 83 was comparable this may indicate that a high level of immune maturity had been reached. A titre reflecting such a large dilution factor reflects a successful immunisation program resulting in an antibody of good concentration and affinity. No absorbance was observed where the plate was coated with BSA only or where dilutions of pre-immunisation serum were added. This confirms that the signal arises due the binding of the acquired antibody in the serum to the [Os(bpy)₂dcbpy] attached to the ELISA plate

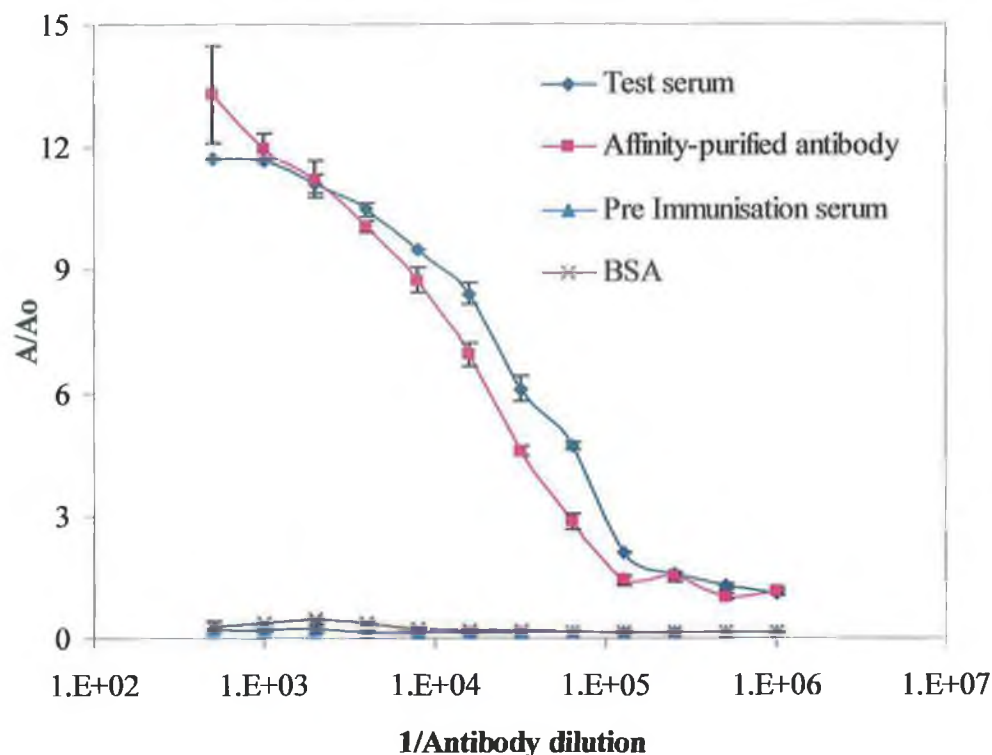


Figure 2.26. Plot showing the antibody titre of the test serum and final affinity purified antibody. The test serum was taken 12 days after the second boost (day 55) to check the immunisation response. The final serum was taken 10 days after the last immunisation (day 83).

2.4.8 Competition ELISA

Competition assays were performed as illustrated in Figure 2.6. Determination of the antibody working dilution and optimal conjugate loading density was first carried out as per section 2.3.15. This was done to obtain the optimum concentration of antibody relative to the hapten of the plate-bound conjugate, thereby resulting in components which would yield a sensitive competition. Figure 2.27 shows that although all the conjugate concentrations gave a similarly wide working range, maximum signal response was obtained at $3 \mu\text{g ml}^{-1}$. The optimal antibody dilution determined from the mid point of the linear portion of the sigmoidal curve was 1/2,400.

The $[\text{Os}(\text{bpy})_2\text{dcbpy}]\text{-BSA}$ conjugate ($3 \mu\text{g ml}^{-1}$) was then coated to the wells of an ELISA plate. The fixed concentration of antibody binds to either free competitive antigen in solution or the antigen on the plate. The competition ELISA displayed as Figure 2.28 showed that as the concentration of the free complex increased, the absorbance signal decreased indicating the displacement of antibody from the conjugate on the plate by the competing antigen. This confirms the presence of antibodies specific to the hapten $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ but also cross-reactive to $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$. This was to be expected as the metal ligand bond lengths for analogous complexes of Os(II) and Ru(II) are very similar due to the lanthanide contraction. This minimises the differences in steric effects (Kober *et al.*, 1988). The competition ELISA in Figure 2.28 also shows that the antibody was capable of binding $[\text{Ru}(\text{bpy})_3]^{2+}$ although the affinity was not as strong as when the carboxyl groups were present.

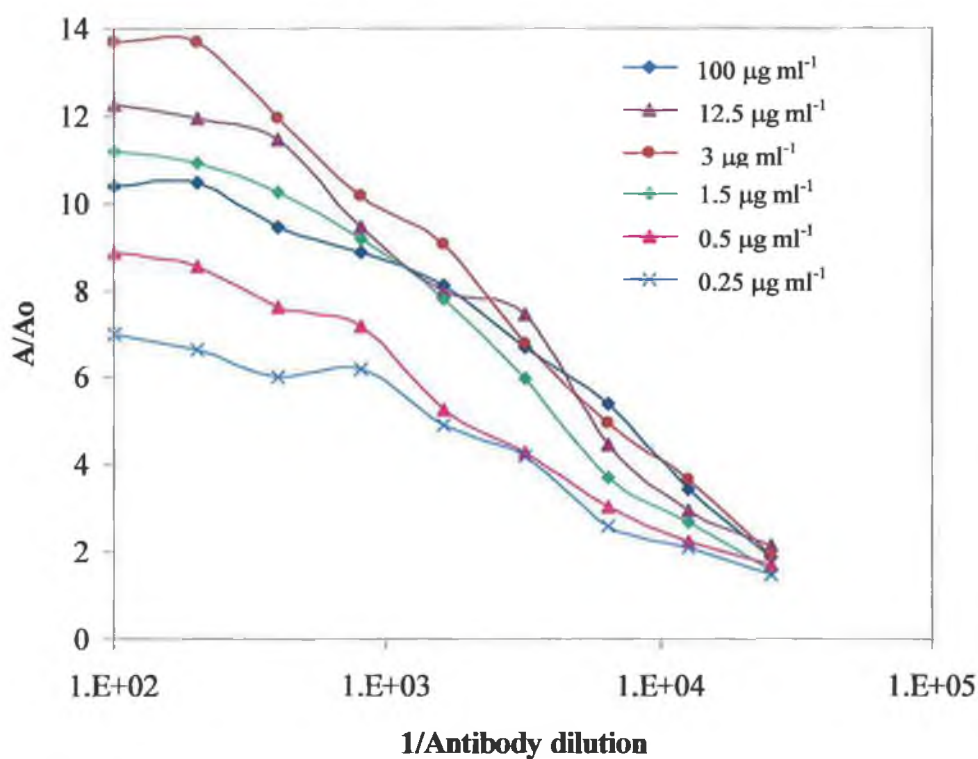


Figure 2.27. "Checkerboard" ELISA to determine optimal concentration of conjugate and antibody dilution. The effect of serial antibody dilution on a concentration of BSA-hapten in the range between 100 and $0.25 \mu\text{g ml}^{-1}$ coated on the ELISA plate was determined.

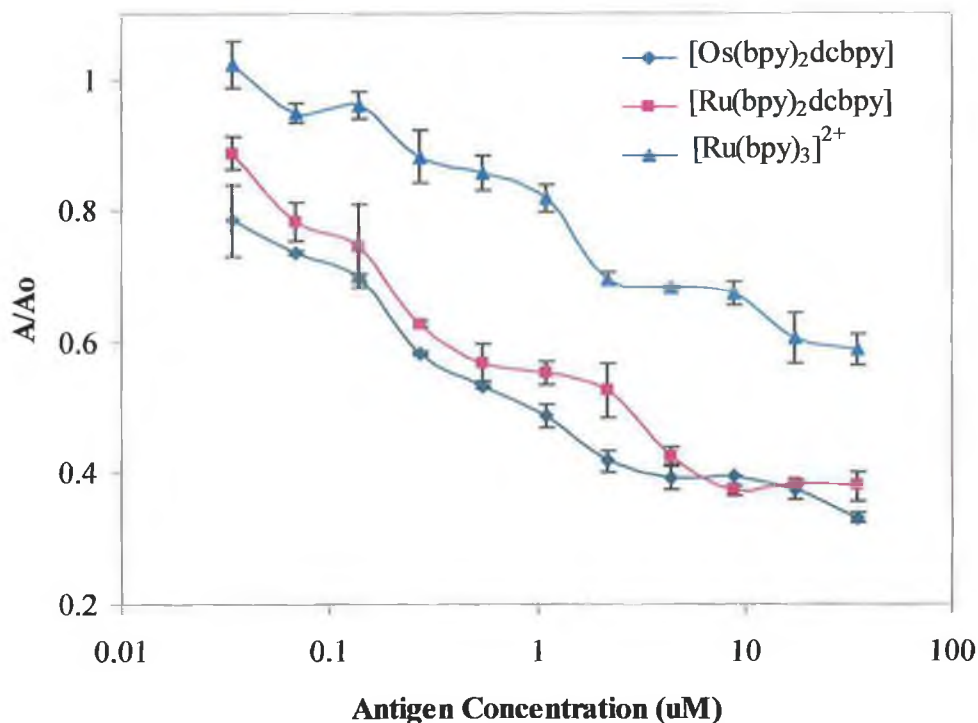


Figure 2.28. Competitive ELISA showing the recognition of the antibody towards free $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_3]^{2+}$. Error bars cover a range of $n=3$.

The cross-reactivity of the antibody towards $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ was also investigated using competition ELISA, where p2p is 1,2-bis(4-pyridyl)ethane. This complex is capable of self-assembly on platinum or gold electrodes. Cross-reactivity may be defined as a measure of the antibody response to structurally related molecules as a result of shared epitopes. A cross-reacting species generally has lower affinity and a higher concentration is required to displace 50% of the labelled antigen. Cross-reactivity is usually expressed as the relative concentration of cross reactant required for 50% displacement of the labelled antigen (Tijssen, 1985):

$$\% \text{ cross-reactivity} = \frac{\text{Concentration of antigen giving 50\% decrease in signal}}{\text{Concentration of cross-reactant giving 50\% decrease in signal}} \times 100$$

The competition ELISA of Figure 2.29 indicated a cross reactivity of the antibody towards $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ of approx. 10%.

The antibody may therefore be capable of recognising other complexes in which at least two bipyridyl groups are present as long as the remaining ligands do not sterically hinder close approach of the antibody. According to Landsteiner's principle the antibody specificity is directed primarily at the portion of the hapten further removed from the functional group that is used to link it to the carrier protein (Law, 1996).

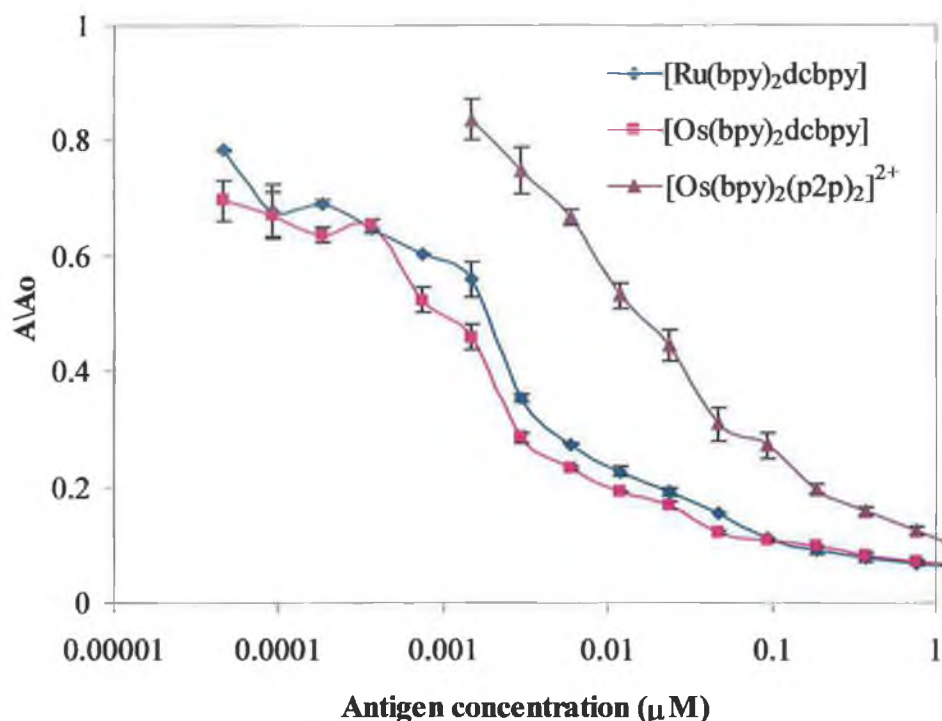


Figure 2.29. Competitive ELISA showing antibody cross-reactivity with $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$, $n=3$

2.5 CONCLUSIONS

In this Chapter a series of Os and Ru polypyridyl complexes were synthesised and characterised including $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$. $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ -thyroglobulin and BSA conjugates were prepared and characterised. Conjugation of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ to the proteins and removal of all non-conjugated complex was confirmed using high pressure size exclusion chromatography. Conjugation

of thyroglobulin and BSA affected the UV-Vis and emission spectra of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ in a similar way. The UV-Vis spectra experienced a broadening and slight red shift of the $^3\text{MLCT}$ bands. The emission maxima of both thyroglobulin and BSA conjugates showed a red shift of approx. 20 nm with similar decreases in intensity. The lifetimes of the conjugated complex were only slightly affected. A successful immunisation programme against a $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ -thyroglobulin conjugate with a final substitution ratio of 83:1 was performed. This resulted in polyclonal antibodies with a titre greater than 1/64,000. The antibodies were purified by ammonium sulphate precipitation and on a protein A column. A competitive ELISA using a $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ -BSA conjugate determined that the antibody had a strong affinity for both $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_3]^{2+}$. It was also shown by competitive ELISA that the antibody had a lower affinity for $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ with a cross-reactivity of the antibody towards $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ as approx. 10%.

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CHAPTER 3

Characterisation of polyclonal antibody-bound osmium(II) and ruthenium(II) polypyridyl complexes

3.1 INTRODUCTION

In the previous Chapter, a polyclonal antibody was raised against a $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ -thyroglobulin conjugate. In this Chapter, the effects of antibody binding on the luminescent properties of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ were studied using lifetime and steady state luminescence. Association equilibrium constants were derived from luminescence titration data. Further lifetime analysis in deuterium oxide was performed to probe the extent that the antibody shielded the complex from excited state deactivation by the aqueous solvent. The possibility of energy transfer from $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ to $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ when both were bound to the same antibody was investigated, as were the effects of antibody binding to a self-assembled layer of $[\text{Os}(\text{bpy})_2(\text{p2p})_2](\text{PF}_6)_2$.

3.2 MATERIALS, INSTRUMENTATION AND METHODS

3.2.1 Materials

Platinum microelectrodes sealed in soft glass with a diameter of 25 μm were prepared as described previously (Faulkner *et al.*, 1990).

3.2.2 Instrumentation

3.2.2.1 Emission measurements

Emission spectra were recorded using a Perkin-Elmer LS50B luminescence spectrophotometer, which was equipped with a red sensitive Hamamatsu R298 PMT detector and interfaced with an Elonex PC 466 employing Perkin-Elmer Fl Winlab software. Emission spectra were uncorrected for photomultiplier response. Emission and excitation slit widths were 15 nm.

The transient emission spectra was measured using the third harmonic (355 nm) of a Spectron Q-switched Nd:YAG laser for excitation. Emission was detected in a right-

angled configuration to the laser using an Oriel model IS520 gated intensified CCD coupled to an Oriel model MS125 spectrograph. The step size was 45 ns. The gatewidth was 60 ns with 100 integrations.

3.2.2.2 Lifetime Measurements

Luminescence lifetime measurements were obtained using an Edinburgh Analytical Instrument time-correlated single-photon counting apparatus (TCSPC). This consisted of two model J-y A monochromators (emission and excitation), a single photon photomultiplier detection system model 5300, and an F900 nanosecond flashlamp (N₂ filled at 1.1 or 0.3 atm. pressure, 40 Hz) interfaced with a personal computer via a Norland MCA card. Data correction and manipulation were carried out using EAI F900 software version 5.1.3. All lifetime measurements were carried out in 0.01 M PBS, pH 7.4. Lifetime decay data analysis was performed as per section 2.2.2.6.1.

3.2.2.3 Electrochemistry

Cyclic voltammetry was performed using a CHI 660 or CHI 660a instrument and a conventional three-electrode cell. Potentials were measured against a sodium chloride saturated silver/silver chloride (Ag/AgCl) reference electrode. The auxiliary electrode was a platinum wire. All solutions were degassed using nitrogen and a blanket of nitrogen was maintained over the solution during all experiments. The electrolyte solution was 0.01 M PBS, pH 7.4.

3.2.3 Methods

3.2.3.1 Luminescence titrations

Luminescence titrations were carried out in 0.01 M PBS, pH 7.4 by addition of an increasing amount of protein A purified antibody to a constant concentration of complex until saturation of the antibody binding sites was obtained. The conditions were as

follows; in the case of $[\text{Os}(\text{bpy})_2(\text{dcbpy})]$, 20 μM was excited at 445 nm with the addition of 0-19 μM antibody, 1 μM of $[\text{Ru}(\text{bpy})_2(\text{dcbpy})]$ was excited at 460 nm with the addition of 0-1.35 μM antibody, 1 μM of $[\text{Ru}(\text{bpy})_3]^{2+}$ was excited at 455 nm with the addition of 0-1.6 μM antibody and 3 μM of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ was excited at 440 nm with the addition of 0-10.5 μM antibody. The titrations were carried out such that the concentration of the complexes remained constant throughout the titration.

3.2.3.2 Calculation of association equilibrium constants

Association equilibrium constants (K_a) were derived from the steady-state luminescence titrations. At equilibrium, antibody (Ab) antigen (Ag) binding can be described as:



where k_1 is the association rate constant, k_2 is the dissociation rate constant and $[\text{AgAb}]$ is the concentration of the antibody-bound metal complex. $[\text{Ab}]$ is the concentration of the antibody in binding sites. From equation (3.1) at equilibrium:

$$K_a = \frac{k_1}{k_2} = \frac{[\text{AgAb}]}{[\text{Ag}][\text{Ab}]} \quad (3.2)$$

This can be rewritten as:

$$K_a = \frac{[\text{AgAb}]}{[\text{Ag}-\text{AgAb}][\text{Ab}-\text{AgAb}]} \quad (3.3)$$

In the luminescence titration data where increasing concentration of antibody is added to a constant concentration of complex:

$$E = E_c \frac{[\text{AgAb}]}{[\text{Ag}]} \quad (3.4)$$

where E is the integrated emission intensity of the complex at a particular point in the titration, E_c is the total difference in the relative integrated emission intensity between the

antibody bound and free metal complexes. $[AgAb]/[Ag]$ represents the fraction of the complex that is antibody-bound. Equation 3.5 was obtained when an expression for $[AgAb]$ was solved in equation 3.3 and substituted into equation 3.4. A plot of E vs. $[Ab]$ was drawn and solver used to determine the best fit based on equation 3.5 and thereby ascertaining a value for K_a (Shreder *et al.*, 1996).

$$E = \frac{(K_a[Ab] + K_a[Ag] + 1 - \sqrt{(K_a[Ab] + K_a[Ag] + 1)^2 - 4K_a^2[AbAg]})E_c}{2K_a[Ag]} \quad (3.5)$$

3.2.3.3 Preparation of $[Os(bpy)_2(p2p)_2]^{2+}$ self assembled electrodes

The platinum micro-electrodes were cleaned by successive polishing with 0.3 and 0.05 μm alumina. They were then cycled in 0.5 M H_2SO_4 between the limits of -0.2 and 1.4 V (10 cycles). The electrodes were then cycled between 0.1 and 0.9 V in 0.1 M $LiClO_4$ until hydrogen desorption was complete. Monolayers were formed by deposition on the electrode overnight in a 1 mM solution of $[Os(bpy)_2(p2p)_2](PF_6)_2$ in $2:1$ (v/v) $H_2O:DMF$. The electrodes were then rinsed in the electrolyte solution which was 0.01 M PBS, pH 7.4 . Where antibody was bound, the self-assembled monolayer electrodes were immersed in a 1 mg ml^{-1} purified antibody solution for 30 min, the electrodes were then gently rinsed with electrolyte solution.

3.3 RESULTS AND DISCUSSION

3.3.1 Luminescence titrations

The effect of adding increasing amounts of the polyclonal antibody raised against $[Os(bpy)_2dcbpy]$, on the emission properties of $[Os(bpy)_2dcbpy]$, $[Ru(bpy)_2dcbpy]$, $[Ru(bpy)_3]^{2+}$ and $[Os(bpy)_2(p2p)_2]^{2+}$ was studied. The emission spectra of all the complexes experienced a significant blue shift to higher energy and a substantial increase in luminescence intensity on binding of the antibody (Table 3.1). To confirm that this was due to the specific nature of antibody binding and not non-specific protein association,

the complexes were also titrated with BSA. No change in the emission intensity or wavelength maxima occurred. This confirms that the increase in emission intensity and shift to higher energy was due to the interaction of the complexes with the antibody.

Complex	Emission maxima (nm)		Emission intensity enhancement
	Free	Antibody intercalated	
[Os(bpy) ₂ dcbpy]	771	734 (37)	7.2
[Os(bpy) ₂ (p2p) ₂] ²⁺	757	730 (27)	5.6
[Ru(bpy) ₂ dcbpy]	642	619 (23)	3.9
[Ru(bpy) ₃] ²⁺	612	599 (13)	3.0

Table 3.1. Changes in emission maxima and integrated intensity upon association with saturation concentrations of antibody. All measurements were carried out in 0.01 M PBS, pH 7.4. Values in brackets show shift to higher energy of maximum. Rsd was less than 3.4% for integrated intensity increases.

The emission spectrum of the hapten [Os(bpy)₂dcbpy] experienced the greatest change upon saturation with antibody. A blue shift of 37 nm as well as a 7.2-fold increase in emission was observed (Table 3.1, Figures 3.1 and 3.2). This contrasts with the 20 nm and 24 nm red shift which was observed on covalent binding of this complex to thyroglobulin and BSA respectively (Figure 2.24). This illustrates the radical change in environment which occurs when [Os(bpy)₂dcbpy] is rigidly buried in a hydrophobic antibody binding cleft as opposed to the predominantly hydrophilic environment achieved on covalent binding.

An increase of 23 nm to higher energy was observed when [Ru(bpy)₂dcbpy] was fully saturated with antibody. This was accompanied by a 3.9-fold increase in emission intensity (Table 3.1, Figures 3.3 and 3.4). A sharp endpoint was obtained when [Os(bpy)₂dcbpy] and [Ru(bpy)₂dcbpy] were titrated with the antibody indicating a strong affinity. A similar ratio of complex to antibody achieved the endpoint of both titrations, which was 1:0.9 for [Os(bpy)₂dcbpy] and 1:1.06 for [Ru(bpy)₂dcbpy]. The molar binding ratio of antibody to antigen is dependent on the affinity of the antibody. This suggests

that the antibody had a similar affinity for both complexes with perhaps a slightly higher affinity for the osmium complex. A similar affinity is expected as the metal ligand bond lengths for analogous complexes of Os(II) and Ru(II) are very similar as mentioned previously due to the lanthanide contraction, minimising differences in steric effects and solvation (Kober et al., 1988).

The antibody was purified on a protein A column and so some non-specific IgG may have been present. Using the complex:antibody ratio at saturation and assuming that one MLC binds per antibody binding site leads to the conclusion that 55% of the polyclonal IgG had bound to $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and 47% to $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$. Voss *et al.* (2001) have reported on the yields of specific antibodies in ten different IgG populations of rabbit polyclonal anti-fluorescein antibodies. Yields of specific Fab fragments ranged from 12.7 to 84.1%. The yields obtained showed both significant animal variation and differences between different bleedings of the same rabbit. The high yields indicated that in the late hyperimmune state, a large proportion of the IgG immunoglobulins were specific for the fluorescein hapten. In this light, the high yield of 55% of the isolated IgG which bound to $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ obtained here is not unreasonable.

To avoid the presence of non-specific IgG, purification with affinity chromatography incorporating specific antigen could be used. Difficulties in affinity chromatography are often encountered with regards to separating and eluting high affinity sub-populations of antibody from the column-bound antigen. To avoid loss of this fraction, purification via protein A was chosen. The controls have shown that the non-specific IgG should not affect the spectroscopic properties of the complexes.

Antibody binding of $[\text{Ru}(\text{bpy})_3]^{2+}$ resulted in a three-fold increase in intensity accompanied by a 13 nm red shift in wavelength (Table 3.1, Figures 3.5 and 3.6). A ratio of complex to antibody of 1:1.6 was required in this case to saturate the complex. Lower affinity binding was expected for this complex as reflected by the larger excess of antibody needed to achieve saturation. Alternatively, it can be stated that 31% of the polyconal IgG present bound to $[\text{Ru}(\text{bpy})_3]^{2+}$.

The titration of $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ showed a large increase in emission of 5.6 times the original integrated intensity of the unbound species in the presence of 3.5 times molar excess of antibody (Table 3.1, Figures 3.7 and 3.8). Again, a lower affinity binding

was indicated by the larger excess of antibody needed to achieve saturation as was expected due to the structural differences between this complex and the hapten [Os(bpy)₂dcbpy]. A gradual tailing of the intensity was achieved rather than a sharp endpoint so that the emission was still increasing slowly at the end of the titration, although most of the increase had taken place by the time a ratio of 1:1.5 [Os(bpy)₂(p2p)₂]²⁺ antibody had been reached. A 3.5-fold molar excess of antibody relates to 14% of the total IgG present binding to this complex. The extent of the increase in emission is surprising given the antibodies lower affinity for this complex. A 27 nm blue shift of maximum wavelength was observed at the end of the titration.

It was interesting to note that the changes in emission intensity occurred instantaneously on addition of antibody. When the emission intensity of antibody-saturated [Ru(bpy)₂dcbpy] was monitored over four hours no further increase was observed. Although it has been reported that the secondary binding processes may not be complete for several hours (Davies, 1994), it was the initial binding event that had the significant effect in the changes in spectroscopic properties of the complex noted here.

Titration of the complexes with non-specific protein BSA, served to confirm that the spectral changes observed were due to the specific nature of the antibody binding. As already outlined, antigen/antibody association involves electrostatic, Van der Waals and hydrogen bonding interactions (Mayforth, 1993). These cooperate to provide a close approach of the two molecules resulting in effective water elimination. The non-radiative rate constant (k_{nr}) and emission energy of Os(II) and Ru(II) polypyridyls are strongly solvent-dependent. k_{nr} experiences a dramatic increase in hydroxylic solvents with a corresponding decrease in emission energy (Masuda *et al.*, 1998, Hauenstein *et al.*, 1983). Therefore, the results were consistent with elimination of water molecules from the solvation shell of the complex on antibody binding. Other reasons may include protection of the complex by the antibody from O₂ collisional quenching and a decrease in the mobility of the complex when bound to the high molecular weight antibody.

Shreder *et al.* (1996) raised a monoclonal antibody against a tris(2,2'-bipyridine)cobalt(III)-methyl viologen hapten. The largest change in emission obtained when the antibody was bound to a series of Ru(II) complexes was a 2.6-fold increase which was considerably smaller than the results achieved here. Although the authors

reported that a slight blue shift was achieved, the magnitude of the shift was not mentioned. This is in contrast to the large changes in emission maxima to higher energy observed here. This group's report of binding of $[\text{Ru}(\text{bpy})_3]^{2+}$ to a polyclonal antibody raised against a tris(2,2'-bipyridine)cobalt(III)-methyl viologen hapten (Shreder *et al.* 1995), displayed an emission increase of 3.3 times, which was closer to the values that were obtained here. No further information was given on the magnitude of the blue shift. This highlights the variable nature of the affinity of antibodies raised using different immunisation protocols and indicates that the spectral properties of the bound complex are influenced by the particular environment provided by the binding pocket.

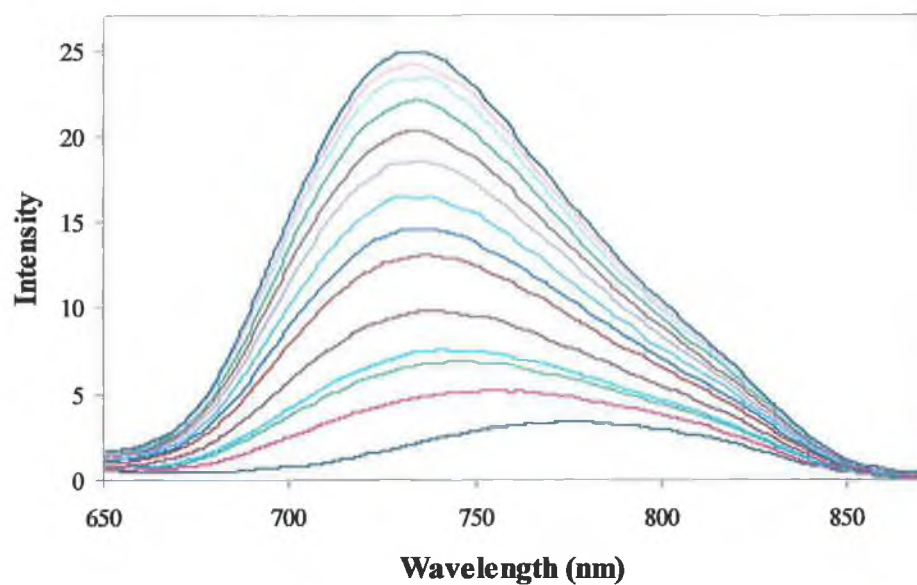


Figure 3.1. Emission spectra of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ (20 μM), showing increasing intensity with increasing concentration of antibody (0-19 μM). No further increase in emission was obtained when the ratio of complex:antibody was greater than 1:0.9.

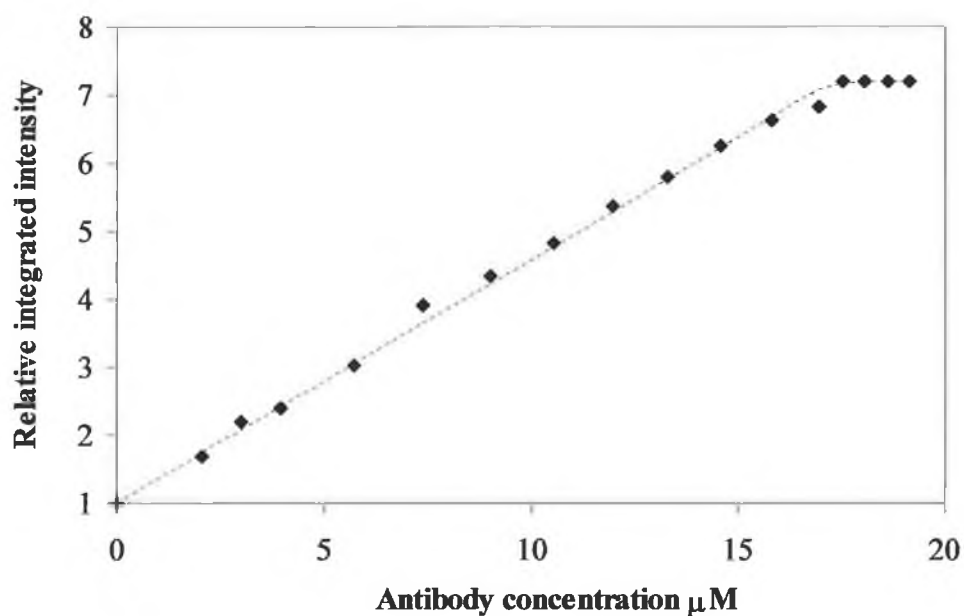


Figure 3.2. Luminescence titration of 20 μM of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ with increasing concentration of antibody.

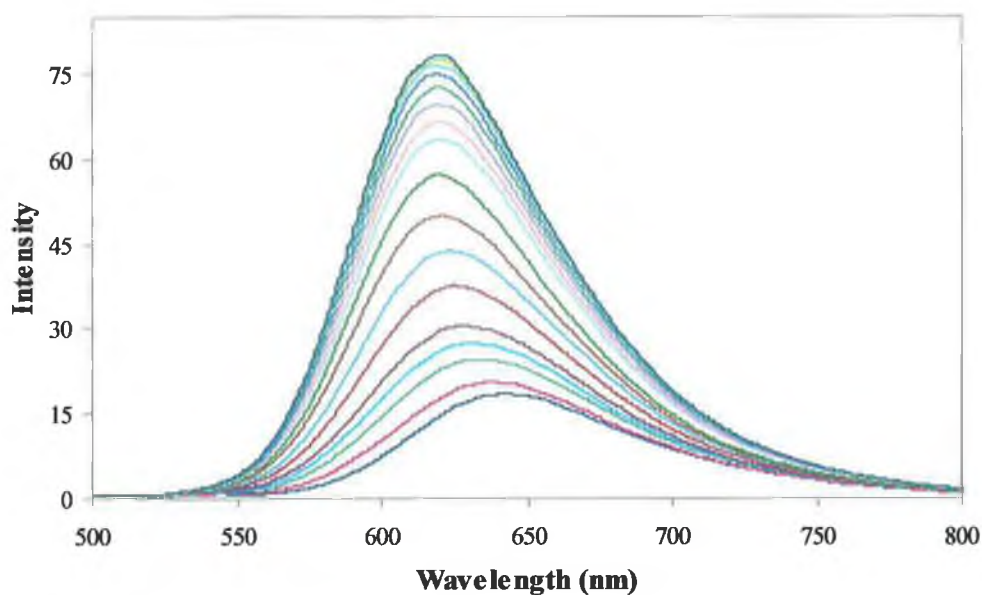


Figure 3.3. Emission spectra of 1 μM $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ showing increasing intensity with increasing concentration of antibody (0-1.35 μM). No further increase in emission was obtained when the ratio of complex:antibody was greater than 1:1.06.

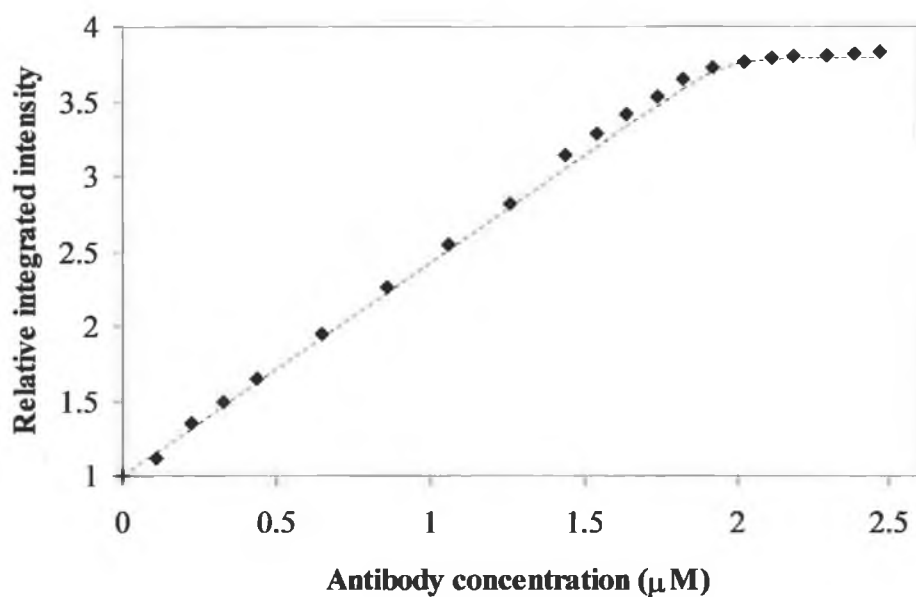


Figure 3.4. Luminescence titration of 1 μM of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ with increasing concentration of antibody (0-1.35 μM).

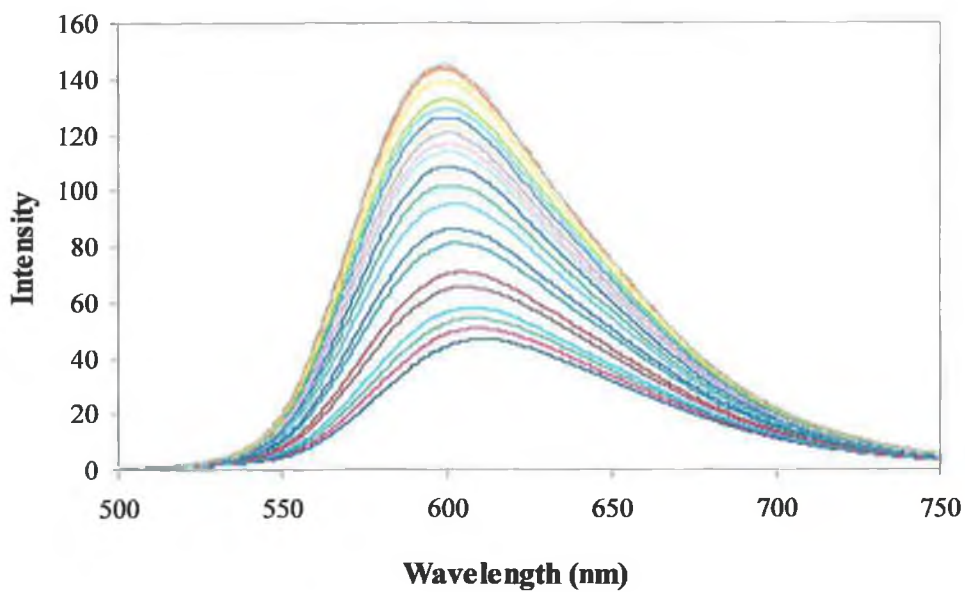


Figure 3.5. Emission spectra of $1 \mu\text{M}$ $[\text{Ru}(\text{bpy})_3]^{2+}$ excited at 455 nm showing increasing intensity with increasing concentration of antibody (0 - $1.6 \mu\text{M}$).

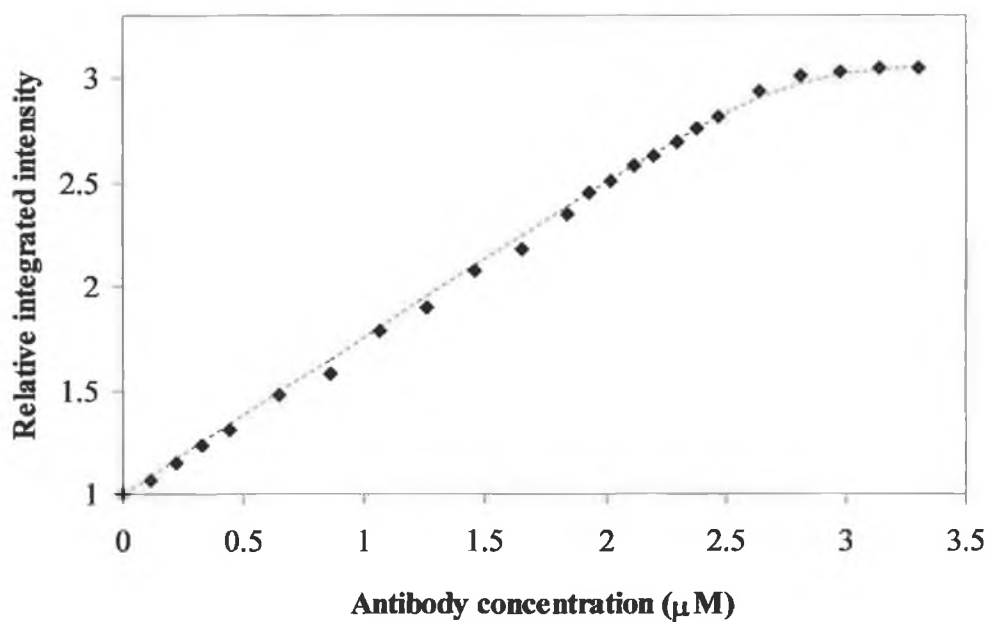


Figure 3.6. Luminescence titration of $1 \mu\text{M}$ $[\text{Ru}(\text{bpy})_3]^{2+}$ with increasing concentration of antibody.

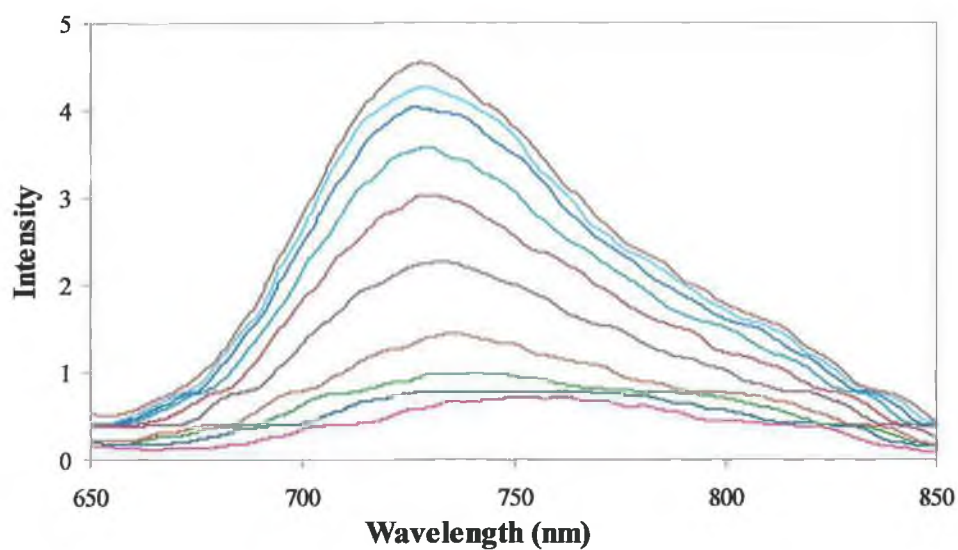


Figure 3.7. Emission spectra of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ ($3 \mu\text{M}$), showing increasing intensity with increasing concentration of antibody (0 – $10.5 \mu\text{M}$).

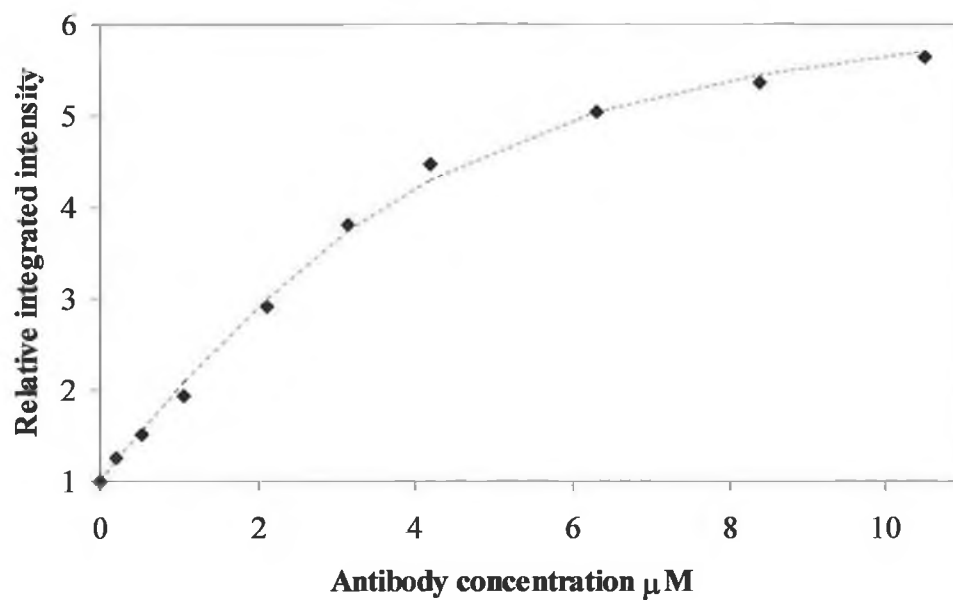


Figure 3.8. Luminescence titration of $3 \mu\text{M}$ of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ with antibody.

3.3.2 Calculation of Association equilibrium constants

Association equilibrium affinity constants (K_a) were derived from the steady-state luminescence titrations. A plot of integrated emission intensity vs. antibody concentration was drawn and the solver function of Microsoft Excel used to determine the best fit based on equation 3.5, thereby ascertaining a value for K_a . Solver returns a value for K_a by attempting to minimise the residual error between the test and predicted data (Diamond and Hanratty, 1997). The fit obtained for each titration is shown as the broken line in Figures 3.2, 3.4, 3.6 and 3.8. Polyclonal antibodies are produced as a mixed population with varying affinities. Equilibrium constants represent an average of these values. Equilibrium constants in the range of 10^6 to 10^{12} M^{-1} are commonly encountered.

As expected the K_a obtained for $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ were very similar being $5.6 \times 10^8 \text{ M}^{-1}$ and $5.0 \times 10^8 \text{ M}^{-1}$, respectively, which represents a high affinity. The equilibrium constants obtained here decrease with increasing structural difference of the complex towards the hapten ($[\text{Os}(\text{bpy})_2\text{dcbpy}]$), i.e. the K_a obtained for $[\text{Ru}(\text{bpy})_3]^{2+}$ was almost one order of magnitude lower at $7.1 \times 10^7 \text{ M}^{-1}$ and that of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ was significantly lower at $3.6 \times 10^5 \text{ M}^{-1}$ (Table 3.2). The K_a value obtained for $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ from the luminescence titration data may have been lower than expected on the basis of the competition ELISA results, which showed an affinity difference in the region of one order of magnitude (section 2.4.8).

In vivo, the production of antibodies of varying affinities are dependent on several factors, including physical-chemical properties of the immunogen, route of immunisation, immunogen dosage, duration of immunisation and species or genetic strain of the host. *In vitro* the affinity of the antibody depends predominantly on the complementarity of the antigen or cross-reacting species and the avidity. A lack of fit between the antigen and the surface presented by the CDRs prevents the close approach of the two molecules which is necessary for the weak attractive forces to be effective. This explains the lower K_a that was obtained for $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ relative to $[\text{Os}(\text{bpy})_2\text{dcbpy}]$. While affinity is a measure of the strength of interaction between one site of the antibody and the antigen, avidity is the overall strength of binding of the antibody molecule as a whole taking into account multi-valency in the antibody and antigen. The antibody-antigen interaction is a

continuous association-dissociation process during which the antibody and antigen may become separated. An avidity bonus is obtained due to the superior association which occurs when antibodies attach to a multivalent antigen since the multiple bonds do not separate synchronously, making it less likely for the complex to become separated (Tijssen, 1985). Also, re-association of the antigen from one binding site to another has a greater likelihood in a multivalent antibody. As noted earlier IgG antibodies are divalent and this will contribute to the K_a values obtained.

The largest K_a reported by Shreder *et al.* (1996) when a monoclonal antibody was titrated against a series of Ru complexes was $5 \times 10^7 \text{ M}^{-1}$ and that of a polyclonal antibody for $[\text{Ru}(\text{bpy})_3]^{2+}$ was $5 \times 10^8 \text{ M}^{-1}$. These are comparable to the results obtained here.

Complex	$K_a (\text{M}^{-1})$
$[\text{Os}(\text{bpy})_2\text{dcbpy}]$	5.6×10^8
$[\text{Ru}(\text{bpy})_2\text{dcbpy}]$	5.0×10^8
$[\text{Ru}(\text{bpy})_3]^{2+}$	7.1×10^7
$[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$	3.6×10^5

Table 3.2. Association equilibrium constants derived from luminescence titrations.

3.3.3 Lifetime studies

Time-resolved luminescence spectroscopy was used to study the interaction of the antibody with $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$. As the lifetime varies with temperature and the amount of oxygen present in an aerated species, the lifetime of aerated $[\text{Ru}(\text{bpy})_3]^{2+}$ was taken at the start of each measurement session as a control. The lifetime of this complex was found to be $382 \pm 11 \text{ ns}$. This indicated that variations in temperature of 20-25 °C and instrumental response result in a standard deviation of less than 5% for a monoexponential decay. It is thus assumed that the lifetimes represented by a monoexponential decay shown here are within $\pm 5\%$.

The lifetimes of the unbound complexes all displayed monoexponential decays. Control samples were prepared which contained a mixture of the complex with non-specific protein (BSA). A monoexponential decay was observed with no significant change in lifetime.

Complex	Lifetime (ns)	
	Aerated	Deaerated
[Os(bpy) ₂ dcbpy]	20	20
[Os(bpy) ₂ dcbpy] + antibody	75 (3.7)	77 (3.9)
[Ru(bpy) ₂ dcbpy]	399	528
[Ru(bpy) ₂ dcbpy] + antibody	1299 (3.3)	1572 (3.0)
[Ru(bpy) ₃] ²⁺	387	658
[Ru(bpy) ₃] ⁺ + antibody	1124 (2.9)	1375 (2.1)
[Os(bpy) ₂ (p2p) ₂] ²⁺	17	nd
[Os(bpy) ₂ (p2p) ₂] ²⁺ + antibody	53 (3.1)	nd

Table 3.3. Emission decay lifetimes for free and antibody-bound complex ($n=3$, $rsd < 3\%$). Values in brackets show the enhancement of lifetime upon antibody binding. All lifetimes measured in 0.01 M PBS, pH 7.4.

The lifetime of unbound [Os(bpy)₂dcbpy] in PBS was 20 ns (Table 3.3). No significant change was observed when degassed with argon as was reported in the literature (Terpetschnig *et al.*, 1996). Although the lifetimes were almost unaffected when covalently bound to BSA or thyroglobulin as seen in Table 2.5, a 3.7-fold increase to 75 ns was observed when the complex was saturated with antibody (Figure 3.9). This represents the largest increase of the complexes studied, showing the relationship between a close steric fit and enhancement of lifetime. A close steric fit is necessary for effective electrostatic, Van der Waals and hydrogen bonding between the complex and antibody. These forces combine to exclude water molecules from the binding cleft, resulting in the emission and lifetime increases. When the antibody-saturated

[Os(bpy)₂dcbpy] solution was degassed, the lifetime was found to be 77 ns which was not a significant change from the aerated measurement. On complete saturation of [Ru(bpy)₂dcbpy] with antibody a 3.3-fold increase in lifetime to 1299 ± 12 ns with a monoexponential decay was observed in aerated buffer (Table 3.3, Figure 3.10).

A transient emission decay profile of degassed antibody-saturated [Ru(bpy)₂dcbpy] is shown in Figure 3.11. As expected from a monoexponential decay no change in the wavelength of maximum emission was observed throughout the decay. All spectra had a wavelength of maximum emission at 612 nm which is characteristic of the antibody-bound complex.

The lifetime of an antibody-saturated solution of [Os(bpy)₂(p2p)₂]²⁺ increased by a factor of 3.1 from 17 to 53 ns (Table 3.3). This shows that even lower affinity antibody binding can also result in a large increase in lifetime.

The increase in lifetime experienced for an aerated sample of [Ru(bpy)₃]²⁺ was 2.9-fold, this was smaller than the 3.3-fold increase experienced by [Ru(bpy)₂dcbpy] again showing the importance of a close steric fit between antibody and antigen for effective electrostatic interactions and hydrogen bonding and the subsequent exclusion of water from the binding cleft.

The lifetime of the antibody-bound [Ru(bpy)₂dcbpy] and [Ru(bpy)₃]²⁺ increased further when the samples were deaerated (Table 3.3). This showed that the antibody did not completely protect the complex from collisional quenching by O₂.

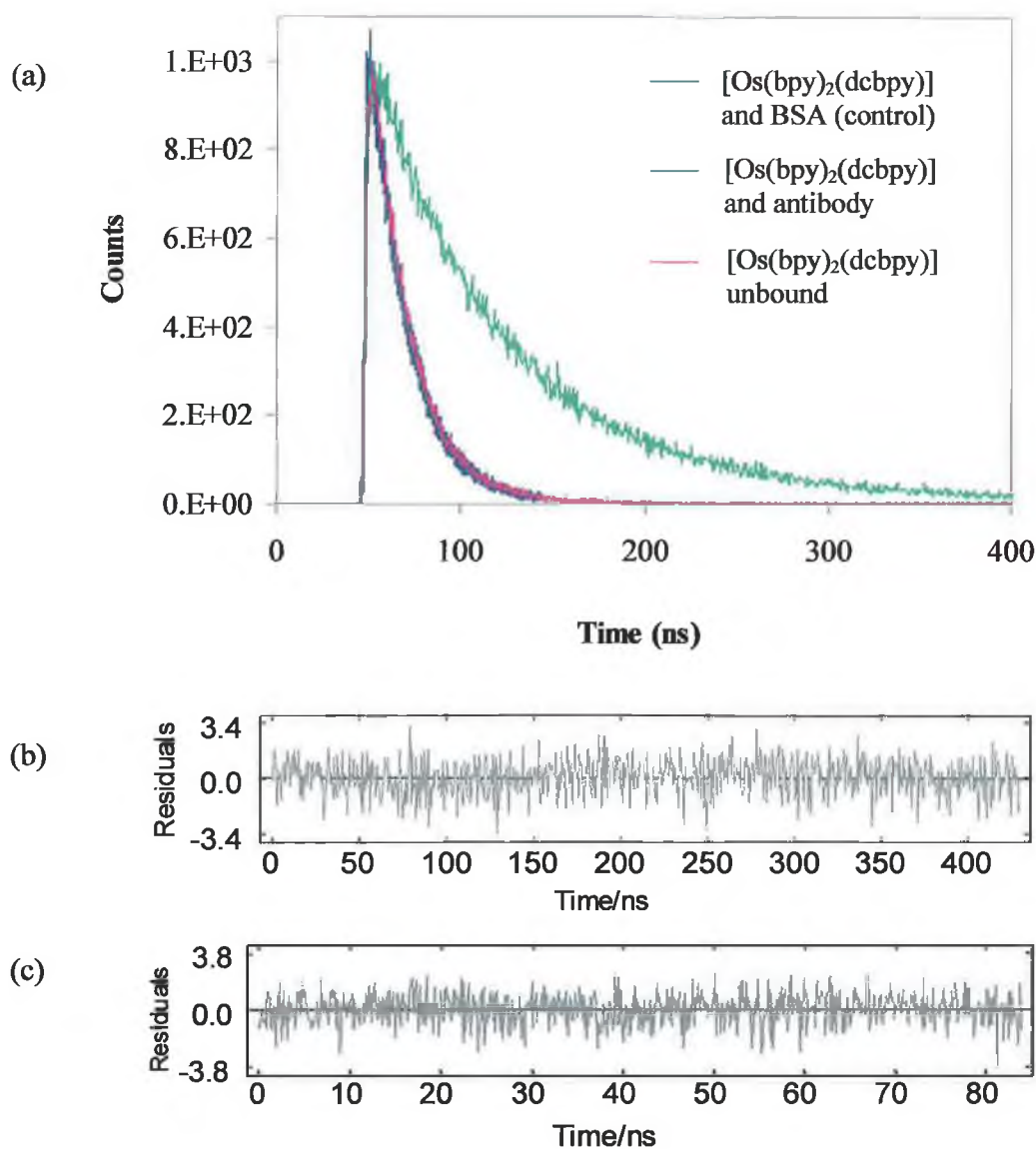


Figure 3.9. (a) Emission decay profile of free and antibody-saturated $[\text{Os}(\text{bpy})_2\text{dcbpy}]$. The lifetime of the complex increased from 20 to 75 ns upon antibody binding, the control sample with added BSA did not show an increase in lifetime. (b) Residual analysis for the decay of antibody-bound $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, (c) Residual analysis for the decay of unbound $[\text{Os}(\text{bpy})_2\text{dcbpy}]$.

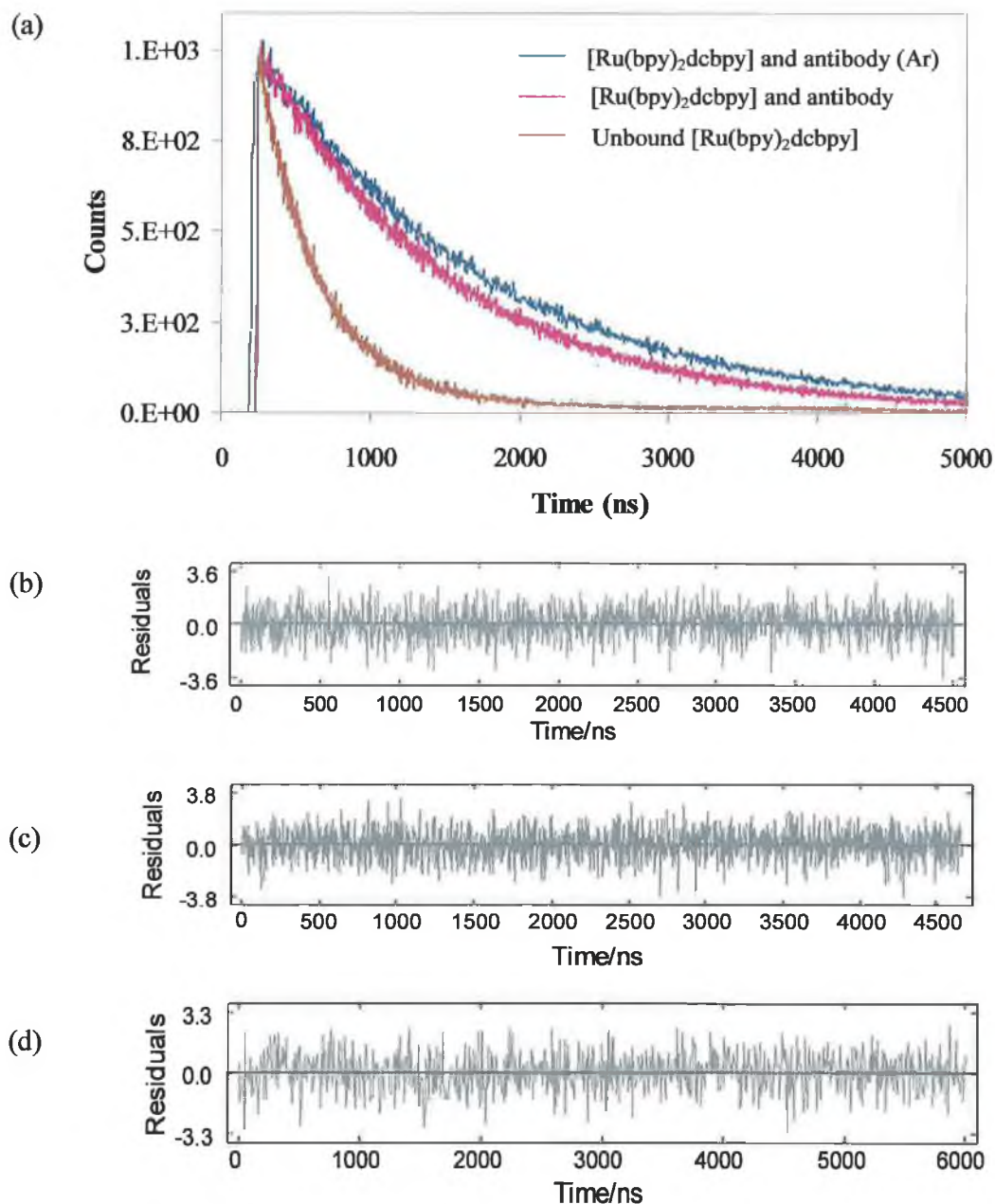


Figure 3.10. (a) Emission decay profile of free and antibody-bound $[Ru(bpy)_2dcbpy]$. The lifetime of the unbound complex increased from 399 ns to 1299 ns upon antibody binding. This value increased further to 1572 ns when deaerated. Residual analysis for (b) aerated unbound $[Ru(bpy)_2dcbpy]$ (c) aerated antibody-bound $[Ru(bpy)_2dcbpy]$ and (d). deaerated antibody-bound $[Ru(bpy)_2dcbpy]$.

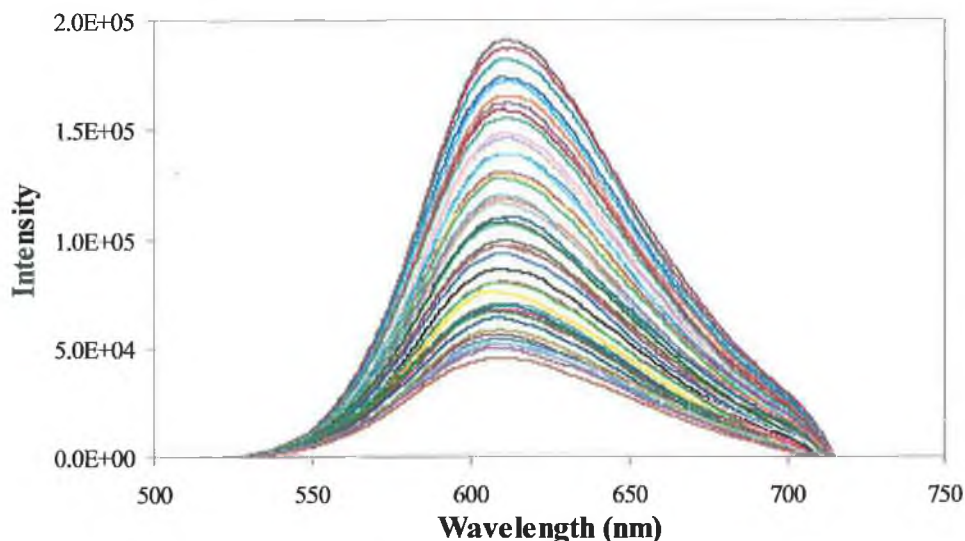


Figure 3.11. Transient emission decay of a deaerated solution of antibody saturated $[Ru(bpy)_2dcbpy]$, step size 45 ns.

Lifetime analyses were carried out in a series of solutions of $[Os(bpy)_2dcbpy]$ which had increasing concentrations of antibody (Table 3.4). Samples which contained an unsaturated concentration of antibody, resulted in biexponential decays. The short lifetime component of the decay corresponded to the lifetime of the unbound species. The much longer second component seemed to correlate with the lifetime of the antibody-bound species. Increasing the concentration of the antibody resulted in a biexponential decay with increasing percentage of the longer component as indicated by the preexponential decay value (A_2) (Figure 3.12). Lifetime titrations allow the antibody-bound species to be identified separate from the unbound species. This is why the increase in the percentage of the longer-lived component (antibody-bound species) shows a steeper gradient over the linear increase observed in the luminescence titration.

Counts in the region of 10^4 photons must be generated before reliable preexponential decay values are generated for a multi exponential decay. The 10^3 counts accumulated in these experiments could be obtained in a reasonable time frame given the relatively lower quantum yield of $[Os(bpy)_2dcbpy]$ and limitations of antibody concentration. 10^3 counts may not have yielded precise preexponential values but served

the purpose of indicating that an equilibrium of bound and unbound species exists in an unsaturated antibody complex solution. It can be seen from Table 3.4 that accurate deconvolution of the biexponential decay was more difficult where there was a small percentage of one component relative to the second. This is where the lifetime deviated most from that obtained in each component's monoexponential decay. However, the average lifetime of the shorter component throughout the titration was 22 ± 2 ns and for the longer component $75 \text{ ns} \pm 5 \text{ ns}$. This compares well with the lifetimes obtained for the free and antibody-saturated complex. The lifetime titration serves to indicate that there were no other intermediary states that could be detected, only bound and unbound complex.

The longer lifetimes of the antibody-bound complexes were to be expected given the increase in intensity observed in the steady-state luminescence titrations. The factors outlined previously to account for the higher emission in the presence of antibody also explain the increase in lifetime. These include protection from excited state deactivation by solvent and O_2 and the greater rigidity and decrease in mobility experienced by the antibody-bound complex. Shreder *et al.* (1996) reported lifetime increases between 2.2 and 4.6-fold for a series of monoclonal antibody-Ru(II) complexes. A polyclonal antibody-bound $[\text{Ru}(\text{bpy})_3]^{2+}$ resulted in a 3.3-fold increase. This degree of enhancement was similar to the values obtained here.

Ratio [Os(bpy) ₂ dcbpy]: Antibody	τ_1 (ns)	% A ₁	τ_2 (ns)	% A ₂	χ^2
1 : 0	20	(100)			1.049
1 : 0.02	21	(81.2)	67	(18.8)	1.101
1 : 0.04	20	(62.2)	71	(37.8)	1.077
1 : 0.1	23	(27.2)	80	(72.8)	1.03
1 : 0.3	23	(15.3)	80	(84.6)	1.079
1 : 0.4	25	(9.3)	78	(90.7)	1.069
1 : 0.9			75	(100)	1.074

Table 3.4. Decay data for [Os(bpy)₂dcbpy] with increasing concentration of antibody. Increasing the antibody concentration increased the proportion of the longer lifetime component as indicated by % A₁ and A₂, the preexponential factors. All lifetimes measured in 0.01 M PBS, pH 7.4.

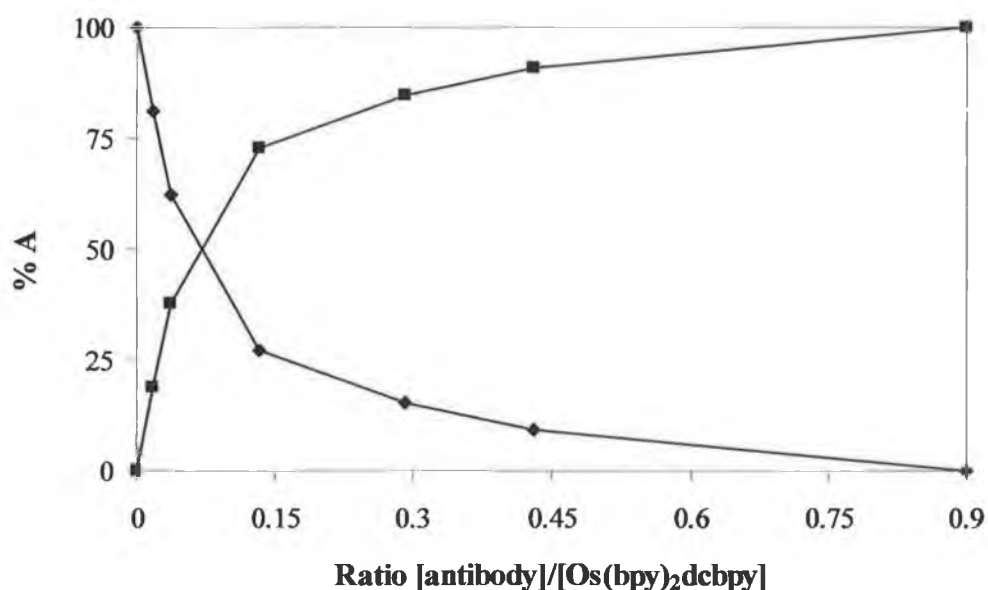


Figure 3.12. Titration of [Os(bpy)₂dcbpy] with increasing concentration of antibody, showing the relative % of the short (decreasing) and long (increasing) lifetime components of the biexponential decay. The relative % of each component was determined from the % A₁ and A₂ pre-exponential factors.

3.3.4 Solvent accessibility

The close approach of antibody and antigen on binding results in effective water elimination from the binding cleft, thereby altering the nature of the solvation of the complex. The linear dependence of the reciprocal lifetime (τ^{-1}) on the mole fraction of H₂O in H₂O/D₂O solutions of [Ru(bpy)₃]²⁺ has indicated that a H₂O-specific quenching process is involved (Hauenstein *et al.*, 1983). A probable explanation for a specific H₂O quenching process has been suggested in the case of [Os(bpy)₃]²⁺ (Masuda *et al.*, 1998). It has been reported that the overtones of the vibrationally excited states of H₂O lie close to the ³MLCT state of [Os(bpy)₃]²⁺ and are expected to act as energy accepting levels. Energy transfer via dipole-dipole interaction between the transition of the Os(II) complex and that of the included water will increase the k_{nr} .

The linear relationship between τ^{-1} and the mole fraction of H₂O in H₂O/D₂O mixtures allows one to calculate F, the fraction of complex not shielded from the aqueous phase by antibody binding. Measurements of the lifetime of the complex in deaerated solutions of H₂O-PBS (τ_H), D₂O-PBS (τ_D) and bound by antibody in H₂O-PBS (τ_{HA}) and D₂O-PBS (τ_{DA}) are required:

$$F = \frac{[\tau^{-1}_{HA} - \tau^{-1}_{DA}]}{[\tau^{-1}_H - \tau^{-1}_D]} \quad (3.6)$$

This method was developed by Hauenstein *et al.*, (1983) to measure solvent accessibility of Ru (II) and Os (II) polypyridyls in sodium lauryl sulphate micelles.

Lifetimes of free and antibody-bound complexes in deoxygenated D₂O are given in Table 3.5. As expected the lifetimes of [Os(bpy)₂dcbpy] and [Ru(bpy)₂dcbpy] increase significantly in D₂O, both by a factor of 2.3. Use of equation 3.6 determined that 82% of the antibody-bound [Os(bpy)₂dcbpy] was inaccessible to excited state deactivation via interaction with solvent, while 80% of antibody-bound [Ru(bpy)₂dcbpy] was protected. Again this shows little discrimination of the antibody between the Os and Ru metal centre. While this method confirms that the antibody significantly protects the complexes from solvent, it also indicates that other important contributory factors are involved in the enhancement of lifetime on antibody binding. For example the lifetime of

[Os(bpy)₂dcbpy] in PBS is 20 ns, which increased to 46 ns in D₂O/PBS, but the lifetime of antibody-bound [Os(bpy)₂dcbpy] was larger again in PBS at 77 ns. Other considerations include the protection by the antibody from O₂ quenching and a decrease in the mobility of the complex when bound to the high molecular weight antibody.

Complex	Lifetime (ns)
[Os(bpy) ₂ dcbpy]	46
[Os(bpy) ₂ dcbpy] + antibody	121
[Ru(bpy) ₂ dcbpy]	1177
[Ru(bpy) ₂ dcbpy] + antibody	2289

Table 3.5. Emission decay lifetimes for free and antibody-bound complexes in D₂O. All solutions were deaerated under argon. All lifetimes measured in 0.01 M D₂O-PBS, pH 7.4.

3.3.5 Investigation of energy transfer between antibody bound complexes

The antibody produced here was quite unique in that it could bind two different metal centered luminescent complexes which have the ability to undergo energy transfer with each other when placed in close proximity. This raised the question of whether binding to adjacent binding sites on the same antibody would provide sufficient proximity for energy transfer to occur from [Ru(bpy)₂dcbpy] to [Os(bpy)₂dcbpy].

The two possible quenching pathways which have been reported for [Ru(bpy)₃]²⁺ and [Os(bpy)₃]²⁺ are:



The first process involves an electron transfer process with the reduction of the ^{*}ML₃²⁺ excited state while the second describes an energy transfer process. Reaction rates in the order of 1.5 x 10⁹ M⁻¹s⁻¹ have been reported (Creutz *et al.*, 1980). The

electron transfer process is also called diffusional or dynamic quenching as it requires molecular contact. Resonance energy transfer (RET) describes a process in which a fluorescent donor molecule transfers energy via a non-radiative dipole-dipole interaction to an acceptor molecule and is a standard spectroscopic technique for measuring distances in the 10-80 Å range.

To investigate the possibility of energy transfer between [Ru(bpy)₂dcbpy] and [Os(bpy)₂dcbpy] when bound to two binding sites on the same antibody, a series of solutions were prepared in which the ratio of [Ru(bpy)₂dcbpy] to [Os(bpy)₂dcbpy] was varied in the presence of a constant concentration of antibody. The antibody concentration was estimated to be just sufficient to contain enough antibody binding sites for all the metal complexes present. Presumably, a number of the antibodies around the 50:50 ratio would have their two binding sites bound by a mixture of the complexes. To avoid mistaking energy transfer with diffusional quenching, a low concentration of metal complex was used so that the total concentration of both complexes present totalled to 1 µM in each solution. It can be seen from Figure 3.13 that at these concentrations the emission from [Ru(bpy)₂dcbpy] was not affected when [Os(bpy)₂dcbpy] was added. The graph also shows that the emission intensity of the antibody-bound [Ru(bpy)₂dcbpy] did not change in the presence of [Os(bpy)₂dcbpy] for any of the ratios used. This would indicate that energy transfer did not take place. The emission maximum of [Ru(bpy)₂dcbpy] on its own or when antibody-bound was unchanged when compared to the same solutions with [Os(bpy)₂dcbpy]. Lifetime analyses were performed on all of these solutions. In the absence of energy transfer a monoexponential decay of the antibody-bound Os complex and the antibody bound Ru complex would be expected. A monoexponential decay with a lifetime of an average of 1292 ± 29 ns was found in the solutions, which was comparable to that obtained previously for [Ru(bpy)₂dcbpy] (1299 ± 12 ns) when saturated with antibody in the absence of [Os(bpy)₂dcbpy] (Table 3.3). Unchanged lifetime of the antibody-bound [Ru(bpy)₂dcbpy] supports the luminescence evidence that detectable energy transfer did not occur.

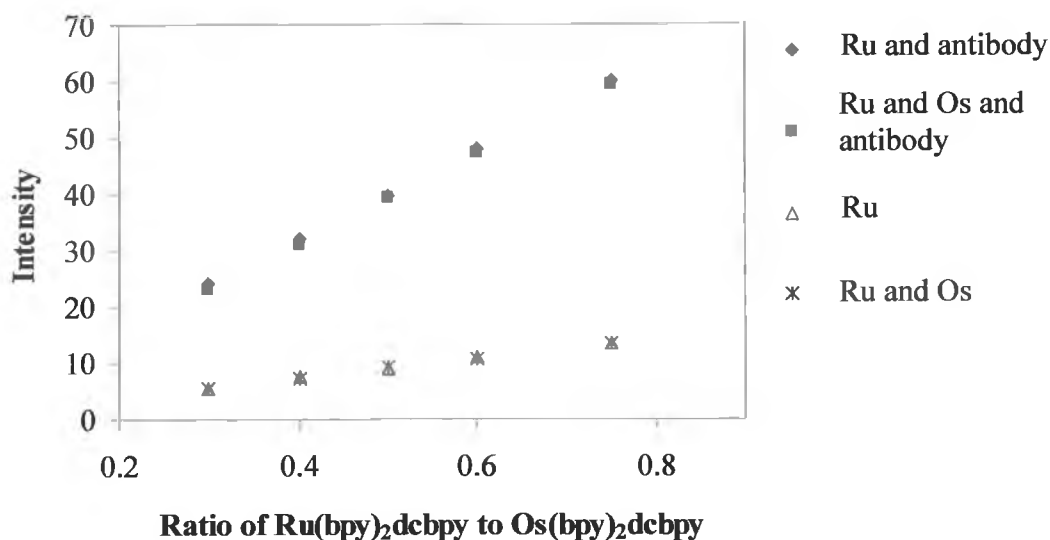


Figure 3.13. Emission intensity of $[Ru(bpy)_2dcbpy]$ (Ru) in a series of solutions in which the ratio of $Ru(bpy)_2dcbpy$ and $[Os(bpy)_2dcbpy]$ (Os) were varied in the presence and absence of a saturation concentration of antibody. Since the emission of antibody-bound $[Ru(bpy)_2dcbpy]$ did not show a decrease in the presence of $[Os(bpy)_2dcbpy]$, energy transfer was not observed.

The absence of energy transfer in this system may not be unexpected. The rate of energy transfer depends strongly on distance being inversely proportional to r^6 where r is the distance between the donor and acceptor. The distance at which RET is 50% efficient is called the Förster distance (R_0). The donor-acceptor distance must be comparable to R_0 for efficient RET. For example, it can be calculated that if r is twice the Förster distance the transfer efficiency is 1.56% (Lakowicz, 1999). Förster distances are typically in the range of 20-60 Å and in most cases are limited to 30 Å (Wu and Brand, 1994). Measurements of the distances between the antigen-binding sites of the antibody have resulted in values at least twice this. For example, Sosnick *et al.* (1992) used neutron scattering from deuterated antigens complexed with proteated IgG to measure the distance between the antigen binding sites of three different murine immunoglobulins (IgG subclasses 1, 2a and 2b). For all three IgG subclasses, the mean distance was

between 117 and 134 Å with a large variance of approx. 40 Å, indicating a high degree of flexibility of the Fab arms.

Similar studies of the investigation of energy transfer of antibody-bound luminescent complexes have been performed on a covalently-linked hybrid rabbit IgG containing one anti-lactose site and one anti-DNS site (5-dimethylaminonaphthalene-1-sulfonamido) (Ludedtke *et al.*, 1980). A hybridisation scheme was employed in which complete reoxidation of the L-H disulphide bond was retained and 80% reoxidation of the inter-heavy-chain disulphides. It is important that the inter-heavy chain disulphide bonds in the hybrid IgG were retained otherwise additional modes of flexibility will be imparted increasing the distance between the antigen binding sites. It was found that the fluorescence lifetime (24 ns) and quantum yield (0.57) of the bound DNS group was unaffected by the presence of the acceptor (derivatives of *p*-aminophenyl β-lactoside) in the adjacent binding site demonstrating the absence of energy transfer. It was estimated that the minimum distance of separation between antibody binding sites was in the range 5.5-7.0 nm. It is possible that energy transfer from IgG tyrosine and tryptophan residues to the bound Os(II) or Ru(II) complexes could occur. However this was not investigated here.

3.3.6 Interaction of antibody with self-assembled [Os(bpy)₂(p2p)₂](PF₆)₂

3.3.6.1 Preparation of electrodes

After polishing with 0.3 and 0.05 μm alumina the 12.5 μm radius platinum microelectrodes were cycled in 0.5 M H₂SO₄ to electrochemically clean the electrode surface by first oxidising and then reducing the surface of the platinum electrode. The current-potential curve for a platinum electrode in H₂SO₄ shows peaks for the formation and oxidation of both adsorbed hydrogen and oxygen (Bard and Faulkner, 2001). The anodic cycle builds an oxide film and is thought to destroy adsorbed organics, the cathodic cycle is thought to reduce the oxide to clean, active platinum (Faulkner, 1983). Well-defined hydrogen and oxygen peaks indicate a pristine surface. A typical CV is shown in Figure 3.14.

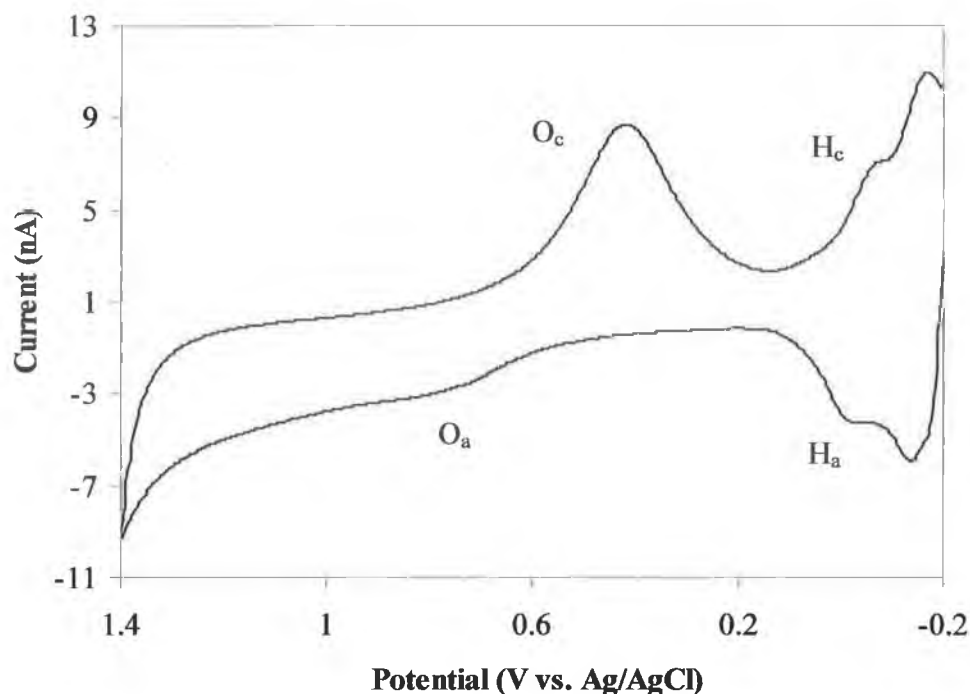


Figure 3.14. Cyclic voltammetry of a $12.5 \mu\text{m}$ radius platinum electrode in $0.5 \text{ M H}_2\text{SO}_4$ with a scan rate of 0.5 Vs^{-1} . The limits are -0.2 V and 1.4 V with the initial potential set at -0.2 V . Peak H_c shows formation of adsorbed hydrogen; H_a oxidation of adsorbed hydrogen; O_a formation of adsorbed oxygen or platinum oxide layer and O_c reduction of the oxide layer.

Cycling in H_2SO_4 also provides a means of determining the real or microscopic area of the electrode surface based on either oxygen or hydrogen adsorption. The oxygen adsorption method was used here. Determining the electrode area is based on the assumption that oxygen is chemisorbed in a monoatomic layer prior to O_2 evolution with a one-to-one correspondence with surface metal atoms (Trasatti *et al.*, 1992). The charge (Q_0) associated with the formation or reduction of the layer is:

$$Q_0 = 2eN_A\Gamma_0A \quad (3.9)$$

where N_A is the Avogadro constant, Γ_o , the surface concentration of atomic oxygen assumed to be equal to the surface density of metal atoms and A is the true surface area. The true surface area (A) can then be derived from:

$$A = \frac{Q_o}{Q_o^s} \quad (3.10)$$

The accepted reference value for Q_o^s for polycrystalline platinum is $420 \mu\text{C cm}^{-2}$ (Trasatti *et al.*, 1992). Only electrodes with a surface roughness of less than 2 were used, typically the surface roughness was between 1.4 and 1.7. After the electrochemical areas were measured, the electrodes were cycled in 0.1 M LiClO_4 to desorb the adsorbed hydrogen. The electrodes were then ready to form self-assembled layers.

3.3.6.2 Formation of monolayers

Figure 3.15 shows a cyclic voltammogram of a monolayer of $[\text{Os}(\text{bpy})_2(\text{p2p})_2](\text{PF}_6)_2$ self assembled on a platinum microelectrode through a pendent nitrogen of the p2p ligand. Monolayers were formed by depositing the electrode overnight in a 1 mM solution of $[\text{Os}(\text{bpy})_2(\text{p2p})_2](\text{PF}_6)_2$ in 2:1 $\text{H}_2\text{O}:\text{DMF}$. The $E_{1/2}$ for a monolayer in 0.01 M PBS, pH 7.4 was found to be $0.581 \pm .008 \text{ V}$. The surface coverage (Γ) was determined by integrating the area under the cathodic voltammetric peak. This represents the charge associated with the reduction of the adsorbed species and can be used to calculate Γ according to:

$$\Gamma = \frac{Q}{nFA} \quad (3.11)$$

where Q is the charge under cathodic peak in coulombs, n is the number of electrons transferred, F is Faraday's constant and A is the true surface area. The surface coverage of a monolayer of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ was found to be $1.1 \pm 0.15 \times 10^{-10} \text{ mol cm}^{-2}$. This compared well with that reported for similar complexes. Typical monolayer surface coverages for complexes with the form $[\text{Os}(\text{bpy})_2(\text{LCl})]^+$ or $[\text{Os}(\text{bpy})_2(\text{LL})]^{2+}$ where L is a pyridine-type ligand such as 4,4-trimethyllenedipyridine (p3p) (Forster and Kelly,

1996) or trans-1,2-bis(4-pyridyl)ethylene (bpe) (Forster *et al.*, 2000) have been reported to be between $0.7 - 1.1 \times 10^{-10} \text{ mol cm}^{-2}$.

The voltammetric response of self-assembled monolayers of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ is consistent with that expected for an electrochemically reversible reaction involving a surface-confined species. For example, the peak height scaled linearly with scan rate, at least over the range 0.5 to 20 Vs^{-1} (Figure 3.16). The slopes are similar indicating a highly reversible system. This is in contrast to the $v^{1/2}$ dependence expected for the complex in solution.

Where there are no lateral interactions between surface-confined redox centres and a rapid equilibrium is established with the electrode, a zero peak to peak splitting and a full width at half maximum (fwhm) of 90.6 mV are expected for a reaction involving the transfer of a single electron. A non-zero peak-to-peak splitting was observed for a monolayer of adsorbed $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ even at low scan rates e.g., ΔE_p was $17 \pm 4 \text{ mV}$ at 0.5 V s^{-1} . The fwhm for a monolayer ranged between 135 and 165 mV . Non-zero peak-to-peak splitting and larger fwhm are features of these types of monolayers. This can be explained by adsorbate-adsorbate interactions. Assemblies of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ have a $+3$ charge in the oxidised form and a $+2$ charge in the reduced form. This results in repulsions which are larger in the oxidised form than in the reduced form.

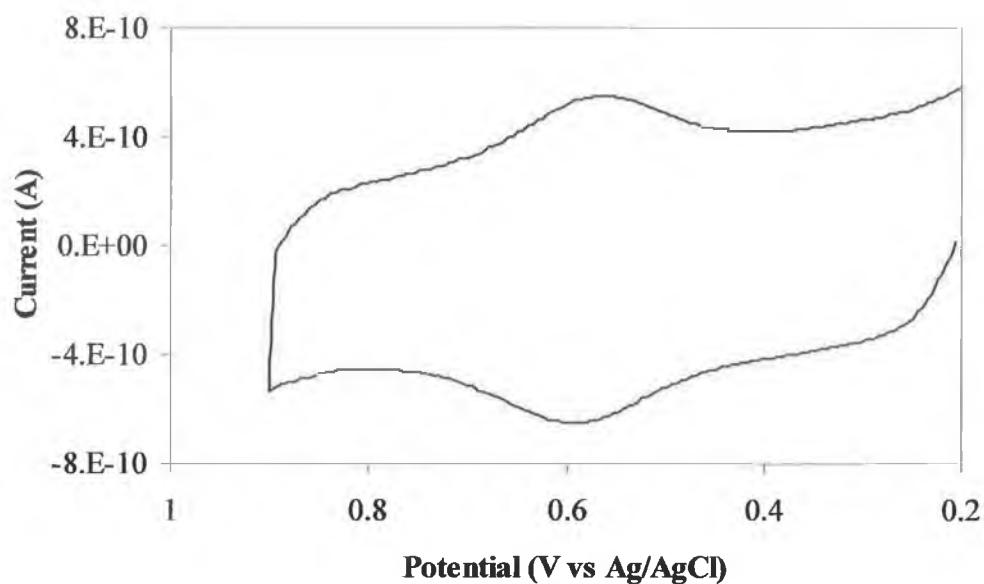


Figure 3.15. CV of self-assembled monolayer of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ ($0.97 \times 10^{-11} \text{ mol cm}^{-1}$) on a $12.5 \mu\text{m}$ radius microelectrode. The electrolyte solution was 0.01 M PBS , $\text{pH } 7.4$ at a scan rate of 0.5 V s^{-1} . Cathodic peaks are positive and anodic peaks are negative.

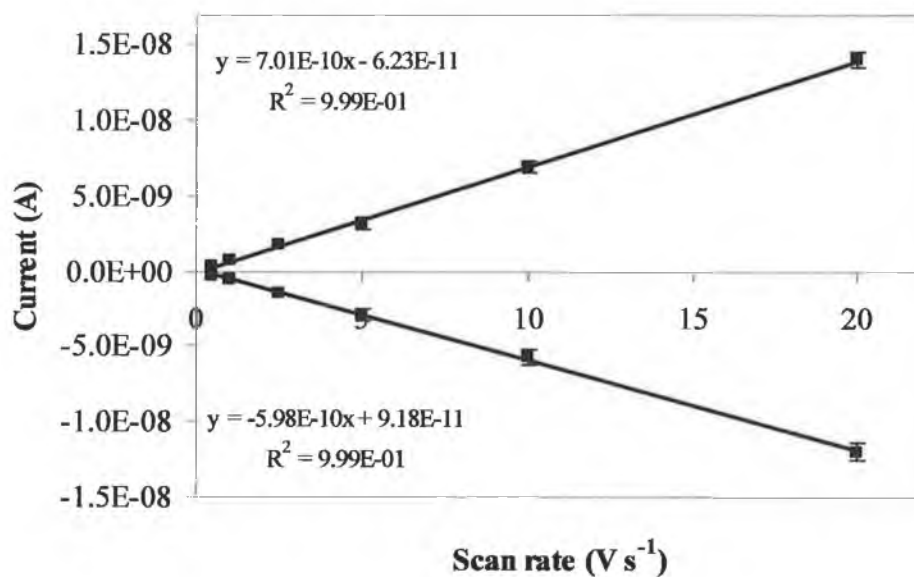


Figure 3.16. Scan rate dependence of a monolayer of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ up to 20 V s^{-1} deposited on a $12.5 \mu\text{m}$ radius platinum microelectrode. The electrolyte solution was 0.01 M PBS , $\text{pH } 7.4$.

3.3.6.3. The effect of antibody binding to $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ monolayers

Ruthenium(II) and osmium(II) polypyridyl complexes in self-assembled films act as probes of local microenvironment as their half wave potential is sensitive to the physicochemical surroundings. The half wave potential of self assembled monolayers of osmium(II) polypyridyl complexes have previously been found to be sensitive to the monolayer surface coverage, the concentration and type of electrolyte and the solvent (Acevedo *et al.*, 1991). Monolayers with higher surface coverage have a more positive $E_{1/2}$ due to increased coulombic repulsions among the headgroups making oxidation more difficult. Ion pair formation between the monolayer and electrolyte anion results in partial neutralisation of the layer thereby minimising repulsion between sites. The solvent can effect intermolecular interactions in two ways. One of these is the screening of charge between headgroups resulting in less repulsion between sites. This effect is more pronounced in solvents with higher dielectric constants. However, the formation of ion pairs is more pronounced in solvents with low dielectric constants.

The effect of antibody binding to $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ is reduced by its structural differences to the hapten $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ as evidenced by the smaller K_a value. In section 3.3.2 the K_a of the antibody towards $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ was determined as $5.6 \times 10^8 \text{ M}^{-1}$ and that towards $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ was $3.6 \times 10^5 \text{ M}^{-1}$. However, as noted earlier there was sufficient binding in solution to result in a 5.6-fold increase in luminescence and a 3.1-fold increase in lifetime. The electrochemical effect of antibody binding on the self-assembled complex may also depend on the degree that the solvation shell of the complex has already been altered on adsorption to the electrode surface. It has been suggested that the redox centres within the monolayer are solvated to a large extent since only a 20-30 mV difference between the half wave potentials of the solution phase and self-assembled monolayer complex are observed (Forster and O'Kelly, 1996).

The self-assembled $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ electrodes were incubated in a 1 mg ml^{-1} solution of antibody for 30 min. A CV was performed in 0.01 M PBS, pH 7.4, before and after antibody incubation. It was found that monolayers with full surface coverage ($1.1 \times 10^{-10} \text{ mol cm}^{-2}$) experienced no significant change in the appearance of the CV with addition of antibody. This may indicate that the densely packed monolayer at full surface

coverage sterically hinders antibody binding. However, submonolayer coverages in the region of $6.7 \pm 0.5 \times 10^{-11} \text{ mol cm}^{-2}$ resulted in a shift to a more positive potential of $18 \pm 9 \text{ mV}$ at 0.5 Vs^{-1} after antibody incubation with an average increase in the fwhm of 14 mV (Figure 3.17). These changes would imply greater repulsive interactions and increased difficulty in the oxidation of the self-assembled complex. Possible reasons for the slight changes in the CV may be due to alterations in the solvation shell of the antibody-bound complex or to the ability of the antibody to limit anion pairing of the electrolyte. When an even lower submonolayer coverage of $4.5 \times 10^{-11} \text{ mol cm}^{-2}$ was incubated with antibody, no significant change in the CV was noted (Figure 3.18). This showed that the changes in the CV observed in Figure 3.17 appeared to depend on the amount of complex assembled.

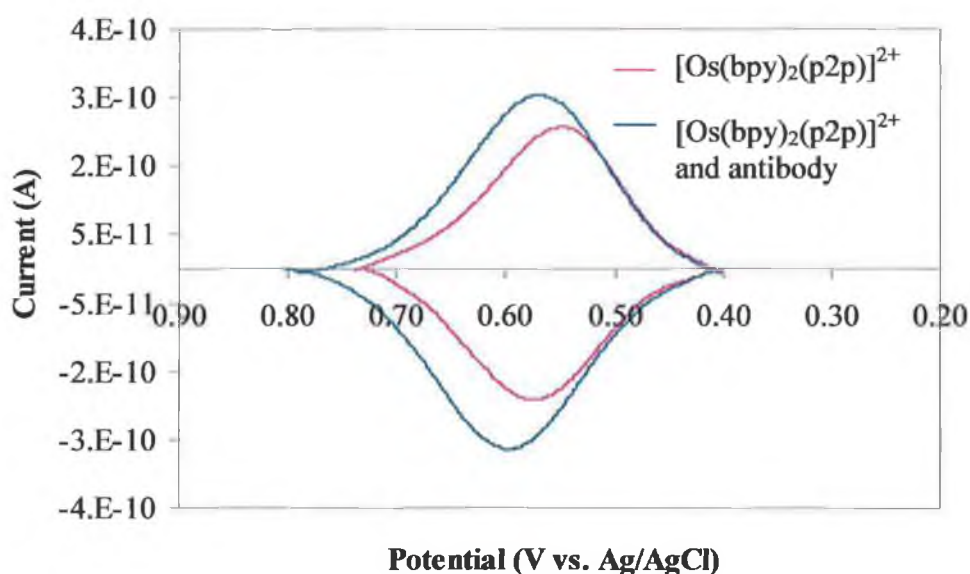


Figure 3.17. Background corrected CV of self-assembled submonolayer ($6.2 \times 10^{-11} \text{ mol cm}^{-2}$) of $[\text{Os}(\text{bpy})_2(\text{p2p})]^{2+}$ on a $12.5 \mu\text{m}$ radius microelectrode before and after incubation in antibody. The electrolyte solution was 0.01 M PBS , $\text{pH } 7.4$ at a scan rate of 0.5 V s^{-1} . Cathodic peaks are positive and anodic peaks are negative.

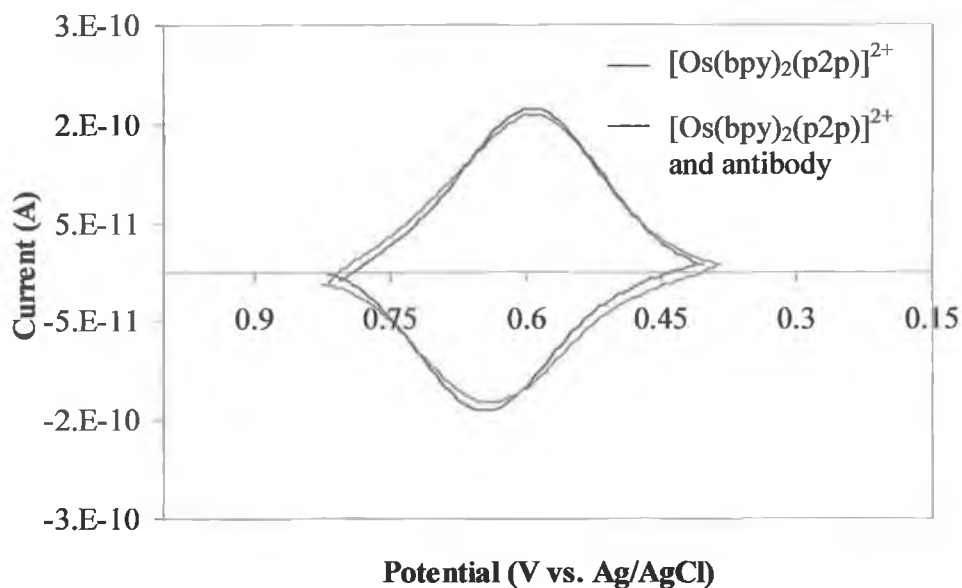


Figure 3.18. Background corrected CV of self-assembled submonolayer ($4.5 \times 10^{-11} \text{ mol cm}^{-2}$) on a $12.5 \mu\text{m}$ radius microelectrode before and after incubation with antibody. The electrolyte solution was 0.01 M phosphate, $\text{pH } 7.4$ at a scan rate of 0.5 V s^{-1} . Cathodic peaks are positive and anodic peaks are negative.

3.3.6.4 Determination of the standard rate constant

The possible effects of antibody binding on the self-assembled $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ properties were further investigated by determining the standard rate constant (k^0) before and after antibody incubation. k^0 is a measure of the kinetic facility of a redox couple. For example, a system with a large rate constant achieves equilibrium in a short time-scale and may describe a process involving only electron transfer and resolution. In this study, the Laviron method (Laviron, 1979) was used to estimate k^0 of $[\text{Os}(\text{bpy})_2(\text{p2p})_2](\text{PF}_6)_2$ when self-assembled on a platinum electrode with and without the presence of antibody. This method is based on the following equation;

$$k = \frac{\alpha n F v_c}{RT} = \frac{(1-\alpha) n F v_a}{RT} \quad (3.12)$$

where α is the transfer coefficient, n is the number of electrons transferred in the redox reaction, v_c and v_a are the scan rates where the cathodic and anodic points intersect the horizontal ($E_{1/2}$) and R , T , and F have their usual meaning. The transfer coefficient α is assumed to be 0.5. A plot of the peak potential of the anodic and cathodic waves as a function of $\log v$ is drawn.

Figure 3.19a shows a graph of E_p vs. $\log$ of the scan rate for a sub monolayer ($6.7 \pm 0.5 \times 10^{-11} \text{ mol cm}^{-2}$) of $[\text{Os}(\text{bpy})(\text{p2p})_2]^{2+}$. Both the cathodic and anodic lines intersect the horizontal ($E_{1/2}$) at a $\log v$ value of 2.4. When the antilog of this number (251 Vs^{-1}) was substituted into Equation 3.12, the rate constant was determined as $(4.9 \pm 0.2) \times 10^3 \text{ s}^{-1}$ for both the cathodic and anodic processes. In the presence of antibody, the rate constant was determined as $(2.5 \pm 0.2) \times 10^3 \text{ s}^{-1}$ and $(4.4 \pm 0.2) \times 10^3 \text{ s}^{-1}$ for the cathodic and anodic processes, respectively (Figure 3.19b). It would appear from these measurements that antibody binding has not significantly influenced electron transfer from the metal centre to the electrode or the resolution processes involved. A series of experiments on monolayers of $[\text{Os}(\text{bpy})_2(\text{pNp})(\text{Cl})]^+$, where pNp is 1,2-bis(4-pyridyl)ethane or 4,4'-trimethylenedipyridine, has shown that electrode kinetics are influenced by solvent but not significantly by ion-pairing over the range of supporting electrolyte of concentration 0.1 M to 1 M (Forster and Faulkner, 1994). This may indicate that possible effects of antibody binding on ion-pairing may not be reflected in the rate constant. Also, the effects of antibody binding on the rate constant may be limited by the lower affinity of the antibody for this complex.

These values are slower than the rate of $2.5 \times 10^4 \text{ s}^{-1}$ that has been obtained for a similar complex $[\text{Os}(\text{bpy})(\text{p3p})_2]^{2+}$ where p3p is 4,4'-trimethylenedipyridine in 0.1 M LiClO_4 , using potential step chronoamperometry with an overpotential of 50 mV (Forster and O'Kelly, 1996). One of the possible sources of error in the rate constant values obtained here is iR drop. When Faradaic and charging currents flow through a solution, they generate a potential that acts to weaken the applied potential by an amount iR , where i is the total current and R is the cell resistance. Reduced ohmic effects are achieved at smaller electrodes since although the resistance increases with decreasing radius the current decreases as the square of the radius. For example using a $12.5 \mu\text{m}$ radius platinum electrode at $5,000 \text{ V s}^{-1}$, it was estimated that the iR drop was 11%. Another

source of error is that the Laviron method assumes a Langmuir isotherm i.e. that there are no interactions among the adsorbed particles. This is not the case here since ΔE_{fwhm} is much larger than 90.6 mV.

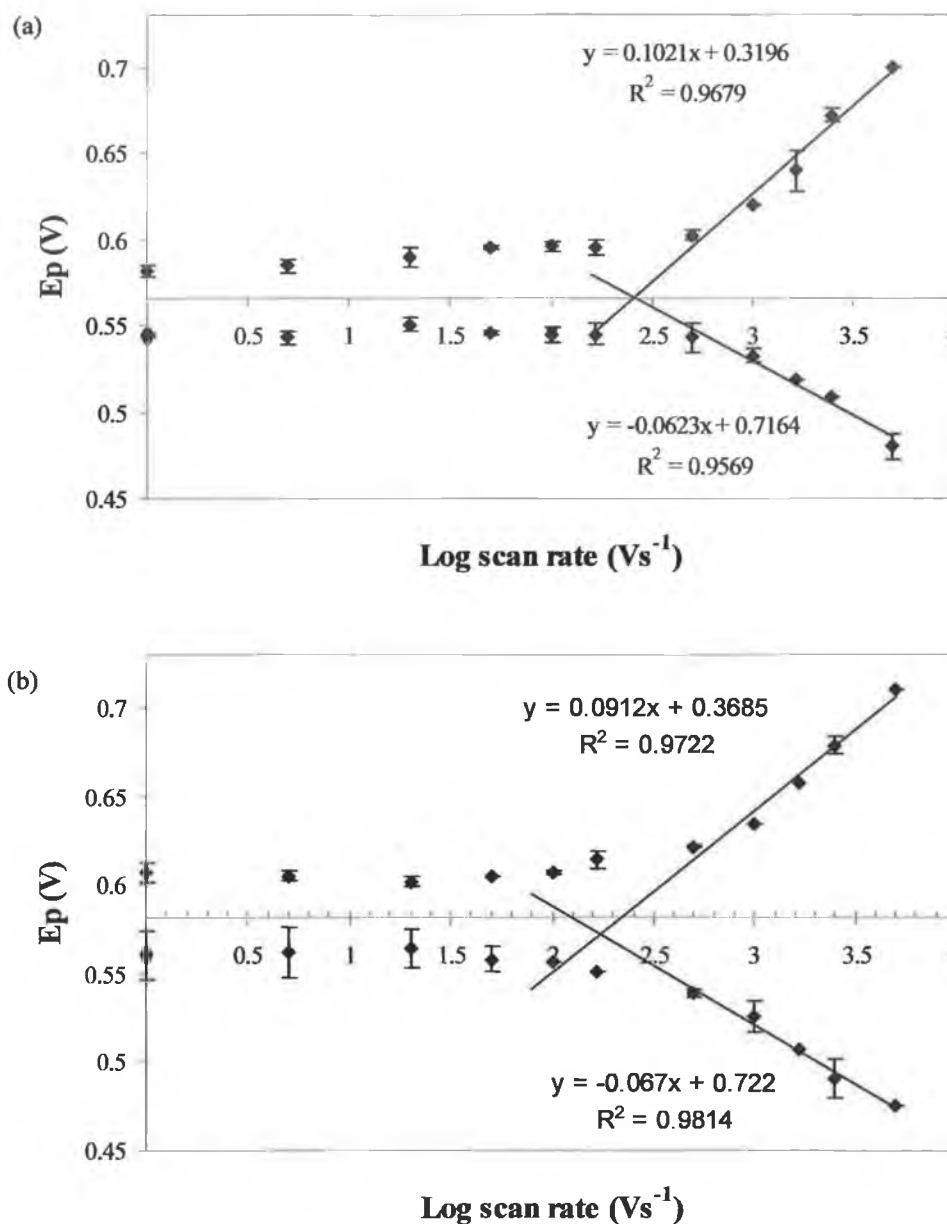


Figure 3.19. Laviron model for the determination of the heterogeneous electron transfer rate constant for $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]^{2+}$ ($\Gamma = 6.7 \pm 0.5 \times 10^{-11} \text{ mol cm}^{-2}$) adsorbed on a 12.5 μm radius platinum microelectrode (a) in the absence and (b) presence of 1 mg ml^{-1} antibody. The electrolyte is 0.01M PBS, pH 7.4.

3.4 CONCLUSIONS

Binding of the polyclonal antibody raised against $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ substantially changed the emission intensity and wavelength maxima and lifetime of all the MLC measured. The integrated emission increase varied from 7.2 for the high affinity binding of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ to 5.6 for the lower affinity binding of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$. A 3.9 increase in emission intensity was found on antibody binding of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$. All of the complexes experienced a blue shift of wavelength of emission maxima varying from 37 nm in the case of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ to 13 nm on antibody binding of $[\text{Ru}(\text{bpy})_3]^{2+}$. The lifetime increased by at least a factor of 2.3 for all the complexes measured with the largest increase experienced by $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ with a 3.7-fold increase.

A reduction of the hydration of the complex upon antibody binding was found to be a contributing factor to the changes in the spectrochemical properties. Lifetime measurements in deaerated H_2O -PBS and D_2O -PBS confirmed that 82% of the antibody-bound $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ was inaccessible to excited state deactivation via interaction with solvent, while 80% of antibody-bound $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ was protected.

Association constants were derived from the luminescence titration data and were determined to be 5.6×10^8 and 5.0×10^8 for $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$, respectively, 3×10^8 for $[\text{Ru}(\text{bpy})_3]^{2+}$ and 3.6×10^5 for $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$. The K_a values decrease with increasing structural difference of the complex towards the hapten $[\text{Os}(\text{bpy})_2\text{dcbpy}]$. This confirms the importance of a close steric fit for high affinity antibody-antigen binding.

The distances between the antigen binding site of the antibody are too large to permit effective energy transfer between $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ when bound to the antibody.

The effect of antibody binding on $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ self-assembled on platinum microelectrodes was investigated. Changes in the CV appeared to depend on the amount of complex assembled. Submonolayer coverages in the region of $6.7 \pm 0.4 \times 10^{-11} \text{ mol cm}^{-2}$ resulted in a shift to a more positive potential of $18 \pm 9 \text{ mV}$ at 0.5 Vs^{-1} in the presence of antibody with an average increase in the fwhm of 14 mV. These changes imply increased difficulty in the oxidation of the self-assembled complex. Possible

reasons for the slight changes in the CV may be due to alterations in the solvation shell of the antibody-bound complex or to the ability of the antibody to limit anion pairing of the electrolyte.

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CHAPTER 4

The development of a mediated amperometric immunosensor based on carbon nanotube forests

4.1 INTRODUCTION

Carbon nanotubes (CNTs) are new and exciting members of the carbon family potentially offering unique structural, electronic and mechanical properties. Their nanometer dimensions, combined with high surface area and electrical conductivity promise excellent construction materials for nanoscale sensors. Among the anticipated applications of CNTs is their use as components in biological devices. They have been shown capable of direct electron transfer to and from redox proteins (Xue *et al.*, 2003, Yamamoto *et al.*, 2003) and have allowed improved electrochemical communication with a growing number of redox active biomolecules (Musameh *et al.*, 2002, Wang *et al.*, 2002a). This ability to behave as a molecular wire may potentially serve to simplify sensor construction since mediating materials could be minimised. The structure-dependent metallic character of CNTs allows them to promote electron transfer reactions at low overpotentials (Sotiropoulou *et al.*, 2003). Low operational potential is preferable to minimise interference from electroactive species.

The use of CNT-modified electrodes has resulted in the improved electrochemical communication of redox proteins. Previously when single wall nanotubes (SWNTs) were assembled on pyrolytic graphite and HRP or myoglobin immobilised a limit of detection of H_2O_2 of 70 nM and 50 nM, respectively was obtained (Yu *et al.*, 2003). Presented here is an extension of that work, whereby amperometric immunosensing based on the SWNT forest assembly technique previously developed is evaluated. The immunosensing strategy tested involved the adsorption of anti-biotin antibody to the carbon nanotube surface. The presence of HRP-labelled biotin was determined via the reduction of hydrogen peroxide in the presence of the soluble mediator hydroquinone. The ability of a mutant catalase negative *E. coli* bacteria to produce H_2O_2 was also investigated by immobilisation of the *E. coli* cells with HRP to the SWNT forest.

This Chapter begins with a review of the unique structure and distinguishing properties and synthesis of CNTs. The application of CNTs thus far in biosensing is illustrated including their interaction with redox proteins, DNA and other physiologically important biomolecules such as NADH, ascorbic and uric acid.

The experimental work described in this Chapter was performed in the Department of Chemistry at the University of Connecticut under the supervision of Prof. James Rusling and in collaboration with the Nanomaterials Optoelectronics Laboratory also at the University of Connecticut.

4.1.1 THE STRUCTURE AND PROPERTIES OF CARBON NANOTUBES AND THEIR APPLICATION IN BIOSENSING

4.1.1.1 Structure

Carbon nanotubes are cylindrical structures composed of a hexagonal lattice of sp^2 hybridised carbon atoms (Harris, 1999). Hexagons and pentagons form the end cap structures. It can be derived from Euler's theorem that 12 pentagons are needed to completely close the hexagonal lattice (6 in each cap). There are various ways in which the hexagons can be arranged around the circumference. These include armchair, zigzag and chiral or helical structures. The armchair structure is characterised by hexagons with two sides perpendicular to the tube axis whereas zigzag structures have the two sides parallel. In chiral nanotubes each pair of the hexagon sides is arranged at an angle other than 0 or 90° relative to the nanotube axis. This subtle difference in configuration is important as nanotube properties change as a function of helicity and tube diameter.

SWNT are single layered cylinders with diameters that range narrowly between 1 and 2 nm. Multi-wall nanotubes (MWNT) are made of concentric cylinders placed around a common central hollow. These range in outer diameter from about 2.5-30 nm. Each MWNT "needle" can contain typically between 4 and 25 layers. MWNTs have an interlayer spacing of 0.34-0.36 nm, which is close to the typical atomic spacing of graphite (Mamalis *et al.*, 2004). The small diameter and long length of both MWNTs and SWNTs leads to very large aspect ratios that make them almost one-dimensional structures (Ajayan *et al.*, 1999). SWNTs appear to have a higher degree of structural perfection, whereas MWNTs can suffer frequent defects along their length which limits their properties (Colbert *et al.*, 1999). Moreover SWNTs can become defect-free after high-temperature annealing in inert media (Rakov *et al.*, 2001).

Nanotubes tend to form relatively stable aggregates (also referred to as bundles or ropes), in which the axes of individual nanotubes are parallel to one another, the shortest distance between them being ~ 0.32 nm (Thess *et al.*, 1996). These aggregates arise due to Van der Waals forces.

4.1.1.2 Distinguishing properties

The combination of dimension, structure and topology of carbon nanotubes confers a range of superior properties such high electrical conductivity and mechanical strength. As mentioned previously, the electronic properties of CNT depends on their helicity and diameter (Avouris, 2002). Nanotube folding can be defined by the chirality vector $C = na_1 + ma_2 \equiv (n, m)$ where a_1 and a_2 are the unit vectors of the hexagonal lattice. When a point B is brought over a point A, a tube of circumference C is generated. The differences in conducting properties are caused by the molecular structure that results in a different band structure and thus a different band gap. The general rule is that the tubes exhibit metallic or semiconducting behaviour, depending on (n,m). Calculations have predicted that all the armchair tubes are metallic ($n=m$). Zigzag and helical tubes are either metallic or semi conducting (tubes with $n-m = 3i$, where i is an integer, have a small curvature-induced band gap and CNT with $n-m \neq 3i$ are semi-conducting). As the diameter of the tubes increase, the bandgap (which varies inversely with the tube diameter) tends to zero, yielding a zero-gap semiconductor electronically equivalent to the planar graphene sheet. The one-dimensional (1D) nature of the electron system in the nanotubes has been confirmed by resonant Raman scattering experiments (Rao *et al.*, 1997). In addition, scanning tunneling microscopy has allowed the measurement of the density of electronic states (DOS), tube diameter and helicity to be performed simultaneously (Wilder *et al.*, 1998). Sharp peaks in the DOS were observed, which are characteristic of the 1D nature of conduction within a 1D system. A 1D system implies drastically reduced carrier scattering and the possibility of ballistic devices (Avouris *et al.*, 2002). In ballistic transport the electrons can be considered as moving freely through the structure, without any scattering from atoms or defects. The reduced scattering in metallic CNTs means they can carry enormous current densities up to 10^9 A cm⁻² without being destroyed. This

density is about 2-3 orders of magnitude higher than is possible in metals such as aluminum or copper.

Carbon nanotubes are characterised by high mechanical strength making them one of the strongest and stiffest materials known (Ajayan, 1999). This is due to their low density of defects and the intrinsic strength of the carbon-carbon sp^2 bond which gives the nanotube a large Young's modulus value similar to that of graphite in-plane (~ 1000 GPa) (Salvetat-Delmotte *et al.*, 2002). A comparison of the Young's modulus of MWNTs grown by the arc discharge method and by the catalytic decomposition of hydrocarbons found that the latter was smaller by one to two orders of magnitude (Salvetat-Delmotte *et al.*, 2002). This confirms that only highly ordered and well-graphitised nanotubes have stiffness comparable to graphite. Tensile load experiments have been performed for MWNT (Yu *et al.*, 2000a) and SWNT (Yu *et al.*, 2000b) ropes reporting tensile strengths in the range of 11 to 63 GPa. In comparison, high strength steel alloys break at about 2 GPa.

The hollow structure and closed topology of nanotubes produces a distinct mechanical response compared to other graphitic structures. In simulations when carbon nanotubes are subjected to large deformations, they switch into different shapes that come with an abrupt release of stress-energy (Salvetat-Delmotte *et al.*, 1999). The bending is fully reversible up to very large bending angles despite the occurrence of kinks and highly strained tubule regions. This flexibility property stems from the ability of the sp^2 network C-C to reversibly change hybridisation when deformed out of plane. The higher the curvature, the stronger the sp^3 character of the C-C bonds in the deformed region.

4.1.1.3 Synthesis

Several techniques exist for the production of carbon nanotubes. Three of the main methods are electric arc discharge, laser ablation and chemical vapour deposition.

Arc discharge is the classic method as used by Iijima in the discovery of MWNTs (Iijima, 1991). This method was initially used for the synthesis of C_{60} fullerenes. CNTs are created through arc-vaporisation of two C rods placed end to end, separated by

approx. 1 mm in an enclosure that is filled with inert gas at low pressure (between 50 and 700 mbar) (Ebbesen *et al.*, 1992). A direct current of 50 to 100 A driven by approx. 20 V creates a high temperature discharge between the two electrodes. The discharge vaporises one of the carbon rods and forms a small rod-shaped deposit on the other.

Numerous variables govern the yield and quality of CNTs produced by this method (Rakov, 2000). These involve the main arc discharge variables (the voltage between the electrode, the current strength and density, temperature of the plasma) and also characteristics of the inert gas, dimensions of the reacting chamber and configuration of cooling devices.

SWNTs can also be made by this method. In 1993, Iijima and Ichicashi and Bethane *et al.* independently discovered SWNTs while attempting to produce metallofullerenes in an arc discharge. To synthesize SWNTs the anode must be doped with a metal catalyst. Several metals (Fe, Co, Ni, Y or Mo) and combinations of metals have been tried. Good yields have been obtained from mixtures of Ni-Y and Co-Y with the content of SWNT in the deposits reaching 70-90% (Journet *et al.*, 1997).

The mechanism of the catalytic action of metals in the formation of single-walled nanotubes implies adsorption of C atoms on the surface of metal particles and their free migration over the surface to the base of a growing nanotube (Maiti *et al.*, 1997). This mechanism has been shown probable by molecular modeling. It has been reported that this theory may explain why MWNTs are not formed in the presence of catalytic additives (this would require cooperative processes, associated with consistent interaction of numerous particles), why SWNT have relatively small diameters and why mixed catalysts are often more efficient than one-component systems (due to the enhancement of carbon adsorption, to the change in the activation energy of the NT growth and to the formation of a surface with a large number of bulges).

The use of laser ablation for the growth of carbon nanotubes was first reported by Guo *et al.* (1995a). A graphite target rod was vaporised by a laser with the condensing carbon-metal vapour swept down a heated flow tube, depositing on the cooler walls downstream. This group also proved that by using a target rod composed of graphite and various metals, SWNTs could be synthesised with this method (Guo *et al.*, 1995b). Further optimisation of the process was reported giving SWNT yields of 70-90% of the

carbonaceous material in the samples (Thess *et al.*, 1996). This method involved the initial laser pulse being followed 50 ns later by a second pulse to provide a more uniform evaporation of the graphite/cobalt/nickel target. The SWNTs obtained were very uniform in diameter (1.38 nm) and had a greater tendency to form aligned bundles (100-500 SWNT) than those prepared using arc evaporation. Rinzler *et al.* (1998) attempted to scale up this laser-assisted production by altering the flow tube dimensions. This resulted in 1 g per day production with the SWNT yield ranging from 60 to 90% of the carbonaceous product.

Laser ablation is similar to arc discharge since the optimum background gas and catalyst mix is the same as in the arc discharge process. In contrast to the arc method, direct laser vaporisation allows for greater control over growth conditions, permits continuous operation and produces nanotubes in higher yields and of better quality (Guo *et al.*, 1995b). A disadvantage of this method is the use of expensive lasers.

In 1996 a chemical vapour deposition (CVD) method emerged as a new candidate for large scale nanotube synthesis (Li *et al.*, 1996). In this process a gaseous carbon source such as methane, ethylene, acetylene or carbon monoxide is introduced into a reaction chamber. An energy source such as a plasma or a resistively heated coil is used to decompose the hydrocarbon at temperatures between 700-900 °C. The carbon diffuses towards the substrate, which is heated and coated with catalyst. Catalysts commonly employed include first row transition metals such as Ni, Fe or Co. A perpendicularly aligned film of CNTs are formed on the substrate. Substrates used include silicon, glass and alumina. These can be coated with catalysts by solution deposition, electron beam evaporation or physical sputtering. Chemical etching or thermal annealing is used to induce catalyst particle nucleation. Advantages of CVD are the excellent alignment and positional control on the nanometer scale. Control over the diameter of both MWNT and SWNT, as well as the growth rate of the nanotubes can be maintained (Daenen *et al.*, 2003). The appropriate metal catalyst can preferentially grow single rather than multi-walled nanotubes. This method produces nanotubes at much lower temperatures, although this is at the cost of lower quality. Typical yields for CVD are approximately 30%.

The SWNTs used in the immunosensor development described in the following section were produced by the high pressure CO disproportionation process (HiPCO). This technique involves the production of SWNTs in a continuous gas phase using CO as the carbon source and $\text{Fe}(\text{CO})_5$ as the iron-containing catalyst precursor (Nikolaev *et al.*, 1999). While in CVD methods, the metal catalysts are deposited on the substrate before the deposition of the carbon, the HiPCO process involves the introduction of both the catalyst and hydrocarbon in the gas phase into the furnace.

After production the CNT material contains many impurities. The main impurities are graphite sheets, amorphous carbon, metal catalyst and smaller fullerenes. Purification techniques used include oxidation, acid treatment, annealing, micro filtration and chromatography techniques (Rakov, 2000). For SWNTs, standard methods to eliminate catalyst particles and amorphous carbon involve re-fluxing the raw material in acid followed by centrifugation or cross-flow filtration. Depending on the acid used and exposure time this method can also result in shortened open tip SWNTs.

4.1.1.4 Ordered assembly of SWNT

Many of the preparation methods described above produce samples in which the nanotubes are randomly oriented. Although the tubes are often grouped into bundles, the bundles themselves are not generally aligned with respect to each other. The ability to assemble CNTs perpendicular to the surface is important as for most applications well ordered arrays of nanotubes are highly desirable. Catalytic methods for preparing aligned tubes have already been described, but techniques for aligning pre-synthesised samples of CNT are also required and have been reported.

A self-assembly method on gold was described by Liu *et al.* (2000). Acid pretreated and shortened SWNTs were thiol-derivatised when the carboxyl-terminated ends were reacted with $\text{NH}_2(\text{CH}_2)_2\text{SH}$ using the condensation reagent DCC. The ordered perpendicular orientation of the thiol-modified self-assembled SWNTs was confirmed by tapping mode AFM. The lengths of the self-assembled SWNTs were in the range 5-25 nm while the diameter of the bundles fell within the range 20-60 nm. It was predicted that there may be as many as eight thiol groups at each end of a 1.3 nm SWNT of (16,0)

zigzag structure. This resulted in a stable assembly resistant to desorption when ultra-sonicated. The bundle size of the SWNTs increased with increasing adsorption time. The larger nanotubes adsorbed more slowly than smaller ones. Disadvantages of this method were the long adsorption time needed and incomplete surface coverage obtained.

A later publication from this group used this technique to form patterned nanotube assemblies on gold surfaces (Nan *et al.*, 2002). To prepare a patterned nanotube assembly, the gold substrate was prepatterned with $\text{NH}_2(\text{CH}_2)_{11}\text{SH}$ and $\text{CH}_3(\text{CH}_2)_{11}\text{SH}$ SAMs using a microcontact printing technique before being subjected to the surface condensation reaction. Raman spectroscopy and AFM were used to confirm the presence of SWNTs on the substrate.

Yu *et al.* (2000) were the first group to report the use of metal-assisted assembly to align SWNTs normal to substrates. The first step of their method involved the formation of a monolayer of 11-mercaptoundecanoic acid [$\text{HS}(\text{CH}_2)_{10}\text{COOH}$] on a gold film. The monolayer was treated with a ZnSO_4 solution. Zinc carboxylate is expected to form $(\text{Zn}(\text{RCOOH})_2)$. When SWNTs were placed on the surface, new ZnO bonds with the COOH of the SWNT complete the assembly process. This assembly also produced a SWNT layer that was stable and resistant to ultra-sonication. The thickness and tilt angle of the SWNT layer was determined by angle-resolved X-ray photoelectron spectroscopy (ARXPS). It was found that at least two hours were needed to form a well-ordered monolayer of SWNTs. At longer adsorption times, the thickness of the SWNT layer increased and reached a saturated state. There was little difference in the film between 4 and 6.5 hours of immersion time. At these times the intensity of $\text{Zn}2p_{3/2}$ had reached its lowest value, the SWNT layer was the thickest (82 and 84 Å, respectively) and the tilt angle was in its lowest degree (38° and 36°). The diameters as measured by AFM fall into the range 15-50 nm. It was estimated that the tip broadening effect contributed significantly to this measurement and after deconvolution, a tube diameter of 1-2 nm was obtained which was consistent with the preassembled measurements obtained by high resolution electron microscopy.

Wu *et al.* (2001) proposed a technique for the assembly of oxidatively shortened SWNTs on a silver surface. Silver films on silicon wafer substrates were prepared by thermal evaporation of silver in a vacuum chamber and were then dipped into the

colloidal SWNT suspension. The absence of a $\nu(\text{C}=\text{O})$ peak and appearance of a new peak of $\nu_{\text{as}}(\text{COO}^-)$ in reflection absorption infrared spectroscopy showed that the oxidised SWNT were being adsorbed on silver surface through COOH groups by releasing a proton and forming a chemical bond between SWNTs and the silver/silver oxide. The AFM images suggested a perpendicular orientation with 85% of the SWNT falling into a 20-60 nm height range. The lateral dimensions were in the range of 80-300 nm. Further information was obtained from transmission electron microscopy images of the SWNT assembly on a silver wire. The typical lengths obtained from these images were between 60-150 nm and diameters between 2-15 nm. The majority (80%) of the SWNTs had a similar bundle size (6.5 ± 0.5 nm). This suggested that the silver surface can selectively immobilise the SWNTs into specific bundle sizes possibly related to the silver lattice structure.

Chattopadhyay *et al.* (2001) reported on a metal-assisted assembly of acid oxidised SWNT based on Fe^{3+} . The assembly was successfully performed on substrates such as glass, (100) silicon wafers with native oxide and gold quartz crystal microbalance resonators. The surface was coated with a polyelectrolyte film before Fe^{3+} was adsorbed. This was followed by immersion in DMF dispersed SWNT. The assembly mechanism was attributed to both metal-assisted chelation and electrostatic interactions. The elevated pH used for both the DMF washing step and SWNT dispersion was expected to cause the surface Fe^{3+} layer to transform into its basic hydroxide form, providing an initial driving force (acid-base neutralisation) for the assembly to occur. AFM characterisation showed a monolayer of densely packed, needlelike domains after 30 min immersion in SWNTs with an average domain width of 93 ± 22 nm. This increased to 387 ± 34 nm when the immersion time was increased to 4 hours. AFM and spectroscopic ellipsometry showed that an apparent increase in average film thickness with time is accompanied by a substantial enlargement in average domain width. However, QCM studies show that film density seemed to plateau after ~ 1 hour of immersion in SWNT. This may indicate that the increase in average thickness and domain width is most likely associated with a dynamic exchange between the shorter SWNTs that quickly diffuse and organise on the surface versus the longer ones, rather than a densification process. Polarisation Raman studies confirmed that the orientation was normal to the substrate with a 57-fold intensity

enhancement at 1593 cm^{-1} when polarisation of the incident light was parallel to the nanotube axis.

Cai *et al.* (2002) have reported on assemblies of SWNTs on functionalised self-assembled monolayers of thiolated conjugated oligo(phenyleneethynylene) on gold. A chemical and physical-based assembly method was outlined. Both involved ozonation pretreatment of SWNTs (O_3 -SWNT). This produced oxygenated functional groups including carboxylic acid, ester and quinine moieties. These can be removed from the surface by heating at $600\text{--}800^\circ\text{C}$. The chemical assembly was based on a condensation reaction between the carboxylic acid functionalities of O_3 -SWNT and the amine functionalities of SAMs to form amides. The physical adsorption method was based on layer-by-layer deposition with bridging of metal cations, i.e. Fe^{3+} on carboxylate-terminated SAMs or Cu^{2+} on thiol-terminated SAMs. In the case of the chemical assembly the condensation reaction and electrostatic force resulted in a stable and high density but parallel coverage of O_3 -SWNT on the surface. AFM images of metal-assisted deposition after 20 hours immersion in DMF solution of SWNTs indicated that O_3 -SWNT assembled perpendicular to the gold surface. AFM images of the Fe^{3+} -based SWNT assembly had SWNT widths between $35\text{--}55\text{ nm}$ and height between $30\text{--}45\text{ nm}$. Cu^{2+} -mediated SWNTs were between $70\text{--}120\text{ nm}$ in domain width and $20\text{--}45\text{ nm}$ in height. The coverage density was found to be lower when Cu^{2+} was used in the assembly process instead of Fe^{3+} .

The mechanism of assembly is as a result of coordination of the carboxylic groups of O_3 -SWNT to the Fe^{3+} ions on the modified SAM surface. This provides an initial nucleation core where strong hydrophobic attractive interactions between the walls of the O_3 -SWNTs occur. The surface-bound Fe^{3+} ions are in their hydroxylic forms in weak basic solution. The increased pH provides a driving force for acid base neutralisation with carboxylic O_3 -SWNT. Moreover, the chelation effect and electrostatic interactions facilitate the assembly.

Gooding *et al.* (2003) have also reported on the assembly of shortened carboxylic acid functionalised SWNTs. A self-assembled monolayer of cysteamine was formed on a gold electrode. This was then immersed in a DMF dispersion of SWNTs which had been treated with the condensation reagent DCC. This resulted in the alignment of SWNT

normal to the electrode surface. Direct electron transfer was observed from microperoxidase MP-11, a small redox protein obtained by proteolytic digestion of horse heart cytochrome C. The rate constant for electron transfer for the nanotube-modified electrodes as determined by the Laviron method, was similar to that for MP-11 attached directly to a cysteamine-modified gold electrode.

4.1.1.5 Immobilisation methods of biomolecules onto carbon nanotubes

Various methods have been used to attach biomolecules onto CNTs. These include hydrophobic or electrostatic interactions with the sidewalls (Balavoine *et al.*, 1999) or by functionalisation of the nanotube sides by hydrophobic molecules (Chen *et al.*, 2001) or a polymer coating (Chen *et al.*, 2003). It is important to functionalise the sidewalls in noncovalent ways to preserve the sp^2 nanotube structure and electronic characteristics. Attachment via covalent bonding with COOH functional groups obtained on the severed ends of acid-cut nanotubes also provides a convenient method (Xue *et al.*, 2003).

Balavoine *et al.* (1999) have presented a detailed study of the helical crystallisation of streptavidin when adsorbed onto the outer surface of MWNTs. Electron microscopy images showed that while most of the streptavidin molecules bound to the NT in a manner that did not form any ordered arrangements, in some instances the nanotubes showed lateral striations that were regularly spaced at 6.4 nm with an angle of 71° to the tube axis. Perpendicular striations were also visible, which suggested that the streptavidin molecules were organised in a square lattice. The outer diameter of the streptavidin-covered tube was constant at 27 ± 2 nm. This showed that the size of the tube was an essential parameter for helical organisation. The diameter of the underlying MWNT was about 16-17 nm. The 5 nm of protein layer is consistent with a monolayer of streptavidin. All the crystalline arrays observed the same helical repeat unit.

The helical organisation of HuPR were also found to occur. HuPR is a DNA binding protein produced by the photosynthetic bacterium *Rhodobacter capsulatus*. Ordered arrays were found on a wider range of MWNT diameters although a minimum value of about 12 nm was required. Striations that made an angle close to 81° with the tube axis were observed.

Glucose oxidase (GOD) has been adsorbed onto the surface of MWNTs. Sotiropoulou *et al.* (2003) reported that it is probable that upon oxidation of the MWNT array, the introduced carboxylic groups at the open ends provide a stabilising hydrophilic environment that allows for the adsorption of the enzyme while preserving its functionality.

Attachment of biomolecules via functionalisation of the sidewalls has been achieved using 1-pyrenebutanoic acid succinimidyl ester (Chen *et al.*, 2001). This group reported that it irreversibly adsorbed onto the sidewall of a SWNT via π -stacking and was highly stable against desorption in aqueous solutions. The succinimidyl ester groups of the reagent are highly reactive to nucleophilic substitution by primary and secondary amines that exist in abundance on the surface of most proteins resulting in the formation of an amide bond. The authors successfully attached both ferritin and streptavidin as confirmed from transmission electron microscopy (TEM) and AFM.

The irreversible adsorption of biomolecule conjugates of Tween 20 on nanotubes to prevent non-specific binding while at the same time enabling the binding of specific proteins of interest has been described (Chen *et al.*, 2003). Tween 20 is a surfactant comprising a linear aliphatic chain and three polyethylene oxide (PEO) branches. The PEO portion of the molecule renders the surface highly hydrophilic and charge neutral, thereby eliminating hydrophobic interactions and electrostatic binding with proteins. The Van der Waals interaction between the hydrophobic segment of the molecules and the nanotube sidewall is robust against desorption in aqueous solutions because of favourable hydrophobic-hydrophobic association. Biotinylation of the adsorbed Tween 20 was achieved by activating the hydroxyl termini of Tween towards nucleophilic addition with 1,1-carbonyldiimidazole.

4.1.1.6 Electrochemical behaviour of biomolecules on carbon nanotubes

To date, specific applications of CNTs in biosensing have mainly centered around the demonstration of improved electrochemical communication from redox proteins (Table 4.1), catecholamine neurotransmitters such as dopamine (Wang *et al.*, 2002b) and other physiologically important biomolecules such as uric acid, ascorbic acid (Wang *et al.*,

2002a) and NADH (Musameh *et al.*, 2002). Sensitive detection of target DNA has also been achieved (Wang *et al.*, 2003).

4.1.1.6.1 Carbon nanotubes and redox proteins

Table 4.1 summarises reports of the electrochemical activity of various redox enzymes on CNTs. CNTs displayed good communication with redox proteins where the redox active centre is close to the surface such as HRP (Yamamoto *et al.*, 2003) and Mb (Yu *et al.*, 2003) and also where it is embedded deep within the protein such as GOD (Xue *et al.*, 2003). The biosensor design outlined in these publications varies with respect to the electrode used, the deposition method of the nanotubes, the method of attachment of the biomolecule, or the use of a mediator. As discussed previously, the redox protein may be retained on the nanotube via adsorption or covalent attachment. Some of these reports use CVD or a self-assembly technique so that the CNTs are aligned perpendicular to the electrode surface. The other reports employed cast films of non-oriented single or multi-walled CNTs. Of these sensors the lowest detection limit for glucose was 10 μM and for H_2O_2 it was 50 nM.

<i>Nanotube</i>	<i>Electrode & CNT deposition</i>	<i>Enzyme & immobilisation method</i>	<i>Performance</i>	<i>Reference</i>
MWNT	Gold CVD	GOD adsorbed	LOD = 0.01 mM glucose	Wang <i>et al.</i> , 2003
MWNT	Platinum CVD	GOD adsorbed	LOD = 0.19 mM glucose sensitivity = 93.9 $\mu\text{A mM}^{-1}$	Sotiropoulou <i>et al.</i> , 2003
MWNT	Glassy carbon Drop cast	HRP adsorbed	LOD = 1 $\mu\text{M H}_2\text{O}_2$ (methylene blue mediator)	Xu <i>et al.</i> , 2003
MWNT	Glassy carbon Drop cast	HRP glutaraldehyde Crosslinked	LOD = 0.1 $\mu\text{M H}_2\text{O}_2$	Yamamoto <i>et al.</i> , 2003
MWNT	Glassy carbon Drop cast	Mb adsorbed	LOD = 4.2 μM	Zhao <i>et al.</i> , 2004
SWNT	Pt Drop cast	GOD covalently attached	Linear range = 0-12 mM sensitivity = 18.7 $\text{mAM}^{-1}\text{cm}^{-2}$	Xue <i>et al.</i> , 2003
SWNT	Pyrolytic graphite Self assembly on Fe_2O_3	Mb, HRP covalently attached	LOD Mb= 70 nM HRP= 50 nM	Yu <i>et al.</i> , 2003

Table 4.1. Preparation and performance of enzyme-CNT modified electrodes.

LOD = limit of detection

4.1.1.6.2 Carbon nanotubes and some physiologically important biomolecules

Musameh *et al.* (2002) have reported on the improved electrocatalytic response of CNT-modified glassy carbon electrodes towards β -nicotinamide adenine dinucleotide (NADH). SWNT and MWNT-coated electrodes were prepared by casting from concentrated sulphuric acid dispersions. A substantial (490 mV) decrease in the

overvoltage of the NADH oxidation reaction was observed in both SWNT and MWNT coatings compared to unmodified electrodes. Furthermore, the NADH amperometric response of the coated electrodes was extremely stable with 96% of SWNTs and 90% of MWNTs initial activity remaining after 60 min stirring of 2×10^{-4} M NADH solutions compared to 20% at the bare surface. The cyclic voltammograms of the modified electrodes were accompanied by 1.6-fold (MWNT) and 2.3-fold (SWNT) increases in peak current over the current obtained at the bare surface. The authors concluded that CNT-modified glassy carbon electrodes offer a stable, low potential amperometric detection of NADH and suggest that the oxygen-rich groups on the CNT surface may be partially responsible for the electrocatalytic behaviour.

Wang *et al.* (2002a) have successfully detected uric acid in the presence of a high concentration of ascorbic acid on MWNT cast electrodes. Uric acid is the primary end product of purine metabolism. Uric and ascorbic acid are both present in biological fluids such as blood and urine. In differential pulse voltammetric (DPV) experiments the anodic peaks (E_{pa}) of uric and ascorbic acid were unresolved on a graphite electrode showing only a broad peak at 0.3 V. However, the CNT-modified electrode showed well-defined peaks with E_{pa} for uric acid at 0.4 V and ascorbic acid at 0.012 V. The sensitive detection of uric acid was further improved on a β -cyclodextrin-MWNT coated electrode. β -cyclodextrin is a macrocyclic glucose oligomer, consisting of seven D-glucose units which is capable of forming a supramolecular complex with uric acid. A linear calibration curve was obtained for 5×10^{-7} to 5×10^{-5} M uric acid in acetate buffer, pH 4.5. The limit of detection was 0.2 μ M.

MWNTs have also been used to sensitively detect dopamine in the presence of ascorbic acid (Wang *et al.*, 2002b). Dopamine cannot be discriminated from ascorbic acid on a bare graphite electrode. An anodic peak separation (ΔE_{pa}) of 270 mV was achieved with both a MWNT-coated graphite electrode and a MWNT-intercalated graphite electrode. The latter was formed when the soft graphite layers were ground with a suitable amount of MWNT powder. The sensitivity of the latter preparation was reported to be slightly higher. The ΔE_{pa} decreased and the anodic peak current ratio of dopamine to ascorbic acid increased when the MWNTs were oxidised. The authors attributed this to the increased negativity of the MWNT when oxygen-rich groups were introduced. This

enhanced the electrostatic repulsion towards ascorbic acid. The detection limit for dopamine on the CNT-intercalated graphite electrodes was determined to be 0.1 μM and that for ascorbic acid to be 0.02 mM. It was concluded that the resolution of dopamine and ascorbic acid was mainly attributable to the stereo porous interfacial layer formed from aggregated pores and inner cavities of the MWNTs.

MWNT-modified basal plane pyrolytic graphite electrodes have also been used to separate the oxidation peaks of epinephrine and ascorbic acid (by 220 mV) (Salimi *et al.*, 2004). MWNTs were abrasively immobilised onto the electrode by gently rubbing the surface on filter paper containing the CNTs. This proved to be a stable preparation method as the amperometric current remained unchanged after the electrodes were stored for three weeks in PBS.

4.1.1.6.3 CNTs and DNA hybridisation

Wang *et al.* (2003) have described a new electrical sandwich hybridisation assay which combined the use of SWNTs carrying a large number of Cadmium sulphide (CdS) particle tracers with an ultrasensitive electrochemical stripping detection of the CdS nanoparticle tracers. A detection limit of 40 pg ml^{-1} of target DNA was achieved which is 500-times lower than the detection limit of 20 ng ml^{-1} reported for the analogous electrical protocol based on the use of single CdS tags.

The assay was conducted in a streptavidin coated 96-well microplate to which the biotinylated DNA probe 1 was immobilised. A sandwich complex was formed with addition of target solution and SWNT-CdS labelled probe 2. Stripping voltammetric detection of the dissolved cadmium ion was performed. The cadmium stripping signal was proportional to the concentration of the target DNA. The low detection limit obtained was attributed to the high density and strong hydrophobic anchoring of the octadecanethiol-capped CdS nanoparticles to the acetone-activated CNT. A coverage of approx. 500 CdS spheres for every CNT (with an average length of 2 μm) was estimated from SEM images.

4.1.1.6.4 CNTs and immunosensing

To date, only a few reports have been published concerning the application of CNTs in the field of immunosensing. Chen *et al.* (2003) developed sensitive CNT-based immunoassays based on two different transduction mechanisms. In the first, a SWNT film was formed on a quartz crystal surface and mass changes were measured on a quartz crystal microbalance. The second involved a transistor configuration featuring a layer of interconnected SWNTs bridging two Ti/Au electrodes. The antigen, a U1A RNA splicing factor, was conjugated to Tween 20 which adsorbed to the SWNTs. Detection of an auto-antibody specific for this factor was achieved by both systems at concentrations ≤ 1 nM. However, with regards to the transistor configuration, subsequent work showed that proteins were capable of adsorption to metal-nanotube junctions of similar devices and this was the cause of the observed resistance changes. Most proteins which adsorbed only to the carbon nanotubes gave no change in resistance (Chen *et al.*, 2004).

A CNT-poly(ethylene vinylacetate) (EVA) composite has been used in the development of an electrochemiluminescence (ECL)-based immunoassay (Wohlstadter *et al.*, 2003). Disks of the nanotube composite were acid oxidised. Subsequently, streptavidin was covalently bound and biotinylated anti α -fetoprotein (AFP) immobilised. Introduction of AFP and an anti-AFP monoclonal antibody labelled with $[\text{Ru}(\text{bpy})_3]^{2+}$ led to the formation of a sandwich complex. The ECL signal obtained led to a limit of detection of 0.1 nM (7 ng ml⁻¹) of AFP.

4.2 MATERIALS AND INSTRUMENTATION

4.2.1 Materials

SWNTs, (HiPCO) were purchased from Tubes@rice (90% pure). The *E. coli* mutant (strain UM2) was obtained from the *E. coli* Genetic stock centre, Yale University, New Haven, USA. All other chemicals were reagent grade and purchased from Sigma-Aldrich. Anti-biotin antibody was a goat polyclonal (B-3640) and horseradish peroxidase-labelled biotin (Biotin-HRP) was biotinamidocaproyl-labelled peroxidase (P-9568).

4.2.2 Instrumentation

4.2.2.1 Electrochemical measurements

Cyclic voltammetry and amperometry were performed with a CHI 660 potentiostat. A three-electrode cell was used employing a saturated calomel reference electrode, a platinum wire as counter electrode and basal plane pyrolytic graphite as working electrode. (Advanced Ceramics, area of 0.2 cm²).

4.2.2.2 Atomic Force Microscopy

Tapping mode AFM measurements were performed with a Nanoscope IV scanning probe microscope on (100) silicon wafers.

4.2.2.3 Resonance Raman measurements

Resonance Raman measurements were performed using a Renishaw Ramanscope 2000, using a 785 nm (1.58 eV) Argon ion laser focused on a 1 μ m spot by a 100 X objective lens.

4.3 METHODS

4.3.1 Assembly of SWNT forests

SWNT forests were constructed on basal plane pyrolytic graphite disk electrodes that were first abraded on 400 grit SiC paper, then ultra-sonicated in water for 1 min. SWNT were carboxylic acid functionalised and shortened by sonication in a mixture of HNO₃ and H₂SO₄ for 4 hours at 70 °C. The electrodes were dipped in 1 mg ml⁻¹ Nafion solution in 9:1 methanol:water for 15 min. This was quickly washed with water and then placed in 5 mg ml⁻¹ of FeCl₃·6H₂O for 15 min. After washing with water the electrodes were then placed in a freshly sonicated DMF suspension of SWNTs.

4.3.2 Immunoassay procedure

Anti-biotin antibody was prepared at 0.5 mg ml^{-1} (unless otherwise stated) in 0.01 M phosphate buffer, 0.137 M NaCl, 2.7 mM KCl, pH 7.4 (PBS) and allowed to incubate on SWNT surface for 3 hours. Excess antibody was removed by washing with PBS with 0.05% (v/v) Tween 20, followed by washing with PBS. This washing procedure was repeated after each incubation. The surface was then blocked with 2% (v/v) BSA in PBS. The competition step involved incubating biotin (dissolved in PBS) and biotin-HRP simultaneously on the antibody-immobilised and BSA-blocked SWNT surface for 1 hour.

4.3.3 Electroanalytical procedure

Rotating disk amperometry was performed at -0.3 V vs. SCE at 2000 rpm with 1 mM hydroquinone and $400 \text{ }\mu\text{M}$ H_2O_2 unless otherwise stated. The electrochemical buffer was phosphate buffer, 0.1 M, 0.137 M NaCl and 2.7 mM KCl, pH 6.8. The buffers (10 ml) were purged with purified nitrogen and a nitrogen environment was maintained in the cell during experiments.

4.3.4 Immobilisation of HRP/*Escherichia coli*

The *E. coli* catalase negative UM2 mutant was provided by the Genetic stock centre located at Yale University New Haven. The culture was grown aerobically at the Environmental Engineering department, University of Connecticut, in LB media. The cells were then washed with 0.1 M phosphate buffer, pH 7.0 by centrifuging at 5000 g for 10 min. The supernatant was discarded. The pellet was then resuspended in phosphate buffer again and the process was repeated three times. To ensure that the mutant strain did not contain the catalase enzyme, both the mutant and the wildtype were exposed to H_2O_2 . A positive reaction was observed only in the wildtype. Since it contains catalase it consumes H_2O_2 releasing bubbles of O_2 . HRP-immobilised SWNT electrodes were prepared according to Yu *et al.* (2003). A 20 μl droplet of freshly prepared 0.4 M of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 M of *N*-

hydroxysulphosuccinimide (NHSS) was placed on the SWNT forest electrodes. This was immediately followed by adding 30 μl of 3 mg ml^{-1} HRP in 0.01 M phosphate buffer, pH 7.5. The EDC/NHSS and HRP were mixed carefully with a syringe needle, reacted for 4 hours and then washed with water. 10 μl of 2% (w/v) agarose was mixed with 10 μl of the *E. coli* cells from the highly concentrated pellet and immediately placed on the HRP-modified electrode surface. The concentration of cells were in the region of 10^{15} cells ml^{-1} . Three types of SWNT electrodes were prepared; one with HRP only, another with HRP and the catalase-negative mutant *E. coli* and the last with HRP and wild-type catalase positive *E. coli*. The electrodes were all placed in the same electrochemical cell containing 10 ml of 20 mM phosphate buffer, pH 6.2 with 50 mM NaCl. The buffer had been deaerated with nitrogen gas but was exposed to a headspace of piped ambient air during the experiments. Amperometry was performed at -0.2 V vs. SCE.

4.4 RESULTS AND DISCUSSION

4.4.1 SWNT assembly and characterisation

4.4.1.1 SWNT assembly

Shortened SWNTs (s-SWNT) were achieved by sonicating in a 3:1 mixture of HNO_3 and H_2SO_4 (Liu *et al.*, 1998). This is a very effective method since sonication produces holes in the tube sides, which are then further attacked by the acids cutting the tube completely. Even in this acid mixture alone without sonication, the average cut nanotube has been reported to shorten at a rate of approx. 130 nm per hour. Acid oxidation also serves to form COOH functional groups at the open edges and results in nanotubes which are capable of forming stable suspensions in solvents. While the bundles tend to separate during acid pretreatment, afterwards the SWNT tend to form thick bundles again. However, the oxidation process reduces the cross-sectional diameter of the bundles (Martinez *et al.*, 2003).

The assembly process used here and developed previously (Chattopadhyay *et al.*, 2001) involved the self-assembly of s-SWNTs onto Fe^{3+} /Nafion underlayers (Figure 4.1).

Nafion 117 is a cation-exchange membrane consisting of a polytetrafluoroethylene backbone and long fluorovinyl ether pendant side-chains regularly spaced and terminated by a sulphonate ionic group. The electrodes were coated with Nafion to achieve a uniform negatively charged surface suitable for formation of a high surface coverage of Fe^{3+} . The layer of Fe^{3+} was formed by immersion of the electrode in an aqueous solution of FeCl_3 .

Several factors may come into play to produce a successful assembly of s-SWNTs. Previously, it was reported that the surface-immobilised Fe^{3+} in its basic hydroxide form may provide the initial driving force (acid-base neutralisation) for the assembly to occur (Chattopadhyay *et al.*, 2001). In practice, more dense assemblies of s-SWNTs have been formed upon ageing the aqueous solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ for at least a week. Therefore, it would appear that the nature of the iron precipitate on the electrode surface would seem to be an important factor. When ferric chloride is dissolved in water the chloride ions are displaced and a hydrated ferric ion is formed (Meyer *et al.*, 2000). Protons may be released by coordinated water molecules or from coordinated hydroxyl groups as a consequence of condensation reactions involving intermediate hydrolysis products. This leads to a rapid decrease in observed pH ($\text{pH} < 2$). Polycondensation reactions cause the initial yellow-coloured solution, characteristic of monomer species, to change to a deep red/brown colour. Solid hydrous iron oxides also form. Akaganeite $\beta\text{-FeOOH}$ is initially produced. It has been shown that after seven days of ageing at room temperature, haematite Fe_2O_3 is the main component of the precipitate (Suigimoto *et al.*, 1993). Since carboxylic acids can be deprotonated by various metal oxides, the s-SWNT assembly process may also be promoted by coulombic forces between the carboxylate anion headgroup and metal oxides coated on the substrate. This mechanism has been proposed for the assembly of s-SWNTs on zinc (Yu *et al.*, 2000c) and silver oxide (Wu *et al.*, 2001) surfaces.

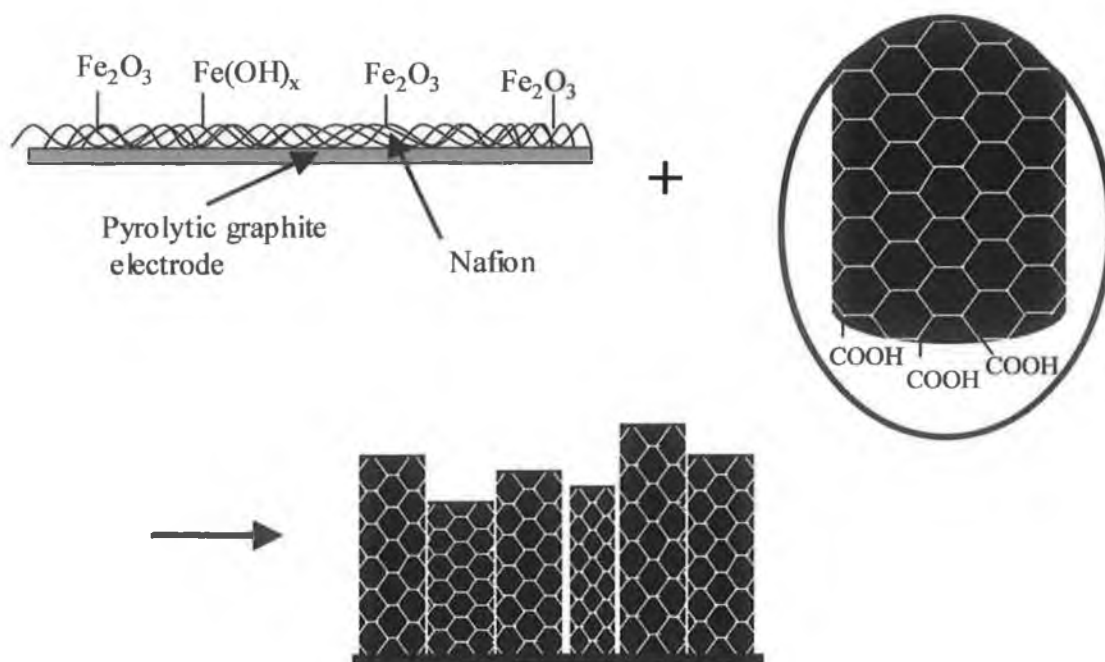


Figure 4.1. Schematic representation of the s-SWNT self-assembly process onto Fe^{3+} /Nafion underlayers.

4.4.1.2 Atomic force microscopy

Tapping mode atomic force microscopy (AFM) images were taken on smooth silicon wafers at each step of the assembly, i.e. after deposition of Nafion, Fe_2O_3 and SWNT layers and also after antibody adsorption (Figure 4.2-4.7). Section analysis of the Nafion layer showed peak heights between 1-2 nm (Figure 4.2a). A large contrast to this can be seen on addition of the Fe_2O_3 layer (Figure 4.2b). The peak height from section analysis of Nafion/ Fe_2O_3 layer showed a height of 29 ± 10 nm. The domain width of the iron islands was 209 ± 32 nm. After assembly of the SWNTs the section analysis showed a densely packed layer with protrusion height of 26 ± 6 nm (Figure 4.3a). The top view showed a dense granular appearance of the SWNTs with dimensions of $96 \pm 25 \times 30 \pm 10$ nm (Figure 4.6b). Although there were most likely contributions to these measurements from the AFM tip broadening effect, this may also suggest the formation of SWNT bundles due to hydrophobic forces. The section view of the SWNT assembly may suggest a perpendicular orientation of the nanotubes. However, the greatest indication of

perpendicular orientation using this assembly method has been found previously with the use of polarisation Raman spectroscopy (Chattopadhyay *et al.*, 2001). This technique can be used to provide an insight into the orientation of SWNTs on a surface since it is known that maximum intensity of the G band (1593 cm^{-1}) is obtained when polarisation of the incident laser is parallel to the nanotube axis. The signal is minimal when the polarisation is perpendicular to the nanotube axis. It was found that a 57-fold intensity enhancement at 1593 cm^{-1} was obtained, when polarisation of the incident light was parallel to the nanotube axis. This indicates a significant degree of nanotube alignment normal to the surface is achieved by using this assembly method.

Adsorption of antibody resulted in an AFM image with a more aggregated appearance (Figures 4.3 and 4.7). The domain width of these aggregates was found to be $187 \pm 44\text{ nm}$. A uniform surface coverage of the antibody was observed.

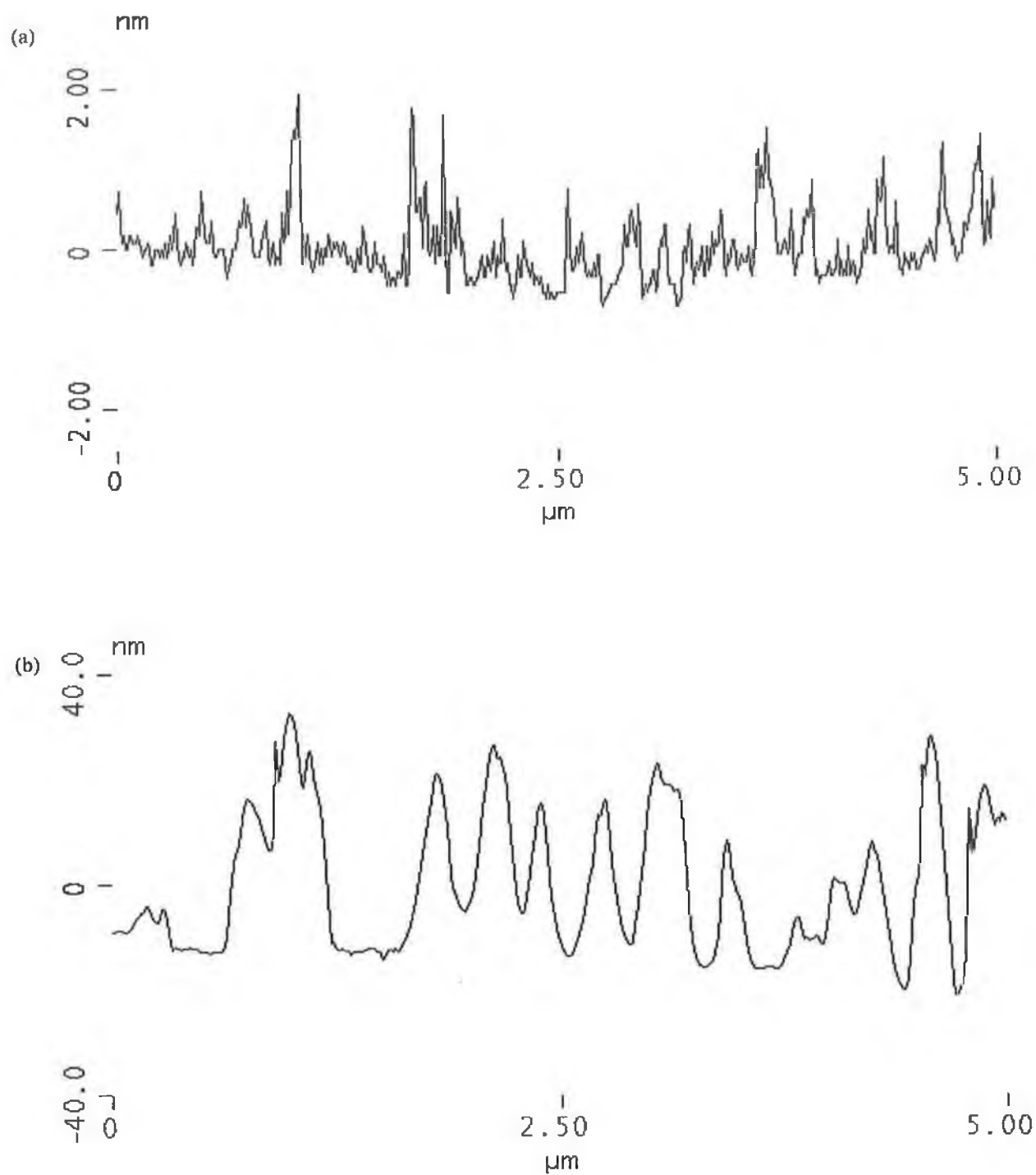


Figure 4.2. Tapping mode AFM section analysis on smooth silicon of (a) Nafion and (b) Nafion/Fe₂O₃.

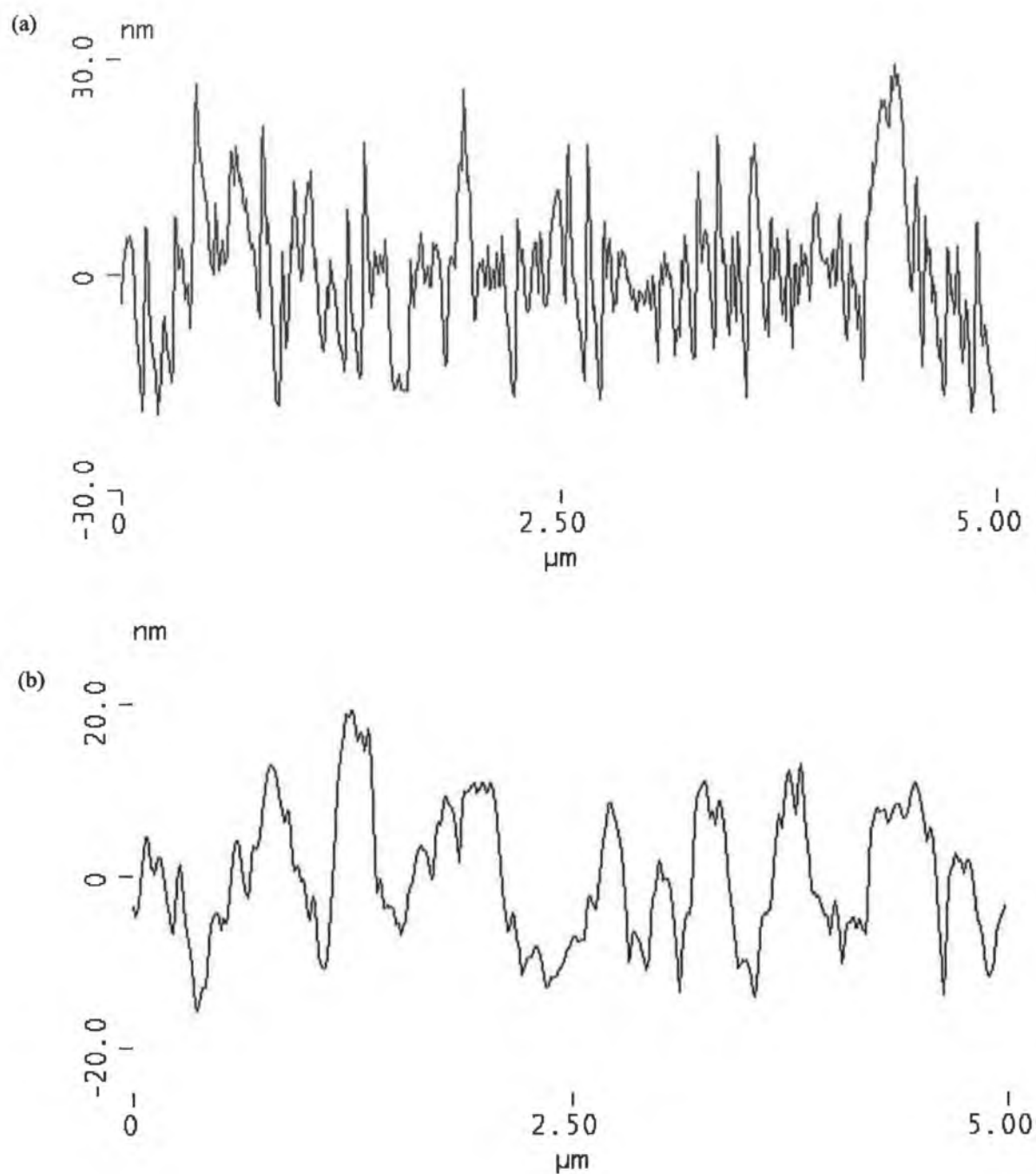


Figure 4.3. Tapping mode AFM section analysis on smooth silicon of (a) Nafion/ Fe_2O_3 /s-SWNT and (b) Nafion/ Fe_2O_3 /s-SWNT/anti-biotin antibody.

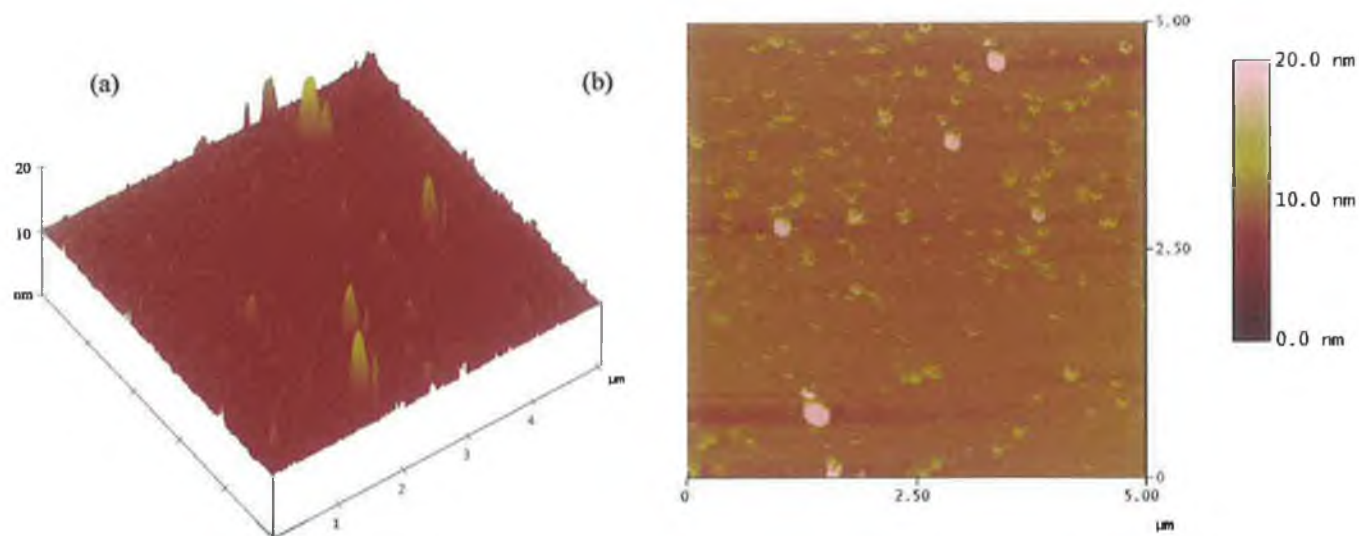


Figure 4.4. Tapping mode AFM of Nafion on smooth silicon (a) 3D image and (b) top view.

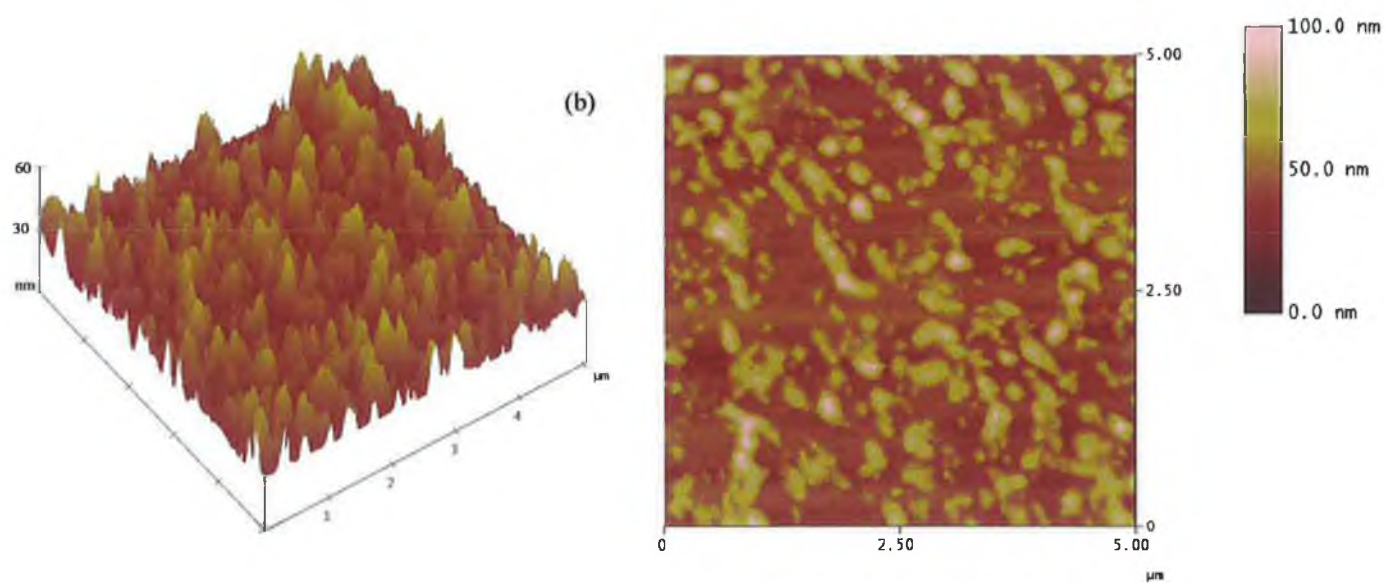


Figure 4.5. Tapping mode AFM of Nafion/Fe₂O₃ on smooth silicon (a) 3D image and (b) top view.

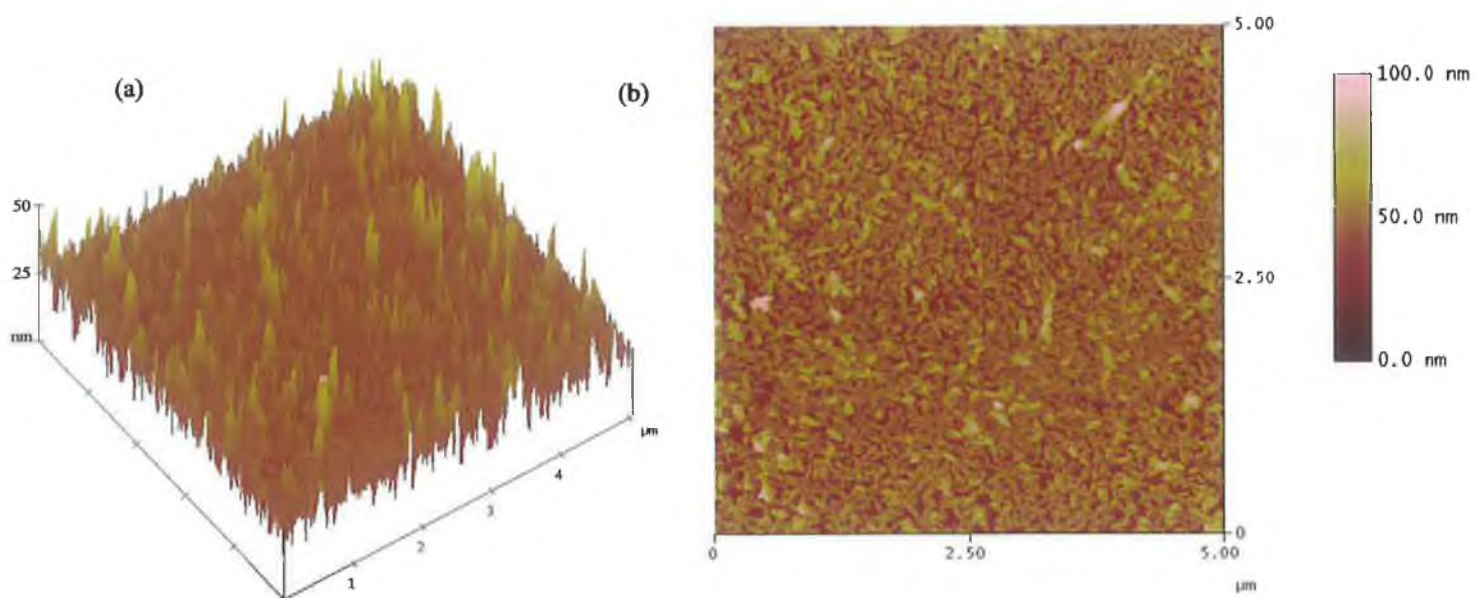


Figure 4.6. Tapping mode AFM image of Nafion/Fe₂O₃/s-SWNT (a) 3D view and (b) top view.

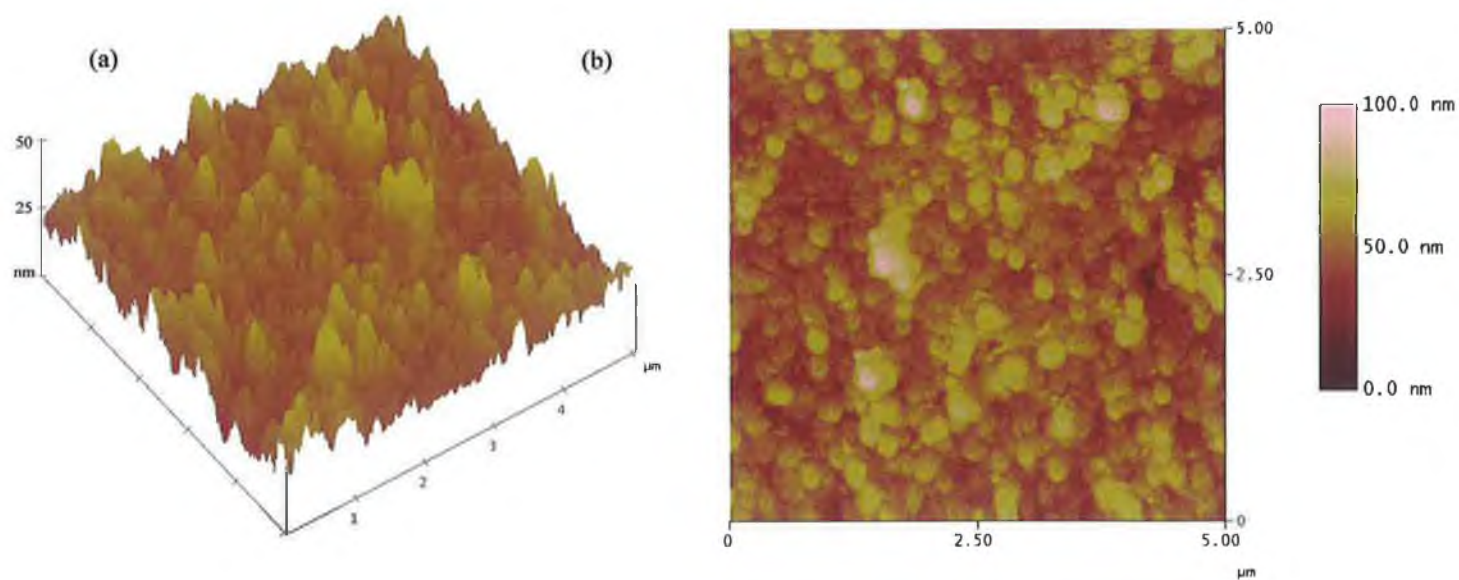


Figure 4.7. Tapping mode AFM of Nafion/Fe₂O₃/s-SWNT/anti-biotin antibody (a) 3D view and (b) top view.

4.4.1.3 Resonance Raman spectroscopy

Raman spectroscopy is a promising tool for the identification and probing of carbon nanotubes. SWNTs have 15 or 16 Raman active vibrational modes. The exact number depends on the symmetry of the tube but is independent of the diameter (Kurti *et al.*, 1998). Enhancement of the Raman intensity is observed as a function of laser frequency when the excitation frequency is close to the frequency of high optical absorption and this effect is called the resonant Raman effect (Saito *et al.*, 1998). Four of the Raman bands are strongly resonance-enhanced. Three of these are located around 1600 cm^{-1} and correspond to the characteristic A, E_1 and E_2 modes of the graphene sheet. The fourth band at around 200 cm^{-1} is caused by the radial breathing mode (RBM), where all atoms move in phase perpendicular to the tube axis changing the radius of the tube. For an isolated tube, the radial frequency (ω_r) is inversely proportional to the tube diameter (d) where:

$$\omega_r = 224 (\text{cm}^{-1}\text{nm})/d (\text{nm}) \quad (4.1)$$

The radial frequency is sensitive to the diameter only and not to the helicity of the nanotube. However, in a rope of SWNTs this correlation between frequency and diameter breaks down as the tube–tube interactions shift the frequency of the RBM upwards by as much as 8-10% (Lambin *et al.*, 2002). In the case of bundled nanotubes the relationship between (ω_r) and diameter has been described as (Rao *et al.*, 2001):

$$\omega_r = 224 (\text{cm}^{-1}\text{nm})/d (\text{nm}) + 14\text{ cm}^{-1} \quad (4.2)$$

The SWNTs used here were produced by the HiPCO method. This method produces tubes with relatively narrow diameters depending on the process parameters and a relatively broad diameter distribution (Zhou *et al.*, 2001). The average diameter of HiPCO nanotubes is approx. 1.1 nm (Daenen *et al.*, 2003). The radial breathing mode frequency obtained in Figure 4.7 show the presence of SWNT with diameters in the range 1-1.1 nm.

Since the RBM is unique to nanotubes without any counterpart in graphite it can be used to confirm the assembly of SWNTs on pyrolytic graphite electrodes. Figure 4.8 shows that a peak with maximum intensity at 230 cm^{-1} is present in the 1.58 eV (785 nm) resonance Raman spectra of the SWNT assembly. This is characteristic of RBM of SWNTs and is not present in the spectra of either the bare pyrolytic graphite electrode or pyrolytic graphite/Nafion/ Fe^{3+} control. Although the laser of the Ramanscope focuses on only a $1\text{ }\mu\text{m}$ spot, several different spots were measured showing the presence of SWNTs on each. This indicated a distribution of SWNT throughout the electrode surface. A resonance Raman spectra was also taken after all the immunocomponents had been assembled on the SWNT electrodes and placed in PBS buffer at 4°C for one week (Figure 4.8). The RBM peak was still present after this time indicating good stability of the SWNT assembly. The slight difference in radial breathing mode frequency between the two samples is not unusual and also occurs within these samples. It reflects a variation in the distribution of SWNT diameters in each spot.

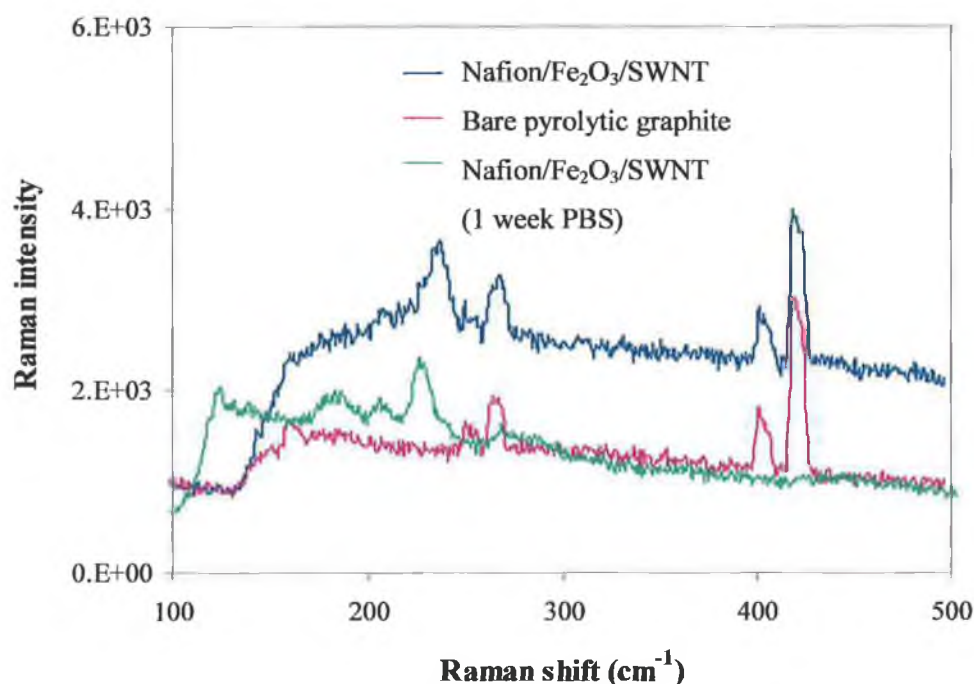


Figure 4.8. Resonance Raman spectra (1.58 eV / 785 nm) of bare pyrolytic graphite and Nafion/Iron oxide and Nafion/Iron oxide/SWNT on pyrolytic graphite.

4.4.1.4 Cyclic voltammetry of SWNT assembly

Figure 4.9 shows a cyclic voltammogram of the Nafion/ Fe^{3+} layers in the absence and presence of s-SWNTs. Both show broad oxidation and reduction peaks, the oxidation peak centered at -0.14 V and the reduction peak at 0.1 V vs. SCE. Previously, oxidation and reduction peaks of -0.126 and -0.024 V vs. SCE, respectively, were obtained from a cast film of acid-pretreated SWNTs on glassy carbon and were attributed to the carboxylic acid functionalities (Luo *et al.*, 2001). However, in the case of SWNTs self-assembled on pyrolytic graphite, it would appear difficult to make such a distinction since surface functional groups already present on pyrolytic graphite form broad oxidation and reduction peaks in this region.

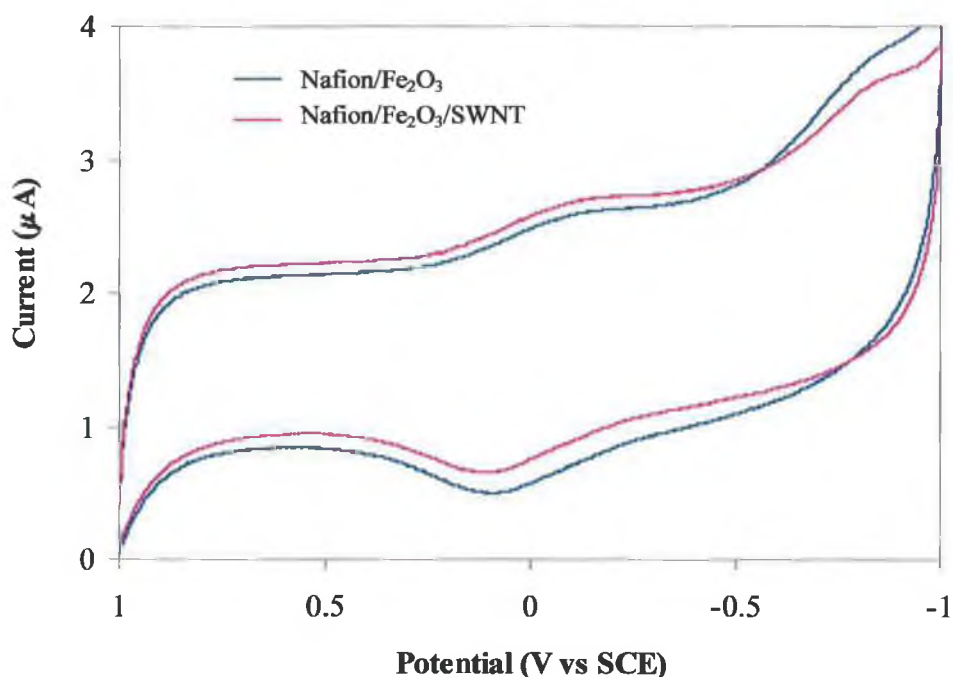


Figure 4.9. Cyclic voltammogram of Nafion/ Fe^{3+} on pyrolytic graphite, in the absence and presence of s-SWNTs, at 0.1 V s^{-1} , 0.1 M PBS , pH 6.8.

4.4.2 Development of immunoassay for biotin

4.4.2.1 Antibody concentration

The SWNT electrodes were incubated with anti-biotin antibody in the concentration range between 0.1 and 1.5 mg ml⁻¹ and the immunoassay procedure carried out as outlined in section 4.3.2 using 1 µg ml⁻¹ biotin-HRP. Figure 4.10 shows that while a large signal was obtained with incubation of 0.1 mg ml⁻¹, saturation of the surface was achieved at 0.5 mg ml⁻¹ antibody.

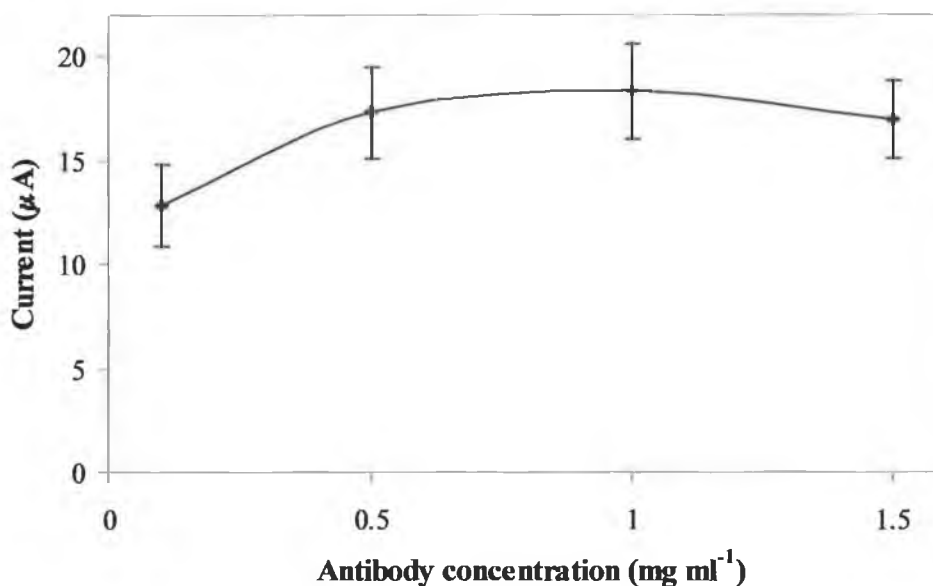


Figure 4.10. Effect of antibody concentration on amperometric signal (1 µg ml⁻¹ biotin-HRP, 300 µM hydroquinone, 150 µM H₂O₂, 2000 rpm, n=3).

4.4.2.2 Optimisation of Biotin–HRP concentration

Varying concentrations of biotin-HRP in the range 0.05 to 100 µg ml⁻¹ were incubated with the antibody-immobilised and BSA-blocked electrodes. Figures 4.11–4.13 show that the current resulted in a linear increase with increasing concentration of biotin-HRP until 1 µg ml⁻¹ was reached. At concentrations greater than 3 µg ml⁻¹ a decrease in current was observed. This indicated that the diffusion of hydroquinone may be impeded by steric hindrance of increasing concentration of biotin-HRP. This is a general characteristic of

diffusion-controlled mediators whereby their redox reaction is suppressed due to a decrease in the electrode active area and/or an increase in the distance that marker ions can approach the electrode active surface (Kuramitz *et al.*, 2003). The optimum concentration of biotin-HRP for use in the competition assay was therefore chosen as 1 $\mu\text{g ml}^{-1}$. The detection limit of biotin-HRP calculated as the analyte concentration yielding a signal three times the standard deviation of the blank response, was determined as 0.1 $\mu\text{g ml}^{-1}$ (Figure 4.13). This was comparable to the limit of detection achieved from a traditional colorimetric ELISA where the detection limit was found to be 0.06 $\mu\text{g ml}^{-1}$ (data not shown).

The non-specific adsorption of biotin-HRP was assessed. This was determined by incubating a mouse anti-IgG on the surface, blocking with 2% (v/v) BSA in the usual way and measuring the current after incubation with biotin-HRP. Since this antibody was not specific for biotin-HRP, any catalytic current generated in this case was indicative of random adsorption of biotin-HRP on the surface. Very low current was observed showing that at a biotin-HRP concentration of 1 $\mu\text{g ml}^{-1}$, only 0.1% of the current was due to non-specific adsorption of biotin-HRP.

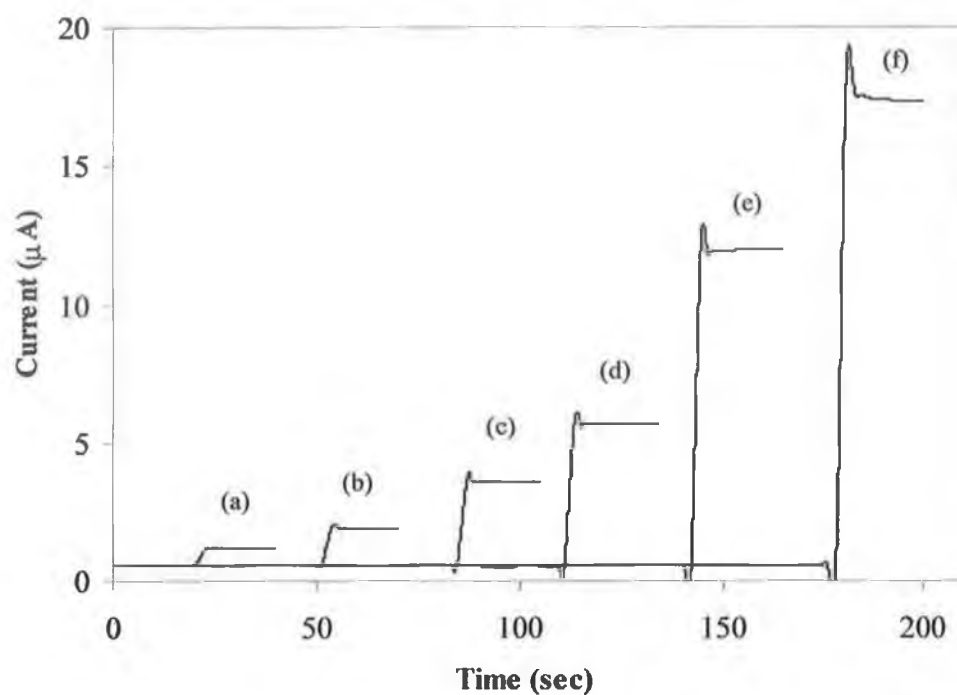


Figure 4.10. Amperograms showing the effect of biotin-HRP concentration on catalytic current. These were obtained at 2000 rpm, 300 μM hydroquinone and 150 μM H_2O_2 at -0.3 V. The anti-biotin concentration was 0.5 mg ml^{-1} . The concentrations of biotin-HRP were (a) 0.05 (b) 0.1 (c) 0.2 (d) 0.4 (e) 0.6 (f) 1 $\mu\text{g ml}^{-1}$.

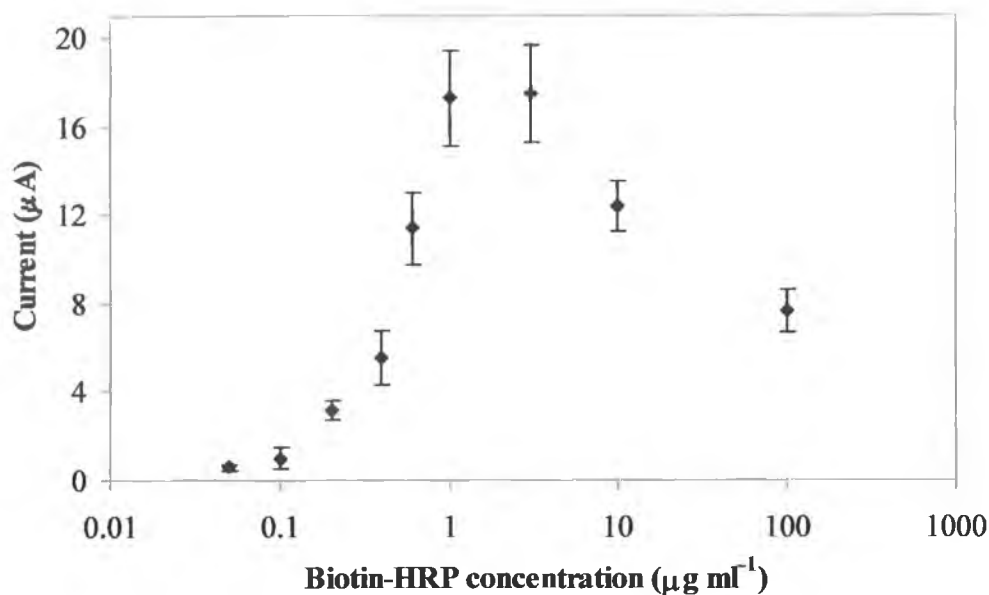


Figure 4.11. Effect of biotin-HRP on rotating electrode amperometric current following addition of $300 \mu\text{M}$ hydroquinone and $150 \mu\text{M}$ H_2O_2 , 2000 rpm, $n=3$.

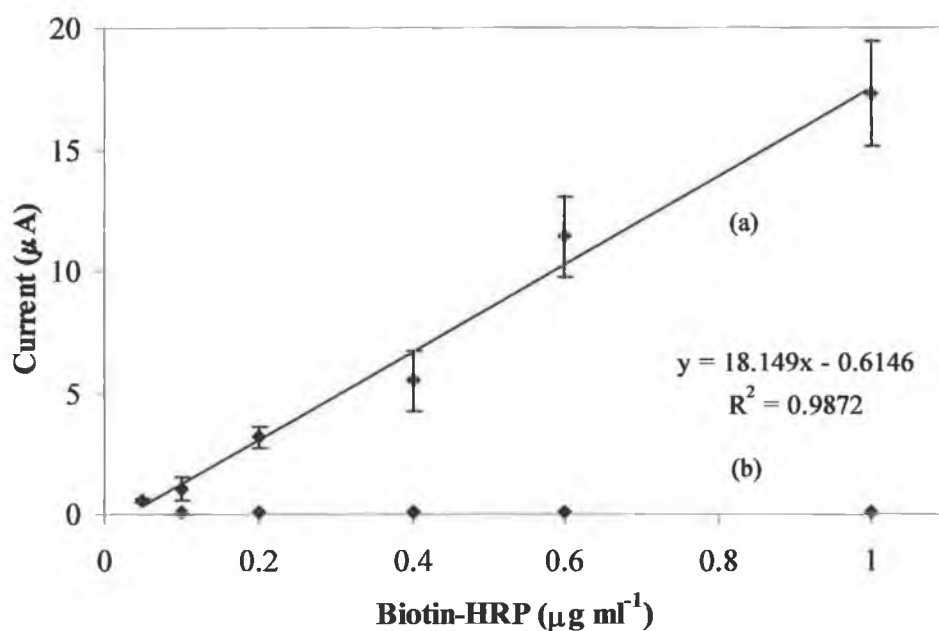


Figure 4.12. Effect of biotin-HRP on rotating electrode amperometric current (linear region) following addition of $300 \mu\text{M}$ hydroquinone and $150 \mu\text{M}$ H_2O_2 , 2000 rpm, $n=3$, where (a) 0.5 mg ml^{-1} anti-biotin antibody was adsorbed and (b) 0.5 mg ml^{-1} mouse anti-IgG was adsorbed on surface (non-specific adsorption control).

4.4.2.3 Hydroquinone as mediator

To overcome the slow heterogeneous electron transfer of peroxidases, mediators which are small redox-active molecules with inherent high heterogeneous electron transfer rates are frequently required. Mediators are especially useful when the peroxidase is at a distance from the electrode surface as in the case of immunoassays. Hydroquinone (1,4 dihydroxybenzene) is one of the most efficient electron donors to HRP with a reaction rate as high as $1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Ruzgas *et al.*, 1996). A simplified catalytic scheme is shown in Figure 4.14.

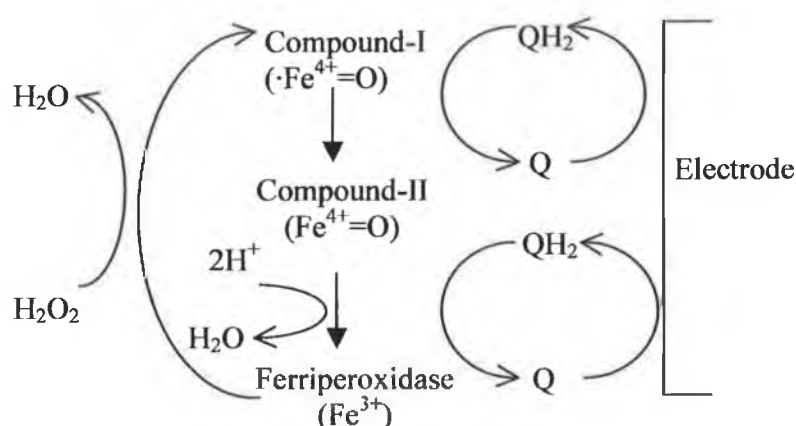
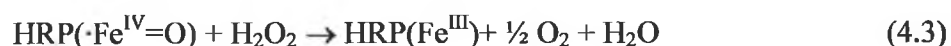


Figure 4.14. Mechanism of mediated bioelectrocatalytic reduction of H_2O_2 at peroxidase-modified electrodes. QH_2 and Q are the reduced and oxidised forms of the mediator (hydroquinone), respectively (from Ruzgas *et al.*, 1996).

Native HRP (Fe^{3+}) is oxidised by H_2O_2 through a formally two-electron-transfer reaction to compound I, in which the heme group is oxidised to an unstable oxyferryl radical intermediate ($\cdot\text{Fe}^{4+}=\text{O}$) with a formal oxidation state of 5+ (Schmidt *et al.*, 2002). The enzyme is regenerated in two one-electron-transfer reactions by the electron donor (QH_2) involving a non radical intermediate ($\text{Fe}^{4+}=\text{O}$) with an oxidation state of 4+. The benzoquinone (Q) is subsequently reduced back to hydroquinone (QH_2) by a rapid reaction involving the acceptance of two electrons from the electrode. The scheme above is simplified as the catalytic response of peroxidases to H_2O_2 may involve significant

contributions from the catalytic reduction of oxygen generated by reaction of H_2O_2 with compound I (Zhang *et al.*, 2002) as expressed below:



The cyclic voltammogram in Figure 4.15 shows that with the addition of $400 \mu\text{M}$ of H_2O_2 the reduction current increased and the oxidation current decreased. This is the expected result of benzoquinone (Q) being reduced at the electrode surface.

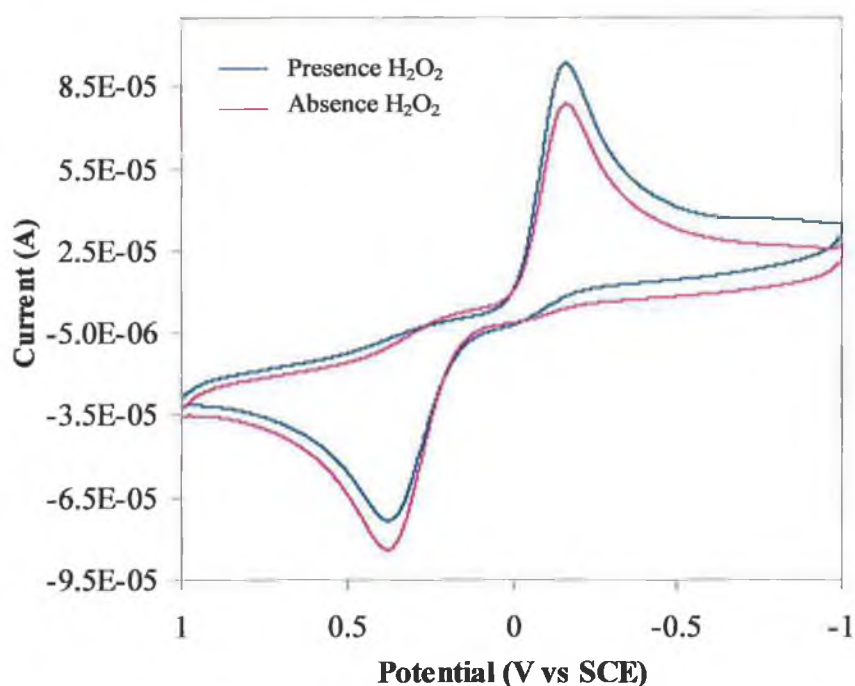


Figure 4.15. Cyclic voltammogram of antibody/biotin-HRP-immobilised SWNT electrode at 0.3 V s^{-1} with 1 mM hydroquinone before and after addition of $400 \mu\text{M}$ H_2O_2 . Cathodic peaks are positive and anodic peaks are negative.

Figure 4.16 shows that, catalytic current undergoes large increases with addition of $100 \mu\text{M}$ hydroquinone and levels off at 1 mM where saturation is reached. 1 mM was therefore chosen for use in the competitive assay.

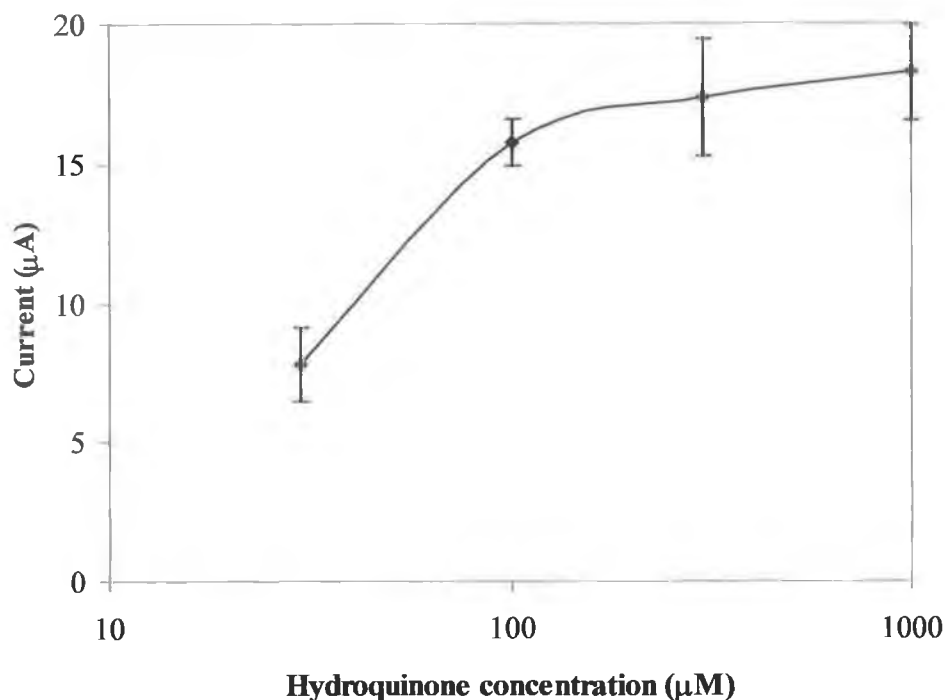


Figure 4.16. Effect of hydroquinone concentration on amperometric signal (0.5 mg mL^{-1} antibody, $1 \text{ } \mu\text{g mL}^{-1}$ biotin-HRP, $150 \text{ } \mu\text{M H}_2\text{O}_2$, 2000 rpm , $n=3$).

4.4.2.4 Hydrogen peroxide concentration

On addition of H_2O_2 , the current increased at approx. $0.08 \text{ } \mu\text{A } \mu\text{M}^{-1} \text{ H}_2\text{O}_2$ for concentrations of H_2O_2 between $200 \text{ } \mu\text{M}$ and 1 mM (Figure 4.17-4.18). It can be seen from Figure 4.18 that the rsd also increased with each successive addition of H_2O_2 . With addition of $200 \text{ } \mu\text{M H}_2\text{O}_2$ the rsd was 11.9% but this had increased to an rsd of 13.6% when the concentration of H_2O_2 had reached 1 mM . A concentration of $400 \text{ } \mu\text{M}$ was chosen for future work as a compromise between obtaining a high signal and better reproducibility. HRP is known to show a decreasing activity when H_2O_2 is present with the degree of inactivation being dependent on the incubation time and the hydrogen peroxide concentration (Schmidt *et al.*, 2002). The decreasing activity is due to the formation of HRP compound III which is formed via compound II with an excess of H_2O_2 and is catalytically inactive. This effect may have attributed to the increasing rsd with increasing H_2O_2 .

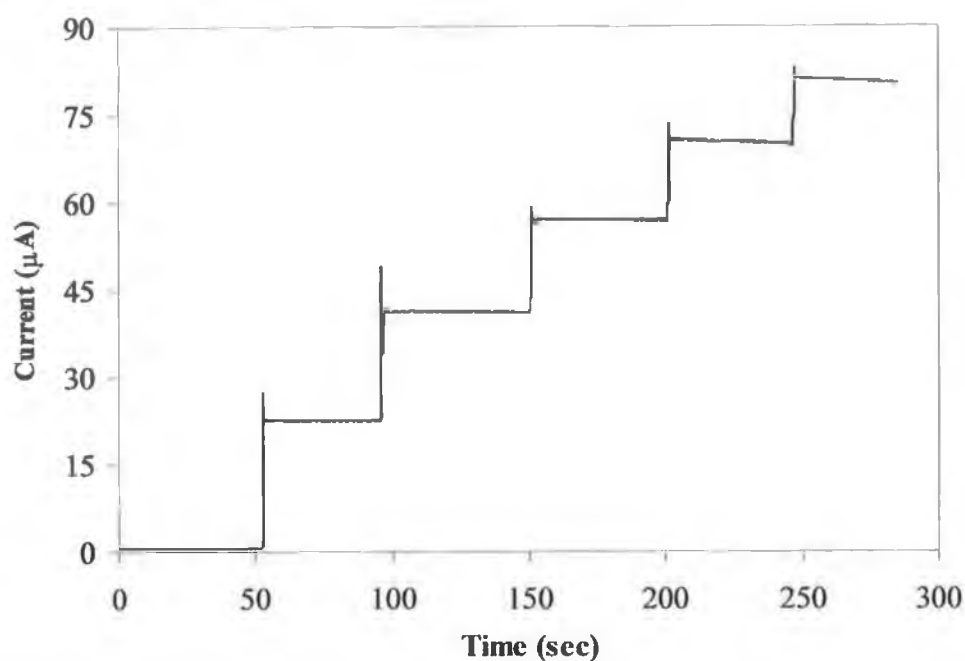


Figure 4.17. Amperometric current vs. time where each step represents addition of 200 μM aliquot of H_2O_2 (0.5 mg mL^{-1} antibody, 1 $\mu\text{g mL}^{-1}$ biotin-HRP, 1 mM hydroquinone, 2000 rpm, $n=3$).

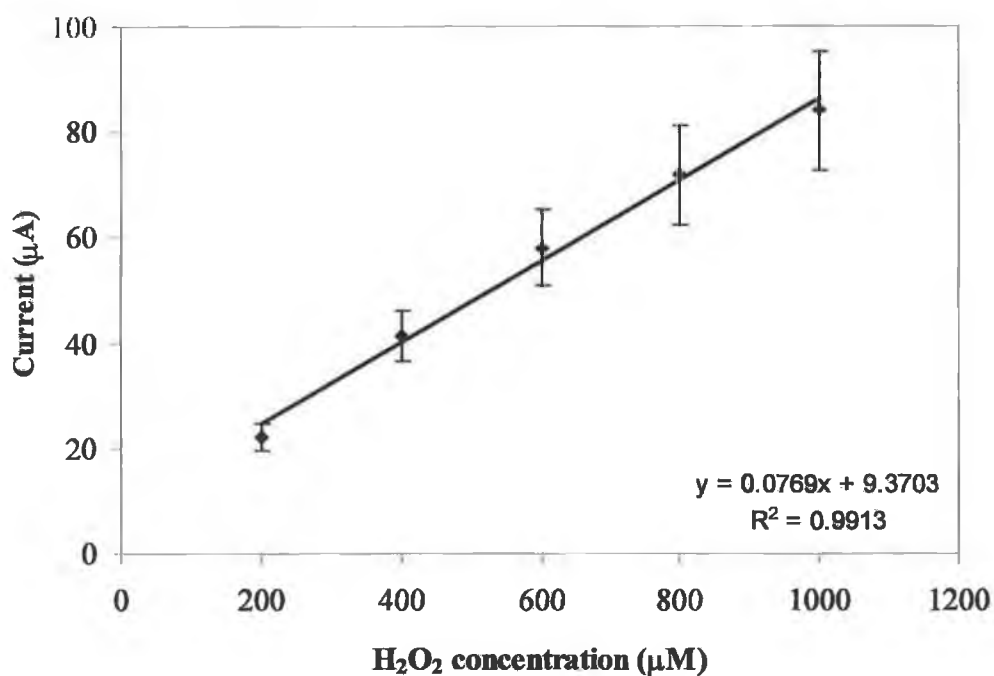


Figure 4.18. Amperometric current vs. H_2O_2 concentration (0.5 mg mL^{-1} antibody, 1 $\mu\text{g mL}^{-1}$ biotin-HRP, 1 mM hydroquinone, 2000 rpm, $n=3$).

4.4.2.5 Biotin competition assay

The semi-logarithmic graph of current vs. biotin concentration in Figure 4.19 showed that the response decreased as less enzyme conjugate bound to the immobilised antibody, as the concentration of free biotin in solution increased. A linear range can be seen within the biotin concentrations from $1 \mu\text{g ml}^{-1}$ to $30 \mu\text{g ml}^{-1}$. The limit of detection was defined as the analyte concentration corresponding to the blank signal (biotin-HRP only) minus three times the standard deviation of the blank. This resulted in a detection limit of $4 \mu\text{g ml}^{-1}$. The average rsd of the assay was relatively high at 12%. This may be due to deviations in the surface area of the home-made pyrolytic-graphite electrodes on which the SWNT forest was assembled or differences in the amount of antibody adsorbed. Compared to a related method carried out recently (Yu *et al.*, 2004) where unmediated amperometric detection of biotin-HRP was achieved, the detection limit of the present mediated system was about 10-fold better. This is likely due to the more efficient electron transfer between the HRP and electrode surface in the presence of hydroquinone. While very sensitive detection limits of biotin-HRP were reached, the detection limit of biotin obtained here was higher than that obtained from a traditional colorimetric ELISA experiment where the detection limit was found to be 50 ng ml^{-1} . This was carried out on a 96 well ELISA plate using opd as the substrate. The assay may have benefited from pre-incubation of biotin before biotin-HRP was added in an inhibition assay format (Killard *et al.*, 1999).

4.4.2.6 Stability

SWNT-assembled electrodes were prepared for immunoassay by adsorbing 0.5 mg ml^{-1} antibody, blocking with 2% (w/v) BSA and binding with $1 \mu\text{g ml}^{-1}$ biotin-HRP. Amperometry was performed on these electrodes one week after they had been stored in PBS buffer or in a humid chamber at 4°C as an indication of the stability of the adsorbed antibody on the SWNT-assembled electrodes. After one week in PBS buffer the average amperometric current generated was $9.9 \pm 1.3 \mu\text{A}$ ($n=4$, $\text{rsd}= 13.4\%$). This represented a decrease of 43% of the original signal. When the electrodes were stored for one week in a

humid chamber without being immersed in PBS buffer the amperometric current was $16.7 \pm 1.6 \mu\text{A}$ ($n=5$, $\text{rsd}= 9.6\%$). This was not significantly different from the current generated immediately after preparation of the electrodes ($17.3 \pm 2.2 \mu\text{A}$, $n=6$, $\text{rsd} = 12.6\%$). This indicates that the decrease observed when the electrodes were stored in PBS may have been due to desorption and not due to degradation of HRP.

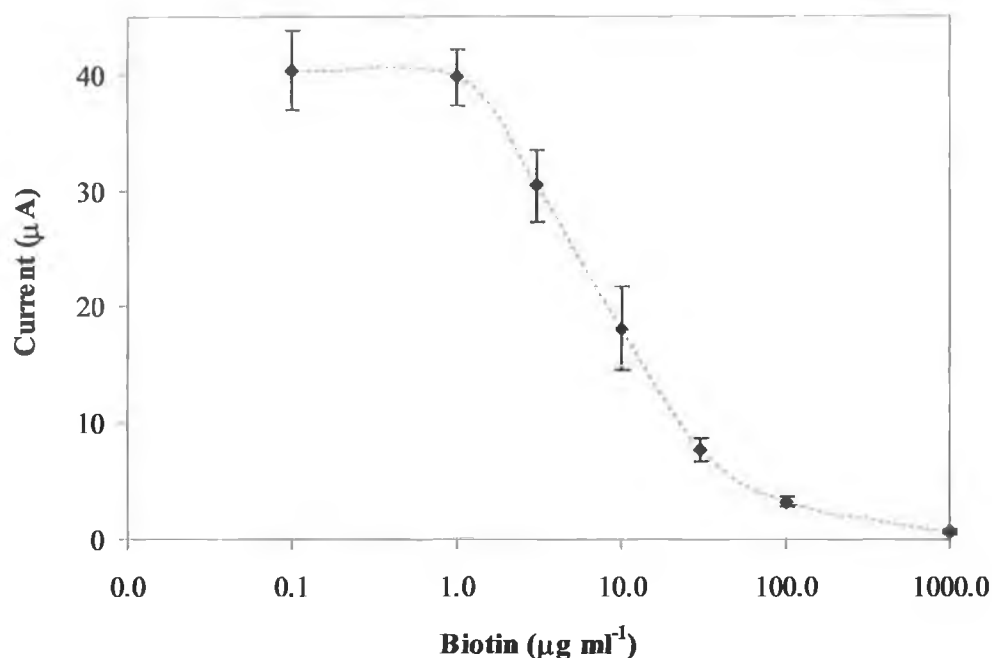


Figure 4.19. Amperometric current vs. biotin concentration with $1 \mu\text{g ml}^{-1}$ biotin-HRP, $400 \mu\text{M H}_2\text{O}_2$ and 1 mM hydroquinone, 2000 rpm , $n=3$.

4.4.3 The electrochemical behaviour of SWNT-HRP modified electrodes in the presence of *E. coli* bacteria

Previously, the immobilisation of HRP on SWNT forest pyrolytic graphite electrodes resulted in the sensitive detection of H_2O_2 by direct electron transfer at a detection limit of 50 nM (Yu *et al.*, 2003). It was further investigated here whether these electrodes could detect the real-time production of H_2O_2 as it was released from a mutant *E. coli* which lacked the catalase enzyme. Catalase is a heme protein and is a very efficient

catalyst for the decomposition of H_2O_2 , which is a product of many cellular reactions and within cells is a harmful oxidant. Catalase catalyses the following reaction:



Catalase has an extremely high turnover rate of $> 40,000$ molecules per second (Mathews *et al.*, 2000). Therefore it is expected that *E. coli* cells without the catalase enzyme should have undecomposed H_2O_2 , which it can release from the cell.

HRP-immobilised SWNT electrodes were prepared according to Yu *et al.* (2003). A CV in 20 mM phosphate buffer with 50 mM NaCl, pH 6.5 resulted in a redox peak with a $E_{1/2}$ value of $-0.3 \text{ V} \pm 0.015 \text{ V}$ (Figure 4.20). This is characteristic of the heme $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple of the enzyme. The peak current increased linearly with increasing scan rate in the range $0.025\text{--}0.5 \text{ V s}^{-1}$ (correlation coefficient, $r^2 = 99.6\%$) with a non-zero intercept (Figure 4.20). This was indicative of a thin film adsorbed electroactive species. The peak-to-peak separation of the adsorbed layer of HRP was typically 0.055 V . This was larger than the zero peak-to-peak separation which occurs for an ideal nernstian reaction under Langmuir isotherm conditions. The full width at half maximum was also larger than the nernstian ideal of 90.6 mV for a one electron process and was found to be approx. 200 mV at 0.2 Vs^{-1} . Protein monolayers and films frequently exhibit non-ideal CV characteristics suggesting interactions between redox sites (Rusling, 2003).

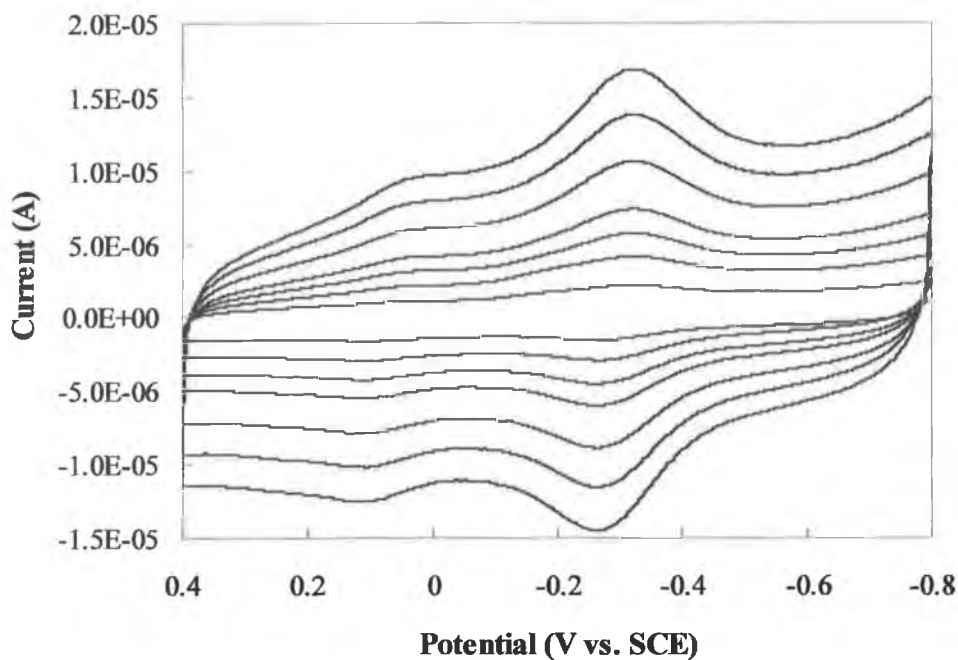


Figure 4.20. Cyclic Voltammogram of HRP immobilised on a SWNT-coated electrode in 20 mM phosphate buffer, 50mM NaCl, pH 6.5. The scan rates are (from top to bottom) 0.5, 0.4, 0.3, 0.2, 0.15, 0.1, 0.05 $V s^{-1}$.

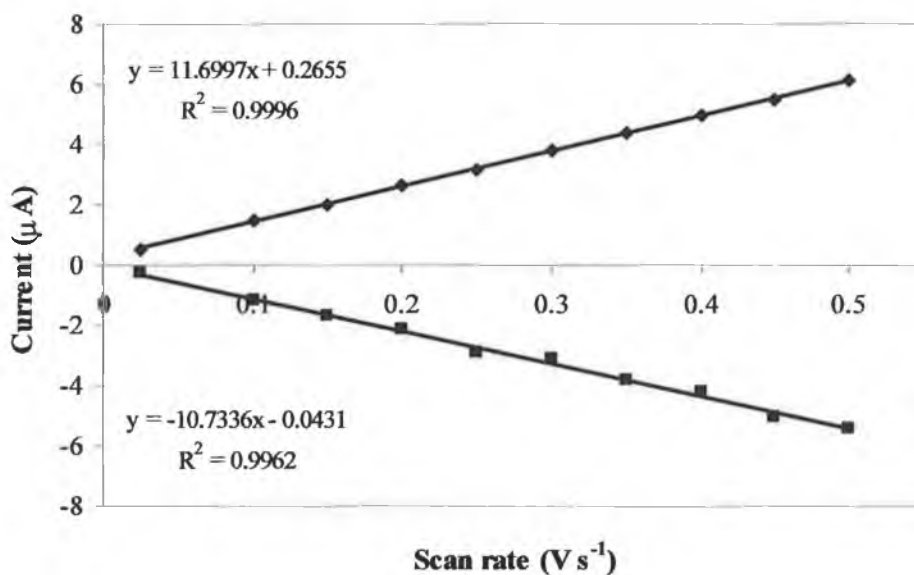


Figure 4.21. Scan rate dependence of the peak current of HRP immobilised on a SWNT-assembled electrode. The electrolytic solution was 20 mM phosphate buffer, 50 mM NaCl, pH 6.8.

The surface concentration Γ^* of an adsorbed electroactive species undergoing nernstian reaction, may be estimated from equation 4.5:

$$\frac{I}{\nu} = \frac{n^2 F^2 A \Gamma^*}{4RT} \quad (4.5)$$

Where I is the anodic or cathodic peak current, ν is the scan rate, n is the number of electrons transferred, F is the Faraday constant, A is the area of the electrode, R is the gas constant and T is the absolute temperature of the system (298 K). Therefore, the slope of the linear I vs. ν plot is equal to the right-hand side of the above equation. This was found to be $57 \pm 9 \text{ pmol cm}^{-2}$ based on an electrode surface area of 0.2 cm^2 . This is less than that reported previously for HRP immobilised on SWNT-self assembled pyrolytic graphite (170 pmol cm^{-1}) (Yu *et al.*, 2003). The CV also contained a background peak with $E_{1/2}$ of 0.045 V. This compares well with the peak noticed earlier on a bare pyrolytic graphite electrode which was due to oxygen-containing functional groups on the pyrolytic graphite (Figure 4.9).

The SWNT electrodes were assembled and immobilised with either HRP only, HRP and mutant *E. coli* or HRP and wild-type *E. coli*. Electrodes were placed in the same electrochemical cell and monitored simultaneously using a multi-channel potentiostat. A CV was taken before the start of the experiment i.e. before the addition of glucose or introduction of ambient air (Figure 4.22a). The characteristic reversible redox peak of HRP was present from each electrode as already described with a $E_{1/2}$ value of $-0.3 \text{ V} \pm 0.015 \text{ V}$ and a surface coverage of $57 \pm 9 \text{ pmol cm}^{-2}$.

Amperometry was initiated at a potential of -0.2 V . When steady state was attained, 1 mM glucose was added and ambient air was introduced into the headspace of the previously degassed buffer to encourage metabolism of the *E. coli* cells. The current was monitored for approx. 1 hour. No change in current was observed for the mutant or wildtype *E. coli* whereas an increasing signal was observed for the HRP only electrode. This result was reflected in the cyclic voltammogram taken directly after the amperometry (Figure 4.22b). The CV of the mutant and wildtype *E. coli* remained unchanged with reversible oxidation and reduction peaks. The CV of the HRP only SWNT electrode showed a large increase in the reduction peak and disappearance of the

oxidation peak. This is the expected result of catalytic reduction of oxygen by HRP. Given that there was no increase in the reduction peak of HRP when either type of *E. coli* was present indicates that the *E. coli* were consuming oxygen in the surrounding environment of the HRP maintaining reversible redox behaviour. Therefore the *E. coli* was viable and in good proximity to the HRP where it had been fixed by agarose. Since no change was found in the amperometric current or CV of mutant *E. coli*, it would appear that insufficient H_2O_2 was produced for HRP detection in the time frame of the experiment.

Although the HRP/SWNT electrodes did not detect the production of H_2O_2 in mutant *E. coli*, the system showed the effective consumption of oxygen by the *E. coli* cells. Perhaps these modified electrodes could prove useful in providing a simple way to study the real time ability of *E. coli* and other bacteria to consume oxygen under varying conditions.

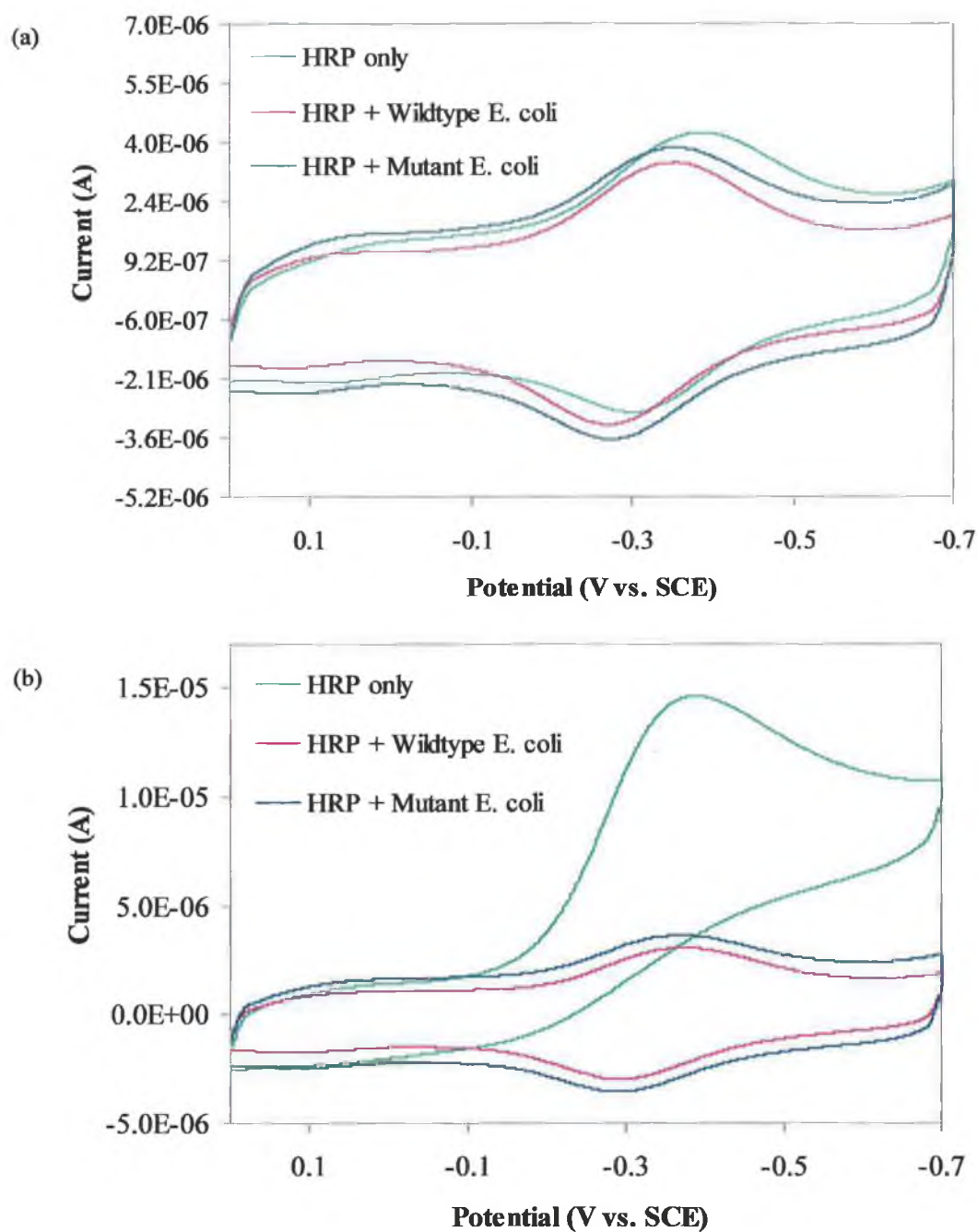


Figure 4.22. Cyclic voltammogram of SWNT-electrodes immobilised with either HRP only, or HRP with mutant *E. coli* or HRP with wild-type *E. coli* (a) before the start of the experiment i.e. before addition of glucose or oxygenation of buffer (b) one hour after introduction of glucose and ambient air. Scan rate was 150 mV s^{-1} . Electrolyte solution was, 20 mM phosphate buffer, 50 mM NaCl, pH 6.5.

4.5 CONCLUSIONS

This work showed that s-SWNT forests could be conveniently assembled and used to develop a sensitive amperometric immunosensor. The forests were assembled from oxidatively shortened SWNTs onto Nafion/iron oxide coated pyrolytic graphite electrodes. The assembly was characterised by both AFM and resonance Raman spectroscopy. A densely packed and uniform surface coverage of the SWNTs was found with a protrusion height of the SWNTs determined by AFM as 29 ± 10 nm. Anti-biotin antibodies adsorbed on the surface of the SWNT forest enabling the detection of biotin in a competitive assay format with an average rsd of $\sim 12\%$. The limit of detection of biotin-HRP was $0.1 \mu\text{g ml}^{-1}$ and that of biotin was $4 \mu\text{g ml}^{-1}$. The surface of the nanotubes were effectively blocked with BSA to result in low non-specific binding of biotin-HRP (approx. 0.1%). While the limits of detection achieved here might be further improved, for example by using high affinity monoclonal antibodies, the feasibility of high-sensitivity mediated immunosensing on SWNT forest platforms has been illustrated. Further improvements involving the isolation and use of metallic SWNTs and improvements in the assembly technique may allow for sensitive direct unmediated amperometric immunoassays to be developed.

HRP was immobilised on the surface of a SWNT forest pyrolytic graphite electrode as previously described. The surface coverage of HRP was determined to be $57 \pm 9 \text{ pmol cm}^{-2}$. Catalase mutant or wildtype *E. coli* were fixed to the HRP electrodes with agarose. Both mutant and wildtype *E. coli* maintained reversible redox conditions for HRP at the electrode surface. However, no change was found in the amperometric current or in the cyclic voltammogram of mutant *E. coli*, and it would appear that insufficient H_2O_2 was produced for HRP detection in the time frame of the experiment.

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CHAPTER 5

Future directions

5.1 Further exploration and application of the interaction of antibodies with Os(II) and Ru(II) polypyridyl complexes

Chapter 2 is concerned with the synthesis and characterisation of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, its conjugation to thyroglobulin and the production of a polyclonal antibody using $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ -thyroglobulin as an immunogen. Related complexes were synthesised such as $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ to give more information on the nature and effects of the antibody-antigen binding interactions on the complexes. A competitive ELISA determined that the antibody has a strong affinity for both $[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ and $[\text{Ru}(\text{bpy})_3]^{2+}$. It was also shown by competitive ELISA that the antibody had a lower affinity for $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$.

Chapter 3 found that binding of the polyclonal antibody raised against $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ substantially changed the emission intensity and wavelength maxima and lifetime of all the MLCs measured. Of the complexes measured ($[\text{Os}(\text{bpy})_2\text{dcbpy}]$, $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$, $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ and $[\text{Ru}(\text{bpy})_3]^{2+}$), the lifetime increased by at least a factor of 2.3 for all to a maximum of 3.7 times in the case of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$. The emission intensity increases for the complexes ranged between 3 and 7.2. These changes in the spectrochemical properties can be attributed to the reduction of the hydration of the complex upon antibody binding, the protection by the antibody from O_2 quenching and a decrease in the mobility of the complex when bound to the high molecular weight antibody.

Polyclonal antibodies consist of a heterogeneous population which vary with regards to affinity and the exact (epitope) to which the antibody binds. The polyclonal antibody produced here seemed to result in a homogeneous response with the antibody-bound complex exhibiting a monoexponential decay. However, monoclonal antibodies do not contain any ambiguity of binding and this may be a factor considered in future immunisations using these complexes.

The contribution towards the enhancement of emission intensity and lifetime from the decreased rotational motion of the Os(II) and Ru(II) polypyridyls when bound to the antibody binding site has yet to be explored. Determining the steady state anisotropy and correlation time of luminescent probes when bound to proteins serves to give information

on the rotational motions of proteins. $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ has previously been noted as a very suitable anisotropy probe (Terpetschnig *et al.*, 1995). This has been attributed to its long lifetime and less than symmetrical structure which enables high anisotropies in the absence of rotational motions. Very symmetrical complexes can have low polarisation due to randomisation of the excited state among the three ligands. Its lifetime of approx. 400 ns is very long in comparison to the lifetime of typical fluorophores which are in the range of 1 to 10 ns. Information on the rotational motion is available over a time range extending to about three times the fluorescence lifetime, after which there is too little signal for accurate anisotropy measurements. Consequently, the long lifetime of $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$ will allow the determination of long correlation times associated with large molecular weight proteins such as antibodies.

As mentioned in Chapter 1, antibodies to luminescent molecules have provided a sensitive means to determine the nature of the antibody's active site, the binding interactions involved and the hydrodynamic behaviour of immunoglobulins and their fragments. Os(II) and Ru(II) probes can now be used in similar studies. Again, their longer lifetimes especially of Ru(II) would be an advantage. This is in contrast to fluorescein which is commonly used in these studies and which has a lifetime of only 2-4 ns. Also, Ru(II) and Os(II) polypyridyls, especially those of Os(II), can be excited at long excitation wavelengths eliminating auto-fluorescence from biological molecules. Both Os(II) and Ru(II) complexes are very stable depending on the ligands used.

The change in emission intensity that can occur when fluorophores bind to their specific antibody has also been applied in the development of homogeneous immunoassays. The spectroscopic changes which occur when Os(II) and Ru(II) polypyridyls are antibody bound may also have applications in this area. For example antibodies could be produced in the traditional way or engineered so that the antibody binding site binds an Os(II) or Ru(II) MLC and all or at least part of an analyte. In this way binding of the analyte to the antibody excludes the binding of the MLC-analyte conjugate with a resulting decrease in emission intensity.

Another suitable immunoassay format could involve the incubation of analyte, labelled antigen, where the label is a Ru(II) or Os(II) MLC and anti-analyte. When equilibrium is attained an excess of anti-MLC is added. The success of the assay depends

on the binding of anti-MLC to the labelled antigen being sterically hindered when the labelled antigen is already bound by the first antibody. This renders the assay suitable only to the detection of large molecules such as proteins. Anti-MLC will only bind to and enhance the MLC in the free fraction. This assay design was developed based on fluorescein as the label allowing the detection of human serum albumin (HSA), human immunoglobulin G and human placental lactogen at approx $1 \mu\text{g ml}^{-1}$ (Nargessi *et al.*, 1979).

Since the properties of Os(II) and Ru(II) complexes vary widely with the choice of ligands other polypyridyl complexes may show an even more pronounced effect on antibody binding. An interesting area for further work would be the production of antibodies to complexes such as $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ or $[\text{Os}(\text{phen})_2\text{dppz}]^{2+}$ where dppz is dipyrrodo [3,2-a:2',3'-c] phenazine. These complexes display no detectable emission in water but do emit in more non polar solvents (Olofsson *et al.*, 2002). This may allow antibody binding to act as a complete light switch.

In Chapter 3 the effect of antibody binding on self-assembled monolayers of $[\text{Os}(\text{bpy})_2(\text{p2p})_2]^{2+}$ was investigated. Although the antibody was found to be cross-reactive with this complex, the affinity of the antibody towards this complex was less than that of $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ or $[\text{Ru}(\text{bpy})_3]^{2+}$. At certain submonolayer coverages antibody binding resulted in slight changes in the $E_{1/2}$ and fwhm values. However, the antibody may have a greater effect on the properties of a monolayer of $[\text{Os}(\text{bpy})_2\text{Qbpy}]^{2+}$ where Qbpy is 2,2':4,4'':4'4''-quaterpyridyl as this is closer in structure to the hapten. Also monolayers of $[\text{Os}(\text{bpy})_2\text{Qbpy}]^{2+}$ and $[\text{Ru}(\text{bpy})_2\text{Qbpy}]^{2+}$ are luminescent (Forster *et al.*, 1998) enabling the effect of antibody binding on the spectrochemical properties of surface bound species to be investigated. Porter *et al.* (2001) reported on the development of an electro-active system of immuno-assay (EASI assay) based on a self-assembled monolayer of a carbazole dimer (3,3(N-6-thiol hexyl)carbazole)N-ethyl carbazole monolayer. The immunoassay format involved the production of a bifunctional antibody of which one binding site was specific for the carbazole dimer and the other for estrone-3-glucuronide (E3G). Free E3G analyte in the sample displaced the bispecific antibody fragment from an immuno-surface leaving it free to bind the carbazole monolayer surface. Changes in the surface electrochemistry of the carbazole on binding of the

bifunctional antibody allowed detection of E3G down to 10 ng ml^{-1} . This illustrates how electrochemical changes obtained on antibody binding to a monolayer can act as the basis for a reagentless immunoassay.

In Chapter 3 it was found that the distance between the antibody binding sites appeared to be too far apart for detectable energy transfer between Ru and Os complexes bound to adjacent binding sites of the same antibody. With antibody engineering techniques it is possible to link single chain Fv fragments (scFv) thus limiting the distance between the antibody binding sites. The possibility of energy transfer between fluorophores which are brought into proximity by association with these binding sites, brings new possibilities for separation free immunoassays. For example the immunoassay could be based on one of the binding sites being specific for a Ru(II) complex and the other for the analyte of interest. If the analyte is labelled with a fluorophore that can undergo energy transfer with the bound Ru(II) complex then the antibody-analyte interaction can be detected.

5.2 Future directions involving the use of carbon nanotubes in immunosensing

In Chapter 4 it was shown that s-SWNT forests could be conveniently assembled and used to develop a sensitive amperometric immunosensor. This work represents an initial study of the feasibility of SWNT forests as a platform for amperometric immunosensing. One of the main potential advantages of using SWNTs as a platform is that metallic SWNT are extremely conductive. It has been reported that they can carry enormous current densities up to 10^9 A cm^{-2} . However, only one third of carbon nanotubes combine the right diameter and helicity to exhibit metallic behaviour. The remaining two-thirds are semi-metallic. A route for the separation of metallic from semi-metallic SWNTs has been reported (Chattopadhyay *et al.*, 2003). It appeared that physisorption of octadecylamine (ODA) experienced additional stabilisation on semimetallic rather than metallic SWNT. After a degree of solvent evaporation metallic SWNT precipitated while their semiconducting counterparts were retained in the supernatant. This was confirmed by changes in the resonant Raman spectra and conductivity measurements. Use of

metallic SWNT as a platform may lead to improved limits of detection and potentially eliminate the need for mediators.

The assembly format adopted here involved the co-ordination of the oxidatively shortened SWNTs onto Nafion/iron oxide coated pyrolytic graphite electrodes. The electrodes were coated with Nafion to achieve a uniform negatively charged surface suitable for formation of a high surface coverage of Fe_2O_3 . Although this assembly method has been successfully applied in the sensitive detection of the reduction of H_2O_2 by HRP and Mb modified SWNT electrodes, one concern is that the insulating Nafion layer may act as a resistive barrier creating a bottleneck in the electron conduction. One approach to circumvent this is to replace the Nafion with a conducting layer such as sulphonated polyaniline (SPAN). SPAN is water soluble, self-doped and electroactive at neutral pH and above. By using SPAN instead of Nafion as the polymer “glue” to hold the metal oxide nanoparticles onto the graphite surface, the conductance of the junctions between the nanotubes and the graphite may be significantly increased. This may improve the limits of detection achieved here involving the use of SWNT as a platform in amperometric immunosensing.

Antibodies can also be covalently attached to the carboxyl groups of the SWNT. This would result in a more robust immunoassay design suitable to flow injection. To incorporate only covalently attached antibody, steps involving the removal of non-specifically bound antibody, such as the inclusion of detergents, will need to be optimised.

The assembly method which has been used here allows SWNT forests to be easily constructed on single electrodes or in patterns on the micrometer size scale. The ability to pattern SWNT may have applications in the future development of biosensing arrays. One method of constructing the patterns which was recently developed, involved the use of electron-beam lithography. Nafion was coated on a Si wafer, then a TEM grid was placed over the wafer and it was irradiated with an electron beam. This left the desired pattern of Nafion. The assembly of SWNT was continued in the usual way on iron oxide adsorbed on the Nafion. AFM images clearly showed the resulting SWNT forest pattern. This demonstrated that the iron oxide precursor layer required underneath the SWNT forests can be deposited selectively on patterns of anionic polymer.

Since the use of CNT in immunosensing is a new area, there are many fundamental issues which warrant investigation. These include the performance of MWNT vs. SWNT, ordered assemblies vs. a thicker random orientation obtained in a cast layer and the effect of oxidation of the CNTs. While metallic SWNT holds potential for high conductivities, MWNTs have very large surface areas.

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Appendix

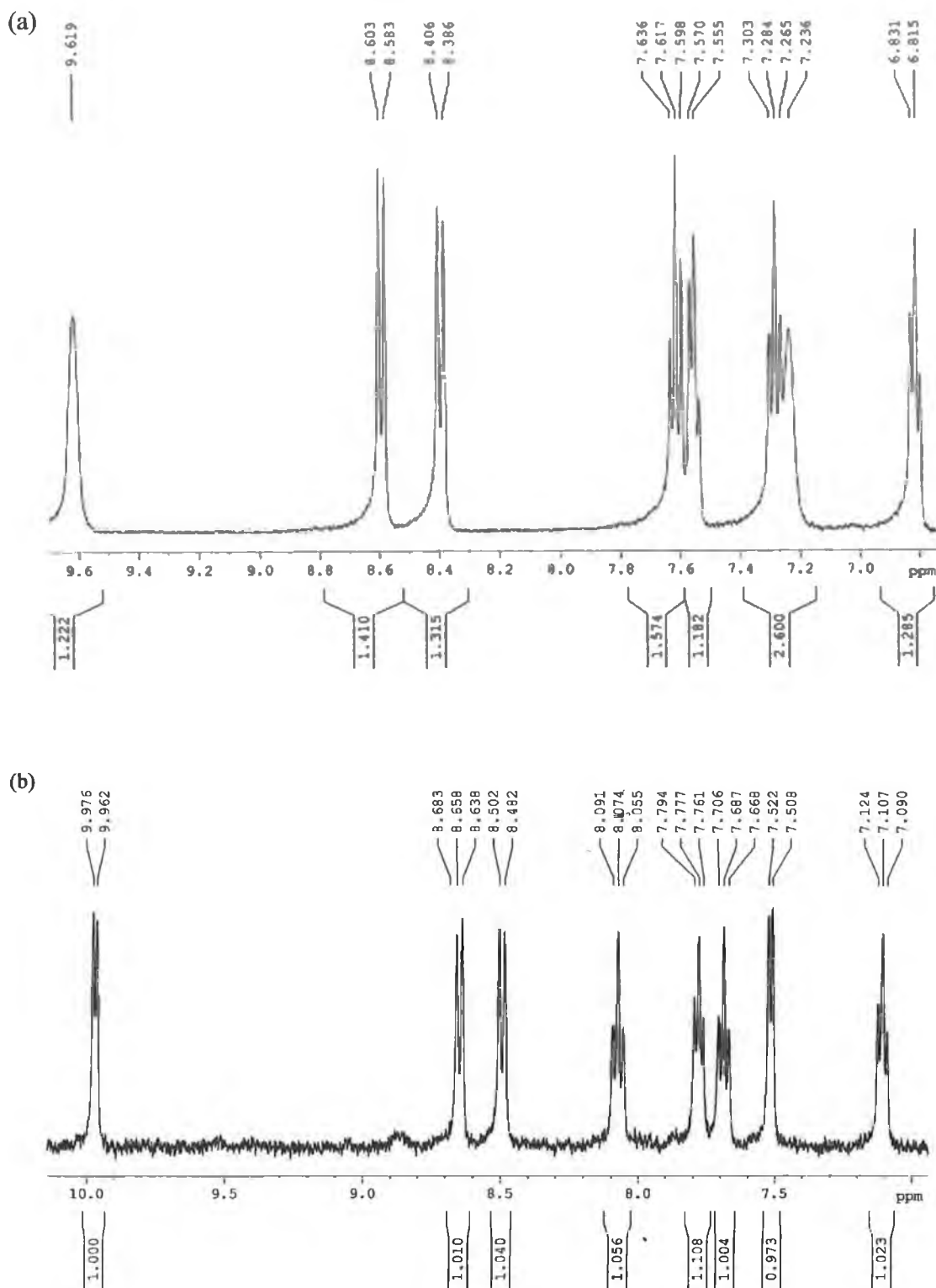


Figure 1. ^1H NMR in $d_6\text{DMSO}$ of (a) $[\text{Os}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$ (b) $[\text{Ru}(\text{bpy})_2\text{Cl}_2]\cdot 2\text{H}_2\text{O}$

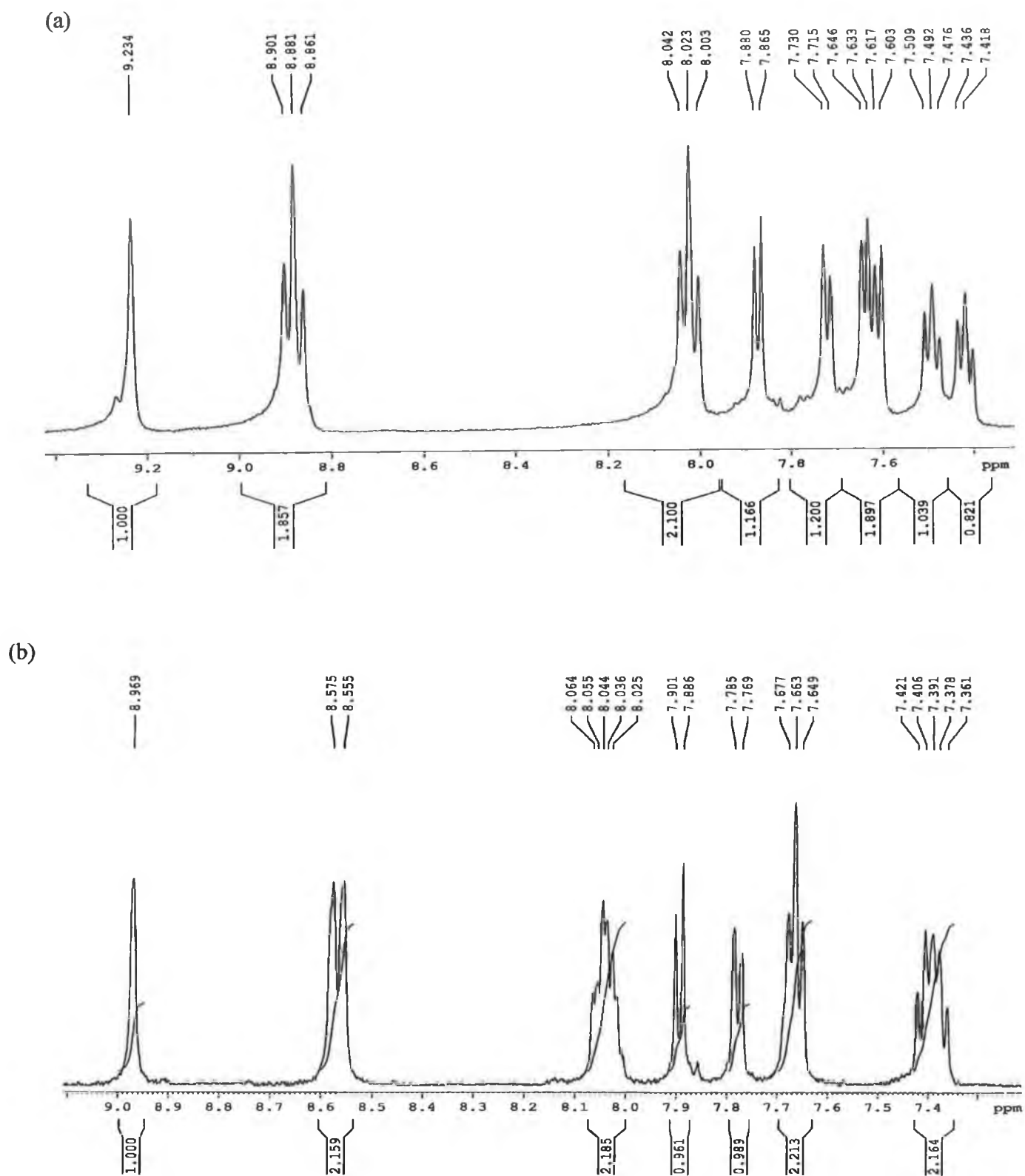
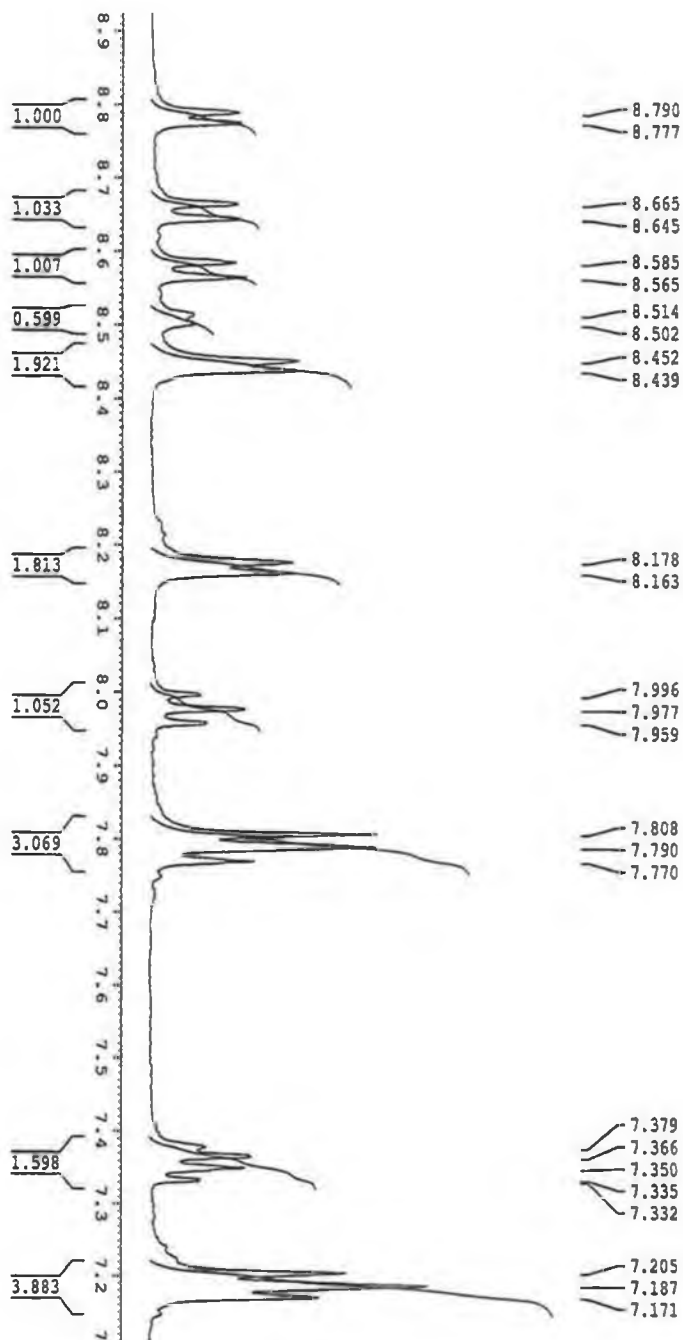


Figure 2. ^1H NMR in $d_6\text{DMSO}$ of (a) $[\text{Os}(\text{bpy})_2\text{dcbpy}]$ (b) $[\text{Ru}(\text{bpy})_2\text{dcbpy}]$.

Figure 3. ^1H NMR in $d_6\text{DMSO}$ of $[\text{Os}(\text{bpy})_2(\text{p}2\text{p})_2]\text{J}^{2+}$



LIST OF PRESENTATIONS

Poster presentations

- 2nd Annual Conference on Analytical Sciences in Ireland. Institute of Technology, Tallaght, Dublin, 4-5 April 2002.
“Production and characterisation of a polyclonal antibody specific for Os(II) and Ru(II) polypyridyl complexes.”
Máire O’Connor, Anthony J. Killard, Robert J. Forster, Malcolm R. Smyth.
- Analytical Research Forum Incorporating Research and development Topics. Kingston University, Kingston upon Thames, London, UK, 15-17 July 2002.
“Production and characterisation of a polyclonal antibody for Os(II) and Ru(II) polypyridyl complexes.”
Máire O’Connor, Anthony J. Killard, Robert J. Forster, Malcolm R. Smyth.
- 55th Irish Universities Chemistry Research Colloquium. Trinity college Dublin, Ireland, 14-16 May 2003.
“The characterisation of polyclonal antibody bound Os(II) and Ru(II) polypyridyl complexes.”
Máire O’Connor, Anthony J. Killard, Robert J. Forster, Malcolm R. Smyth.
- 10th International Conference on Electroanalysis. National University of Ireland, Galway, 6-10 June 2004.
“Single-walled carbon nanotube forests for amperometric immunosensing.”
Máire O’Connor, Sang Nyon Kim, Anthony Killard, Robert Forster, Fotios Papadimitrakopoulos, James Rusling, Malcolm Smyth.

Oral Presentation.

- 205th Meeting of the Electrochemical society, San Antonio, Texas, 11-14 May 2004.

"Single-walled carbon nanotube forests for amperometric immunosensing."

Máire O'Connor, Sang Nyon Kim, Anthony Killard, Robert Forster, Fotios Papadimitrakopoulos, James Rusling, Malcolm Smyth.

PAPERS

- *"Production and characterisation of a polyclonal antibody for Os(II) and Ru(II) polypyridyl complexes."*

Máire O'Connor, Anthony J. Killard, Robert J. Forster, Malcolm R. Smyth.

To be submitted

- *"Mediated amperometric immunosensing using single walled carbon nanotube forests"*

Máire O'Connor, Sang Nyon Kim, Anthony J. Killard, Robert J. Forster, Malcolm R.

Smyth, Fotios Papadimitrakopoulos, James F. Rusling

To be submitted