DNA Interactions of Bimetallic Ruthenium Complexes Containing Tridentate Ligands
with Extended π-Systems

THESIS

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Abstract

Ruthenium complexes containing extended π-systems have been shown to intercalate between the DNA bases. [Ru(tpy)(pydppn)]^{2+}, (pydppn = (3-(pyrid-2'-yl)-4,5,9,16-tetraaza-dibenzo[a,c]napthacene and tpy = [2,2';6',2'']-terpyridine), has a long lived (~20μs) \(^3\pi\pi^*\) lowest energy excited state. Because of the long lifetime, this Ru(II) complex is able to produce singlet oxygen with ~100% efficiency. The high efficiency singlet oxygen production and strong binding to DNA make for a promising photodynamic therapy (PDT) agent. However, the absorption maximum of 474 nm is outside of the therapeutic PDT window of 600-800 nm. In order to red-shift the absorption into the PDT window, a series of bimetallic Ru(II) complexes were synthesized. The complexes [(tpy)Ru(dpyp)Ru(tpy)]^{4+} (1), [(tpy)Ru(dpyp)Ru(pydppn)]^{4+} (2), [(pydppn)Ru(dpyp)Ru(pydppn)]^{4+} (3) all have absorption maxima of approximately 630nm, (dpyp =2,5-di[2’-(1’,10’)-phenanthrolinyl]pyrazine). The DNA interactions of these complexes were investigated.
Acknowledgments

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Ruthenium polypyridyl complexes have been investigated extensively due to their unique photophysical properties that allow for their use as photosensitizers in numerous applications, including photodynamic therapy (PDT). PDT is a treatment that can destroy cancer cells whilst leaving healthy cells unharmed. PDT uses a photosensitizer, visible light, and oxygen to selectively kill cancer cells.\textsuperscript{1} When irradiated with visible light, the ground state ($^{1}\text{GS}$) photosensitizer (PS) is promoted to a singlet excited state ($^{1}\text{ES}$), then intersystem crossing (ISC) will lead to population of the triplet excited state ($^{3}\text{ES}$).\textsuperscript{2} If the $^{3}\text{ES}$ is long lived there can be two different reactions involving oxygen.\textsuperscript{3} First, a Type I reaction involves electron transfer to O$_2$, which generates harmful reactive oxygen species (ROS). The second reaction, Type II, occurs when there is energy transfer from the $^{3}\text{ES}$ of the photosensitizer to ground state molecular oxygen ($^{3}\text{O}_2$). This results in the production of reactive singlet oxygen ($^{1}\text{O}_2$). The Type II mechanism is shown in Figure 1.
In order for a molecule to be a good PDT photosensitizer there needs to be a high yield of the desired photochemical event, commonly $^1\text{O}_2$ production, under irradiation and for the molecule to be unreactive in the dark. It is also desirable for the photosensitizer to absorb strongly in the PDT window of 600-800 nm. This therapeutic window is where light has the greatest penetration depth through tissue and still have enough energy to excite the photosensitizer.

Hematoporphyrin (Figure 2) and its derivatives are currently used as
photosensitizers in clinical PDT cancer treatments. Photofrin® is a FDA approved hematoporphyrin photosensitizer used for the treatment of esophageal, bladder, and lung cancers via a Type II mechanism.¹

![Structure of hematoporphyrin](image)

Figure 2: Structure of hematoporphyrin.

Even though Photofrin® is commonly used, there is room for improvement in photosensitizer design. The O₂ quantum yield of the monomer is 0.65 and the dimer is 0.11, due to non-unity population of the ES state. The active components are difficult to separate so the quantum yield is diminished. Also, the only absorption peak of Photofrin® in the PDT window is weak. Ruthenium polypyridyl complexes are desirable as alternative photosensitizers because of the tunable light absorption properties, their greater singlet oxygen yields, and the ability to intercalate and electrostatically bind to DNA.

O₂ causes oxidative damage to DNA, leading to cell death through apoptosis or necrosis. The double-helical structure of DNA is composed of a sugar-phosphate backbone with four possible bases bonded to the backbone. The bases adenine (A),
cytosine (C), guanine (G), and thymine (T) are held together with hydrogen bonds in specific base pair arrangements. Metal bonding to DNA can occur through several different ways. Intercalative binding involves metal complexes with aromatic ligands inserting between base pairs and interacting via $\pi-\pi$ stacking. There is also electrostatic attraction between cationic metal complexes and the anionic DNA.

$$[\text{Ru(tpy)(pydppn)}]^2^+, \quad \text{pydppn} = (3-(\text{pyrid-2'-yl})-4,5,9,16-\text{tetraaza-dibenzo}[a,c]\text{naphthacene and tpy} = [2,2',6',2'']-\text{terpyridine}),$$

was shown to have a ligand centered (LC) $3\pi\pi^*$ lowest energy excited state with $\tau = 20.1$ μs. This long lifetime leads to a high $^1\text{O}_2$ quantum yield, $\Phi_{^1\text{O}_2}$, of 0.92 $\pm$ 2. This is much greater than that previously reported for Photofrin®. The DNA interactions of $[\text{Ru(tpy)(pydppn)}]^2^+$ were also investigated previously, and the DNA binding constant was determined to be $K_b = 3.5 \times 10^5$ M$^{-1}$ ($s = 056$). This DNA binding constant is comparable to those of known intercalators, such as ethidium bromide ($1.7 \times 10^5$ M$^{-1}$). The pUC18 plasmid DNA photocleavage by $[\text{Ru(tpy)(pydppn)}]^2^+$ was shown to be very efficient when irradiated with $\lambda_{\text{air}} > 395$ nm for 10 minutes. The photocleavage was due to sensitized $^1\text{O}_2$ and there was no cleavage in the dark. The high quantum yield of $^1\text{O}_2$, strong intercalative binding to DNA, and DNA photocleavage made $[\text{Ru(tpy)(pydppn)}]^2^+$ a possibly effective PDT photosensitizer. However, the absorption maximum of $[\text{Ru(tpy)(pydppn)}]^2^+$ is at 474 nm, such that its excitation would not be possible with irradiation in the PDT window of 600-800 nm.

In order to extend the absorption maximum of this complex into the PDT window, new bimetallic complexes were synthesized. The complexes, shown in Figure 1,

$$[(\text{tpy})\text{Ru(dppy)Ru(tpy)}]^4^+, \quad (1), \quad [(\text{tpy})\text{Ru(dppy)Ru(pydppn)}]^4^+ \quad (2),$$
[(pydppn)Ru(dppy)Ru(pydppn)\(^{4+}\)] (3) all have two ruthenium metal centers with a diphenpyrazine bridging ligand, \((\text{dppy}=2,5\text{-di}[2'-(1',10')\text{-phenanthroinyl}]\text{pyrazine})\). Complex 1 has no intercalating pydppn ligand, whereas 2 and 3 have one and two respectively. The absorption maxima for all three complexes was red-shifted into the PDT window, ~630 nm. The excited state lifetimes of these complexes are shorter (~100-300 ns) than the long lived (20.1 \(\mu\)s) \(^3\pi\pi^*\) excited state of \([\text{Ru(tpy)(pydppn)}]^{2+}\). Electronic structure calculations for complexes 1 – 3 are consistent with a metal-to-ligand charge transfer (MLCT) excited state from the ruthenium metal center to the pyrazine moiety of the bridging ligand. The lack of a long lived \(^3\pi\pi^*\) excited state could lead to a lower yield of singlet oxygen, however, their absorption in the PDT window provides an great advantage over the mononuclear compounds.\(^9\)
The goal of the present work is the investigation of the interactions of 1 – 3 with DNA. Establishing how a potential PDT photosensitizer interacts with DNA is important before studying how a complex behaves in a cell. Thermal denaturation studies and viscosity measurements were taken to determine if the complexes are intercalating between the base pairs of DNA. DNA binding constants were determined, and the ability of the complexes to photocleave DNA upon irradiation in the PDT window was also investigated.
Chapter 2: Experimental Methods

2.1 Materials

The three diruthenium complexes were prepared by the Thummel research group and were used without further purification (University of Houston). Water was purified by a Barnstead ultrapure water purification system. The two complementary DNA oligonucleotides, (5’-CTCTCAACTTCC-3’) and (5’-GGAAGTTGAGAG-3’), were purchased from The Midland Certified Reagent Company and were used as received. Calf-thymus DNA, sodium chloride, ethidium bromide, agarose, and potassium phosphate were obtained from Sigma, tris(hydroxymethyl)-aminomethane was purchased from Aldrich, and pUC18 Plasmid DNA was purchased from Bayou Biolabs, all of which were used without additional purification.

2.2 Instrumentation and Methods

Absorption spectra were obtained using a HP photodiode array spectrometer and a 1 cm pathlength quartz cuvette. Thermal denaturation experiments were done according to literature methods. The two complementary DNA oligonucleotides, (5’-CTCTCAACTTCC-3’) and (5’-GGAAGTTGAGAG-3’) were annealed in a 40 mM K_2HPO_4 buffer (pH 6.2) with 300 mM NaCl. The absorbance at 260 nm was recorded for the double stranded DNA (2 μM) alone and with each complex (2 μM) added. The
temperature was increased from 20 – 70 °C by 1 °C increments using a Peltier temperature control unit. Graphing absorbance versus temperature resulted in a melting curve where the half-way point is the melting temperature, $T_m$. Each melting curve was fitted in Igor Pro 6.32A with a sigmoid function.

A Cannon-Manning Semi-Micro Viscometer (No. 50-N88) was used for measuring viscosity of the three complexes and calf-thymus DNA. Varying concentrations of complex, dissolved in acetonitrile, were added to a 1 mM (per base-pair) solution of calf-thymus DNA in 50 mM NaCl 5 mM Tris buffer. The calf-thymus DNA and complex solution was sonicated before each measurement, thus reducing the structural complexity of the DNA. The viscometer was placed inside a circulating water bath to maintain a constant temperature and the viscosity was measured by using a pipet bulb to suction the solution up the capillary side of the viscometer, then allowed to flow. A stopwatch was used to record the time the sample took to fall between the upper and lower mark, shown in figure 4.
Each flow time was measured three times, and the average was calculated. The flow times were converted to viscosity and graphed as $(\eta/\eta_0)^{1/3}$ versus $[\text{Complex}]/[\text{DNA}]$,\textsuperscript{12} where $\eta_0$ represents the viscosity of the DNA alone and $\eta$ the viscosity of DNA in the presence of a given concentration of complex.

Absorption binding titration experiments followed a reported procedure.\textsuperscript{13} Calf-thymus DNA was added to a fixed concentration of complex (10 $\mu$M) and the changes in the absorption of the complex at 260 nm were used in equations 2.1 and 2.2 to calculate a binding constant, $K_b$.\textsuperscript{14} In equations 2.1 and 2.2, $C$ represents the concentration of the complex, $[\text{DNA}]$ is the DNA concentration in base pairs, $\varepsilon_a$ is the apparent absorption of complex with DNA, $\varepsilon_f$ is the extinction coefficient of complex without DNA, and $\varepsilon_b$ is the extinction coefficient of complex completely bound to DNA. The concentration of DNA was measured using $\varepsilon_{260} = 13,100 \text{ M}^{-1} \text{ cm}^{-1}$. 

Figure 4: Viscometer Diagram
\[ \frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{\sqrt{b - (b^2 - 2K^2C_t [DNA]/s)}}{2KC_t} \]  

(2.1)

\[ b = 1 + KC_t + K [DNA]/2s \]  

(2.2)
Chapter 3: Results and Discussion

3.1 Light Absorption

The absorption spectra for complexes 1, 2, and 3 in acetonitrile are shown in Figure 1. All three complexes absorb in the UV region due to intraligand $\pi-\pi^*$ transitions. The metal-to-ligand charge transfer (MLCT) transitions are observed in the visible region of the spectrum. The previously reported absorption spectrum for the monomer [Ru(tpy)(pydppn)]$^{2+}$(4) is shown in Figure 2. Table 1 lists the electronic absorption maxima and intensities for complexes 1-4.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$\lambda_{abs}$/nm ($\varepsilon \times 10^3$ cm$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^a$</td>
<td>389 (89.3), 449 (22.4), 636 (22.0)</td>
</tr>
<tr>
<td>2$^a$</td>
<td>387 (76.6), 452 (25.3), 633 (19.1)</td>
</tr>
<tr>
<td>3$^a$</td>
<td>388 (85.4), 452 (26.0), 637 (21.5)</td>
</tr>
<tr>
<td>4$^b$</td>
<td>331(60.1), 390(12.6), 414 (11.5), 474 (16.8)</td>
</tr>
</tbody>
</table>

Table 1: $^a$Absorption Maxima and Intensities for Complexes 1-3 measured in CH$_3$CN from Ref. 7. $^b$Absorption Maxima of Complex 4 measured in H$_2$O from Ref. 8.
The MLCT absorption maxima of the diruthenium complexes (~630 nm) is red-shifted compared to [Ru(tpy)(pydppn)]^{2+} (474 nm). This same shift is seen in similar ruthenium complexes containing tridentate ligands. [Ru(tpy)(tppz)]^{2+}, (tppz = 2,3,5,6-tetrakis(2-pyridyl)pyrazine), has a MLCT absorption maximum of 472 nm, while the bimetallic complex [(tpy)Ru(tppz)Ru(tpy)]^{2+} has a MLCT absorption maximum of 547 nm. These bathochromic shifts are caused by the second ruthenium center stabilizing the bridging ligand orbitals, therefore lowering the energy of the MLCT transition. The monometallic ruthenium complexes have a MLCT from the ruthenium to the pydppn ligand.

Figure 5: Electronic absorption spectra of 1, 2, and 3. Solutions are in acetonitrile.
Figure 6: Electronic absorption spectrum of 4 dissolved in acetonitrile.
3.2 Thermal Denaturation

Intercalators have been shown to raise the melting temperature of DNA.\textsuperscript{16,17} The raise in temperature is due to stabilization of the DNA helix.\textsuperscript{6} Kang et al. reported a melting temperature of 47 ± 1°C for the annealed oligonucleotide sequences (5’-CTCTCAACTTCC-3’) and (5’-GGAAGTTGAGAG-3’).\textsuperscript{Error! Bookmark not defined.} The melting curve for the same DNA sequence is shown in Figure 3. The measured T\textsubscript{m} for DNA alone is 49 ± 1°C. Buffer pH differences could account for the 2 degree difference in melting temperature. Complex 1 had a ΔT\textsubscript{m} = 0 °C and therefore did not affect the thermal stability of the DNA, consistent with lack of intercalation. This result was expected because 1 does not have a ligand with the ability to intercalate DNA. Complex 2 (ΔT\textsubscript{m} = +6 °C) and 3 (ΔT\textsubscript{m} = +2 °C) both increased the T\textsubscript{m}. This is most likely because of the intercalative binding ability of the pydppn ligand. Ethidium bromide, a known intercalator of DNA, exhibits ΔT\textsubscript{m} = +5 °C.\textsuperscript{18} A possible reason for 2 being a better intercalator of DNA than 3 is that the latter is sterically hindered from intercalating DNA with two pydppn ligands.
Figure 7: The melting temperature of the annealed ds-DNA alone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_m$ (±1°C)</th>
<th>$\Delta T_m$ (±1°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA only</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>DNA+ Complex1</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>DNA+ Complex2</td>
<td>55</td>
<td>6</td>
</tr>
<tr>
<td>DNA+ Complex3</td>
<td>51</td>
<td>2</td>
</tr>
<tr>
<td>DNA+ Acetonitrile</td>
<td>49</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2: Summary of the thermal denaturation data.

3.3 Relative Viscosity Measurements

The measurement of relative viscosity is a common method to evaluate whether or not a complex intercalates into DNA. The viscosity of a solution of CT-DNA alone was measured and compared to the viscosity of CT-DNA titrated with varying concentrations of 1, 2, and 3. Intercalation of a complex into DNA lengthens and increases the surface area of the DNA duplex, resulting in an increase in relative viscosity.\textsuperscript{19}

The plot of $(\eta/\eta_0)^{1/3}$ versus [Complexes]/[CT-DNA] is shown in Figure 4. Ethidium Bromide (EtBr), a known intercalator of DNA, is also shown on the plot as a control. The relative viscosity of CT-DNA increases as more EtBr is added. Complex 2 matches the
intercalating ability of EtBr. This is because of the pydppn ligand having an extended aromatic structure. Complex 3 is shown to intercalate with a positive slope, however less so than 2. Complex 3 contains two pydppn ligands, which could result in steric hindrance that would diminish the intercalation into DNA. Also, if a complex is self-stacking there will be less intercalation between DNA base pairs. Complex 3 has two pydppn ligands and therefore more chance of self-stacking.

Complex 1 does not contain an intercalating ligand, and therefore an increase in relative viscosity was not expected. Instead, the relative viscosity of DNA was observed to decrease as the concentration of 1 was successively increased. Complexes that covalently bind to DNA are known to decrease the relative viscosity of DNA because the helix is distorted and the surface area of the DNA duplex is decreased.\textsuperscript{20} However, 1 is not able to covalently bind to DNA.
3.4 Binding Titrations

The DNA binding constants, $K_b$, were determined from the absorption titrations. The $K_b$ values were $K_b = 1 \times 10^6 \text{ M}^{-1}$ ($s = 1.8$), $3 \times 10^7 \text{ M}^{-1}$ ($s = 0.7$), and $8 \times 10^6 \text{ M}^{-1}$ ($s = 0.32$) for complexes 1, 2, and 3, respectively. These binding constants are similar to the complexes $[\text{Ru(bpy)}_2(\text{dppz})]^{2+}$, $10^6$-$10^7 \text{ M}^{-1}$, and $[\text{Ru(tpy})(\text{pydppn})]^{2+}$, $4.6 \times 10^6 \text{ M}^{-1}$ ($s = 1.34$). The binding constant for EtBr with DNA is $10^6$-$10^7 \text{ M}^{-1}$.

Bimetallic ruthenium complexes have sometimes been shown to bind more strongly to DNA than their corresponding monometallic complex. The monometallic complex $\Delta$-
[Ru(phen)$_2$(dppz)]$^{2+}$ has a reported binding constant of $K_b = 6 \times 10^7 \text{M}^{-1}$. A bimetallic analogue, $\Delta \Delta -[(\text{phen})_2\text{Ru} \{\text{dppz}(11-11')\text{dppz}\} \text{Ru}(\text{phen})_2]^{4+}$, has been reported to have a binding constant of $K_b = 5 \times 10^{11} \text{M}^{-1}$.

Complex 1 has a relatively high binding constant for a complex without intercalating ligands. This could be due to the $+4$ charge of the complex and the electrostatic interaction with the anionic DNA. The binding constants for 2 and 3 also have a $+4$ charge, but the binding constants are greater because of the intercalating pydppn ligand.

### 3.5 DNA Photocleavage

Since complex 2 had the highest binding constant of all three complexes and the strongest evidence of intercalation, its DNA photocleaving ability was studied. The photocleavage of supercoiled pUC19 plasmid DNA by 2 was investigated through agarose gel electrophoresis. Irradiation of 2 (5 μM) with $\lambda > 550$ nm for 15 minutes with pUC19 plasmid (100 μM) results in photocleavage of DNA, shown in Figure 8 (Lane 3). No cleavage was observed when the reaction occurred in the dark (Lane 2) or when the pUC19 plasmid was alone in water (Lane 1).
Photocleavage was not inhibited in the presence of 20 mM sodium azide, a singlet oxygen ($^1\text{O}_2$) scavenger. This is shown by the intense nicked band in lane 4. Dimethylsulfoxide (DMSO), which scavenges hydroxyl radicals, shows some inhibition of the photocleavage in lane 5. Superoxide dismutase (SOD), which inhibits superoxide, did not show any inhibition of photocleavage (Lane 6). This data shown in Figure 8 shows that $^1\text{O}_2$ production is not the sole DNA photocleavage mechanism.

**Chapter 3.6 Guanine Oxidation**

Several Ru(II) complexes have excited states that oxidize guanine, leading to DNA cleavage.\textsuperscript{26} Using the equation 3.1, the excited state reduction potential ($E_{\text{red}}^*$) of 2 is calculated. $E_{1/2}(2^{4+/3+})$ is the ground state reduction potential of 2. The cyclic
voltammogram of 2 was used to determine \( E_{1/2}(\mathbb{[2]}^{4+/3+}) = -0.024 \text{ V vs. NHE.} \) \( E_{00} \), the energy of the transition between the ground state and the \(^3\)MLCT, is estimated by emission maxima.\(^{27}\) With \( E_{00} \sim 1.46 \text{ eV} \), the \( E_{\text{red}}^* \) for 2 is estimated to be \( \sim 1.44 \text{ V vs. NHE.} \) The oxidation potential of guanine is \( +1.29 \text{ V vs. NHE in water at pH = 7} \).\(^{28}\) This makes guanine oxidation from the \(^3\)MLCT of 2 favorable by -0.15 V.

\[
E_{\text{red}}^* = E_{1/2}(\mathbb{[2]}^{4+/3+}) + E_{00} \quad (3.1)
\]
Chapter 4: Conclusions

This thesis work investigated the interactions of a series of bimetallic ruthenium polypyridyl complexes with DNA. The complexes are potentially useful as PDT agents because of their strong absorbance in the therapeutic window. Complex 1, having zero intercalating ligands, was shown to not intercalate between the base pairs of DNA. Complex 2, containing one intercalating ligand, stabilized the DNA helix because of the strong intercalating ability. Complex 3, containing two intercalating ligands, did not have as strong of interaction with DNA compared to complex 2. Steric bulk or self-aggregation could result in the diminished interaction with DNA. All three complexes likely exhibit electrostatic interaction with DNA due to their 4+ charge. The strong electrostatic interaction was shown in the measured high binding constants. Evidence of DNA photocleavage by complex 2 was shown. The mechanism for this cleavage is likely not singlet oxygen production but rather guanine oxidation.
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