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Production and characterization of a polyclonal antibody for Os(II) and Ru(II) polypyridyl complexes

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Abstract

The characterization of a polyclonal antibody produced via immunization with an [Os(bpy)$_2$dcbpy] hapten is described. Bpy is 2,2′-bipyridine and dcbpy is 2,2′-bipyridine-4,4′-dicarboxylic acid. The cross-reactivity of the antibody for the Ru(II) analogue of the hapten was also investigated. Large increases in the emission and luminescent lifetime of a series of Os and Ru complexes were observed on binding of the antibody. Association equilibrium constants were derived from luminescence titration data and were found to be 5.6×10^8 and 5.0×10^8 M^{-1} for [Os(bpy)$_2$dcbpy] and [Ru(bpy)$_2$dcbpy], respectively. Spectroscopic changes were likely due to the exclusion of H_2O from the complex/antibody binding cleft and blocking of vibrational relaxation pathways of the Os/Ru excited state. D_2O/H_2O experiments confirmed that the antibody protected approx. 82% of [Os(bpy)$_2$dcbpy] and 80% of [Ru(bpy)$_2$dcbpy] from excited state deactivation by the aqueous solvent.

Keywords: Antibody; Osmium(II); Ruthenium(II); Polypyridyl

1. Introduction

The antigen–antibody interaction is characterized by high specificity and affinity [1]. These properties are fundamental to the success of antibodies as part of the immune response in vertebrates and as a recognition element in assays of medical and environmental importance. Several different forces are involved in the binding of an antigen to the amino acid residues of the antibody's complementary binding pocket. These include electrostatic attraction, Van der Waals forces and hydrogen bonding.

Antibody binding to luminescent molecules may result in either fluorescent quenching or enhancement. The production of antibodies to luminescent haptens has therefore 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 [2–5]. It has also provided a convenient method to study the changes in antibody affinities during the immune response and under varying immunization conditions [6]. Some early papers focused on the use of antibodies with fluorescent probes in the development of homogeneous immunoassays [7,8]. Examples of haptens which undergo fluorescent quenching on antibody binding are fluorescein [2] and anthracycline antibiotics [3]. Haptens which undergo fluorescent enhancement are 5-dimethylaminonaphthalene-1-sulfonamide (DNS) [4] and 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ) [5]. Enhancement or quenching of the antibody-bound fluorophore depends on its interaction with, and the nature of the active site. In the case of fluorescein, fluorescence quenching is due to a complex mechanism involving both static and

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dynamic components by tryptophan and tyrosine amino acids. Fluorescent enhancement is frequently due to the decreased polarity of the binding site.

The present study reports the production for the first time of a polyclonal antibody specific for an Os(II) complex, $[Os(bpy)_2(dcbpy)]$. Polyclonal antibodies consist of a heterogeneous population which vary with regards to affinity and the epitope to which the antibody binds. Monoclonal antibodies do not contain any ambiguity of binding and this may be a factor considered in future immunizations using these complexes. However, the screening of monoclonal antibodies with specific binding characteristics is a lengthy and expensive undertaking. In addition, a monoclonal antibody will bring about a single, defined interaction with the antigen (in this case the polypyridyl complex), the characteristics of which cannot be easily determined or controlled and which may produce unpredictable responses, e.g., in the case of antibodies to Os(II) polypyridyl complexes exhibiting unexpected extremes of fluorescence quenching. The production of polyclonal antibodies provides a convenient path to an initial exploration of the spectroscopic effects of antibody binding to an Os(II) polypyridyl complex. A polyclonal sera will bind to the hapten via a range of configurations. The characteristics of this population of antibodies produces an 'average' measure of the overall binding equilibrium, yielding a fairer picture of the properties of the interaction.

Os(II) and Ru(II) polypyridyl complexes have been shown to be effective and versatile biomolecular probes [9–17]. Both Os(II) and Ru(II) complexes are stable depending on the ligands used. Their lifetimes are usually much longer than that of typical fluorophores which are in the range of between 1 and 10 ns. Their structure and therefore photophysical properties can be tuned to suit particular applications [16,17]. They have been applied as photophysical probes of various types of biomolecules. In the case of DNA they have been used as sequence-specific photo reagents. For example, luminescence quenching of a Ru-labeled oligonucleotide occurred upon hybridization to a complementary target strand where the strand contained guanines [9]. They have also been developed as photosensitizers for strand cleavage in DNA [10]. The complexes have been used to monitor structural changes [11] and rotational motions of proteins [12]. Their sensitivity extends to the interaction of the covalently-bound protein with other protein molecules such as in antibody–antigen interactions [13–15]. The complexes have further useful advantages over other reporter systems such as enzymes as the measurements can be performed repeatedly, with the potential of real-time and kinetic analysis of these biomolecular interactions. Competitive immunoassays for the detection of human serum albumin using Os(II) and Ru(II) polypyridyls based on polarization [14] and fluorescence resonance energy transfer [15] have been successfully developed. This work illustrates the interesting and useful optical phenomena that result upon the interaction of antibodies with Os(II) and Ru(II) polypyridyl complexes. It is hoped that these and other features of the interaction may serve as the basis of novel immunological strategies.

2. Experimental

2.1. Materials

Ruthenium(III) chloride (11808) and potassium hexachloroosmiate (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). Phosphate buffered saline (PBS) was 0.1 M phosphate buffer, pH 7.4, 0.137 M NaCl.

2.2. Methods

2.2.1. Synthesis of complexes

$[Os(bpy)_2Cl_2]\cdot 2H_2O$ was synthesized as reported elsewhere [18]. $[Os(bpy)_2(dcbpy)]Cl_2$ was synthesized following the procedure described by Terpetschnig et al. [19]. $[Ru(bpy)_2(dcbpy)]Cl_2$ was synthesized according to Bard et al. [20]. $[Os(bpy)_2(p2p)_2](PF_6)_2$ where p2p is 1,2-bis-(4-pyridyl)ethane, was synthesized following the procedure described by Acevedo and Abruña [21] with a slight modification; this being the addition of an equal volume of de-ionized water to the dissolved $[Os(bpy)_2Cl_2]\cdot 2H_2O$ in deaerated ethylene glycol. Satisfactory ^1H NMR and CHN analysis were obtained for all complexes.

2.2.2. pK_a measurements

The pK_a of $[Os(bpy)_2dcbpy]$ was determined in Britton-Robinson buffer. The pH was adjusted with conc. H_2SO_4 or conc. NaOH. The pK_a was determined from the point of inflection of the curve obtained upon plotting absorbance versus pH. One inflection point in the pH region 1–12 resulted in a pK_{a1} of 2.7. This also indicated that the pK_{a2} was less than pH 1. pK_a values for $[Ru(bpy)_2dcbpy]$ have been reported as 2.85 and 1.75 [22]. These complexes were therefore expected to have a neutral charge at pH 7.4, the pH at which all measurements were carried out.

2.2.3. Preparation of $[Os(bpy)_2dcbpy]$-thyroglobulin conjugate

Conversion of $[Os(bpy)_2dcbpy]$ to its active succinimide ester and conjugation to protein was carried out according to Terpetschnig et al. [19] with minor modifications. $[Os(bpy)_2dcbpy]$ (26 mg) and a 2 mole excess of *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were dissolved in 1 ml of anhydrous dimethylformamide (DMF). The solution was allowed to stir for 5 h. On ester formation the solution turned red in colour. Thyroglobulin (20 mg) was dissolved in 1 ml of 0.1 M bicarbonate buffer, pH 8.3. The active ester solution was added slowly, the total volume added being equivalent to the desired molar excess of

the ester over the protein. The remaining ester solution could be kept active by storing at −15 °C. The conjugation reaction was allowed to proceed overnight while stirring. Unbound Os complex was removed by the use of gel filtration chromatography on Sephadex G-25 and/or exhaustive dialysis in PBS. The dicyclohexylurea precipitate was removed by centrifugation. Bovine serum albumin (BSA) was also conjugated for the purposes of antibody characterization. The conjugation ratio was defined as the molar ratio of complex to conjugated protein. The amount of complex present was determined by its absorption in PBS at its absorption maximum without correcting for any change in the extinction coefficient upon binding to the biomolecules. The protein concentration was determined using a bicinchoninic acid (BCA) assay (Sigma B-9643). Successful conjugation was confirmed using size exclusion chromatography using a Biosep-sec S3000 column (data not shown).

2.2.4. Immunization procedure and antibody purification

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 immunization. Purification of polyclonal antibody from rabbit serum was initially carried out by precipitation with saturated ammonium sulfate. Since non-specific antibodies did not interfere with the spectroscopic properties of the complexes protein A purification (Pure-1A antibody purification kit, Sigma) was chosen to isolate the IgG fraction thereby retaining a population of antibodies with mixed affinities. Purification of the polyclonal antibody further would in practical terms limit the characterization to a portion of the original antibody population. The purified IgG concentration was determined in mg ml^{-1} as the absorbance reading at 280 nm × 0.7 [23].

2.2.5. Enzyme immunoassays

The antibody working dilution and optimum conjugate loading density was determined as follows. Serial dilutions of BSA-hapten conjugate were prepared in a concentration range from 50 to 0.5 μg ml^{-1} in PBS. A row of each concentration was coated on a 96 well microtitre plate, which was incubated overnight at 4 °C. Plates were then washed three times with PBS containing 0.05% (v/v) Tween-20 and three times with PBS. The plates were blocked with 150 μl of 2% (w/v) non-fat milk powder for 1 h at 37 °C. Serial dilutions of polyclonal antibody in PBS were prepared in the range between 1/500 and 1/1,024,000. These were added in triplicate to the wells and allowed to bind at 37 °C for 1 h. After washing, a 1 in 10,000 dilution of horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody in PBS was incubated on the plate for 1 h at 37 °C. Plates were again washed and 100 μl of Sigma Fast *o*-phenylenediamine (*o*-PD) substrate (P-9187) was added to each well. When 30 min at 37 °C had elapsed, the reaction was stopped on addition of 50 μl of 2 M H_2SO_4. The absorbance was read at 492 nm using a Titretek plate reader. The absorbance readings obtained at each antibody dilution (A) were divided by the absorbance reading determined in the presence of zero antibody (A_o) to give a normalized reading. 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 optimum dilution of purified antibody was determined as the dilution taken from the mid-point of the linear portion of the sigmoidal curve obtained. Competition immunoassays were performed as follows. A 96 well microtitre plate was coated with the optimum conjugate loading density and blocked. Serial dilutions of competing antigen in PBS of a suitable range were prepared. Two hundred microliters of each antigen concentration was incubated with 200 μl of purified antibody at 2 × working dilution, for 1 h at room temperature. Hundred microliters of this solution were transferred in triplicate to the previously coated and blocked microtitre plate. The plate was then developed and read as described.

2.2.6. Instrumentation

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. 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 (N_2 filled at 1.1 or 0.3 atm. pressure, 40 Hz) interfaced with a personal computer via a Norland MCA card. Time resolved intensity decays ($I(t)$) were fit to a single exponential model, $I(t) = A\exp(-t/\tau)$, where A is the preexponential factor and τ the lifetime using EAI F900 software version 5.1.3. All absorption, luminescence and lifetime measurements were carried out in PBS unless otherwise stated. Deaerated samples were obtained by purging with nitrogen for 30 min. Estimated errors in the reported values are as follows: absorption and emission maximums, ±1 nm (±5 nm for weak emitters); emission lifetime ±10%, pK_a, ±0.1.

3. Results and discussion

[Os(bpy)$_2$dcbpy]-thyroglobulin conjugates were prepared as the immunogen. An initial reaction ratio of 200:1 resulted in an actual substitution ratio of 83:1. The

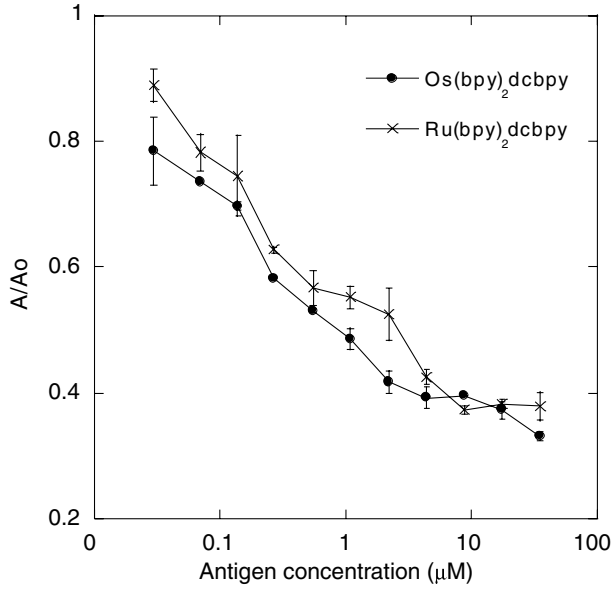

Fig. 1. Immunoassay of *anti*-[Os(bpy)$_2$dcbpy] antibody and its competition between [Os(bpy)$_2$dcbpy]-BSA and free [Os(bpy)$_2$dcbpy] or [Ru(bpy)$_2$dcbpy] complexes ($n = 3$). The titration curves show the typical inverse relationship between antigen concentration and the normalized absorbance response (A/A_o). The slight shift of the Ru complex to higher antigen concentrations shows that the antibody has a slightly lower affinity for it than the Os complex to which the antibody was raised.

amount of complex present was determined by its absorption in PBS while the protein concentration was determined using a bicinchoninic acid (BCA) assay. Conjugation resulted in slight changes to its UV–Vis absorption and emission spectra. The ^{3}MLCT bands at 440 and 485 nm broadened and underwent a slight red shift of 5 nm upon conjugation. This is similar to the changes observed on protein conjugation of [Ru(bpy)$_2$dcbpy] [24]. A 20 nm shift to lower energy and lower intensity emission also occurred. The lifetime of [Os(bpy)$_2$dcbpy] was determined as 20 ± 2 ns and its thyroglobulin conjugate as 18 ± 2 ns.

The immunoassay of the antibody's competition between the [Os(bpy)$_2$dcbpy]-BSA conjugate and both free Os and free Ru complexes (Fig. 1) showed that as the concentration of the free complex increased, the absorbance signal decreased confirming the presence of antibodies specific to the hapten [Os(bpy)$_2$dcbpy] and also to [Ru(bpy)$_2$dcbpy]. This was expected as the metal ligand bond lengths for analogous complexes of Os(II) and Ru(II) are very similar due to the lanthanide contraction [18]. This minimises the differences in steric effects and solvation. However, the response to the Ru(II) complex was of a slightly lower affinity.

The effect of the addition of increasing amounts of antibody on the emission properties of a constant amount of [Os(bpy)$_2$dcbpy], [Ru(bpy)$_2$dcbpy], $[Ru(bpy)_3]^{2+}$ 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 on binding of the antibody (Table 1, Figs. 2–4).

The emission of the hapten [Os(bpy)$_2$dcbpy] experienced the greatest change upon complete saturation with antibody. A blue shift of 37 nm as well as a 7.2-fold increase in emission intensity was observed (Figs. 2 and 3). This contrasts with the 21 nm red shift, which was observed on covalent binding of this complex to thyroglobulin. This illustrates the radical change in environment which occurs when [Os(bpy)$_2$dcbpy] 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)$_2$dcbpy] was fully saturated with antibody. This was accompanied by a 3.9-fold increase in emission intensity (Fig. 4). Sharp endpoints were obtained when [Os(bpy)$_2$dcbpy] and [Ru(bpy)$_2$dcbpy] were titrated with the antibody indicating a strong affinity. Saturation of the Os complex appeared to occur at a slightly lower antibody:complex ratio then for the Ru complex, occurring at 0.9:1 and the latter at 1:1. This may be indicative of the antibody's marginally higher affinity for the Os complex. The emission maximum of $[Ru(bpy)_3]^{2+}$ underwent a 13 nm blue shift with a 3.0-fold increase in emission intensity (Fig. 4), and a saturation ratio of approx. 1.4:1 antibody:complex.

The titration of $[Os(bpy)_2(p2p)_2]^{2+}$ 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 an excess of antibody. The larger excess of antibody needed to achieve saturation may be expected due to the lower affinity of the antibody to this complex as seen by

Table 1
Steady-state emission and lifetimes of free and antibody-bound complexes and association equilibrium constants (K_a)

Complex	Emission		Lifetime (ns)				K_a (M^{-1})
	Maxima (nm)		Aerated		Deoxygenated		
	Free	Bound	Free	Bound	Free	Bound	
[Os(bpy)$_2$dcbpy]	771	734 (7.2)	20	75 (3.7)	20	77 (3.9)	5.6×10^8
[Ru(bpy)$_2$dcbpy]	642	619 (3.9)	399	1299 (3.3)	528	1572 (3.0)	5.0×10^8
$[Ru(bpy)_3]^{2+}$	612	599 (3.0)	387	1124 (2.9)	658	1375 (2.1)	7.1×10^7
$[Os(bpy)_2(p2p)_2]^{2+}$	757	730 (5.6)	17	53 (3.1)	–	–	3.6×10^5

Lifetimes are an average of three measurements, which typically agreed to ±2%. Figures in brackets show the enhancement of integrated emission intensity or lifetime upon antibody binding. All measurements were taken in 0.01 M PBS, pH 7.4.

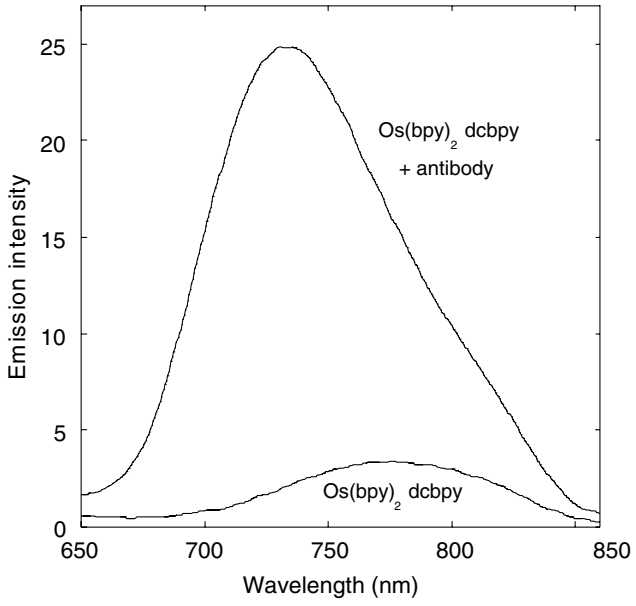

Fig. 2. Emission spectra of $[Os(bpy)_2dcbpy]$ before and after saturation with *anti*-$[Os(bpy)_2dcbpy]$. Upon antibody binding the Os complex showed a 37 nm shift to higher energy and a 7.2-fold increase in emission intensity.

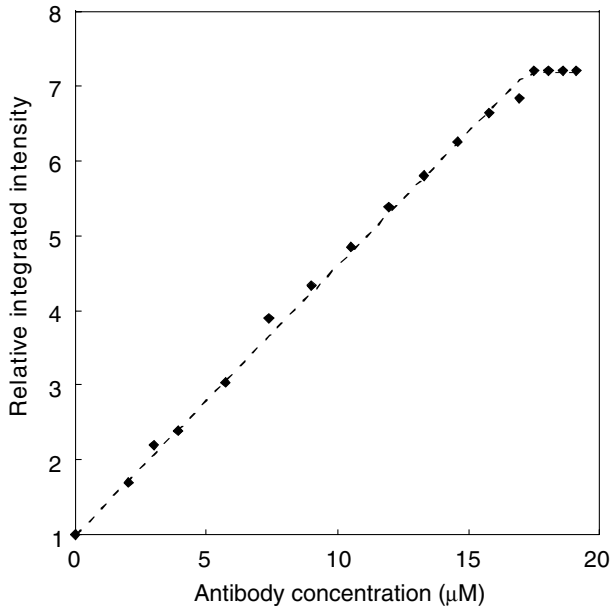

Fig. 3. Increase in relative integrated emission of 20 μM $[Os(bpy)_2dcby]$ with increasing concentrations of antibody. The relationship between the molar ratio of antibody to complex and intensity was linear up to a ratio of 0.9:1 at which point there were no further increases in intensity. This suggests full saturation of the complex at this binding ratio. Broken lines denote the fit for the K_a value of 5.6×10^8 M^{-1}.

competition immunoassay (data not shown). A gradual tailing of the intensity was also 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 1.5:1 antibody to $[Os(bpy)_2(p2p)_2]^{2+}$ had been reached. The extent

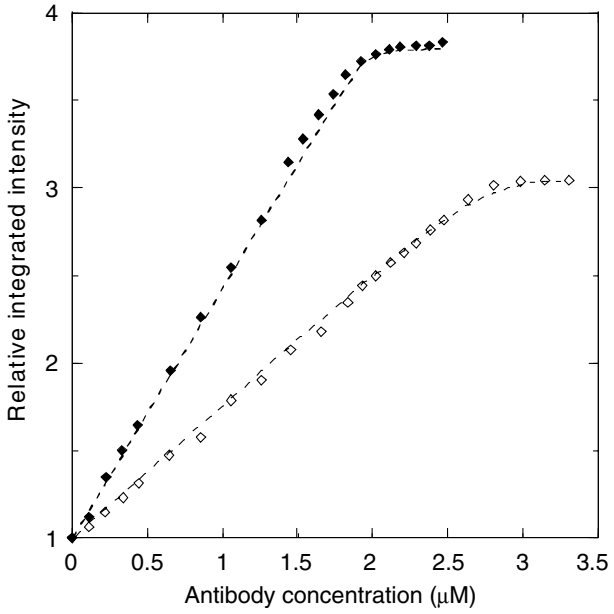

Fig. 4. Increase in relative integrated emission of 1 μM $[Ru(bpy)_2dcby]$ (♦) and 1 μM $[Ru(bpy)_3]^{2+}$ (◇) with increasing concentrations of antibody. Both complexes showed linear responses until saturation was reached. The $[Ru(bpy)_2dcbpy]$ complex appears to have become saturated at an antibody:complex ratio of approx 1:1 and the $[Ru(bpy)_3]^{2+}$ complex at approx. 1.4:1. At saturation, $[Ru(bpy)_2dcbpy]$ has an emission intensity 3.9-fold higher and $[Ru(bpy)_3]^{2+}$ 3.0-fold higher than in the absence of antibody. Broken lines denote the fit for the K_a value of 5.0×10^8 and 7.1×10^7 M^{-1} for antibody binding to $[Ru(bpy)_2dcby]$ and $[Ru(bpy)_3]^{2+}$, respectively.

of the increase in emission was surprising given its expected lower affinity. A 27 nm blue shift of maximum wavelength was observed at the end of the titration (Table 1). Titration of all of the complexes with non-specific IgG served to confirm that the spectral changes observed were due to the specific nature of the antibody binding.

Association equilibrium affinity constants (K_a) were derived from the steady-state luminescence titrations according to Shreder et al. [25]. These constants are, of course only relative as they do not take into account the polyclonal nature of the antibody, avidity effects, etc. As expected the K_a obtained for $[Os(bpy)_2dcbpy]$ and $[Ru(bpy)_2dcbpy]$ were very similar being 5.6 $(\pm 24) \times 10^8$ and 5.0 $(\pm 1.2) \times 10^8$ M^{-1}, respectively, which represents a high affinity. The equilibrium constants obtained here decrease with increasing structural difference of the complex towards the hapten ($[Os(bpy)_2dcbpy]$), the K_a obtained for $[Ru(bpy)_3]^{2+}$ was almost one order of magnitude lower at 7.1 $(\pm 1.7) \times 10^7$ M^{-1} and that of $[Os(bpy)_2(p2p)_2]^{2+}$ was significantly lower at 3.6×10^5 M^{-1} (Table 1). In some cases, the error in the calculation was relatively high due to the small number of data points used to perform the curve fit. Nevertheless, taken in conjunction with the enzyme immunoassay data, a clear picture of the relative affinities of these complexes to the antibody can be seen and related, in turn to the structural characteristics of the complex.

Time-resolved luminescence spectroscopy was also used to study the interaction of the antibody with $[Os(bpy)_2dcbpy]$, $[Ru(bpy)_2dcbpy]$, $[Ru(bpy)_3]^{2+}$ and $[Os(bpy)_2(p2p)_2]^{2+}$. The lifetimes of the unbound and fully antibody-bound complexes all displayed monoexponential decays. As for the luminescence titrations, control samples were prepared which contained a mixture of the complex with non-specific IgG. A monoexponential decay was observed with no significant change in lifetime. The lifetime of unbound $[Os(bpy)_2dcbpy]$ in PBS was 20 ns. No significant change was observed when deaerated with nitrogen as reported in the literature [19]. Although the lifetimes were almost unaffected when covalently-bound to thyroglobulin, a 3.7-fold increase to 75 ns was observed when bound to antibody (Fig. 5). This represents the largest lifetime 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 Waal and hydrogen bonding between the complex and antibody. When the antibody-saturated $[Os(bpy)_2dcbpy]$ solution was deaerated no further significant increase in lifetime was observed (77 ns).

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. This showed that recognition of the 2,2′-bipyridyl ligands by the antibody was sufficient for a substantial increase. The increase in lifetime experienced for an aerated sample of $[Ru(bpy)_3]^{2+}$ was 2.9, which 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.

The lifetime of the antibody-bound $[Ru(bpy)_2dcbpy]$ and $[Ru(bpy)_3]^{2+}$ increased when the samples were deaerated (Table 1). This shows that the antibody does not completely protect the complex from quenching by O_2. The longer lifetimes of the antibody-bound complexes are to be expected given the increase in intensity observed in the luminescence titrations.

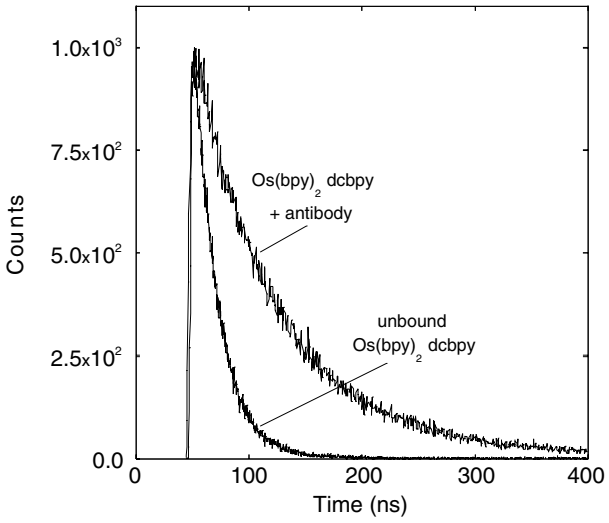

Fig. 5. Monoexponential lifetime decay profile of free and antibody-bound $[Os(bpy)_2dcbpy]$. The lifetime of the complex increased from 20 to 75 ns upon antibody binding.

Antigen–antibody association involves electrostatic, Van der Waals and hydrogen bonding interactions [1]. 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 [26–29]. k_{nr} experiences a dramatic increase in hydroxylic solvents with a corresponding decrease in emission energy. Therefore, the increases in emission intensity and lifetime observed were consistent with elimination of water molecules from the solvation shell of the complexes upon antibody binding. Other reasons may include protection of the complex by the antibody from O_2 collisional quenching and a decrease in the mobility of the complex when bound to the high molecular weight antibody.

The spectroscopic properties of Os(II) and Ru(II) complexes are influenced by the nature of their solvation shell. That of $[Ru(bpy)_3]^{2+}$ has been studied in detail and the presence of several hydrating water molecules, which penetrate between the ligands of $[Ru(bpy)_3]^{2+}$ has been reported [30]. X-ray diffraction studies of aqueous solutions detected about two H_2O molecules at a distance of 3.5–3.6 Å from the central metal atom. Ten or eleven H_2O molecules were found to exist in the region of 5.3–6.3 Å. These may be hydrogen-bonded to the ligands and to adjacent water molecules including the closest ones. The centres of the hydration shell were located around 7.7 Å from the central metal atom of the ion.

It has been found that the k_{nr} values for Os(II) and Ru(II) complexes in hydroxylic solvents are much higher than the trend observed in polar organic solvents, with H_2O having the most dramatic effect [26–29]. It has been shown that the k_{nr} of $[Os(bpy)_3]^{2+}$ and $[Os(phen)_3]^{2+}$ in H_2O are large compared with those in D_2O despite the same emission energy [26] (where phen = 1,10-phenanthroline). The overtones of the vibrationally excited states of H_2O lie close to the ^{3}MLCT state of the Os(II) complex and are expected to act as energy accepting levels. Energy transfer via dipole-dipole interaction between the transition of the Os(II) complex (^{3}MLCT → ground state) and that of the included H_2O (vibrational ground state → its excited state) account for the increase in the k_{nr}.

Hauenstein et al. [27] reported a linear increase of the τ^{-1} of $[Ru(bpy)_3]^{2+}$ on the mole fraction of H_2O in H_2O–D_2O mixtures. The linear dependence of τ^{-1} on the mole fraction of H_2O indicated that a H_2O-specific quenching process also applies to Ru(II) complexes. This relationship also allows us to calculate F the fraction of complex not shielded from the aqueous phase by antibody binding. Measurements of the lifetime of the complex in H_2O–PBS (τ_H), D_2O–PBS (τ_D) and bound by antibody in H_2O–PBS (τ_{HA}) and D_2O–PBS (τ_{DA}) are required.

$$F = \frac{[\tau_{HA}^{-1} - \tau_{DA}^{-1}]}{[\tau_{H}^{-1} - \tau_{D}^{-1}]}$$

It was determined that 82% of the antibody-bound [Os(bpy)$_2$dcbpy] was inaccessible to excited state deactivation via interaction with solvent, while 80% of antibody-bound [Ru(bpy)$_2$dcbpy] was protected. This shows little discrimination of the antibody towards the Ru metal centre.

Shreder et al. [25] have also investigated the change in photophysical properties observed in a series of Ru(II) complexes when bound to a monoclonal antibody raised against a tris(2,2′-bipyridine)cobalt(III)-methyl viologen hapten. The largest increase in emission obtained was 2.6-fold, which was considerably smaller than the 3.8-fold increase for the Ru(II) analogue of the hapten achieved here. The authors reported that a slight blue shift was achieved which is also in contrast to the significant changes in emission maxima observed here. That group's report of binding of $[Ru(bpy)_3]^{2+}$ to a polyclonal antibody raised against a tris(2,2′-bipyridine)cobalt(III)-methyl viologen hapten [31], displayed an emission increase of 3.3, which is closer to the values obtained here. This highlights the variable nature of the affinity of antibodies raised in different immunization schedules and indicates that the spectral properties of the bound complex are influenced by the particular environment provided by the binding pocket. However, the full effect of antibody binding was not seen in these reports since the hapten contained the viologen moiety which led to internal quenching and was relatively non-luminescent. The antibody would be expected to have a lower affinity for the $[Ru(bpy)_3]^{2+}$ portion on its own. This may also account for the lower value of 70% protection against solvent as determined from D_2O/H_2O experiments. Lifetime increases between 2.2- and 4.6-fold were obtained on binding of a series of Ru(II) complexes to a monoclonal antibody [25]. A polyclonal antibody-bound $[Ru(bpy)_3]^{2+}$ resulted in a 3.3-fold increase [31]. This degree of enhancement is similar to the values obtained here.

4. Conclusions

Polyclonal antibodies have been raised against an [Os(bpy)$_2$dcbpy]-thyroglobulin conjugate and shown to have a strong affinity for both [Os(bpy)$_2$dcbpy] and [Ru(bpy)$_2$dcbpy]. Binding of the antibody substantially changed the emission intensity and wavelength maxima and lifetime of the complexes. The integrated emission increase varied from 7.2-fold for the high affinity binding of [Os(bpy)$_2$dcbpy] to 5.6-fold for the lower affinity binding of $[Os(bpy)_2(p2p)_2]^{2+}$. The lifetime increased by at least a factor of 2.3 for all the complexes measured. The greatest change in photophysical properties was experienced by the hapten [Os(bpy)$_2$dcbpy] which also resulted in a 3.7-fold increase in lifetime in aerated buffer and a 37 nm blue shift in wavelength of emission maxima. This confirms the importance of a close steric fit for high affinity antibody–antigen binding. Association constants were derived from the luminescence titration data and were determined to be 5.6×10^8 and $5.0 \times 10^8\ M^{-1}$ for [Os(bpy)$_2$dcbpy] and [Ru(bpy)$_2$dcbpy], respectively, $7.1 \times 10^7\ M^{-1}$ for $[Ru(bpy)_3]^{2+}$ and $3.6 \times 10^5\ M^{-1}$ for $[Os(bpy)(p2p)_2]^{2+}$. The changes in the spectrochemical properties can be attributed to the reduction of the hydration of the complex upon antibody binding. Lifetime measurements in deaerated H_2O–PBS and D_2O–PBS confirmed that 82% of the antibody-bound [Os(bpy)$_2$dcbpy] was inaccessible to excited state deactivation via interaction with solvent, while 80% of antibody-bound [Ru(bpy)$_2$dcbpy] was protected.

5. Abbreviations

BCA	bicinchoninic acid
bpy	2,2′-bipyridine
BSA	bovine serum albumin
CCVJ	9-(2-carboxy-2-cyanovinyl) julolidine
dcbpy	2,2′-bipyridine-4,4′-dicarboxylic acid
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DNS	5-dimethylaminonaphthalene-1-sulfonamide
HRP	horseradish peroxidase
MLCT	metal-ligand charge transfer
NHS	*N*-hydroxysuccinimide
o-PD	*o*-phenylenediamine
PBS	phosphate buffered saline
p2p	1,2-bis-(4-pyridyl)ethane
phen	1,10-phenanthroline
TCSPC	time-correlated single-photon counting
UV–Vis	ultraviolet–visible

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