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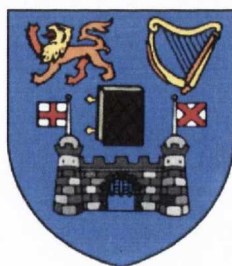
**A Synthetic and Photophysical Study
of Ruthenium Polypyridyl Enantiomers
Binding to DNA**

by

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B.A. Mod. (Medicinal Chemistry)

A thesis presented to the University of Dublin
for the degree of Doctor of Philosophy



Under the supervision of Prof. John M. Kelly

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Thesis 9737

Declaration

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For my parents
Tish and Brendan

Summary

The luminescent DNA probe $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, ruthenium (*bis*-1,10-phenanthroline dipyrido[3,2-*a*:2',3'-*c*]phenazine, was the first *tris*-bidentate ruthenium complex to be shown unequivocally to intercalate to DNA, which it achieves via insertion of the extended dppz ligand. This complex has demonstrated its potential as a DNA probe, courtesy of its luminescent 'light-switch on' effect. Since its discovery and subsequent resolution into its constituent Λ and Δ enantiomers, many analogues of the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complex have been synthesised and scrutinised for any potential attributes. One such analogue is $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, ruthenium (*bis*-1,4,5,8-tetraazaphenanthrene dipyrido[3,2-*a*:2',3'-*c*]phenazine, which demonstrates the 'light-switch off' effect and is sufficiently oxidising in its $^3\text{MLCT}$ excited state to cause photooxidative damage to DNA, leading to strand scission.

The aim of this project was to resolve $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ into its constituent Λ and Δ enantiomers and investigate the binding potential of the separate enantiomers with duplex DNA. Chapter 2 describes the solution studies performed with both enantiomers in the presence of wild type DNA, [poly(dG-dC)] and [poly(dA-dT)]. A variety of spectroscopic methods were employed including UV/vis absorbance, fluorescence, thermal denaturation, circular dichroism and linear dichroism. The investigation culminated in ultrafast experiments utilising nanosecond transient absorption and time resolved infrared spectroscopy to determine the extent of the enantiomeric effect on the back electron transfer process that occurs upon excitation of the complexes in the presence of guanine residues.

A crystallographic study of Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ binding to a self-complementary oligonucleotide, at atomic resolution, is presented in Chapter 3. This is the first such crystal study of a ruthenium complex binding to DNA. The resolved crystallographic model clearly demonstrates the complex binding via intercalation of the dppz ligand and semi-intercalation of the TAP ligands. This latter interaction forms a cross-linking of DNA duplexes in addition to causing a 51° kink in the duplex, reminiscent of the mode through which *cis*-platin induces cytotoxicity. This study also resolves the much debated major/minor groove issue surrounding the binding of ruthenium polypyridyl complexes by demonstrating that Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ binds solely at the minor groove.

Chapters 4 and 5 expand the study to include the methylated and fluorinated analogues of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, and increase molecular complexity via synthesis of a bi-nuclear complex in an attempt to achieve an enantiomerically pure binding agent with a preference for quadruplex DNA. The synthesis and enantiomeric resolution of both $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ was achieved and investigation of the binding potential of the enantiomers with duplex DNA was conducted. The bi-nuclear complex $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ was also synthesised and attempts at resolution into its three optical isomers are described, including stereospecific chromatography and binding affinity-based DNA-assisted stereoisomer separation techniques.

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Abbreviations

$^1\text{O}_2$	Singlet Oxygen
^3S	Triplet excited state
A	Adenine
Å	Angstrom
Abs	Absorbance
ATP	Adenosine triphosphate
Ba	Barium
BET	Back electron transfer
bipym	2,2'-bipyrimidine
bp	base-pair
bpm	2,2'-bipyrimidine
bpy	2,2'-bipyridine
bpypz	2,3-bis(2-pyridyl)pyrazine
Ca	Calcium
CD	Circular dichroism
CH COSY	Carbon-hydrogen correlation Spectroscopy
chrysi	5,6-chrysenequinone diimine
CT-DNA	Calf thymus DNA
Δ	Delta
δ	Delta/chemical shift
d	Doublet
dd	Double doublet
D ₂ O	Deuterium oxide
ΔE	Difference in Energy
ΔG_0	Different in Gibbs Free Energy
DMF	<i>N,N</i> -dimethylformamide
DNA	Deoxyribonucleic acid
dppz	Dipyrido[3,2-a:2',3'-c]phenazine
dpq	Dipyrido[3,2-d:2',3'-f]quinoxaline
EtOH	Ethanol
F ₂ dppz	Difluoro-dipyrido[3,2-a:2',3'-c]phenazine
fs	Femtosecond
G	Guanine
GMP	Guanosine monophosphate
HAT	1,4,5,8,9,12-hexazatriphenylene
HMG	High mobility group
HRMS	High resolution mass spectroscopy
h ν	Light
Hz	Hertz
ICD	Induced circular dichroism
IL	Intra-ligand
ISC	Inter-system conversion
J	Coupling constant (expressed in Hz)

K	Binding Constant
K	Kelvin
kb	kilobases
kcal	kilocal
kJ	kilojoule
k_{nr}	Rate constant of non-radiative decay
KPF ₆	Potassium hexafluorine
k_r	Rate constant of radiative decay
LC	Ligand centered
LD	Linear dichorism
λ	Wavelength
ln	Natural log
MALDI	Matrix-assisted laser desorption/ionization
MC	Metal centered
Me ₂ dppz	Dimethyl-dipyrido[3,2-a:2',3'-c]phenazine
MLCT	Metal ligand charge transfer
MPD	Methyl-2,2-pentane diol
MW	Microwave
Na(CH ₃) ₂ ASO ₂	Sodium cacodylate
NAMS	Nucleic acid mini screen
NaOMe	Sodium methoxide
NMR	Nuclear magnetic resonance
NON-PE	Non-potential energy
OD	Optical density
Os	Osmium
p	Para
P/D	Phosphate/Drug
PAGE	Polyacrylamide gel electrophoresis
PCET	Proton-coupled electron transfer
Pd/C	Palladium on carbon catalyst
PDB ID	Protein database identity
PDT	Photodynamic therapy
PE	Potential energy
PEG	Polyethylene glycol
PET	Photo-induced electron transfer
PF ₆	Hexafluorophosphate
PHEHAT	1,10-phenanthrolino[5,6-b]1,4,5,8,9,12-hexaazatriphenylene
phen	1,10-phenanthroline
PO ₄ ²⁻	Phosphate
ppz	4,7-phenanthroline-5,6:5',6'-pyrazine
Pt	Platinum
pu	Purine
py	Pyrimidine
<i>rac</i>	Racemic

RNA	Ribonucleic acid
Ru	Ruthenium
S ₀	Ground state
s	Second/singlet
SARS	Structural activity relationship
Sr	Strontium
stDNA	Salmon testes DNA
t	Triplet
T	Temperature
T ₁	Triplet State
TA	Transient absorption
TAP	1,4,5,8-tetraazaphenanthrene
TBP	TATA binding protein
τ _{em}	Emission lifetime
T _m	Melting temperature
TOF	Time of flight
TPAC	Tetrapyrido[3,2-a:2',3'-c:3'',2''-h;2''',3'''-j]acridine
TRIR	Time resolved infrared
Tris-HCl	Tris-hydrochloride
tRNA	Transfer RNA
UV/vis	Ultraviolet/visible light

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Chapter 1

General Introduction

1.1 Photodynamic Therapy – DNA as a Target

The origin of light as a therapy in medicine can be traced from antiquity to the modern day. The earliest recorded treatments exploiting a photosensitizer and a light source (in this case sunlight) for medical effect can be found in ancient Egyptian and Indian sources. Annals over 3000 years old report the use of topically applied vegetable and plant substances to produce photoreactions in skin and cause a repigmentation of depigmented skin lesions. The photosensitizing agents used in these ancient therapies have been characterised with modern science as belonging to the psoralen family of chemicals (Figure 1.1), many of which are still used in treatments today.

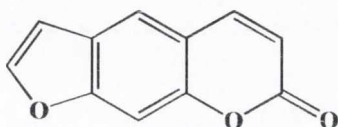


Figure 1.1 – Structural formula of Psoralen

Although the German physician Friedrich Meyer-Betz performed the first scientific study with humans in 1913,¹ photodynamic therapy (PDT) did not mature as a feasible medical technology in the fight against cancer until the early 1980s and only became widespread after Thomas Dougherty initiated clinical trials and formed the International Photodynamic Association in 1986. Since then its realisation as a promising new method for cancer treatment has become progressively well-established as a mode to combat both malignant²⁻⁵ and non-malignant diseases,⁶⁻⁸ characterized by the occurrence of unwanted or harmful cells.

PDT exploits the biological consequences of localized oxidative damage inflicted by photodynamic processes. Three critical elements are required for the initial photodynamic processes to occur: a molecule that can be activated by light (a photosensitizer), light and oxygen.⁹ Interaction of light at the appropriate wavelength with a photosensitizer produces an excited-state photosensitizer that can itself interact with ground-state oxygen via two different pathways, Type I and Type II. A Type I reaction is a radical or redox process in which a photosensitizer (S_0), excited to the triple state (3S) interacts with a

neighbouring molecule (A) through exchange of either an electron or a hydrogen atom. While the Type I process here (Equations 1.1 and 1.2) is shown to utilise oxygen, such reactions are known to also be oxygen independent, as is the case for the reactions of the psoralens compounds with DNA.¹⁰



The photosensitized process of Type II is an energy transfer process. The most common Type II reaction is oxidation via singlet oxygen (1O_2) formation, described in Equations 1.3 and 1.4.



The Type II reaction that gives rise to singlet oxygen (1O_2) is believed to be the dominant pathway in PDT medical treatments, as studies have shown that the elimination or scavenging of 1O_2 from the system essentially eliminates any cytotoxic effects.¹¹⁻¹³ As the highly reactive 1O_2 species has a short lifetime and, therefore, a short radius of action 1O_2 -mediated oxidative damage will occur in the immediate vicinity of the photosensitizer's location. Cell death is thus induced via apoptotic or necrotic pathways.^{14,15}

With the advent of this technology, the difference between normal cells and tumour cells has been the subject of much research. The results of such intensive studies have shown that tumour cells differ substantially in many ways from normal cells; growth, metabolic pathways, apoptotic pathways and expressed enzymes to name but a few.¹⁶⁻¹⁸ Of all the various differences caused by the multiple species of tumour cells, a main constant throughout is the activation and suppression of genes within an otherwise healthy cell. Genetic information is stored in what is perhaps the central constituent of the living cell, deoxyribonucleic acid (DNA). This understanding has resulted in the belief that the selective targeting of DNA and genes will allow for specific elimination of tumour cells

whilst leaving the surrounding healthy cells undisturbed. To this end, a compound that can be tailored to recognise pre-determined DNA sequences, cause localised DNA-scission upon activation and have no adverse toxic effects to the rest of the organism is the holy grail of photodynamic therapy. To date, there has been much research into compounds exhibiting high DNA binding affinities and photo-oxidative properties, with ruthenium complexes being at the forefront of this field. However, to understand the requirements for DNA binding and subsequent photo-oxidation, an appreciation of the target's structure is first required.

1.2 DNA Structure

The elucidation of the double-helical structure of B-DNA by James Watson and Francis Crick in 1953¹⁹ and the presentation of the human genome in 2001²⁰ are two of the milestones that during the past half-century have not only revolutionized the understanding of DNA, but also our ability to modify and utilize this information-rich molecule.

Being a long chain composed of only four types of 'links', termed nucleobases, the DNA polymer has an astonishingly simple chemical structure. The backbone of DNA consists of alternating phosphate and deoxyribose sugar groups, connected via the 5' and 3' carbons of the sugar residue. The 1' carbon is linked to one of four different nucleobases that, together with the phosphate and sugar, forms the repeating unit (the nucleotide, Figure 1.2) in a DNA strand. The nucleobases are adenine (A) and guanine (G), which are purine derivatives, and cytosine (C) and thymine (T), which are pyrimidine derivatives, shown in Figure 1.3(a).

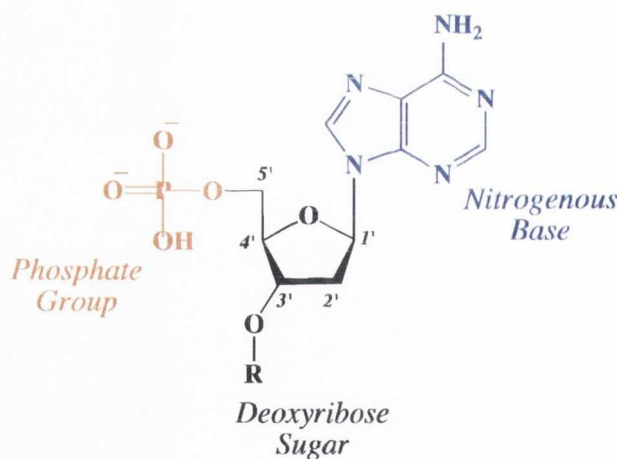


Figure 1.2 – Structure of the repeating nucleotide unit

Two single DNA strands can hybridize to form the characteristic double-stranded helix of two anti-parallel sugar-phosphate backbones wound around each other, with the stacked bases projecting into the interior. According to the Watson-Crick base-pairing scheme, a consequence of homomorphology results in the bonding of adenine to thymine via two

hydrogen bonds and guanine to cytosine via three hydrogen bonds. Thus, a bicyclic purine base is always linked with a smaller monocyclic pyrimidine base to allow the constant diameter of the double helix. This is considered the most common base-bonding configuration found in DNA however reverse-Watson-Crick and Hoogsteen bonding patterns have also been observed in many DNA structures. (Figure 1.3 (b) and (c)).

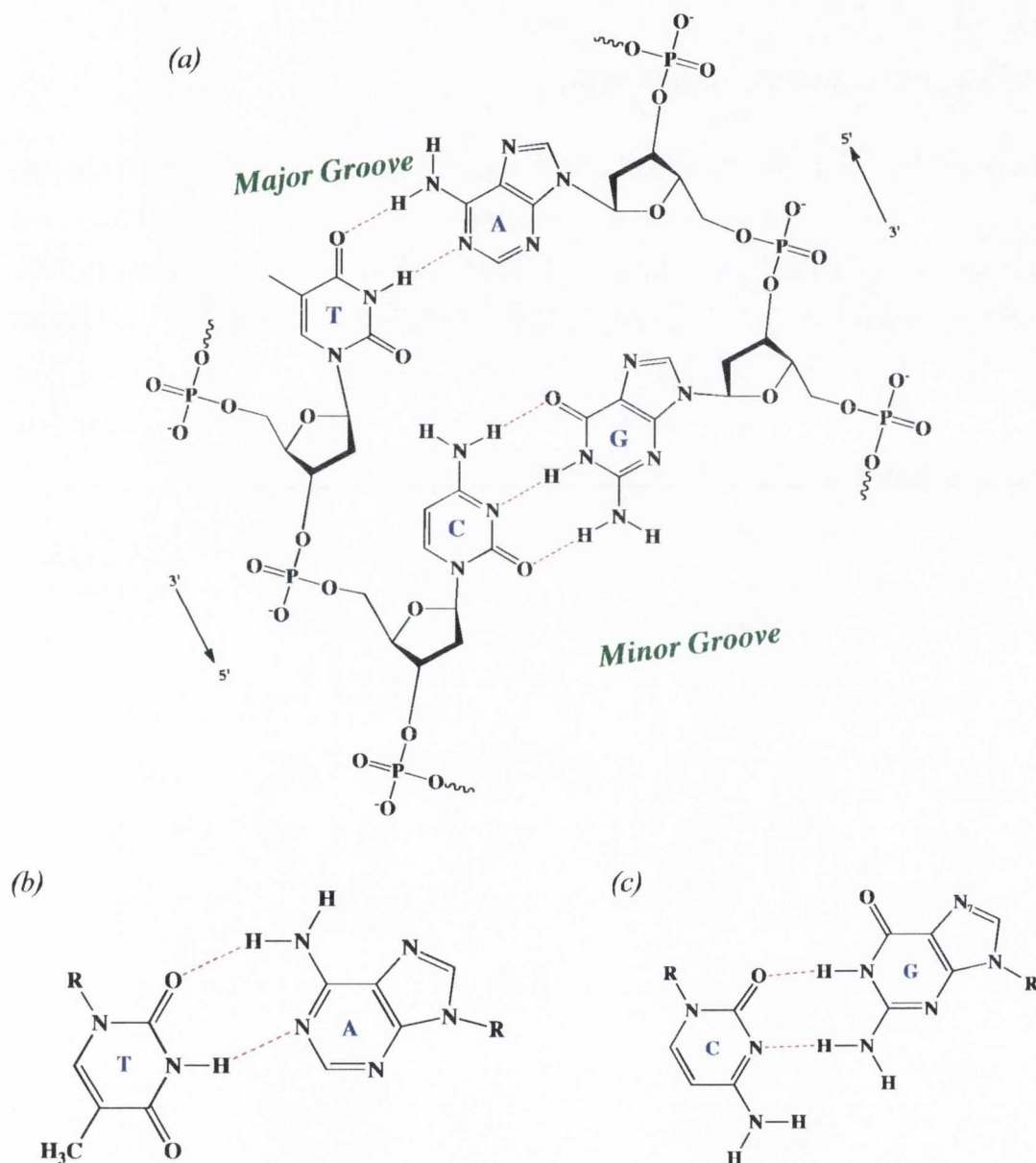


Figure 1.3 – (a) The Watson-Crick base-pairing system. The reverse Watson-Crick bonding between (b) adenine and thymine and (c) guanine and cytosine

The stacked aromatic bases provide a hydrophobic inner environment while the negatively charged phosphate groups, positioned along the outside of the helix, favour interactions with polar surroundings, such as water. Two helical grooves, exposing the edges of the bases, are formed between the intertwined backbones. These are known as the major and minor grooves (see Figure 1.3 (a)) with the major groove being wider and shallower than the minor groove in B-DNA.

1.2.1 The Diversity of the Double Helix

The duplex itself is polymorphic and adopts the conformation that is most favourable with respect to the character of its surrounding environment, local sequence of bases and interactions with other molecules. In the cell, the most abundant conformation is B-DNA (Figure 1.4) in which the bases are separated by a distance of 3.4 Å and positioned almost perpendicular to the helix axis. The DNA strands have, on average, a right-handed twist of 36° per base pair, which results in the helix making a complete turn approximately every 10 base pairs.

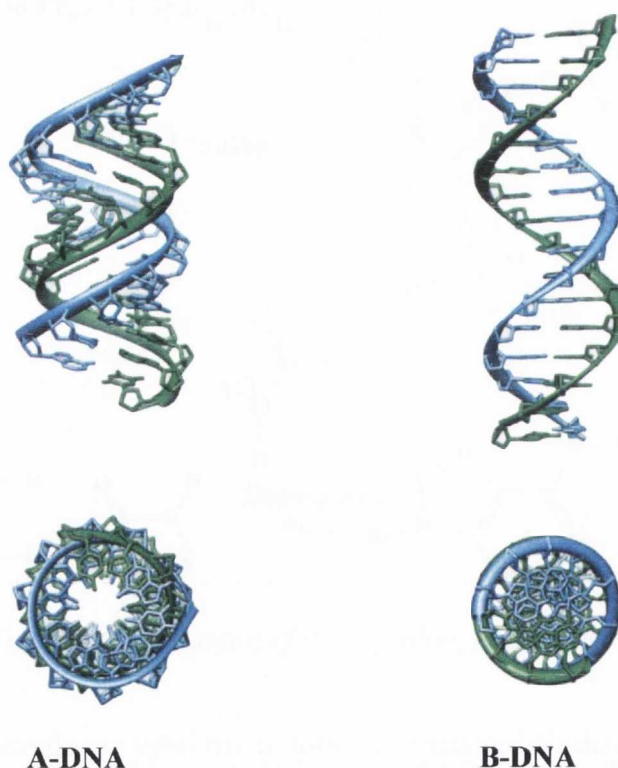


Figure 1.4 – Side and top view representations of the A and B forms of double-helical DNA, each containing 16 bases²¹

In environments of low activity (usually high ionic strength conditions) the helix can adopt a wider, more compact A-DNA conformation. In A-DNA, the stacked bases are strongly tilted and positioned farther from the helix axis, forming a central hollow core (Figure 1.4). The base pair rise is only 2.9 Å and the helix makes a complete turn every 11 base pairs. Double-stranded RNA is commonly found to adopt the A-form as a consequence of the steric restrictions imposed by the presence of a hydroxyl group in the backbone sugar residue ribose. A-DNA has also been shown to play a vital role in the transcription process of cellular DNA. The helix, while normally of the B-form, is found to adopt a partial A-form conformation during the transcription of various proteins.²² This is a remarkable aspect of the DNA helix, and a possible target for the treatment of cancer and other such diseases. If the duplex can be prevented from altering its conformation, the transcription process would cease and inevitably apoptosis would occur.

There is a third form of biologically active DNA commonly found within cells, Z-DNA. It is a left-handed double helical structure in which the double helix winds in a zig-zag pattern. While no definitive biological significance of Z-DNA has been found, it is commonly believed to provide torsional strain relief while DNA transcription occurs.^{23,24} As such the potential to form a Z-DNA structure also correlates with regions of active transcription.²⁵ A summary detailing the similarities and differences between these three secondary structures of DNA thus far mentioned are presented in Table 1.1

	A-DNA	B-DNA	Z-DNA
Helix Sense	right-handed	right-handed	left-handed
Repeating Unit	1 bp	1 bp	2 bp
Rotation/bp	32.7°	35.9°	30°
bp/turn	11	10.5	12
Inclination of bp to axis	+ 19°	- 1.2°	- 9°
Rise/bp along axis	2.9 Å	3.4 Å	3.8 Å
Pitch/turn of helix	32 Å	36 Å	45.6 Å
Sugar pucker	C3'-endo	C2'-endo	C: C2'-endo G: C3'-endo
N-Glycosidic bond angle	syn	anti	C: anti G: syn
Diameter	23 Å	20 Å	18 Å
Major Groove Depth	13 Å	8.5 Å	3.7 Å
Major Groove Width	2.2 Å	12 Å	9 Å
Minor Groove Depth	2.6 Å	2.6 Å	14 Å
Minor Groove Width	11 Å	6 Å	2 Å

*Table 1.1 – Comparison of the geometrical properties of
A-DNA, B-DNA and Z-DNA²⁶⁻²⁹*

1.2.2 Higher Order DNA Structures

After the discovery of double helical DNA in 1953 and subsequent fibre diffraction studies, it was initially assumed that only the A and B conformations existed. However, in the last 25 years, a widening use of new technology has expanded our knowledge about the heterogeneity of DNA structures. DNA is now known to have a number of

polymorphs, and exhibit conformational flexibility, which leads to numerous higher order structural motifs. These structures have begun to be explored for their natural role and biological validation, and are themselves being heralded as possible therapeutic targets. Two such higher-order DNA structures are the Holliday junction and quadruplex DNA.

1.2.2.1 Holliday Junctions

The four stranded DNA structure, now known as the Holliday junction, is a mobile junction between four strands of DNA and was first proposed in 1964 by Robin Holliday. He suggested that a structure of this sort must be formed as an intermediate during the process of homologous recombination, and since then his proposed topology has been recognised as a central recombination intermediate.³⁰

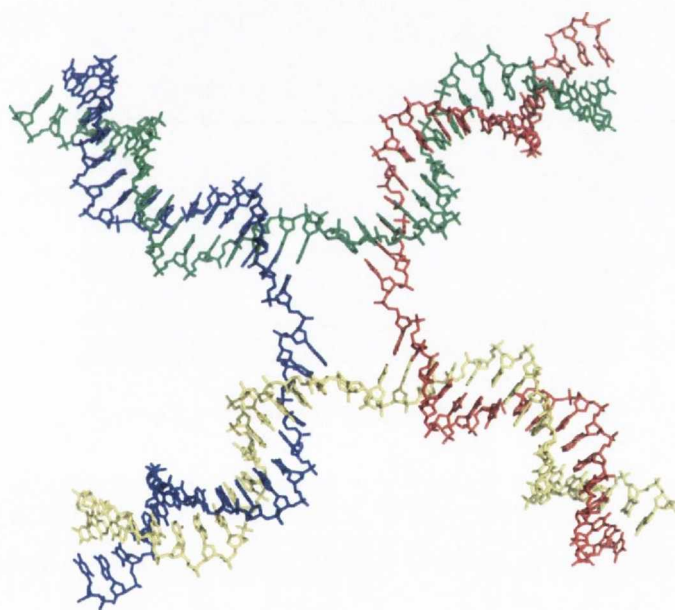


Figure 1.5 – Crystal structure of a Holliday Junction

PDB ID: 3CRX

This DNA structure is formed when two homologous double stranded DNA molecules migrate towards each other and reciprocally exchange DNA strands, generating a crossover link (Figures 1.5 and 1.6). The strand exchange point is called the junction, a four-stranded intermediate that is most central to homologous recombination. As each crossover strand re-anneals to its original partner it displaces the complementary strand

ahead of it, causing the Holliday junction to migrate, thus it is a mobile junction. In the cell, this junction is formed and stabilised by a specific group of helicase proteins (RuvA and RuvB) which use ATP hydrolysis to move the junction up and down the DNA.³¹

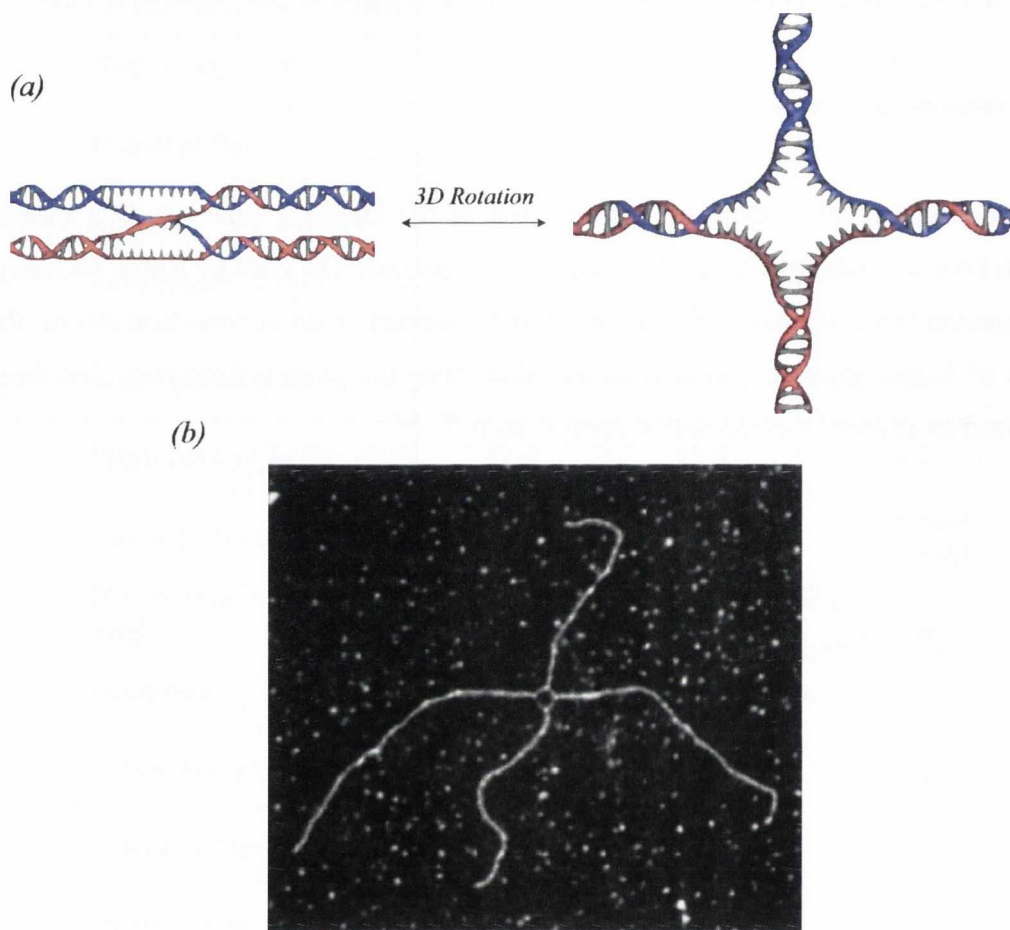


Figure 1.6 – (a) Schematic representation of the two homologous double stranded DNA molecules forming a Holliday junction and (b) an electron microscope image of a Holliday junction within a cell³²

Holliday junctions are an intermediate in genetic recombination that are of importance in maintaining genomic integrity.³³⁻³⁶ For the purposes of the work contained in this thesis an understanding of Holliday Junctions is a secondary matter, but it warrants this brief mention due to its value in Chapter 3, the crystallographic studies conducted with the enantiomeric $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes.

1.2.2.2 Quadruplex DNA

Quadruplexes are higher order DNA structures most often formed from the guanine rich sequences found at the end of chromosomes. These guanine rich sequences tend to fold into a four-stranded (quadruplex) structure *in vitro* having a loop organisation that is stabilised by non-Watson-Crick hydrogen bonds^{37,38}. The basic structural motif of the quadruplex structure is the Hoogsteen paired coplanar guanine tetrad in Figure 1.7(a). These base quartets then stack upon each other to form quadruplex DNA (Figure 1.7(b) and Figure 1.8). Quadruplexes are commonly formed in telomeric DNA which is located at the end of chromosomes, however, non-telomeric quadruplex DNA has also been reported.³⁹

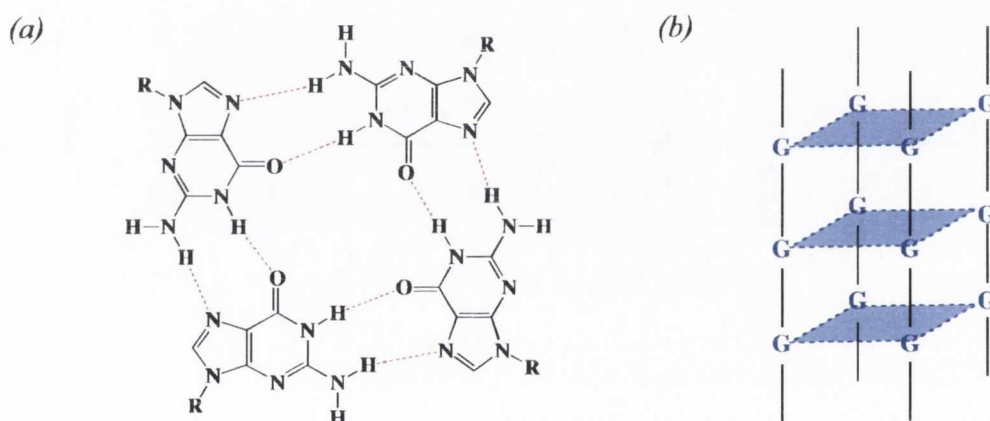


Figure 1.7 – The structural motif of (a) a guanine tetrad and (b) quadruplex DNA

--- represents hydrogen bonding

The natural role and biological significance of these structures has only recently been investigated, and there is particular interest in quadruplex DNA as a target for therapeutic compounds because these structures are formed in the telomeric sequences.⁴⁰⁻⁴⁵ In normal somatic human cells, telomeres function to protect the ends of chromosomes as a disposable buffer. They consist of tandem repeats of the sequence d(TTAGGG) and are typically 5-8 kilobases (kb) long with a single stranded 3'-overhang of 100-200 bases⁴⁶ containing the guanine-rich repeat. Each time the cell undergoes DNA replication, these telomeres are shortened until eventually subsequent replication results in the loss of crucial chromosomal DNA, causing apoptosis and cell death. In contrast, many cancer

cells have the ability to maintain the length of their telomere sequences by increasing the level of activity of the enzyme telomerase. Telomerase is expressed in 85%⁴⁷ of cancer cells and is the ribonucleoprotein that catalyses the de novo synthesis and repair of the telomeric repeats after each DNA replication.⁴⁸ Thus the telomeres of such cells do not shorten, rendering the cells immortal. As a result of this process, research has recently been conducted into developing small molecules that will selectively inhibit this particular enzyme.^{41,42,49-54} Many of the proposed therapeutic molecules act by stabilizing or inducing the formation of the quadruplex structure in telomeric DNA, making the specific targeting of quadruplex DNA a highly desirable attribute of a complex designed to aid in the fight against cancer.

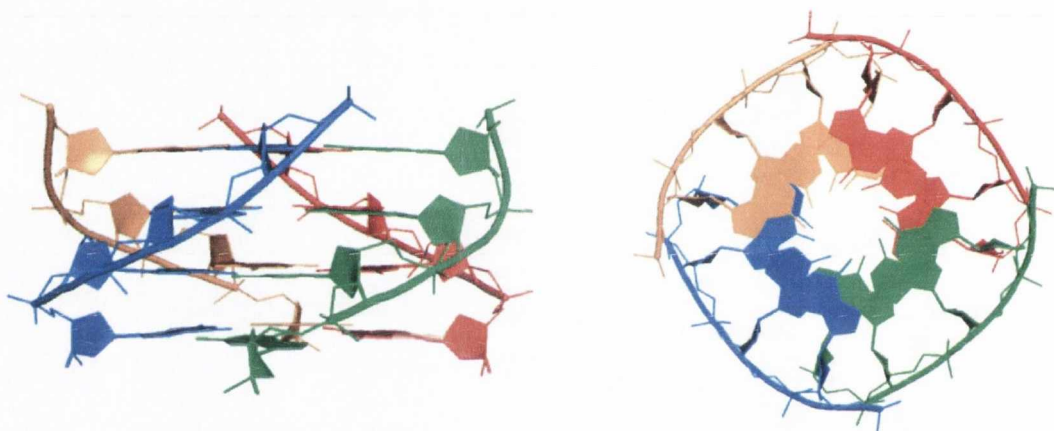


Figure 1.8 – Crystal structure of parallel stranded quadruplex DNA

PDB ID: 139D

While an appreciation of these higher order structures is relevant, for the purpose of the majority of the work contained in this thesis, only B-DNA is considered as the intended target for the binding of complexes. To this end, an understanding of the modes through which a compound may bind to DNA is crucial.

1.3 Binding to DNA

Being essentially a polyanionic macromolecule, DNA will attract most cationic species in an electrostatic binding mode. However, the unique structure of DNA — a polyanionic backbone wrapped around a hydrophobic core of base pairs — allows it to bind a wide range of molecules, both charged and uncharged. There are three ways in which a molecule may bind to DNA; external binding, groove binding and intercalation.

1.3.1 External Binding

External binding occurs when a complex associates with the atmosphere of ions of the DNA polyelectrolyte. This interaction is electrostatic in nature and occurs between the positively charged complex binding to the negatively charged phosphate sugar backbone of DNA. This mode of binding is the least common of the three, but has been proposed as the method of interaction for small compounds such as $[\text{Ru}(\text{bpy})_3]^{2+}$ with duplex DNA.⁵⁵

1.3.2 Groove Binding

Groove binding, as the term suggests, occurs when a compound binds to DNA via either the major or minor groove. The main methods of interaction of this binding mode are hydrophobic attraction and hydrogen bonding. These then stabilise the compound-groove association sufficiently to allow the formation of van der Waals bonds. Groove binders are a large and chemically diverse family of molecules. Many show biological activity and generally display a preference for A–T regions of DNA, due to the narrower, deeper nature of the groove in A–T tracts as compared to G–C.^{56,57} Though the majority of such compounds are minor groove binders, there are an increasing number of major groove binders being reported in the literature. Association at a groove is governed by geometric and steric interactions, resulting in such binding compounds having common structural characteristics;

- Positive charges
- Linked (rather than fused) aromatic/heteroaromatic rings
- An approximate crescent shape in three dimensions

Groove binders generally do not significantly perturb the DNA structure. An example of one such compound is the naturally occurring Netropsin (Figure 1.9).^{58,59} This cationic molecule, considered as a model groove binder, associates at the minor groove and interacts with the DNA via hydrogen bonding between its peptide nitrogens and the donor atoms at the edges of adenine or thymine bases.

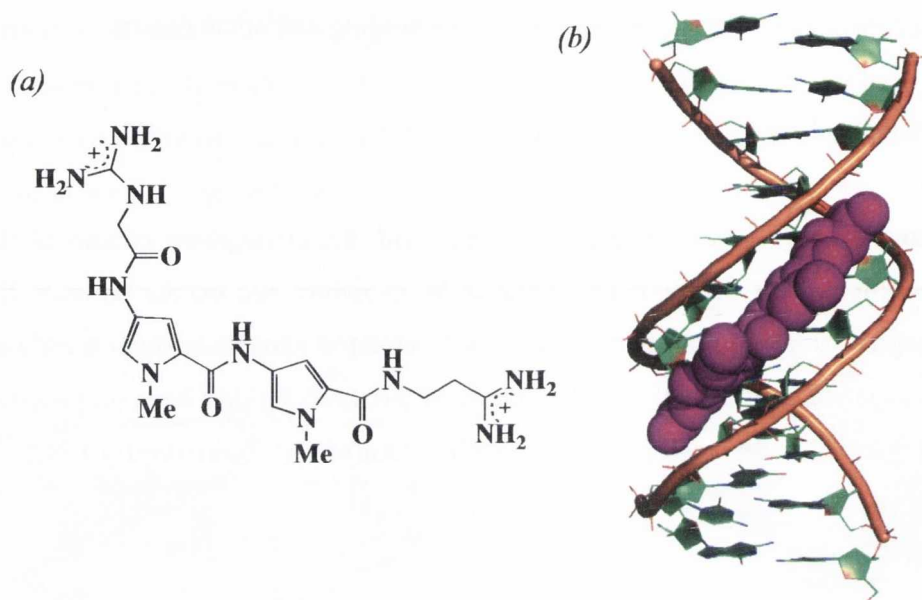


Figure 1.9 – (a) Structural formula of netropsin and (b) the crystal structure of netropsin (pink) bound to the minor groove of B-DNA (PDB ID:101D)

1.3.3 Intercalation

Intercalation is defined as the reversible inclusion of one molecule between two other molecules. In relation to DNA, the intercalative theory, developed by Lerman,⁶⁰ suggests that a planar chromophore of a molecule may be inserted between adjacent base pairs of a DNA strand. As a result, intercalating ligands have common structural characteristics:

- Often polycyclic
- Aromatic
- Planar

The aromatic, planar region of a compound can bind between adjacent base pairs of duplex DNA, stabilized by the π interactions of the purine/pyrimidine bases with the

aromatic rings of the ligand and the associated London van der Waals dispersion interactions. Optimal attractive interactions occur when the intercalating chromophore contains quadrupoles and incorporate one or more heteroatoms such as nitrogen. Electron-deficiency is also an important factor, and a formal cationic charge is commonplace. Some of the more intensely studied DNA intercalators include ethidium bromide and proflavin. Figure 1.10 shows a crystal structure of the intercalative mode of binding of proflavin with a DNA hexamer duplex. Part (b) illustrates the intercalation of the compound between adjacent base pairs while part (c) demonstrates the overlap of the proflavin with the terminal C-G base pair, an interaction stabilised by the π systems of the aromatic rings.

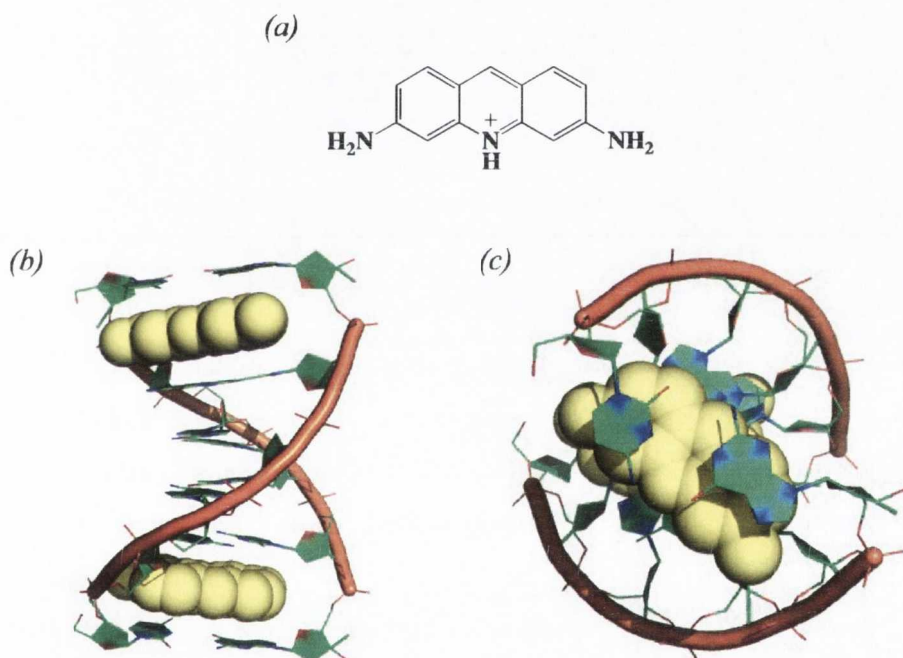


Figure 1.10 – (a) Structural formula of proflavin (b) Side-on view of two proflavin molecules intercalating to duplex DNA (c) Top-down view of two proflavin molecules intercalating to duplex DNA (PDB ID: 3FT6)

In contrast to groove binding, the process of intercalation results in significant changes to the DNA double helix (Figure 1.11). In order for an intercalator to fit between base pairs, the DNA helix must dynamically open a space between its base pairs by unwinding. The degree of unwinding varies depending on the intercalator; proflavin unwinds the duplex by 17° whereas ethidium bromide unwinds it by as much as 26° . This separation of the

base pairs results in an extension of the double helix by approximately 3.4 Å (0.34 nm).⁶¹ The maximum number of intercalating molecules that can bind to a DNA duplex is one for every two base pairs, i.e. the immediate base pair step either side of a binding site cannot bind an intercalating molecule. This is the Neighbour-Exclusion Principle, a consequence of the structural constraints forced on the geometry of the DNA backbone by intercalation.⁶²

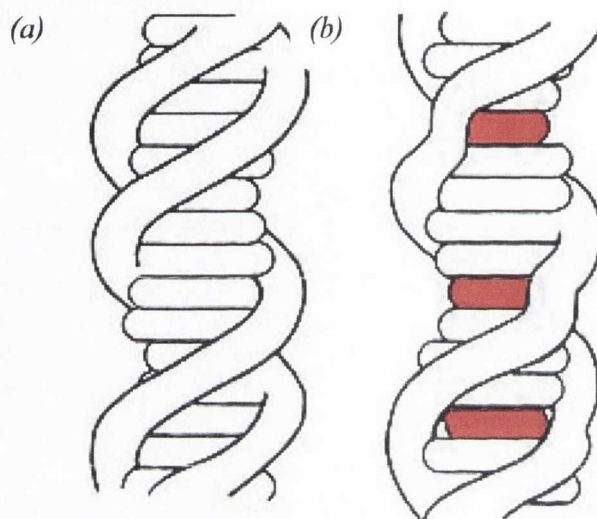


Figure 1.11 – Intercalation induces structural distortions

(a) An unchanged DNA strand, (b) DNA strand intercalated at three regions (red)

The unwinding induces local structural changes to the DNA duplex such as a lengthening of the helix or twisting of the base pairs. These structural modifications can lead to functional changes, occasionally resulting in the inhibition of transcription and replication, and the DNA repair process, making intercalators both potent mutagens and effective anti-tumour agents.

1.4 Ruthenium(II) Polypyridyl Complexes

Ruthenium complexes first became widely researched during the years that followed the energy crisis in the mid 1970s. The unique photophysical properties of such complexes in which a central ruthenium(II) ion coordinates three bipyridine ligands, $[\text{Ru}(\text{bpy})_3]^{2+}$, were anticipated to enable them to catalyse the photochemical splitting of water into hydrogen and oxygen. Though this process in practice turned out to be difficult, the investigations formed a basis for many of the uses of such complexes found today.⁶³

The study of ruthenium polypyridyl complexes as DNA binding agents was first initiated with the study of $[\text{RuL}_3]^{2+}$ (L = bipyridine (bpy) or phenanthroline (phen), Figures 1.12 and 1.15) some twenty-five years ago.⁶⁴ Such complexes were found to possess a number of properties that make them attractive candidates for such application. Firstly, they are generally chemically inert in the sense that interactions with DNA normally are non-covalent and hence, reversible. Additionally, the inversion-stable octahedral coordination of the ruthenium ion provides a rigid scaffold around which ligands can be varied for systematic modification of the complex's physical and chemical properties. The metal centres also exhibit an inherent chirality, the right-handed Δ or the left-handed Λ propeller (Figure 1.12), giving rise to the handedness discrimination found with chiral hosts such as DNA.

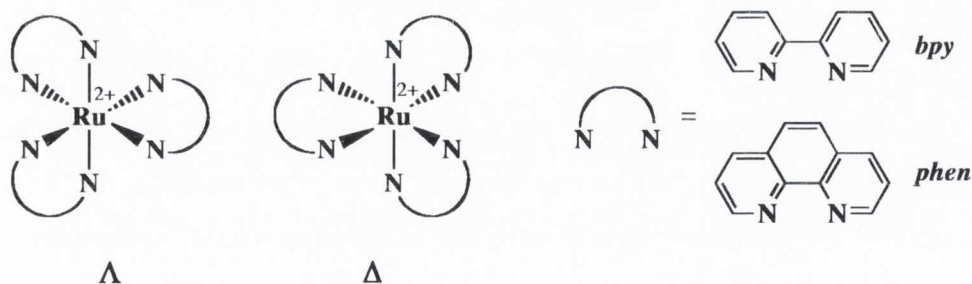


Figure 1.12 – The octahedral coordination of the ruthenium centre, giving rise to two stereoisomeric forms, the left-handed (Λ) and right-handed (Δ) propeller, and two of the most commonly used bi-dentate ligands: bpy = 2,2'-bipyridine and phen = 1,10-phenanthroline

A final important reason that has motivated the extensive use of ruthenium complexes as DNA probes is their rich photophysical repertoire, in particular, the sensitivity of their electronic absorption and emission to different microenvironments.

1.4.1 Photophysical Properties

The photophysical properties and selective photochemistry of ruthenium polypyridyl complexes yield systems that allow the use of relatively simple spectroscopic methods to study their interactions with DNA. However, in order to understand how these complexes interact and photoreact with DNA, an exploration of their photophysics is required.

Perhaps the simplest, yet most widely studied of these complexes is $[\text{Ru}(\text{bpy})_3]^{2+}$.⁶⁵ As such, the basic principles will be explained and illustrated utilizing this model as variations of the electronic environment around the central ion (i.e. varying the nature of the ligands) brings about corresponding changes in the spectral properties of the complex.

1.4.1.1 Absorption

The absorption spectra of ruthenium polypyridyl complexes are dominated by metal-to-ligand charge transfer transitions (MLCTs).⁶⁶ The UV/visible absorption spectrum of $[\text{Ru}(\text{bpy})_3]^{2+}$ is shown in Figure 1.13 and consists of three fundamental types of electronic transitions:

- $(\pi-\pi^*)$: *Ligand-centred (LC)*

These transitions are mainly localised on the ligand and have spectral properties that closely resemble the free ligand states. These bands appear in the ultraviolet region ($\lambda = 200\text{--}400\text{ nm}$) and are often characterised by intense absorptions.

- $(d\pi-d\sigma^*)$: *Metal-centered (MC)*

These ‘dd’ bands, which appear at approx. 300 nm, represent a symmetry-forbidden transition and displays a weak absorption. Also, excitation into MC states often brings about efficient ligand dissociation.⁶⁷

- $(d\pi-\pi^*)$: *Metal-to-ligand charge transfer (MLCT)*

These transitions of lowest excited state energy between a metal centred $d\pi$ ground state and ligand π^* states are often observed in the visible region

($\lambda = 400\text{--}500\text{ nm}$) with middling to high absorbance intensity, and are both solvent and substituent sensitive. In a formal sense, excitation at these wavelengths result in metal oxidation and ligand reduction.⁶⁸

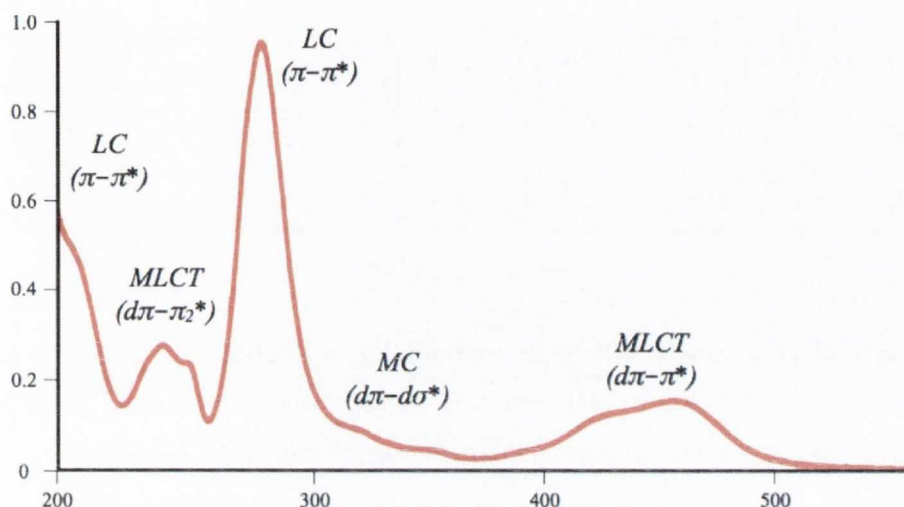


Figure 1.13 – The absorption spectrum of $[\text{Ru}(\text{bpy})_3]^{2+}$ in water with assignments of the various bands

1.4.1.2 Photophysical mechanisms

Upon irradiation to the visible absorption band of $[\text{Ru}(\text{bpy})_3]^{2+}$, a singlet MLCT state is populated. This state deactivates rapidly via intersystem crossing (ISC) to the lowest lying triplet MLCT state ($^3\text{MLCT}$). The ISC is allowed as the ‘heavy atom effect’ of the ruthenium metal induces spin-orbit coupling, which mixes singlet character into triplet states. The resulting $^3\text{MLCT}$ state corresponds to three excited states that are very close in energy and fourth of slightly higher energy. The emission of the complex originates from a $^3\text{MLCT}$ state, which is reasonably long-lived. The various possible deactivation pathways for these excited states are shown in Figure 1.14.^{69,70}

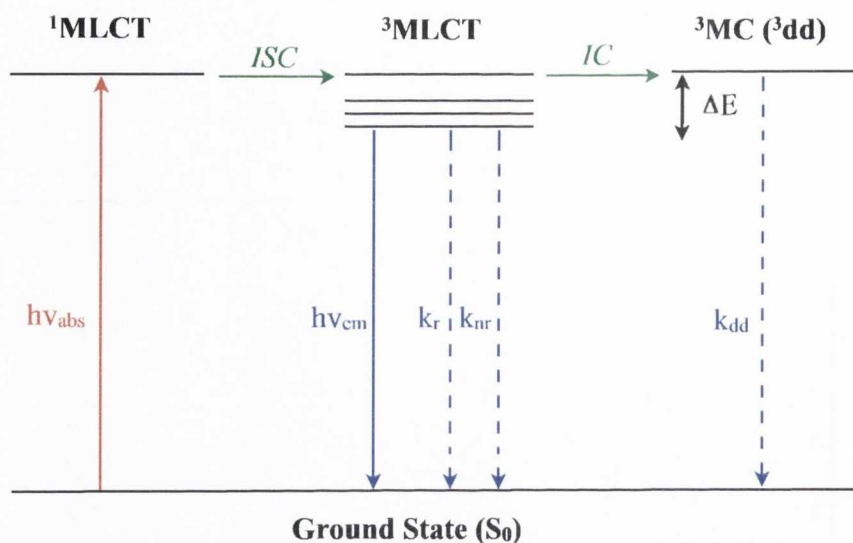


Figure 1.14 – Diagram of the excited state levels of $[Ru(L)_3]^{2+}$ complexes; $h\nu_{abs}$ = absorption, $h\nu_{em}$ = emission, ISC = intersystem crossing, IC = internal conversion. ΔE represents the energy gap between the 3dd and the manifold of 3MLCT emitting states

Interestingly, both the 3MLCT and 3MC states exhibit two types of excited state reactivities. The 3MLCT state generally involves redox processes, exhibiting both oxidation and reduction depending on conditions.⁷¹ Intense activity has been directed towards elucidation of the nature of this state and is now regarded as essentially three non-degenerate states (collectively called the ‘triplet’ state) which, at room temperature, exist in thermal equilibrium.⁶⁹ Thus the resulting emission is considered to arise from an average emitting state with average characteristics. This state also exhibits slow non-radiative decay with a characteristic long luminescence lifetime (in the order of nanoseconds).⁶⁹

In contrast, the 3MC state usually results in the promotion of a non-bonding electron into an anti-bonding σ orbital, which causes distortion along the Ru-N bonds and often leads to the loss of a ligand.⁷¹ This mechanism is responsible for thermal deactivation and the photochemical reactions such as racemisation and photo-substitution.⁷²

1.4.2 Ruthenium Complexes and DNA

Many simple ruthenium polypyridyl complexes exhibit the interesting phenomenon of a high luminescence quantum yield in non-aqueous solvents but a significant luminescence quenching in aqueous environments.⁷³ This characteristic, coupled with the other properties previously mentioned, allows these complexes to be used as detectors of DNA in aqueous solvents as the local non-aqueous environment provided by the double helix increases the quantum yield of the complex.

Initially studied systems were quite simple, based on the bpy and phen derivatives of the ruthenium complex (Figure 1.15). It was observed that while $[\text{Ru}(\text{bpy})_3]^{2+}$ bound to DNA mainly through electrostatic interactions ($K < 10^3 \text{ M}^{-1}$ in 50 mM NaCl)⁷⁴, the binding affinity of the phenanthroline derivative was much stronger ($K = 6.2 \times 10^3 \text{ M}^{-1}$ in 5 mM Tris-HCl + 50 mM NaCl).⁷⁵ This was explained by the cationic nature of both complexes resulting in the attraction for the anionic phosphate backbone of the DNA structure. While the bpy complex binds electrostatically to the groove of the helix,⁷⁶ the extended heteroaromaticity of the phen ligands allows the $[\text{Ru}(\text{phen})_3]^{2+}$ complex to semi-intercalate between the base-pairs, forming a much stronger interaction.⁷⁷

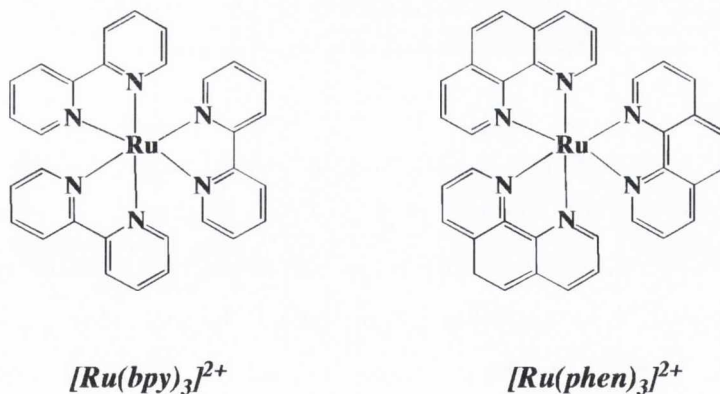


Figure 1.15 – Structural formula of $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$

Being a second row transition metal, ruthenium typically adopts an octahedral conformation. As mentioned previously, chirality around this octahedral metal centre arises from the disposition of the chelating ligands, resulting in either a Λ - or Δ -complex conformation (see Figure 1.12). Enantioselective studies of these complexes revealed the

Δ -[Ru(phen)₃]²⁺ enantiomer to have a higher binding affinity (approximately 30% higher) than that of the Λ -[Ru(phen)₃]²⁺ enantiomer.⁶⁴ This can be rationalised by the differences in the alignment of the complexes' ancillary ligands with regard to the edges of the DNA grooves, as illustrated in Figure 1.16. The resulting differences in steric hindrance affect the ability of the enantiomers to bind to double-helical DNA differently, depending on the nature of the enantiomer.

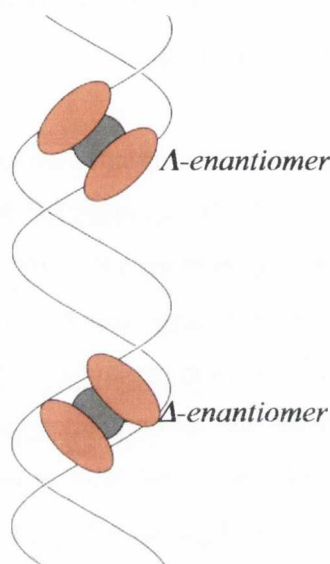


Figure 1.16 – Schematic alignment of Λ - and Δ - enantiomers with a B-DNA helical backbone⁷⁸

While these systems showed remarkable promise in the field of biological probes, they were hindered by their low binding affinity for DNA. However, by altering the ligands surrounding the metal centre, the binding mode and affinities were changed with regard to both nucleic acids and various other biomolecules. The main strategy implemented to increase binding affinity was the extension of the aromatic ligands to provide a platform for binding via intercalation. The presence of an intercalating extended aromatic ligand drastically increased the binding affinity of such ruthenium polypyridyl complexes, for example where [Ru(bpy)₃]²⁺ yielded $K < 10^3 \text{ M}^{-1}$, [Ru(bpy)₂dppz]²⁺ yielded $K < 10^6 \text{ M}^{-1}$.⁶⁵ As a result many similar extended aromatic ligand-containing ruthenium complexes have been investigated, but none as extensively as [Ru(phen)₂dppz]²⁺.

1.4.3 [Ru(phen)₂dppz]²⁺ - The Molecular 'Light-switch'

The ruthenium bis-1,10-phenanthroline dipyrido[3,2-a:2',2'-c]phenazine ($[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$) complex, shown in Figure 1.17, has been the subject of many recent investigations, largely due to the phenomenon known as the molecular 'light switch' effect. This effect is the result of highly efficient quenching of the excited state such that the complex exhibits no observable luminescence in aqueous solutions, but does exhibit luminescence in non-aqueous environments.

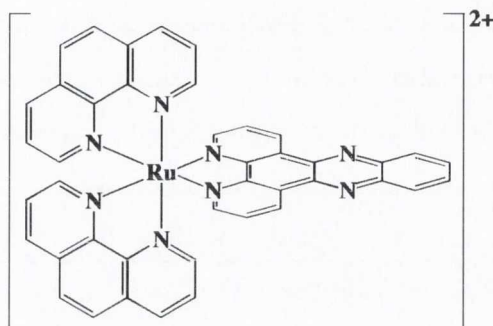


Figure 1.17 – Structural formula of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$

There are a large number of organic fluorophores that exhibit environmentally sensitive luminescence, however there are remarkably few inorganic species that exhibit this phenomenon. In this case, it is the unique combination of the ruthenium centre with the dipyridophenazine ligand and the two bi-dentate aromatic ancillary ligands that create the molecular 'light switch'. Indeed alteration of the central metal or the surrounding ligands has a pronounced effect on the 'light-switch' behaviour of the complex.

There have been a number of theories suggested in an attempt to explain the 'light-switch' phenomenon and while none have been proven unequivocally correct, perhaps the most complete model is that proposed by Lincoln *et al.*⁷⁹ This model suggests that there are four states, each with a characteristic luminescence lifetime (Figure 1.18). Initially, only three states were postulated. The first of these states, State A, occurs immediately after excitation whereby the excited electron lies on a π^* orbital of one of the three polypyridyl ligands. The second state, State B, results from the conversion of State A where the electron moves from its initial excited state across the three ligands to lie in a lower energy state on the extended dppz ligand. Finally, State C, occurs when the solvent has reorganised to hydrogen bond to both phenazine nitrogens. It is accepted that State A

rapidly converts into State B, however there is an entropic/enthalpic equilibrium between States B and C.^{80,81}

Later work, exploring the question of whether or not hydrogen bonding to both phenazine nitrogens is necessary for luminescence quenching, suggested that in addition to the primary state (State A) there are two emissive excited MLCT states, and a third non-emissive MLCT state. These three MLCT states correspond to either both aza-nitrogens being hydrogen bonded to the solvent (fully-quenched state), only one nitrogen hydrogen bonding to the solvent (semi-quenched state) or neither nitrogen hydrogen bonding (full luminescence state). The introduction of a third energy level arose from the calculated energy difference between States B (fully luminescent) and C (fully quenched) being significantly smaller than estimated.⁸² Thus, if State B corresponded to no hydrogen bonds formed, State C must then be reassigned to a mono-hydrogen bonded species, suggesting the existence of State D in which both aza-nitrogens are hydrogen bonded (Figure 1.18).

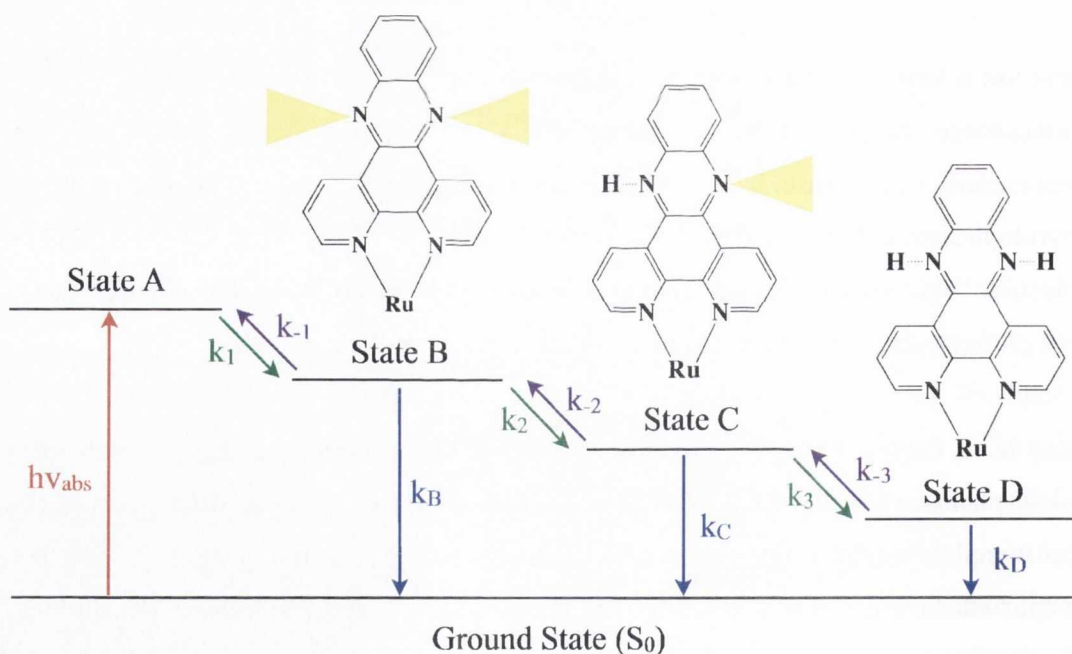


Figure 1.18 - Schematic energy level diagram of the Lincoln light-switch model, taking into account the occurrence of zero (State B), one (State C) or two hydrogen bonds (State D) to the dppz ligand.  indicates luminescence

It is assumed that all hydrogen bonds occur in the excited state as it has previously been taken that they cannot occur in the ground state since the slight negative charge provided by the excited electron is required to assist the formation of these hydrogen bonds.⁸³⁻⁸⁵ Further reasoning for three states is due to there being no change in the emission profile with temperature using water as the solvent — water being such an effective hydrogen bond donor that only mono- and di-hydrogen bonded excited state complexes are present, thus only one weakly luminescing state is observed.⁸² In this environment, the luminescence is said to be ‘off’. Due to the hydrophobic nature of a DNA double helix, intercalation of the dppz moiety between the base-pairs affords the aza-nitrogens a degree of protection from the hydrogen-bonding water molecules. Thus upon excitation, the complex remains in State B, and a strong luminescence is observed. The molecular ‘light-switch’ is now said to be ‘on’ demonstrating the potential of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ as an effective DNA probe.

1.4.4 Ruthenium Complexes as DNA Photocleavage Agents

As mentioned previously, $^1\text{O}_2$ mediated photo-oxidative reactions with tumours is generally the accepted mode of action for most PDT agents.⁸⁶ It was also well known that ruthenium polypyridyl complexes can cause the cleavage of DNA upon photoirradiation.^{60,87} Such species are sufficiently oxidising in their excited state to abstract electrons from species with high oxidation potentials. In the majority of cases, the base oxidation responsible for DNA cleavage is as a result of $^1\text{O}_2$ formation which diffuses along the helix, causing single strand breaks.⁸⁸

Initial work, performed with $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ showed their $^1\text{O}_2$ quantum yields to be quite low,^{87,89} however, the photoredox processes of ruthenium polypyridyl complexes in general can be tuned by alteration of the surrounding ligands. Incorporating tri-dentate ligands and extending the aromatic π systems not only significantly increased the quantum yield of $^1\text{O}_2$ by changing the nature of the lowest excited state (to a $^3\pi-\pi^*$ ligand-centred state localised on the extended aromatic ligand) but increased the complexes’ binding affinity for DNA by incorporating a ligand that can intercalate into the helix.⁹⁰ The high quantum yields of $^1\text{O}_2$ photosensitisation and observed DNA

cleavage ability of such complexes are indicative of the potential of ruthenium polypyridyl complexes in the design of new systems for PDT.

An alternative method of DNA photocleavage is also available by utilising certain ruthenium polypyridyl complexes. Direct reaction in which the photosensitising species can form covalently attached photoadducts with DNA also prevents the process of transcription.⁹¹ For this to occur, the ruthenium complex must be a stronger oxidising agent in its excited state than that previously required for $^1\text{O}_2$ production. This can be achieved through the utilisation of π -electron deficient ligands that have an increased electron accepting potential.

1.4.5 π -Deficient Ruthenium Polyazaaromatic Complexes

Ruthenium complexes that contain at least two π -deficient ligands, such as 1,4,5,8-tetraazaphenanthrene (TAP) or 1,4,5,8,9,12-hexazatriphenylene (HAT), have been shown to be extremely oxidising in their $^3\text{MLCT}$ excited state.⁹²⁻⁹⁵ Interestingly these complexes display an opposite behaviour to that previously observed with the bpy and phen derivatives. In aqueous solutions these complexes display a luminescence, while in the presence of DNA significant quenching is observed.^{93,94}

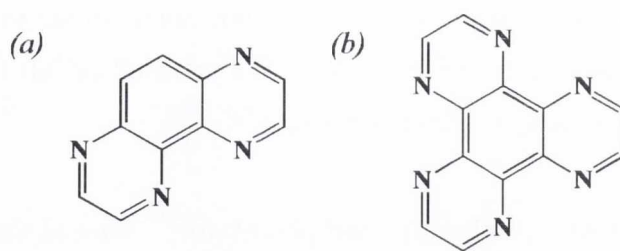


Figure 1.19 – Structure of π -deficient ligands (a) TAP and (b) HAT

Further investigations of ruthenium complexes containing these ligands, such as $[\text{Ru}(\text{TAP})_3]^{2+}$, in the presence of DNA have yielded two different photophysical behaviours. Firstly in the presence of [poly(dA-dT)], the $^3\text{MLCT}$ lifetime increases along with an enhancement in emission as a function of increasing concentrations of polynucleotide.^{55,89} This is due to the protection of the excited state of the complex by the local DNA microenvironment from quenching caused by molecular oxygen. This result

indicates that while oxygen in the solvent does have a quenching effect on the luminescence of the complex, it is not as efficient as that found with its $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ counterparts.

The second photophysical behaviour was found with the same complex in the presence of $[\text{poly}(\text{dG-dC})]$. The emission from the $[\text{Ru}(\text{TAP})_3]^{2+}$ complex was efficiently quenched upon binding, and the $^3\text{MLCT}$ lifetime was significantly shortened.⁸⁹ This quenching effect was shown to be a result of photoinduced-electron transfer (PET) between the $^3\text{MLCT}$ state of the complex and guanine residues of the DNA, due to guanine being the most readily oxidised of the four nucleobases.^{96,97} This charge transfer process, often followed by a back electron transfer (BET), has since been observed by transient absorption spectroscopy,^{98,99} and been shown to occur in the presence of amino acids such as tryptophan.^{100,101} While the BET process is perhaps the most common mechanism of returning to the ground state, another process may compete with this pathway, formation of a covalent adduct between the complex and biomolecule.^{102,103} This photo-adduct originates from the formation of a covalent bond between one chelated TAP ligand and the exocyclic nitrogen of the guanine nucleobase (Figure 1.20) or tryptophan residue.

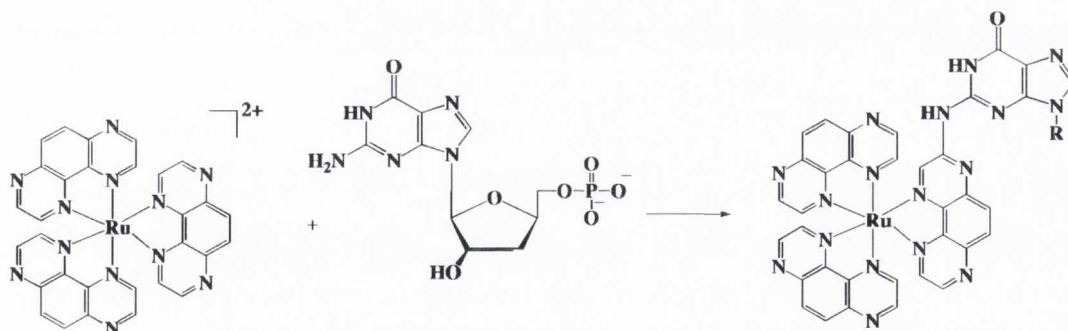


Figure 1.20 – Structure of the photo-adduct formed upon excitation of $[\text{Ru}(\text{TAP})_3]^{2+}$ in the presence of GMP⁹⁵

This adduct is believed to be formed from the recombination of two radicals resulting from the PET, followed by a re-aromatisation of the resultant adduct (Figure 1.21).¹⁰⁴ The photo-adduct with the guanine unit has been confirmed by several techniques, including electrospray mass spectroscopy and polyacrylamide gel electrophoresis (PAGE).^{96,104,105}

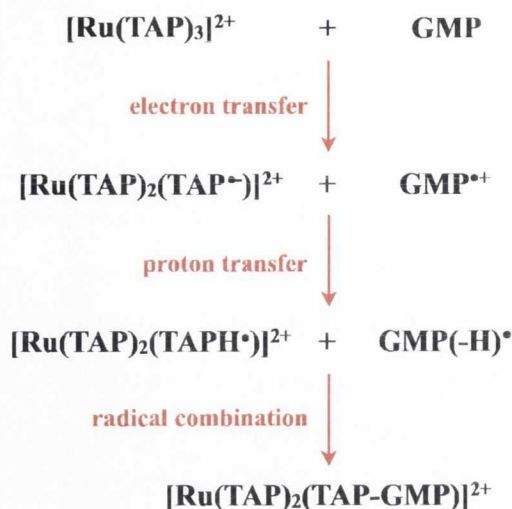


Figure 1.21 – Schematic representation of the proposed photo-electron transfer process between GMP and the excited state of $[\text{Ru}(\text{TAP})_3]^{2+}$

The discovery of this photo-adduct, which would inhibit transcription and whose formation is triggered exclusively by the application of light has significant ramifications for the field of PDT. Indeed these complexes indicate the possibility of precise control over the reaction and subsequent cell damage. Though much research is still required into the practical applications for such compounds, the unique photochemical properties of these ruthenium complexes make them extremely interesting with regard to the field of phototherapeutics.

The majority of the work conducted with π -electron deficient ruthenium polypyridyl complexes has thus far been with the racemic mixture of enantiomers. The following section will discuss the separation and study of enantiomers of such complexes with the aim of providing a molecule of further increased specificity.

1.5 Applications of Chirality

The concept of chirality is a property of asymmetry and is important in several branches of science. The word itself is derived from the Greek word $\chi\epsilon\iota\rho$ (cheir) meaning the 'hand', possibly the most familiar of chiral objects. An object or system is said to be chiral if it cannot be superimposed onto its mirror image, and it is a term first used by Lord Kelvin during an address in Baltimore, 1904;

"I call any geometrical figure, or group of points, 'chiral', and say that it has chirality if its image in a plane mirror, ideally realized, cannot be brought to coincide with itself".¹⁰⁶

While chirality is useful in many aspects of science, it plays a highly significant role in the fields of chemistry and biology in which it usually refers to the structural conformation of molecules. The two mirror images of a chiral molecule are called enantiomers, or optical isomers, with their designation often being either 'right'- or 'left-handed'. While application of chirality is important within the fields of chemical and biochemical research, it more recently has become crucial to the development of medical treatments. A mixture of the two enantiomers is called a racemic mixture and in recent times, a number of drugs were initially developed and utilised as racemates before the realisation that only one enantiomer produces the desired therapeutic effect. The ineffective enantiomers of many such drugs have been shown to have no effect on the body, but a small number have had devastating consequences. Perhaps the most infamous of these drugs is thalidomide (Figure 1.22). The (*R*)-enantiomer is effective against morning sickness while the (*S*)-enantiomer is teratogenic, causing abnormalities in foetal physiological development.

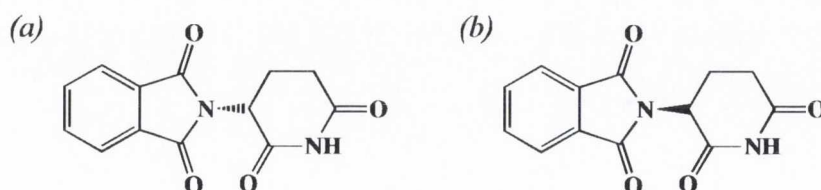


Figure 1.22 – Structural formulae of the two enantiomers of thalidomide

*(a) (*S*)-thalidomide, (b) (*R*)-thalidomide*

The fallout from the shocking effects of such drugs has been to ensure that any future medications, if they possess chirality, must have both enantiomers screened separately to ensure no unwarranted side-effects. The consequence of this has been to not only increase the safety of medications, but has also increased the potency of some drugs in which one enantiomer was essentially therapeutically inactive.

The study of chiral molecules is an integral part of organic chemistry and increasingly inorganic chemistry. As has previously been mentioned, complexes containing octahedral metal centres, such as ruthenium, have an inherent chirality, Λ and Δ . While in most situations, the differences between ligand orientations are irrelevant, application of these chiral compounds to a chiral medium can have very differing effects. This has profound implications for the study of such complexes with DNA, which itself is of 'right-handed' chirality in its most common A- and B-configurations. Considering the previously discussed application of polypyridyl ruthenium complexes with DNA, separation of the enantiomers and investigation with DNA is the next logical step following studies of the racemate. In this regard, perhaps the most widely studied enantiomers are those of the molecular 'light-switch' molecule $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$.

1.5.1 Λ - and Δ -enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$

The discovery of the remarkably high binding affinity of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ in the presence of DNA ($K = 6 \times 10^7 \text{ M}^{-1}$)¹⁰⁷ has led to extensive investigation of this complex as a molecular probe. Following the realisation that the Δ -enantiomers of such ruthenium polypyridyl complexes are generally better binding agents than their Λ counterparts, the separation of the Λ - and Δ -enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ was completed by Lincoln *et al.* via stereoselective recrystallisation¹⁰⁷ and later by Barton *et al.* via ion-exchange chromatography.¹⁰⁸ The two enantiomers were characterised by circular dichroism (CD), one of the few analytical methods available to identify otherwise identical chiral compounds (Figure 1.23).

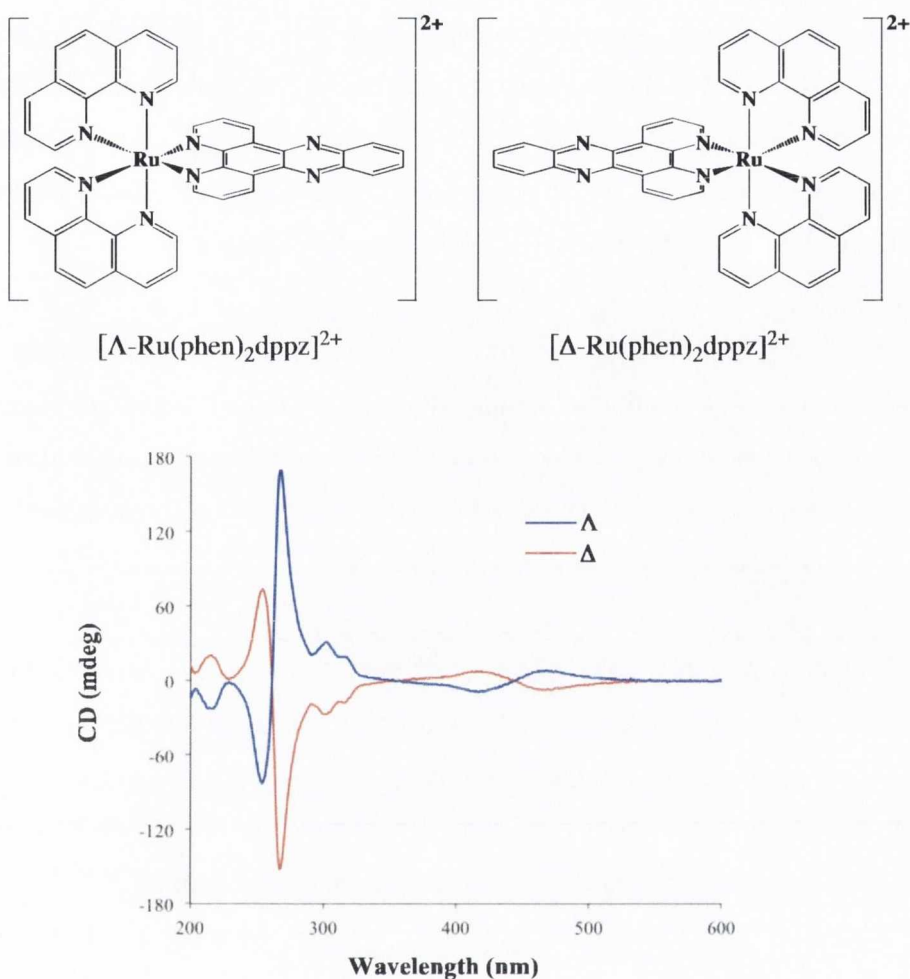


Figure 1.23 - Structural formula of Λ - and Δ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ and the CD spectra of the two enantiomers

Subsequent investigations of the two enantiomers in the presence of DNA have yielded some very interesting results and suggest subtle differences in their binding geometries.¹⁰⁷ Their binding strength is attributed to their ability to intercalate into the DNA helix as evidenced by unwinding studies of the helix,⁶⁵ linear dichroism¹⁰⁷ and calorimetric studies.¹⁰⁹ The binding affinities of both enantiomers, calculated at $K=1.5 \times 10^7 \text{ M}^{-1}$ for Λ , $K=2.4 \times 10^8 \text{ M}^{-1}$ for Δ (10 mM NaCl and 1mM sodium cacodylate buffer) show that the Δ enantiomer has a much greater affinity than that of the Λ enantiomer, however the bound Δ enantiomer exhibits a relative quantum yield 6-10 times greater than that of the Λ .¹⁰⁷ This suggests that while both enantiomers have a similar affinity for DNA, the depth of intercalation experienced by the Δ -dppz ligand is greater than that of the Λ -dppz ligand, thus affording it an increased level of protection from quenching by the water molecules.

What is particularly unusual about the binding of these considerably bulky complexes is that it appears possible for the intercalation sites to be extremely close together (one intercalation site for every two base pairs).^{107,110} This implies that the two phenanthroline ligands play a significant role in influencing the properties of the bound system, and further supports the theory of the different dppz intercalation depths.

Further to this, both enantiomers were found to have a preference for binding to A-T sites¹¹¹ and the angular orientation of the dppz ligand was found to facilitate intercalation of this elongated planar moiety between the DNA base pairs¹⁰⁷ as opposed to the ‘side-on’ partial intercalation previously proposed.¹¹² Also, each of the enantiomers exhibits bi-exponential luminescence decay upon binding to DNA.

There has been considerable debate as to whether the ruthenium complexes bind from the major or the minor groove of the DNA helix. Barton *et al.* suggest that the bi-exponential decay is due to major groove intercalation resulting in two orientations of the dppz ligand, side-on or perpendicular (Figure 1.24). They propose that one orientation is more accessible to bulk solvent and thus has a shorter excited state lifetime.^{65,113} Their nuclear magnetic resonance (NMR) methods utilising selective deuteration of coordinating ligands appear to confirm the major groove binding of the complex.^{108,114}

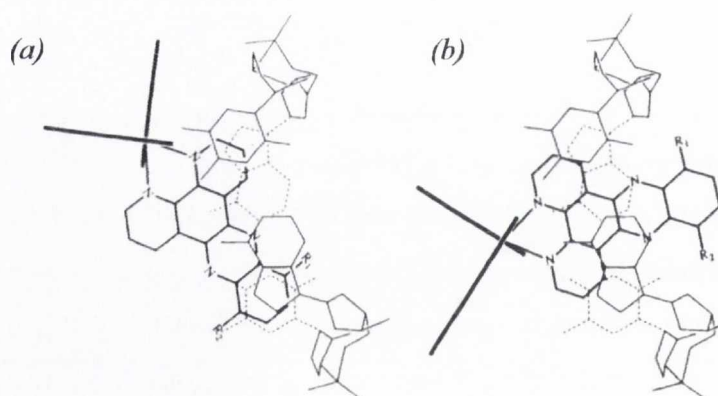


Figure 1.24 – Illustrations of the (a) side-on and (b) perpendicular models proposed for the intercalation of $[Ru(L)_2dppz]^{2+}$ complexes with DNA (major groove is left)

However, work by Lincoln *et al.* suggests the dppz ligand adopts only the perpendicular orientation when binding, and that the bi-exponential decay is due to a dye distribution

effect, with the complex binding almost exclusively in the minor groove as evidenced by lifetimes studies of the complexes with different polynucleotides.^{107,111} Their NMR studies performed also agree with the minor groove intercalation scenario,¹¹⁵ contradicting those results presented earlier by Barton *et al.* Without crystallographic data, however, this dispute cannot be settled easily.

In light of the extensive studies conducted with the enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, and given the rather poorly studied field of enantiomeric ruthenium complexes containing π -electron deficient ligands, the work presented in this thesis is an extensive study of the enantiomeric forms of $[\text{Ru}(\text{TAP})_2(\text{X}_2\text{dppz})]^{2+}$, a ‘reverse light-switch’ complex.

1.6 Work Described in this Thesis

The work described in this thesis is concerned with the separation of the enantiomers of ruthenium polypyridyl complexes containing π -electron deficient ligands and the photophysical investigation of these enantiomers in the presence of various sequences of B-DNA.

In Chapter 2, the successful separation of the Λ - and Δ -enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ is described. Extensive investigations of the enantiomers in the presence of stDNA (salmon testes), $[\text{poly}(\text{dA-dT})]$ and $[\text{poly}(\text{dG-dC})]$ are reported along with nanosecond transient absorption (TA) and time resolved infra-red (TRIR) studies of the two enantiomers in the presence of $[\text{poly}(\text{dG-dC})]$.

Chapter 3 discusses the crystallographic results arising from a collaborative effort with the HeliX Group at the University of Reading. This is the first reported crystal structure of a ruthenium polypyridyl complex with an oligonucleotide, Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with TCGGCGCCGA, and is anticipated to unequivocally show the mode through which such complexes bind to DNA, the orientation of the ligands in the binding site and the groove through which such complexes bind to B-DNA.

Chapter 4 details the synthesis of the methylated and fluorinated analogues of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$, the subsequent separation of the enantiomers and investigation of these enantiomers with stDNA, $[\text{poly}(\text{dA-dT})]$ and $[\text{poly}(\text{dG-dC})]$ in an attempt to determine the affect, if any, of substitution at the dppz ligand may have on the complex's ability to bind to DNA.

Chapter 5 discusses complexes of increased molecular complexity, particularly $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$. Attempts at the separation of the various isomers of this bi-nuclear ruthenium complex are detailed, along with a novel synthesis for the mono-nuclear complex $[(\text{TAP})_2\text{Ru-TPAC}]^{2+}$.

Chapter 6 is comprised of the conclusions drawn from the work discussed in this thesis and suggestions for further studies that may arise from the results presented herein.

Finally, in Chapter 7, general experimental conditions are outlined and the syntheses of each compound discussed in this thesis are detailed.

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Chapter 2

Studies of [Ru(TAP)₂dppz]²⁺ Enantiomers

2.1 Introduction

Since the first synthesis of $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ in 1985,¹ dppz complexes have been the focus of more research than most other Ru(II) complexes.²⁻⁶ The main reason for this is due to these dppz complexes' ability to behave as a DNA light-switch⁷ and thus have been used in several studies with DNA and polynucleotides.^{8,9} Most of these complexes emit extremely weakly in water so their luminescence cannot be detected under standard conditions. Intercalation of the dppz ligand between the DNA base pairs affords the ligand protection from the aqueous solvent molecules and allows the complex to luminesce, thus the 'light-switch' effect of luminescence upon binding to DNA¹⁰. $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ is the classic example exhibiting extremely low emission quantum yields and luminescence lifetimes in aqueous solutions.^{11,12}

$[\text{Ru}(\text{L})_2\text{dppz}]^{2+}$ complexes interact strongly with DNA because of their having this extended aromatic heterocyclic ligand. The large, planar surface allows for intercalation between base pairs of the double helix which, coupled with the complex's luminescent properties, affords DNA recognition and sensing. Variation of the ancillary ligands can dramatically alter the spectroscopic properties of these complexes while retaining their photostability, water solubility and DNA binding constants in the region of $10^6 \text{ (M}^{-1}\text{)}$.¹³

As mentioned in Chapter 1, ruthenium(II) complexes containing π -deficient electron accepting ligands have been shown to make the corresponding excited states more oxidizing than their bpy or phen analogues.¹⁴ This has the effect of altering the lowest ³MLCT state to correspond to a charge transfer from Ru(II) to one of the two TAP ligands instead of the dppz moiety.¹⁵⁻¹⁷ The consequence of this alteration is two-fold. The first is the reversal of the 'light-switch' effect, meaning that in aqueous solutions $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ (Figure 2.1) is highly luminescent as hydrogen bonding to the phenazine nitrogens on the dppz ligand does not cause the non-radiative decay of the ³MLCT.^{4,11} The second is that upon binding to DNA photoinduced-electron transfer (PCET) occurs between a guanine residue and the ³MLCT state of the complex, resulting in a quenching of luminescence ('light-switch off' effect) and can cause single-strand breaks followed by double-strand cleavages. Additionally, the formation of a photo-adduct between such complexes and guanine residues has been reported.^{18,19}

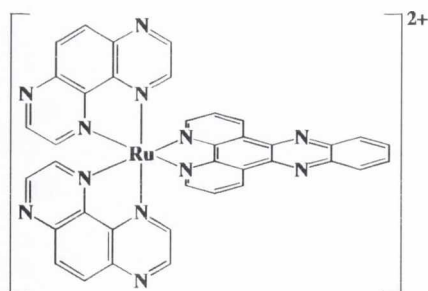


Figure 2.1 – Structure of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

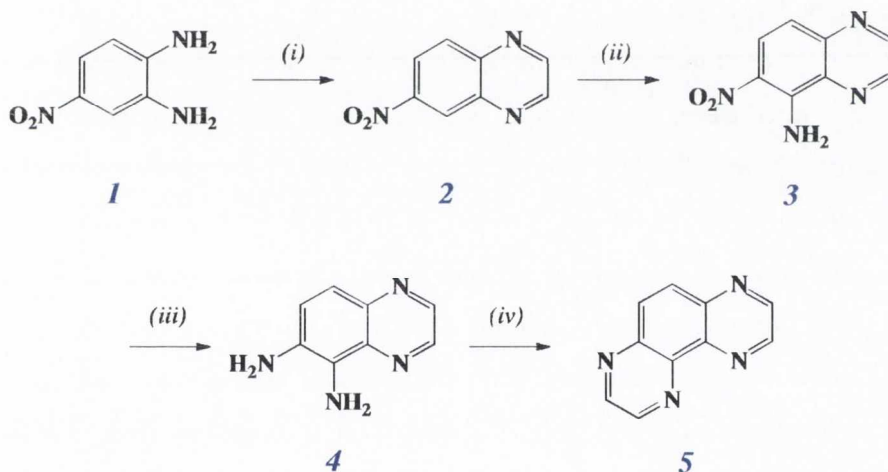
Initial studies of these complexes were conducted using the racemic mixture of the optical isomers but, since then, much work has been conducted with the two enantiomers, Λ and Δ , of $[\text{Ru}(\text{phen})_3]^{2+}$,²⁰⁻²² $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$,²³ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$.^{2,3,5,9} These studies show a significant difference between the binding of the Λ and Δ enantiomers to DNA, as discussed in Chapter 1. Recently picosecond studies were conducted within the Kelly Group, on the *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex which illustrated proton-coupled forward and back electron transfer pathways between the TAP ligands and guanine residues.²⁴ Following this, the objective of the research presented in this Chapter is to separate the Λ and Δ enantiomers of the $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex for the first time, investigate the binding characteristics of each enantiomer with DNA and synthetic polynucleoides, and determine if there is an enantiomeric effect on the forward and back electron transfer pathways, utilising ultra-fast techniques.

2.2 Synthesis

$\text{Rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ was prepared from the reaction of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ with dppz utilizing a novel procedure involving microwave technology. Full experimental details are provided shortly in this chapter and in Chapter 7.

2.2.1 Synthesis of the TAP ligand

The synthesis of the TAP ligand, while not novel, is a tricky synthesis owing to the high number of recrystallization steps required, use of the highly sensitive sodium metal in the second step (to produce NaOMe) and its low yield of $\approx 22\%$ over the four reaction steps. The following synthesis was conducted in collaboration with Dr. Rob Elmes, also of Trinity College Dublin, Scheme 2.1.



Scheme 2.1 – Synthetic pathway of TAP (i) glyoxal, EtOH, reflux; (ii) H_2NOH , NaOMe in MeOH, reflux; (iii) N_2H_4 , 10% Pd/C; (iv) glyoxal, EtOH, reflux

The first step involved the condensation of an aqueous solution of glyoxal with 4-nitrobenzene-1,2-diamine **1** by heating under reflux in ethanol for two hours. The solvent was removed under reduced pressure and the remaining solid was recrystallised from isopropanol to give 6-nitroquinoxaline **2** as golden needles in 79% yield. Amination of **2** was conducted using hydroxylamine in the presence of NaOMe at reflux for 90 minutes. The crude product was recrystallised from a mixture of acetic acid and water (3:1), resulting in bright yellow crystals of 6-nitroquinoxalin-5-amine **3** in 41% yield.

Reduction of the nitro group using hydrazine in the presence of 10% Pd/C gave the pure quinoxaline-5,6-diamine **4** as a red solid in 98% yield. Condensation of **4** with an aqueous solution of glyoxal was conducted by heating under reflux in ethanol for two hours. The solvent was removed under reduced pressure, followed by recrystallization from isopropanol to yield the desired TAP ligand **5** as golden needles in 69% yield. The compound was characterised by ^1H NMR and HRMS which agreed with literature values.^{25,26}

2.2.2 Microwave Assisted Synthesis of $\text{rac}-[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

Previous syntheses of mixed ligand Ru(II) polypyridyl complexes were conducted using the procedure described by Sullivan *et al*²⁷ involving reflux using traditional heat transfer equipment such as oil baths, sand baths and heating jackets. Indeed, prior to this work, the synthesis of $\text{rac}-[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ was conducted in this way; reflux in a water-ethanol (1:1) solution under argon for six hours, resulting in a 46% yield.⁶ These heating techniques are rather slow and a temperature gradient can develop within the sample, leading to local overheating and possible decomposition. Also the longer reaction times increase the probability of side products, often resulting in the requirement of complex purification methods to obtain the desired product. With the advances in modern technology, the use of microwave radiation in chemical syntheses has become increasingly popular as a reliable method of heating a reaction mixture quickly and uniformly. A recent report describes a highly efficient preparation of Ru(II) polypyridyl complexes utilising microwave radiation.²⁸ Given the lower reaction times and the higher yields associated with microwave assisted synthesis, this method was adopted for the synthesis of the $\text{rac}-[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex in this work.

Microwave assisted synthesis in chemistry is based on the efficient heating of materials by microwave dielectric heating. This phenomenon is dependant on the ability of a specific material (usually the solvent) to absorb microwave energy and convert it into heat. Being part of the electromagnetic spectrum, microwave radiation causes heating by two mechanisms: dipole polarisation and ionic conduction. Consequently the sample to be heated must contain dipolar molecules, such as water. A dipole is sensitive to external electric fields so upon application of an electric field from microwave radiation the dipolar molecules will attempt to align themselves by rotation. The oscillating nature of

the microwave electric field, however (Figure 2.2), causes the dipoles to be in perpetual rotation and release energy by molecular friction and collision, giving rise to dielectric heating.²⁹

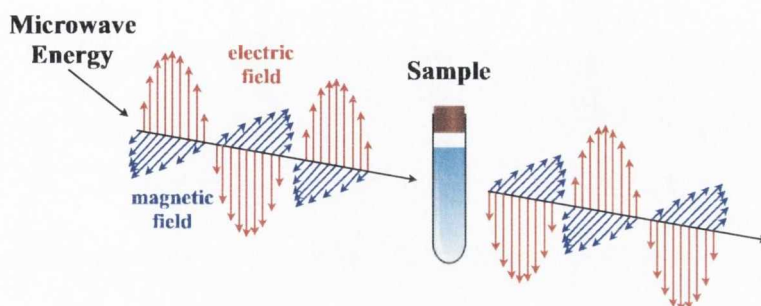


Figure 2.2 – Schematic representation of microwave assisted heating

Ionic conduction is the second major interaction of the electric field component of the microwave. In a solution containing ions, the ions will move under the influence of an electric field, resulting in expenditure of energy due to an increased collision rate thus converting kinetic energy to heat. This conductivity mechanism is a much more significant process than the dipolar mechanism with regard to heat-generating capacity.²⁹

From a practical and synthetic viewpoint, microwave irradiation leads to the direct activation of reactant molecules in solution by localised superheating facilitated by the ability of the microwaves to pass through the walls of the reaction vessel. This results in the temperature increasing uniformly throughout the sample and, in pressurized systems, it is possible to increase rapidly the temperature above the conventional boiling point of the solvent used. In comparison, traditional conductive heating requires the transfer of energy from a heat source to an oil bath, which subsequently heats the outside surface of the reaction vessel before the energy can reach the reaction mixture. Localised heating also occurs within the reaction mixture with the solution nearest the heat source being warmer than that furthest away (see Figure 2.3 for comparisons). This is an extremely inefficient process resulting in long reaction times and side reactions. As such, microwave heating has the ability to reduce reaction times from hours to minutes, minimise the formation of side products, increase yields and improve reproducibility.³⁰

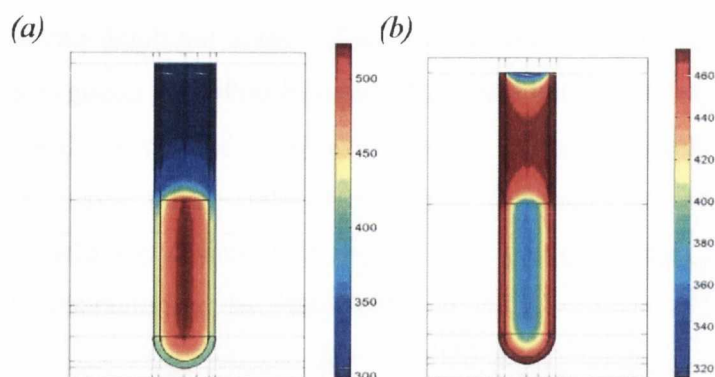
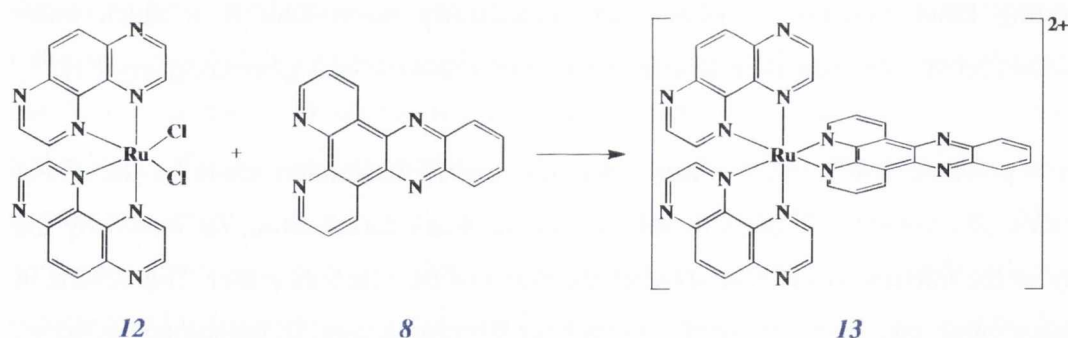


Figure 2.3 – Inverted temperature gradient in (a) microwave vs (b) oil bath heating³¹

Following this, a modified synthesis of $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ incorporating microwave heating was undertaken. The synthesis of the $\text{Ru}(\text{TAP})_2\text{Cl}_2$ and dppz precursors were conducted following literature methods.^{2,28,32} Synthesis of the $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex involved the complexation of a dppz ligand **8** with the $\text{Ru}(\text{TAP})_2\text{Cl}_2$ precursor **12** in a degassed solution of ethanol and water (1:1).



Scheme 2.2 - Synthetic pathway of $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

(i) $\text{EtOH}/\text{H}_2\text{O}$ (1:1), MW, 140°C , 40 mins

Upon forty minutes irradiation in the microwave at 140°C the reaction mixture changed from a violet coloured solution to a deep red/brown solution, indicating the formation of a tris-polypyridyl $\text{Ru}(\text{II})$ complex. Subsequent precipitation of the complex as its PF_6 salt was achieved by the addition of a concentrated aqueous solution of KPF_6 . Isolation of the precipitate yielded a red/brown solid. This was converted to its chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl^- form) and the desired product, **8**, was obtained following purification on an aqueous Sephadex C-25 column with 0.2 M NaCl .

as the mobile phase. The final product was obtained as a red/brown solid in 74% yield. This compares to the 46% yield obtained with the more conventional method of heating,²⁸ showing that the use of microwave heating does in fact increase the final yield while reducing the reaction time significantly from 6 hours to 40 minutes. The main impurities present in the reaction mixture were unreacted $\text{Ru}(\text{TAP})_2\text{Cl}_2$, $[\text{Ru}(\text{TAP})(\text{dppz})_2]^{2+}$ and a highly coloured green/blue compound, most probably a Ru(III) species formed by oxidation. The complex was characterised by ^1H NMR and MALDI TOF spectrometry, both of which agree with the literature values.^{6,28}

2.2.3 Separation of the enantiomers Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

The Λ and Δ enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ have previously been obtained and their binding ability to DNA investigated thoroughly, as discussed in Chapter 1.^{2,3,9} The initial method by which these enantiomers were obtained was through enantio-specific synthesis of the Λ and Δ isomers. The $[\text{Ru}(\text{phen})_2(\text{phen-5,6-dione})]^{2+}$ precursor was synthesised as a racemic mix and separated into its Λ and Δ enantiomers by first crystallising the Λ enantiomer using a boiling solution of sodium arsenyl-L(+)-tartrate, leaving the Δ -enriched mother liquor. The Δ enantiomer was isolated by precipitation with aqueous KPF_6 . Reaction of the separate $[\text{Ru}(\text{phen})_2(\text{phen-5,6-dione})]^{2+}$ enantiomers with 1,2-diaminobenzene yielded the Λ and Δ enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$.²

Unlike the enantiomeric synthesis of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, the Λ and Δ enantiomers in this work were separated from a racemic mix after the fully completed synthesis of *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$, using a column chromatographic method developed by Keene *et al.*^{33,34} This method involves the use of CM Sephadex C-25 cation exchange support as the stationary phase with an electrolytic solution (typically a Na^+ salt) as the mobile phase.³⁵ CM Sephadex is composed of a cross-linked dextran matrix, functionalised with acidic carboxymethyl groups. While coulombic forces dominate the cation-exchange chromatographic process, the charge densities or polarities of the cations³⁶ also exert an influence for species of the same charge. The use of a suitable counter anion in the eluent also has an effect on the second-sphere interactions between cation and anion.^{36,37} Since isomers may differ in their relative interactions with the counter anion, the resultant slight variations in the effective charge and polarity of the species facilitate separation on the

column. Due to the individual units of the CM Sephadex being an α -D-glucopyranoside (Figure 2.4) and each subunit containing five chiral centres, the stationary phase itself imparts some chiral specificity resulting in the chiral resolution of racemic complexes with achiral eluents such as water. Use of chiral counter anions in the eluent will thus either oppose or enhance the resolution imparted by the stationary phase. Correct choice of chiral eluent will increase the efficiency of resolution due to synergistic enhancement.

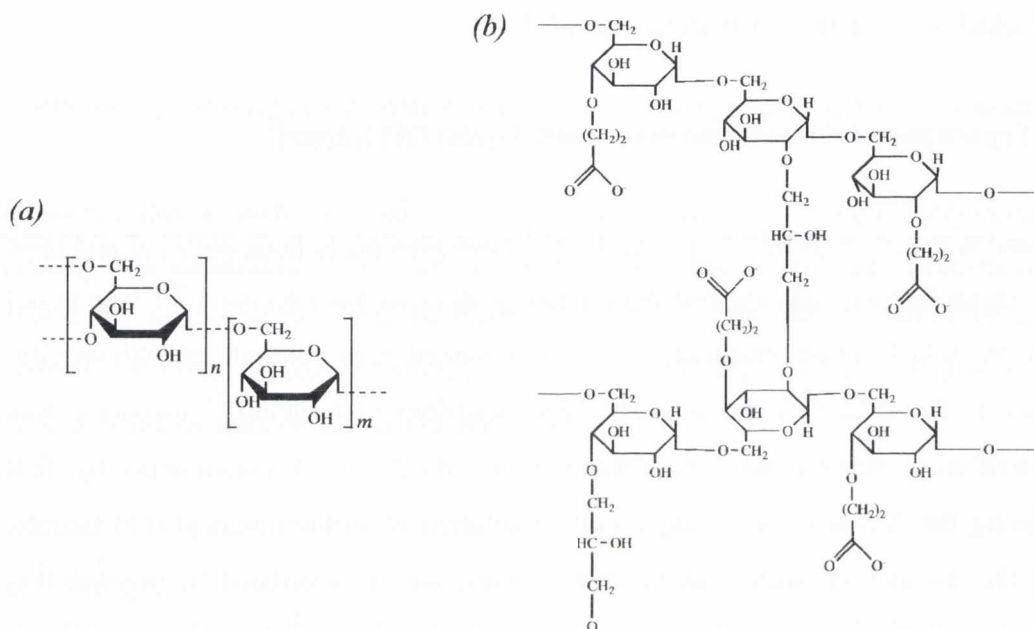


Figure 2.4 – Structural formula of (a) dextran (b) CM Sephadex C-25

There is a selection of counter anions available for separating both mono- and multi-nuclear metallo complexes. For the purposes of this work sodium(-)-dibenzoyl-L-tartrate (0.1 M), neutralised to pH 7, was used as the eluent to greatly improve the resolution of the Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers (Figure 2.6). The $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ was adsorbed onto the aqueous Sephadex stationary phase in a 1 m perspex column (diameter 2 cm) and the cationic mobile phase was applied. This resulted in a slight shrinkage of the Sephadex due to the tartaric acid cations reducing the intramolecular repulsion of the carboxymethyl groups. Upon saturation of the column with the mobile phase, the $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex was allowed to pass through the column four times to facilitate adequate separation of the Λ and Δ enantiomers before they were collected. Due to the nature of the applied anion the Λ -enantiomer eluted before the Δ -

enantiomer. Precipitation of the PF_6 salts was achieved by adding concentrated aqueous solutions of KPF_6 to the collected fractions. Complex purity was confirmed by MALDI mass spectrometry (TOF MS LD+): Found: 747.7317 $\text{C}_{38}\text{H}_{22}\text{N}_{12}\text{Ru}$ (Calc. Mass: 748.1134) and enantiomeric purity was confirmed by circular dichroism analysis (Figure 2.5).

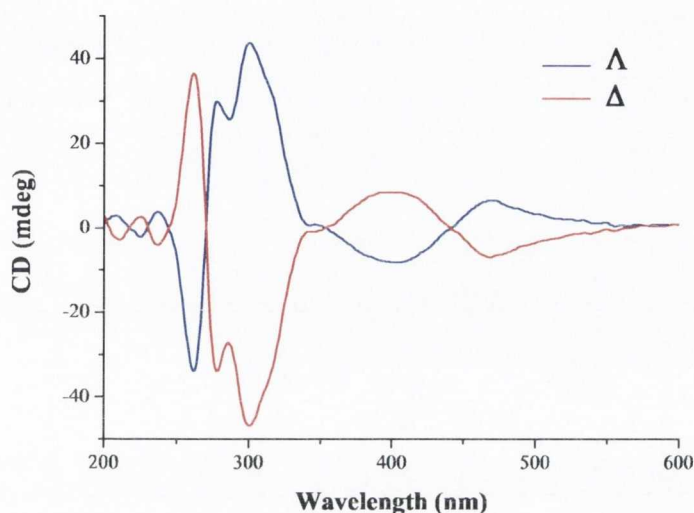


Figure 2.5 – Circular dichroism of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{PF}_6$
 $([\Lambda] = 18.1 \mu\text{M}, [\Delta] = 18.3 \mu\text{M})$ in acetonitrile

Identification of the two enantiomers from their CD spectra was achieved by comparison with the reported spectra of Λ - and Δ - $[\text{Ru}(\text{phen})_3]^{2+}$ and Λ - and Δ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ (Figure 1.23).^{22,38} This assignment of the Λ and Δ enantiomers was later confirmed by the crystallisation studies (see Chapter 3) performed in collaboration with Prof. Christine Cardin and the HeliX Group of the University of Reading which allowed for the direct observation of the Λ enantiomer via its crystal structure.

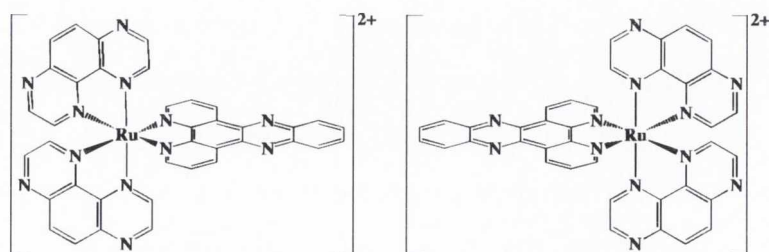


Figure 2.6 – Structural formula of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

2.3 Photophysical Characterisation

Characterisation of the enantiomers Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to confirm that they are identical in every aspect other than chirality was conducted. Initially, small differences were noted between the absorbance and emission spectra of the enantiomers. Rigorous purification via column crystallography of both enantiomers ensued and an impurity was discovered and removed from the Λ enantiomer. The UV/vis absorption and emission spectra are shown in Figure 2.7. Both the enantiomers, once pure, have identical spectra, thus only one line is observed.

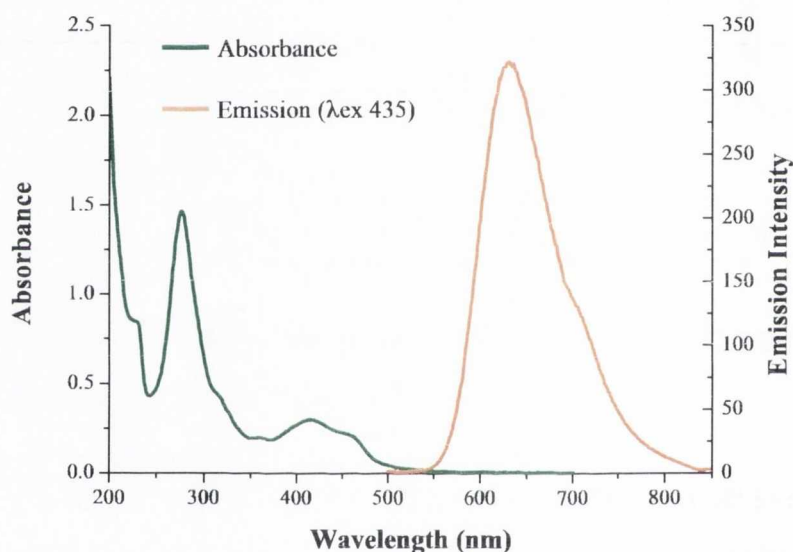


Figure 2.7 – UV/visible absorbance (green) and emission (orange) spectra of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ in water

Five different absorption maxima are shown in the UV/vis absorption spectrum: 230 nm, 275 nm, 362 nm, 412 nm and 456 nm. These compare with the spectrum reported for the racemic mixture which, naturally, has an identical spectrum.⁶ The strong bands at 230 nm and 275 nm are characteristic of the π - π^* intra-ligand transitions of the ancillary TAP ligands, while the very weak absorbance at 362 nm is attributed to the π - π^* transitions of the dppz ligand.⁶ This band is significantly weaker than that seen in the phenanthroline analogue $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ and may be as a result of the aforementioned changes in the electronic structure occurring as a result of containing electron-deficient ancillary ligands. Finally, the bands at 412 nm and 456 nm result from the metal-to-ligand charge transfer

(MLCT) transitions of the Ru(II) centre, the former being the Ru(II) $\rightarrow$ dppz MLCT transition and the latter the Ru(II) $\rightarrow$ TAP MLCT transition.

Excitation into the MLCT bands (at 435 nm – wavelength of the isosbestic point as shown in section 2.4.1) results in an intense MLCT based emission with λ_{max} at 635 nm. This echoes the emission of the racemic mixture and is in stark contrast to the Λ - and Δ - $[Ru(\text{phen})_2\text{dppz}]Cl_2$ complexes which show no emission in aqueous solutions. This is due to the lowest energy excited $^3\text{MLCT}$ state being localised on the TAP ligands as opposed to the dppz ligand,⁶ causing the TAP ligand to act as an electron sink, thus relaxation to the ground state occurs solely via these ligands. As a result, there is relatively little inter-ligand charge transfer to the dppz ligand and significantly less quenching of emission.³⁹

Excited state lifetime measurements (τ_{em}) were also recorded for each of the enantiomers, in water using, time correlated single photon counting ($\lambda_{\text{ex}} = 444$ nm). The data was best fitted to a single exponential decay function, with χ^2 in the range of 1.1 – 1.3. τ_{em} values of 793 ns and 801 ns were recorded for the Λ - and Δ - enantiomers respectively which correlate with the value of 820 ns determined previously for the racemic mixture.⁶ Argon saturated solutions of the enantiomers resulted in an increase in excited state lifetime due to a reduction in the excited state quenching caused by dissolved oxygen, given in Table 2.1.

Enantiomer	τ_{em} (ns) H ₂ O (air)	τ_{em} (ns) H ₂ O (Ar)
Λ	793 $\pm$ 56	920 $\pm$ 64
Δ	801 $\pm$ 48	914 $\pm$ 58

Table 2.1 – Emission properties of Λ - and Δ - $[Ru(TAP)_2dppz]Cl_2$ in water

In summary, the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ display identical spectroscopic and photophysical properties to each other, excluding the CD spectra, and the *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$, as expected. The differences between the two are only highlighted upon interaction with chiral substrates, as seen in their separation using a chiral chromatographic method. Duplex DNA, being a chiral molecule should result in a difference in behaviour between Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$.

2.4 DNA Binding Interactions

Ru(II) complexes are widely reported in the literature as having many different uses with perhaps the most interesting applications have been in the fields of chemotherapeutic agents⁴⁰ and luminescent bioprobes.^{41,42} Polypyridyl Ru(II) complexes containing extended aromatic chromophores, such as dppz, are designed to interact with DNA through a combination of electrostatic and π -stacking interactions with the main mode of binding expected to be via intercalation of the extended aromatic ligand. Depending on the nature of the ancillary ligands, these complexes exhibit either extremely weak or extremely strong photoluminescence at ambient temperature. Upon addition of double helical DNA, however, the photoluminescence is reversed, i.e. where it was previously weak, intense luminescence is observed but where it was previously strong, the luminescence is dramatically quenched. This is the 'light switch' effect and can be either 'switched on' (enhancement of luminescence) or 'switched off' (quenching of luminescence). The changes in the photophysical behaviour of the various complexes can be monitored via spectroscopic titrations and information on the equilibrium binding constant, host-drug stoichiometry and binding site size can be gleaned by analysis of this data. Indeed, interaction with duplex DNA has previously been shown to highlight differences between optical isomers of ruthenium polypyridyl complexes.^{2,20,43}

Previous studies have shown that while enantiomers of such complexes display a preference for A-T binding sites over G-C,^{2,5} they bind extremely strongly to DNA (binding constants in the region of $10^7/10^8 \text{ M}^{-1}$ at low ionic strength)² via intercalation, with the Δ enantiomer having the higher binding affinity. These investigations were conducted with complexes containing ligands with complete π electron systems, such as bpy and phen, but as yet, such a study has not been conducted with the enantiomers of complexes containing electron-accepting ligands such as TAP or HAT. As such, various spectroscopic titrations with Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ were conducted to investigate the nature and binding affinity of these complexes with DNA.

2.4.1 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with duplex DNA in 10 mM sodium phosphate buffer

UV/vis absorption spectroscopy and fluorescence emission spectroscopy were used to examine the titrations conducted between the two enantiomers and DNA. UV/vis spectroscopy is a powerful optical technique that can be used to measure the binding affinity of molecules to DNA. It has a wide range of applications including;

- Investigation of sequence selectivity
- Study of the polyelectrolyte theory⁴⁴ and the dependence of DNA binding on the electrostatic interactions analysed

In this technique the free complexes bind to the DNA macromolecule to form a DNA-ligand complex with a certain binding constant (Figure 2.8). This binding constant is a quantitative measurement of the binding strength of a molecule to DNA, which can be calculated, allowing for comparison with other molecules.

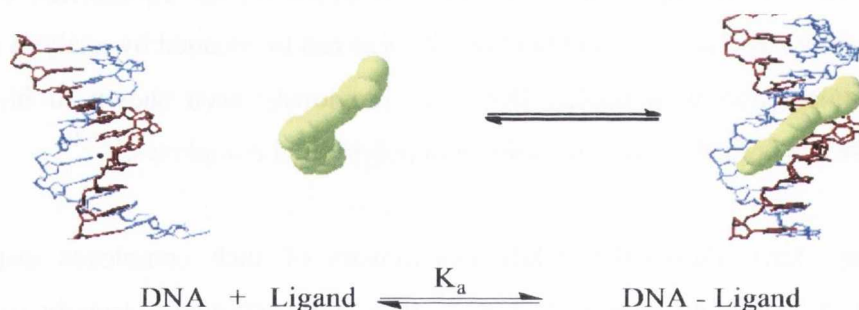
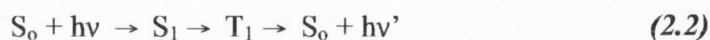
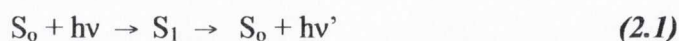


Figure 2.8 – DNA-Ligand binding equilibrium⁴⁵

Fluorescence spectroscopy is another powerful technique in the photophysical chemist's arsenal and is often used to complement absorption spectroscopy. It excites the electrons in molecules and measures any emitted luminescence from the electrons in the form of released photons as they relax from their excited states back to their ground states. A common misunderstanding with ruthenium(II) polypyridyl complexes is the notion that they fluoresce upon excitation. Such complexes do not fluoresce, they phosphoresce. Phosphorescence is a specific type of photoluminescence, related to fluorescence, but does not immediately re-emit radiation due to spin-forbidden transitions (i.e. emission

lifetimes are longer than nanoseconds). As in fluorescence (equation 2.1), phosphorescence results from the relaxation of an excited electron; however, phosphorescence occurs when this excited electron originates from a triplet excited state (equation 2.2) as opposed to the singlet excited state.



Depending on the nature of the compound, intersystem crossings (ISC) can be either allowed or forbidden, with the latter resulting in very slow transitions and often a slow and prolonged luminescence. Within ruthenium(II) polypyridyl complexes, the ISC lifetime is extremely fast (estimated at <1 ps) which results in a rapid phosphorescence upon excitation. The consequence of this is that methods normally reserved for fluorescence measurement can be utilised to study the phosphorescence of said complexes. As the titrations conducted in this work employed a fluorimeter and followed general fluorescent procedures, they have been dubbed ‘fluorescence titrations’ even though it is the phosphorescence of the complexes observed.

The data recorded from these experiments allow for further understanding of a compound’s excited state. Often information regarding aspects such as reaction centres and mechanisms, complexing capacities and stability constants in addition to measuring the binding affinity of a compound to DNA is provided. This technique is particularly useful in demonstrating the ‘light-switch’ effect displayed by the DNA binding of many ruthenium(II) polypyridyl complexes.

The principle behind both the absorption and fluorescence titrations involves the addition of aliquots of DNA to a sample of the molecule in the appropriate buffer while recording the relevant spectra, usually until a plateau in behaviour is reached. When the molecule binds to DNA, spectral changes will be observed in the form of shifts that generally correspond to a decrease in the concentration of the free molecule but an increase in the DNA-ligand complex, which usually has a slightly altered spectra to that of either the DNA or free complex.

Each spectroscopic experiment (i.e. UV/vis and fluorescence titrations, thermal denaturations, circular dichroism titrations and linear dichroism) was conducted a minimum of three times, with the results presented here being those deemed most accurate.

2.4.1.1 UV/visible Absorption Titrations in Low Salt Conditions – 10 mM sodium phosphate buffer

The interaction of the two enantiomers was first examined with stDNA (salmon testes B-DNA) in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4. The titrations were conducted by adding small aliquots of stDNA (at a stock solution concentration of 1×10^{-2} M) to a buffered solution of an enantiomerically pure complex (approx. 2×10^{-5} M) until a plateau in absorbance was reached. For these experiments, DNA additions were continued to a P/D ratio of 25.

The absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific examination of the π - π^* dppz absorption band at 362 nm ($\epsilon = 14,500 \text{ M}^{-1}\text{cm}^{-1}$) and MLCT absorption band at 412 nm ($\epsilon = 22,300 \text{ M}^{-1}\text{cm}^{-1}$), both of which are beyond the DNA absorption at 260 nm, allowing for the observation of any spectral shifts induced by DNA binding.

The titration of both the enantiomers with stDNA resulted in significant changes in their absorption spectra. Figure 2.9 shows the recorded absorbance spectra for the Λ and Δ enantiomers, with Figure 2.10 displaying the shift trends observed and accompanying values are summarised in Table 2.2.

The results obtained for both the enantiomers are similar to those reported for similar titration conducted with the *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex.⁶ However, subtle differences are observed upon careful examination of the enantiomeric absorption spectra (see insets of Figure 2.9)

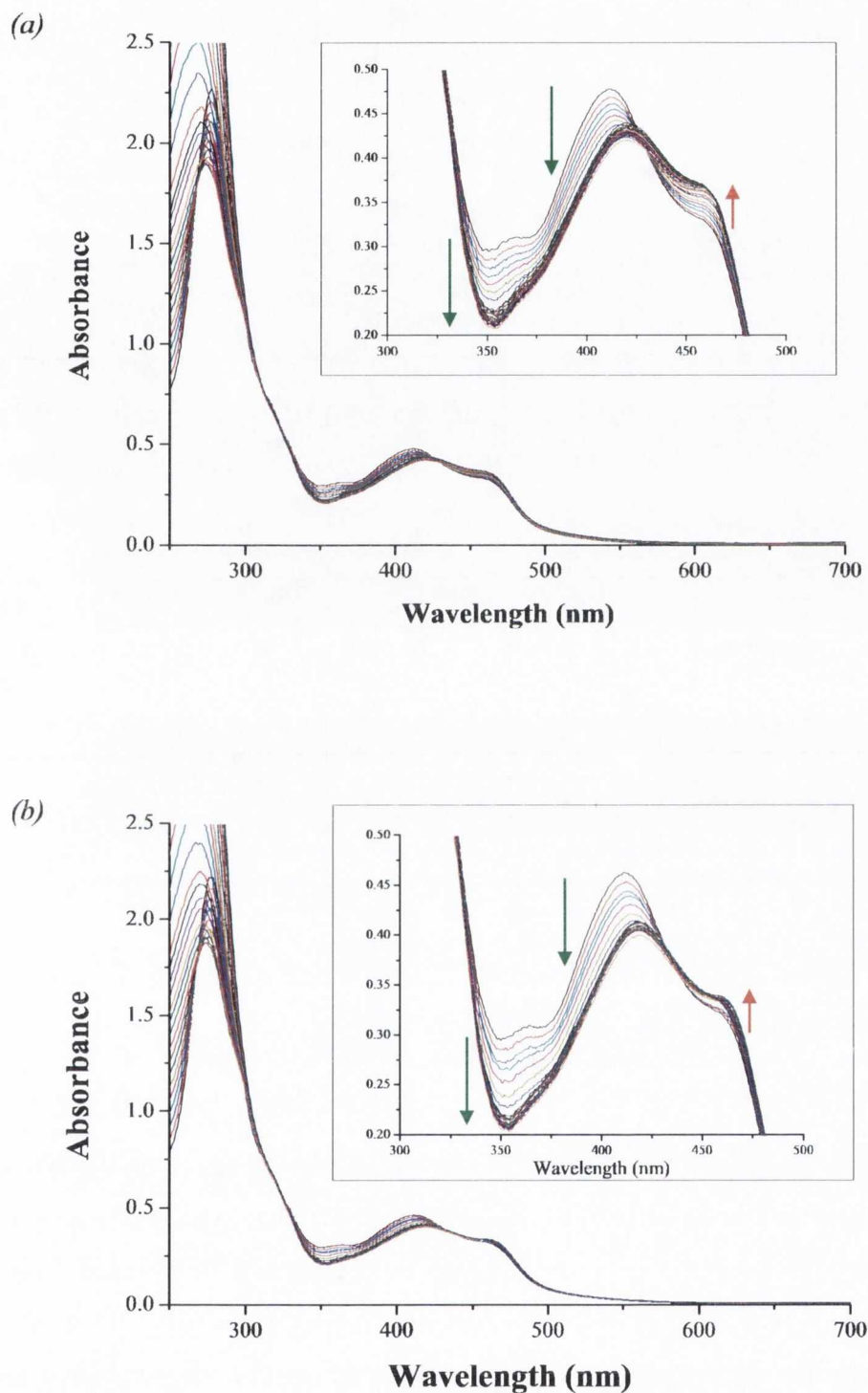


Figure 2.9 – Changes in the absorption spectrum of (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ ($20.1 \mu\text{M}$) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ ($19.9 \mu\text{M}$) with increasing additions of stDNA (to P/D 25) at 10 mM sodium phosphate buffer

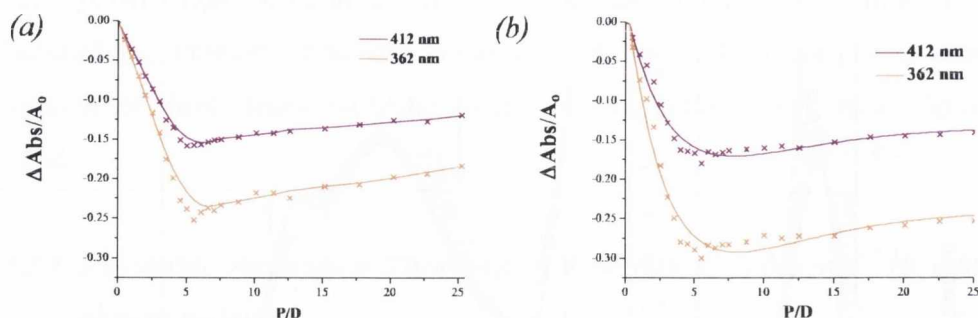


Figure 2.10 – Change in absorbance (ΔAbs) normalised by the absorbance without *stDNA* for (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (20.1 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (19.9 μM) with increasing additions of *stDNA* (to P/D 25) at 10 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs_{362}	Max - 29% Rec - 23%	Max - 30% Rec - 25%	7 nm	8 nm
Abs_{412}	Max - 14% Rec - 10%	Max - 15% Rec - 12%	9 nm	7 nm

Table 2.2 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with *stDNA* (to P/D 25) in 10 mM sodium phosphate buffer

The absorption band of the Λ enantiomer at 275 nm initially decreased until a P/D of 5.5 was reached wherein the absorbance of the DNA (260 nm) became too strong and hid the decay of these transitions. As such, little useful data could be gleaned from this absorbance peak. The band at 362 nm, corresponding to the intra-ligand π - π^* dppz transitions, experienced the most dramatic effects in the form of a hypochromic shift of 29% at P/D 5.5 before recovering to 23% by a P/D of 25. The absorbance band at 412 nm also underwent a hypochromic shift but only by a maximum of 14% before recovering to a final hypochromism of 10%. The band at 456 nm experienced a hyperchromism to a maximum of 12%. Two of the three bands, the 362 nm and 412 nm absorptions, experienced a shift in wavelength, a bathochromic shift of 8 nm and 9 nm respectively.

The absorption band of the Δ enantiomer at 275 nm also initially decreased until a P/D of 5.5 was reached wherein the absorbance of the DNA (260 nm) became too strong and hid the further diminution of these transition strengths. As was seen with the Λ enantiomer, the bands at 362 nm and 412 nm underwent hypochromic shifts upon the addition of stDNA. The absorbance band at 362 nm again experienced the most dramatic effects in the form of a hypochromic shift of 30% at P/D 5.5 before recovering to 25 % by a P/D of 25. The absorbance band at 412 nm also experienced a hypochromic shift, with the maximum of 15% occurring at a P/D of 5.5, followed by a recovery to 12%. Finally, the band at 456 nm experienced a hyperchromic shift of 5%. Unlike the Λ enantiomer, all three of the bands experience a bathochromic shift: 7 nm, 9 nm and 3 nm for the 362 nm band, 412 nm band and 456 nm band respectively.

At 456 nm, only a very small effect upon the addition of stDNA is detectable for both enantiomers. This corroborates the assignment of this band as the Ru(II) $\rightarrow$ TAP MLCT transition as this chromophore does not correspond to the intercalated part of the complex. The hyperchromic shift detected at higher P/D ratios in both compounds is indicative of the reversal of the hypochromicity in absorption, previously attributed to closely bound metal complexes², as the distances between bound complexes grows with the increasing number of free sites available for binding. It is interesting to note that the difference in absorbance is significantly higher for the Λ enantiomer than the Δ , giving the first indication of a subtle difference between the binding modes of the two complexes.

By contrast, at the wavelengths of 362 nm and 412 nm, a pronounced, biphasic absorption behaviour is observed upon stDNA addition. This biphasic trend has been reported in the studies of similar complexes such as *rac*- $[Ru(phen)_2dppz]^{2+}$, *rac*- $[Os(phen)_2dppz]^{2+}$, and *rac*- $[Ru(TAP)_2PHEHAT]^{2+}$.^{2,14,46-48} Phase 1 occurs at low P/D ratios wherein significant hypochromic and bathochromic shifts occur as the absorption decreases linearly to a P/D ratio of 5.5. Beyond this P/D, the second phase (Phase 2) is characterised by a slight absorption increase (referred to as recovery) until a plateau is reached.

There are two theories that attempt to explain this biphasic occurrence. The first claims that the two phases are a result of two different binding modes that occur at different P/D ratios. The second theory argues that only one binding mode is present and the hyperchromicity in Phase 2 is a result of the increasing distances between the complexes, caused by increasing the amount of DNA present, reversing the hypochromic effect attributed to closely bound metal complexes². Both theories do agree on cause of the first phase behaviour. The dramatic decrease in absorption results from the interaction of the dppz ligand intercalating between the base pairs of the DNA helix. This is deduced from the fact that it is the IL dppz $\pi-\pi^*$ transitions (at 362 nm) that are the most affected by hypochromicity as a result of the ligand's intercalation. This also corroborates the assignment of the 412 nm band as the Ru(II) $\rightarrow$ dppz MLCT transition as it too undergoes hypochromicity upon the addition of stDNA, albeit a smaller effect. Non-specific intercalative binding and stacking of the enantiomer complexes are thought to dominate during this phase.

While the notion of the increased absorption in Phase 2 being attributed to increasing distances between complexes is compelling, if this was occurring, the recovery of both the enantiomers should be similar. However, the hypochromicity of the Λ enantiomer recovers more than that of the Δ enantiomer (Table 2.2) indicating that the explanation for this behaviour may indeed be the presence of a second binding mode. This binding mode has been shown to exhibit positive cooperativity and is strongly influenced by the ionic concentration of the buffer solution.⁴⁸ These two characteristics are indicative of an electrostatic binding mode. This has led to the proposal that at higher P/D ratios, after the binding of Phase 1 via intercalation of the complexes has been saturated, the complexes begin to bind to the DNA in a highly cooperative fashion wherein the TAP residues locate in the grooves allowing the dppz moiety to then intercalate only partially between base-pairs.⁴⁸

The hypochromic and bathochromic shifts induced in the spectra are indicative of the intercalation mode of binding,⁴⁹ which is expected of a complex containing an extended planar ligand such as dppz. From the information in Table 2.2 we can conclude that there is a greater effect on the spectroscopic properties of the Δ enantiomer binding to the DNA than the Λ enantiomer. The Δ complex experiences a higher degree of hypochromicity

than the Λ in both the 362 nm and 412 nm absorption bands, as well as experiencing a smaller degree of recovery upon increasing DNA concentration. This is in agreement with the studies conducted with $[Ru(phen)_2dppz]^{2+}$ and $[Ru(bpy)_2dppz]^{2+}$ in which the Δ enantiomer was found to be the more tightly bound of the two, although both enantiomers are extremely efficient DNA intercalating complexes.² The explanation for this effect lies in the different stereochemistry of the two optical isomers. The dppz ligand of both complexes intercalates between the DNA base pairs, however, the orientation of the ancillary TAP ligands determine how deeply the dppz ligand can intercalate. The TAP ligands in the arrangement that result in the Λ enantiomer are aligned in such a way that the complex is ‘left-handed’ in its stereochemistry while the Δ enantiomer has ‘right-handed’ stereochemistry (Figure 2.11).

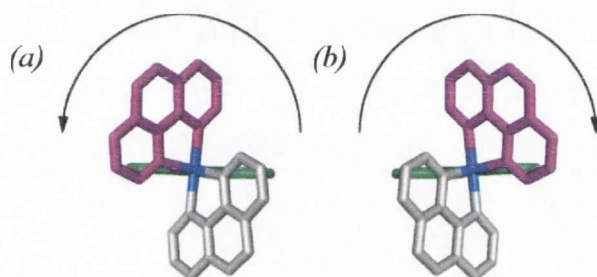


Figure 2.11 – Stereochemistry of (a) Λ - $[Ru(TAP)_2dppz]Cl_2$ and (b) Δ - $[Ru(TAP)_2dppz]Cl_2$

A B-DNA double helix, as is the DNA used in these titrations, also has a ‘right-handed’ stereochemistry. The result of this chirality of DNA is that the intercalative moiety of polypyridyl Δ complexes can penetrate the binding pocket more deeply than Λ complexes because their ancillary ligands are oriented to lie in the direction of the grooves, parallel with the phosphate backbone (Figure 1.16). This allows the distance between the metal centre and DNA helical axis to be shorter, resulting in further penetration of the intercalating ligand and thus a better overlap of the π orbitals between the aromatic surfaces of the ligand and the base pairs. The consequence of this is a tighter binding interaction of the Δ enantiomer than the Λ enantiomer which, as a result of the misalignment of its ancillary ligands with the DNA grooves which cannot facilitate such a deep intercalation of its extended aromatic ligand.

Further evidence of the increased ability of the Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex to bind more tightly to DNA compared to the Λ enantiomer is the appearance of an isosbestic point in the absorbance spectra upon increasing DNA concentrations. The presence of an isosbestic point typically indicates that only two species, varying in concentration, contribute to the absorption around the point. Usually the interaction of a third species in the process results in the spectra intersecting at varying wavelengths as concentrations change, creating the impression that the isosbestic point is 'out of focus'. Indeed this is observed in the two titrations. Addition of stDNA to the Δ complex results in a clear isosbestic point at 435 nm (see the inset of Figure 2.9(b)), however addition of stDNA to the Λ complex does not yield a clear isosbestic point, but one that seems 'out of focus' at approximately 430 nm (inset of Figure 2.9(a)). The implication from this is that with the Δ complex, only two species are present; the free Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex and the bound Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ -DNA complex, while with the Λ enantiomer three complexes are present; the free Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complex, the bound Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ -DNA complex and a third species. The nature of this species remains unknown but we speculate that it may be the cross-linked DNA- Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ -DNA complex arrangement that is discussed in depth in the following chapter. Our crystallisation studies have shown that the Λ enantiomer can cross-link two DNA duplexes via intercalation of the dppz ligand into one duplex and semi-intercalation of an ancillary TAP ligand into a second duplex. The absence of crystals formed with the Δ enantiomer may imply that this complex cannot form these non-covalent cross-linking interactions with DNA as a result of the shortened ruthenium-DNA helical axis distance, reducing the area of the ancillary TAP ligands available for semi-intercalation, preventing this secondary interaction. The consequence of this is the presence of fewer species in the absorbance spectra of the Δ complex and appearance of an isosbestic point.

Naturally, the binding of these complexes to duplex DNA is a complicated arrangement, and of course more than two species are present upon the binding of the Δ enantiomer and more than three species occur with the binding of the Λ enantiomer. For example, the binding of a complex to an A-T/A-T site would undoubtedly yield a slightly different absorption spectrum than the same enantiomer bound to A-T/G-C, G-C/A-T or G-C/G-C sites. However the stark contrasts in the isosbestic points of both enantiomers would

indicate that the binding of the Λ enantiomer is more complicated than that of its enantiomeric counterpart and is further evidence of the different binding modes exhibited by the enantiomers.

2.4.1.2 Fluorescence Titrations in Low Salt Conditions – 10 mM sodium phosphate buffer

As mentioned previously, fluorescence spectroscopy is another useful technique in the investigation of complexes binding to DNA and is often used in conjunction with absorbance spectroscopy. It is a particularly useful method for the study of $[\text{Ru}(\text{polypyridyl})_2\text{dppz}]^{2+}$ complexes which are known for their ‘light switch’ ability, either ‘on’ for complexes such as $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ or ‘off’ as is the situation for the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex.⁶ The ability to record a strong I_0 reference value allows the regular control of the light source intensity during the titration experiments, and therefore better binding data can be collected than for those complexes exhibiting the reverse light switch effect.

The fluorescence titrations between the two enantiomeric compounds and stDNA were conducted in parallel to the absorbance titrations described previously, by addition of small volumes of stDNA to a solution of each complex in the low salt conditions of 10 mM sodium phosphate buffer at pH 7.4. Each spectrum was examined at an excitation wavelength of 435 nm, which corresponds to the isosbestic point observed. The reason for this choice of wavelength is that at the isosbestic point, the fluorescence intensity may be directly related to the relative concentrations of the free and bound enantiomers. The recorded emission spectrum for the Λ complex is shown in Figure 2.12(a) with that of the Δ complex in Figure 2.12(b).

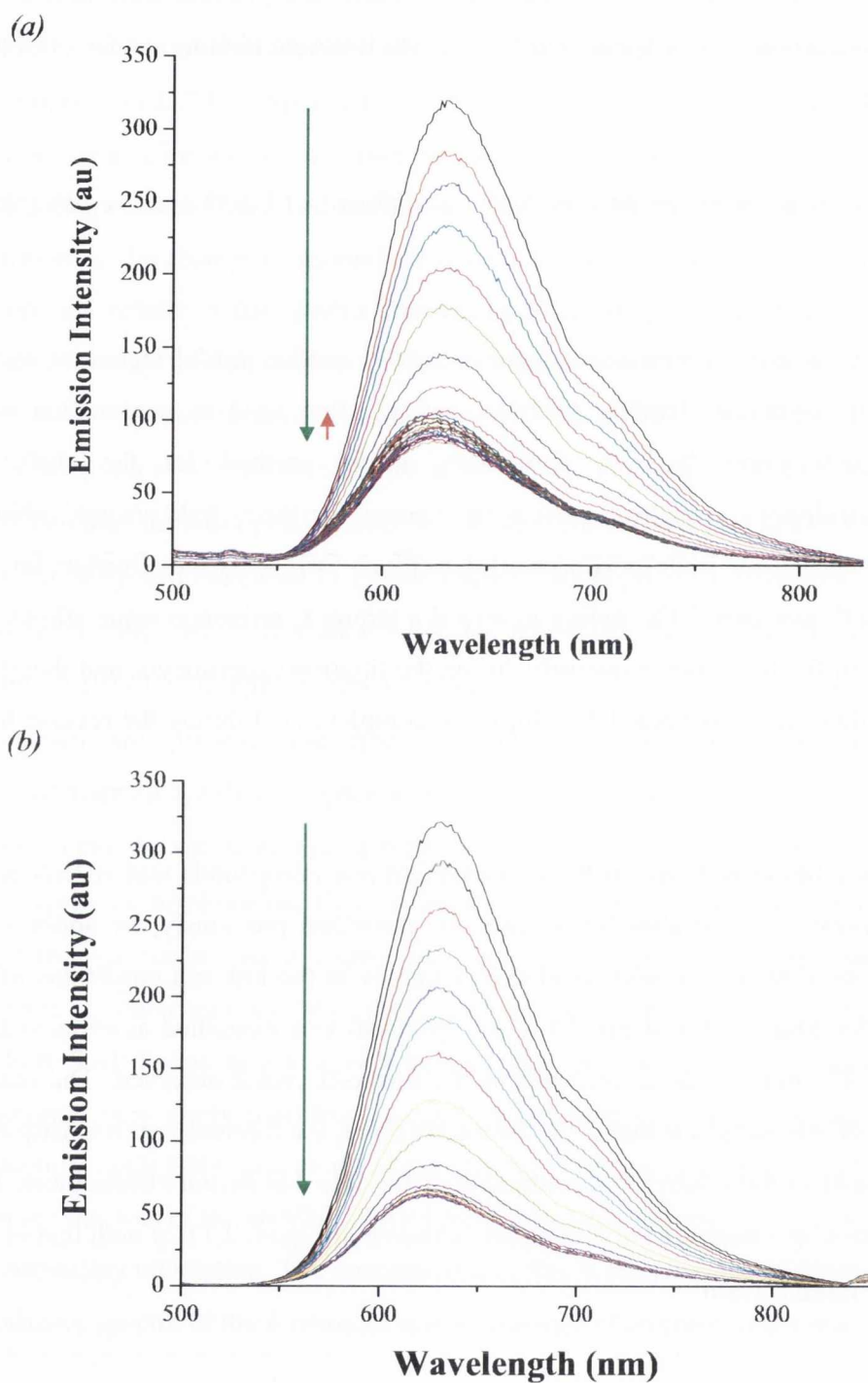


Figure 2.12 - Changes in the emission spectrum of (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ ($20.1 \mu\text{M}$) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ ($19.9 \mu\text{M}$) with increasing additions of stDNA (to P/D 25) in 10 mM sodium phosphate buffer (λ_{ex} 435 nm)

Titration of both the Λ and Δ enantiomers with stDNA (P/D 0 $\rightarrow$ 25) resulted in significant quenching of the emission spectrum compared with the free complexes. It has been shown experimentally that this quenching is due to a proton-coupled electron transfer (PCET) process occurring between the guanine residues in the DNA and the $^3\text{MLCT}$ state of the complexes.^{24,50,51}

Contrasts between the two emission spectra are visible from a simple inspection of the two previous graphs but a comparison of the relative emission intensities I/I_0 below (Figure 2.13), where I_0 is the emission measured in the absence of DNA, highlights these differences.

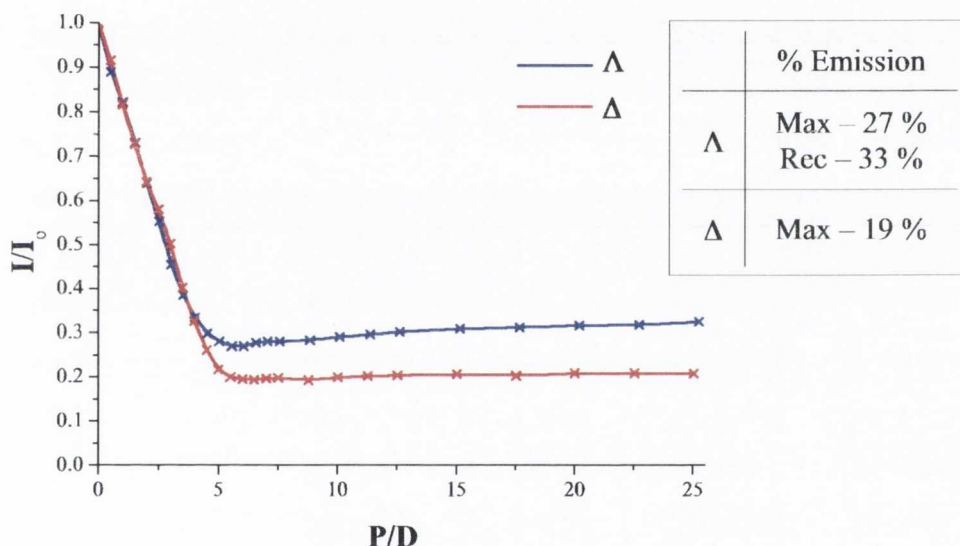


Figure 2.13 – Changes in the relative emission intensities vs increasing ratios of P/D of the Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complexes in 10 mM sodium phosphate buffer (λ_{ex} 435 nm)

The Λ complex was quenched by 73% with the maximum quenching occurring at a P/D of 5.5, mirroring the P/D of the maximum hypochromicity recorded in the absorption spectra. The occurrence of maximum quenching at a P/D of approximately 5.5 supports previous findings in which the nearest neighbour separation for such complexes bound to DNA was found to be two base pairs.⁵ The Δ complex was quenched by 81% with the maximum quenching also occurring at a P/D of 5.5. Thus the Δ enantiomer is quenched to

a higher degree than the Λ enantiomer, complementing the results from the absorbance titrations that the Δ enantiomer binds more tightly to the DNA. Interestingly, while the Δ complex, once a P/D of 5.5 is reached, remains at a constant quenched luminescence, the Λ complex recovers to a luminescence of 0.33 (an increase from 73% to 67% quenching) at the final P/D of 25. Initially one might assume this to be a factor of the stacking effect seen in the recovery of the absorbance spectra but considering there is no recovery of the Δ complex, which has also been shown to stack, this cannot be correct. A possible explanation for this recovery may be found in the base pair preference of the two complexes coupled with their respective binding ability. With the maximum intercalative binding reached after only a P/D of 5.5, both the complexes have an extremely high affinity for binding to DNA, mirroring the result from the absorbance titrations. It is well known, however, that complexes such as $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ have a preference for A-T over G-C base pairs.^{2,3,6} It has also been shown that the luminescence of the *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex, while quenched in the presence of G-C, is enhanced when bound to A-T base pairs (discussed in full later in this chapter). This, coupled with the fact that the Λ enantiomer does not bind as tightly to DNA as the Δ , indicates that increasing the number of A-T binding sites available for the complex may result in the relocation of the Λ enantiomer from the G-C base pairs to which it initially bound to these free A-T sites which enhance the luminescence of the complex, resulting in the observed recovery of the Λ enantiomer.

The question to arise from this relocation theory is why does the Λ luminescence only recover by 6% if all the Λ complexes are bound to A-T sites, which enhance luminescence? The answer to this may lie in the fact that electron transfer from the complexes to the guanine residues can possibly occur between non-nearest neighbours. The lifetime of the excited state in A-T sites is of the magnitude 10^6 s^{-1} thus for quenching by guanines in other base pairs to occur, the reaction needs only a rate constant greater than 10^6 s^{-1} to be effective.⁶ Quenching at G-C sites however, proceeds with a rate of $2 \times 10^9 \text{ s}^{-1}$ thus the Λ complex may still experience luminescence quenching while bound to A-T sites, though the effect is smaller than if it were bound directly to G-C sites, as occurs with the Δ enantiomer upon initial binding but does not relocate.^{12,52,53} Also, salmon testes DNA has a 42% G-C content. This results in only 20% of the binding sites

being A-T/A-T, meaning that the majority of the Λ enantiomers remain bound to a guanine residue after relocation.

The data collected from the absorbance and fluorescence titrations show that the Δ enantiomer binds more tightly than the Λ enantiomer, demonstrated by the ability of the Λ enantiomer to relocate to its preferred A-T sites after initially binding to a mix of A-T and G-C sites, while the Δ enantiomer binds too tightly to allow relocation to these preferred sites. To further confirm these conclusions, thermal denaturation studies between salmon sperm DNA and the two enantiomers were conducted to determine the relative stability imparted by the binding of each of the enantiomers.

2.4.1.3 Thermal Denaturation Studies in Low Salt Conditions – 10 mM sodium phosphate buffer

Thermal denaturation is an optical technique used for measuring the stability of various molecules binding to DNA. It relies on the use of absorption spectroscopy, and measuring the absorbance of a sample of DNA as a function of temperature at a fixed wavelength.

When DNA is in its duplex form, the bases are stacked in the helix by hydrogen bonding between the complementary bases. The transition moment caused by the dipole coupling of such compounds in close proximity often results in hypochromicity in the absorbance spectrum. Thus, when stacked in the double helix, the absorbance of the base-pairs is significantly reduced. However, as the sample of DNA is heated, the hydrogen bonds between the base pairs gain sufficient energy to break. At this point the double helix begins to split giving the bases freedom to rotate and become unstacked. Consequently, the hypochromicity of the aggregation effect is reversed, resulting in an increase in the absorption spectrum of DNA (Figure 2.14).

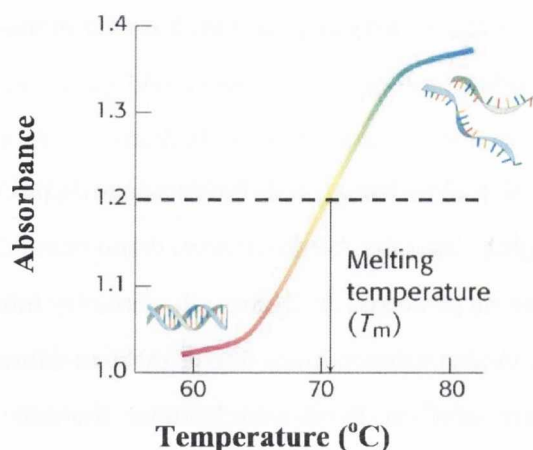


Figure 2.14 - Change in DNA absorbance as a result of thermal denaturation

The midpoint of this increase in a population of DNA molecules is called the melting temperature (T_m) and corresponds to 50% of the DNA being denatured. When a molecule binds to DNA, it generally stabilises the helical structure resulting in more energy being required to separate the strands, and hence modulates the T_m . These experiments are usually run initially with DNA alone followed by experiments with the DNA bound to a ligand and, by comparing the thermal melting temperatures, we can obtain the change in the thermal melting temperature (ΔT_m). These measurements in the presence of DNA binders provide valuable information on the degree of stabilisation of the double stranded form and is an easy method of identifying whether a molecule binds to the DNA helix.

The melting temperature of DNA is dependent on the sequence type. For example, the observed melting temperature for salmon sperm DNA (150 μM , 10 mM sodium phosphate buffer) is 77°C whereas for [poly(dA-dT)], it was found to be 47°C, under the same conditions. The lower melting temperature of this DNA is due to the lack of G-C base pairs with three hydrogen bonds between the bases whereas there are only two hydrogen bonds between the A-T bases.

Thermal denaturation studies were performed with the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ bound to stDNA in 10 mM sodium phosphate buffer, pH 7.4, at a P/D ratio of 20, using degassed solutions. The absorbance at λ_{max} 260 nm was then monitored as the temperature was gradually increased from 20°C to 90°C, at a rate of 1°C per minute. The thermal denaturation curves are shown in Figure 2.15.

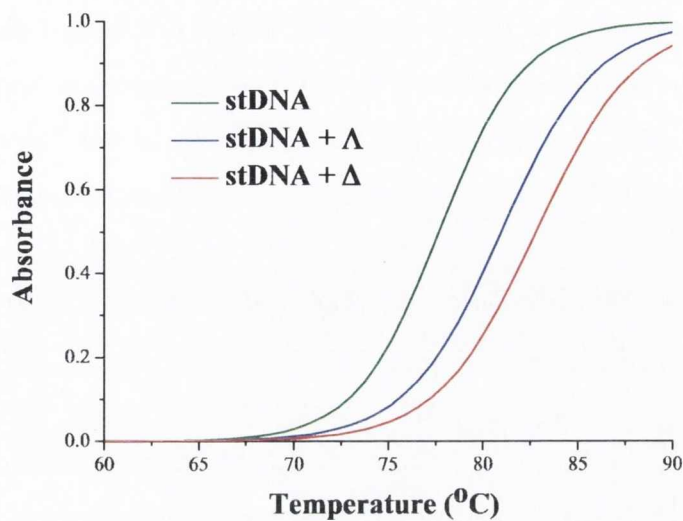


Figure 2.15 – Normalised thermal denaturation curves of stDNA (150 μM) in the absence of any complex (green), the presence of the Λ enantiomer (blue) and the presence of the Δ enantiomer (red) in 10 mM sodium phosphate buffer (P/D 20)

In the absence of metal complex, the T_m value for salmon sperm DNA was recorded as 77°C with significant modulations being measured in the presence of the Λ and Δ enantiomers. Both the enantiomers stabilise the DNA duplex, resulting in increased thermal stability. Binding of the Λ enantiomer increased the T_m temperature by 4°C to 81°C and the Δ enantiomer increased the T_m temperature by 6°C to 83.0°C (Table 2.3).

Complex	T_m	ΔT_m
stDNA	77°C	-
stDNA + Λ	81°C	+ 4°C
stDNA + Δ	83°C	+ 6°C

Table 2.3 – The T_m values from the melting curves in Figure 2.15

These results support the conclusions thus far drawn from the previous photophysical studies, that both complexes bind to and stabilise DNA but the increased ability of the Δ

enantiomer to bind more tightly to DNA and its dppz ligand to penetrate more completely between the base pairs result in the Δ enantiomer having a increased stabilising effect compared to the Λ . It must be noted however, that these thermal denaturation studies do not provide evidence for a particular mode of DNA binding, as stability may arise from the binding of both groove binding molecules⁵⁴ and intercalating compounds.⁵⁵

2.4.1.4 Binding Constant Calculations in Low Salt Conditions – 10 mM sodium phosphate buffer

A determination of the intrinsic binding constant, K_b , for the interaction of the Λ and Δ enantiomers of $[Ru(TAP)_2dppz]^{2+}$ with DNA allows for the evaluation and comparison of their binding affinities under the same specific experimental conditions. In general, K_b can be described as:

$$K_b = \frac{L_b}{L_f S_f} \quad (2.3)$$

where L_b is the concentration of bound ligand, L_f is the concentration of free ligand and S_f is the free site concentration, which is usually taken to be the DNA base concentration divided by the number of DNA bases required for the ligand binding site.

The binding constants for the enantiomers were determined using the luminescence data recorded, by employing the McGhee/von Hippel model.⁵⁶ This method involved K_b being determined using Scatchard plots.⁵⁷ Using luminescence data, the concentration of the bound complex, C_b , and free complex, C_f , at any given concentration where determined from equations 2.4 and 2.5:⁵⁸

$$C_b = \frac{I_f - I}{I_f - I_b} \cdot C \quad (2.4)$$

$$C_f = C - C_b \quad (2.5)$$

I_f and I_b are the fluorescence intensities of the free and bound complex, I is the fluorescence response of the mixture of free and bound complex and C is the total

complex concentration. The absorbance data was treated in a similar manner. Scatchard analysis was then conducted according to the McGhee and von Hippel equation 2.6:

$$\frac{r}{c_f} = K_b(1 - nr) \left(\frac{1 - nr}{1 - (n-1)r} \right)^{n-1} \tag{2.6}$$

where $r = C_b/[DNA]$, ($[DNA]$ is the concentration of DNA in base pairs). The analysis was conducted using the plot of r/C_f versus r and the curve was fitted to equation 2.6 using Origin 8. These curves are shown in Appendix A.

The binding constants for the interaction of the Λ and Δ enantiomers with salmon sperm DNA were determined using the above method applied to the fluorescence data. The figures produced yielded a binding constant of $1.4 \times 10^7 \text{ M}^{-1}$ for the Λ enantiomer and $1.2 \times 10^8 \text{ M}^{-1}$ for the Δ enantiomer (Table 2.4).

Complex	Binding Constant $K_b \text{ (M}^{-1}\text{)}$	Binding Site Size n (base pairs)	R^2
Λ	$1.4 \times 10^7 \text{ } (\pm 0.2)$	$2.0 \text{ } (+ 0.1)$	0.97
Δ	$1.2 \times 10^8 \text{ } (\pm 0.1)$	$2.4 \text{ } (+ 0.1)$	0.99

Table 2.4 – stDNA binding constants calculated from the fluorescence data utilising the McGhee/von Hippel model

From the calculated binding constants, we can confirm that the Δ enantiomer binds to DNA with a higher affinity than the Λ enantiomer. The values calculated for the two enantiomers of $[Ru(TAP)_2dppz]^{2+}$ compare favourably to those reported previously for the Λ and Δ enantiomers of the $[Ru(phen)_2dppz]^{2+}$ complex, $1.5 \times 10^7 \text{ M}^{-1}$ and $2.4 \times 10^8 \text{ M}^{-1}$ respectively (10 mM NaCl and 1 mM sodium cacodylate buffer).² These values, while in agreement with reported literature values, are extraordinarily high binding constants, which suggest that perhaps these complexes bind to DNA too strongly to allow for the

accurate calculation of their binding constants at these conditions. To overcome this, the titrations and subsequent calculations were conducted again, but at the higher salt concentration of 100 mM sodium phosphate buffer.

2.4.2 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with duplex DNA in 100 mM sodium phosphate buffer

Many ruthenium(II) complexes that bind to DNA show a poorer affinity for the macromolecule at high ionic strengths. This is because the binding of many of these systems is driven primarily by electrostatic interactions.⁵⁹ Typically, an increase in ionic strength results in the shortening of a DNA helix as the increased cationic presence in solution reduces the phosphate-phosphate bond repulsion.⁶⁰ Thus in a high ionic strength environment, the affinity of a cationic species binds to DNA is expected to decrease relative to a lower ionic strength environment. The Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ are cationic species with an extended aromatic ligand. The main mode of binding for these complexes is believed to be driven by the intercalation of the dppz ligand, however due to cationic nature of the complexes, electrostatic interactions play a significant role in the DNA binding, both through initial attraction to the anionic DNA helix and once the complex has intercalated, acting as a anchor forming interactions between the positively charged ruthenium centre and the negatively charged phosphate backbone, both on the periphery of the DNA-ligand arrangement. To investigate the extent to which electrostatic interactions influence the binding of the two enantiomers to DNA, titrations were performed between both complexes and stDNA in 100 mM sodium phosphate buffer, pH 7.4.

2.4.2.1 UV/visible Absorbance Titrations in High Salt Conditions – 100 mM sodium phosphate buffer

The titrations at high ionic strength were examined using UV/vis absorption spectroscopy in a method similar to that described in section 2.4.1.1 involving the addition of small aliquots of stDNA (1×10^{-2} M) to a 100 mM sodium phosphate solution of each enantiomer, until a plateau in absorbance was reached. Figure 2.16(a) shows the absorption spectra recorded for the titration of the Λ enantiomer. Figure 2.16(b) gives the same spectra recorded for the titration conducted with the Δ enantiomer.

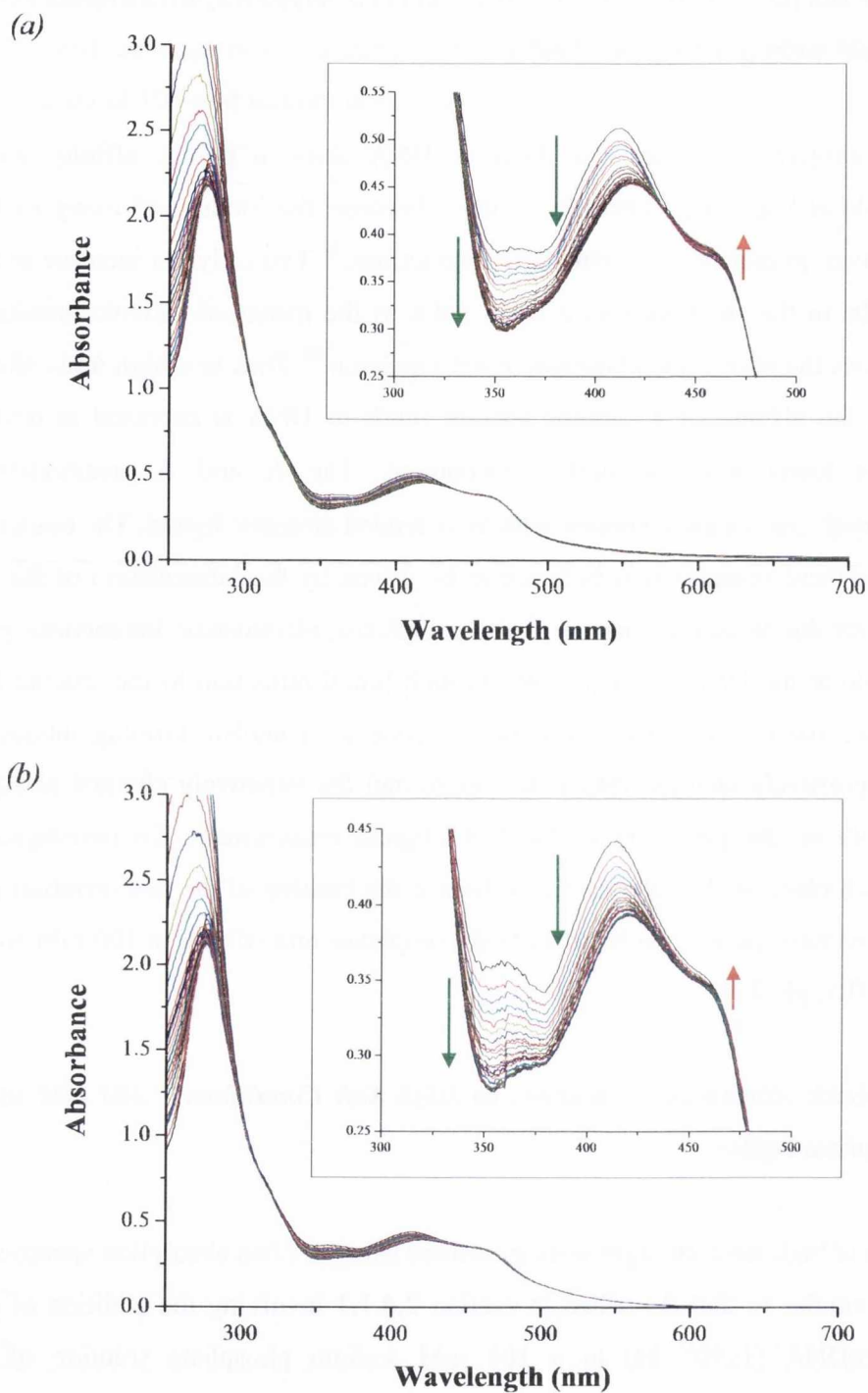


Figure 2.16 – Changes in the absorption spectrum of (a) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (20.2 μM) and (b) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (20.1 μM) with increasing additions of stDNA (P/D 25) in 100 mM sodium phosphate buffer

The results obtained for both the enantiomers are, as expected, generally similar to those recorded for the 10 mM sodium phosphate buffer titration but do show some small differences. The changes in the absorption with the addition of stDNA are shown in Figure 2.17 below, and summarised in Table 2.5.

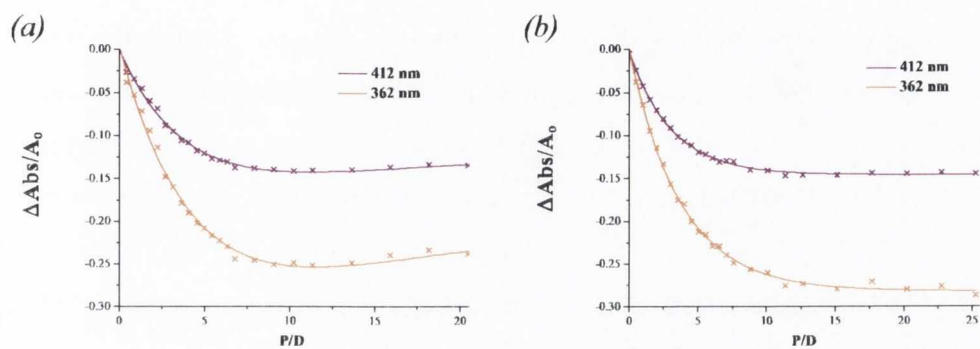


Figure 2.17 – Change in absorbance (ΔAbs) normalised by the absorbance without stDNA for (a) Λ - $[Ru(TAP)_2dppz]Cl_2$ and (b) Δ - $[Ru(TAP)_2dppz]Cl_2$ vs increasing ratios of P/D at different wavelengths in 100 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₆₂	20%	22%	3 nm	2 nm
Abs ₄₁₂	12%	13%	7 nm	8 nm

Table 2.5 – Summary of UV/vis shifts in the ³MLCT absorbance bands for the titrations of Λ - and Δ - $[Ru(TAP)_2dppz]Cl_2$ with stDNA

As was found previously, the absorption bands at 362 nm and 412 nm experience significant changes for both enantiomers upon the addition of stDNA. While both enantiomers again exhibit the biphasic behaviour observed in the low salt titrations, the increased ionic strength from 10 mM to 100 mM causes a remarkable reduction in the amplitude of Phase 2 (see Figure 2.10 for comparison to Figure 2.17).

During Phase 1 of the titration, significant hypochromic and bathochromic shifts occur as the DNA concentration increases. The hypochromic shifts in these high salt

concentrations proceed linearly, as before, however the enantiomers plateau at a higher P/D ratios than observed for the low salt titrations. The Λ enantiomer seems intercalatively saturated at a P/D ratio of 8, after which the absorbance is seen to increase slightly, while the Δ enantiomer continues to a P/D ratio of 12 before reaching a plateau in absorbance. This significant increase in the P/D ratio of the fully intercalated complex for both enantiomers shows that increasing the buffer concentration disrupts the binding ability of the enantiomeric complexes, implying that indeed electrostatic interactions do facilitate the binding of these molecules. The fact that both enantiomers do eventually bind also shows that the intercalative mode is strong enough to overcome the shortened distances between base pairs and the reduced electrostatic attraction. Again, the hypochromicity of the 362 nm and 412 nm bands is larger for the Δ enantiomer, implying that it remains the more effective binder of the two complexes even at these higher buffer concentrations.

Phase two of the high salt absorption profile is quite different from that of the low salt. While the absorption for both enantiomers does increase, the degree of hyperchromicity is much lower in this higher ionic strength environment. This supports the theory of an electrostatically driven second binding mode at these higher P/D ratios. The higher salt concentration in the buffered solution results in increased competition between the remaining free enantiomeric complexes and salt ions for groove binding. This results in fewer complexes binding via this second binding mode, thus a decrease in the hyperchromicity of phase two.

2.4.2.2 Fluorescence Titrations in High Salt Conditions – 100 mM sodium phosphate buffer

An increase in salt concentration typically tends not to overly disturb the fluorescence data recorded due to the non-emissive nature of many salts. As with the absorbance titrations, a decrease in the magnitude of quenching for both enantiomers is expected because their binding affinity for the DNA has decreased, however no such further effects resulting from the increased salt concentration are anticipated.

The fluorescence titrations between the two enantiomeric compounds and stDNA were conducted in parallel to the absorbance titrations described previously, by addition of small volumes of stDNA to a solution of each complex in high salt concentrations of 100 mM sodium phosphate buffer at pH 7.4. Each spectrum was examined at an excitation wavelength of 435 nm. The recorded emission spectrum for the Λ complex is shown in Figure 2.18(a) with that of the Δ complex in Figure 2.18(b). Titration of both the Λ and Δ enantiomers with salmon sperm DNA (P/D 0 $\rightarrow$ 25) resulted in significant quenching of the emission spectrum compared to that of the free complexes. Significant contrasts between the two emission spectra are visible from a simple inspection of the two emission graphs but a comparison of the relative emission intensities I/I_0 (Figure 2.19) highlights these differences.

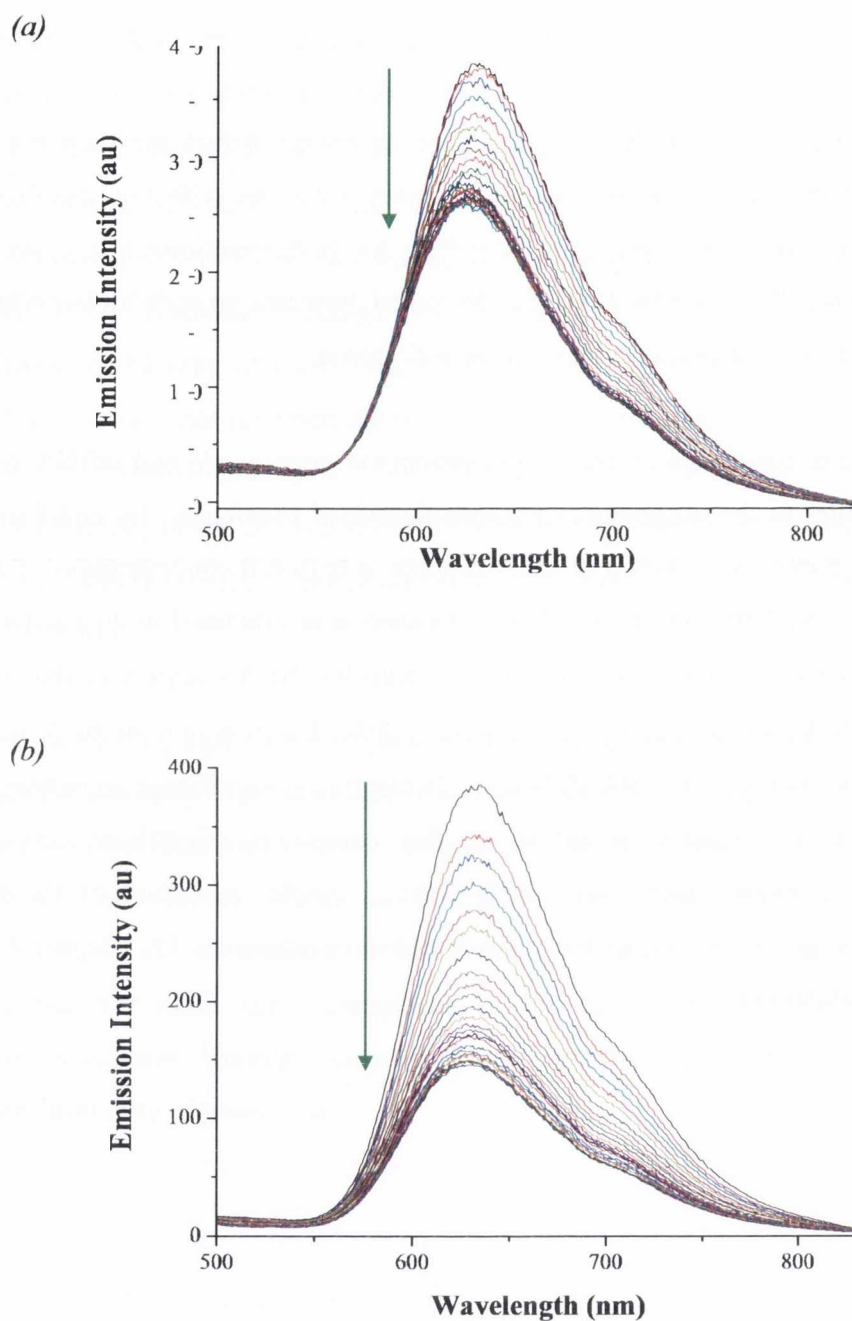


Figure 2.18 - Changes in the emission spectrum of (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (20.2 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (20.1 μM) with increasing additions of stDNA at 100 mM sodium phosphate buffer (λ_{ex} 435 nm)

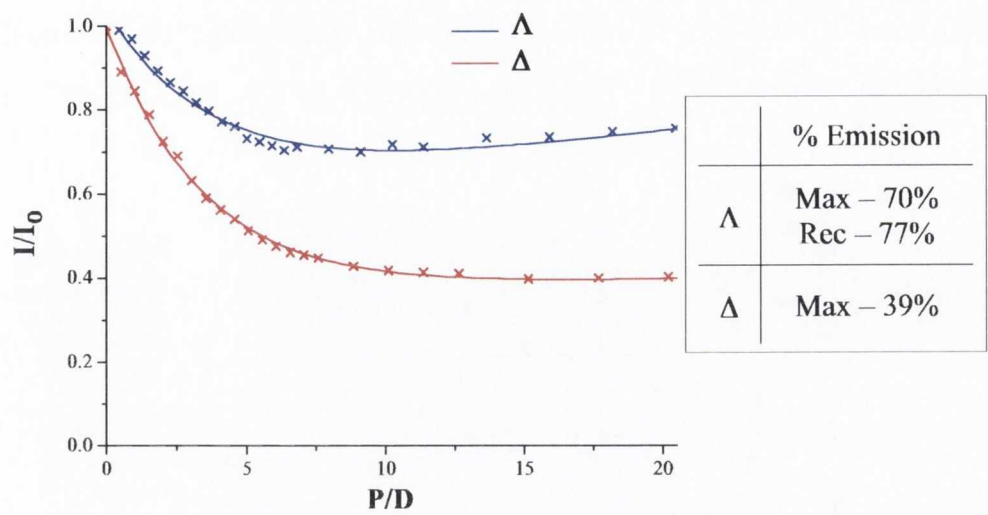


Figure 2.19 – Changes in the relative emission intensities vs increasing ratios of P/D of the Λ- and Δ-[Ru(TAP)₂dppz]Cl₂ complexes in 100 mM sodium phosphate buffer (λ_{ex} 435 nm)

The Λ complex was quenched by only 30% with the maximum quenching occurring at a P/D of 6.5. The Δ complex was quenched by 61% with the maximum quenching also occurring at a P/D of 13.5 but with the majority of the complex also being bound at a P/D of 6.5. Again the Δ enantiomer is quenched to a higher degree than the Λ enantiomer, complementing the hypochromic shifts in the absorbance titrations and the conclusion that the Δ enantiomer binds more tightly to the DNA. As observed in the low salt titrations, once the Δ enantiomer is fully bound, its luminescence remains at this constant quenched level while the Λ enantiomer, once it reaches its maximum quenched level, recovers to a luminescence of 0.77 (an increase from 30% to 23% quenching) at the final P/D of 25, again resulting from the relocation of Λ enantiomers to their preferred A-T binding sites.

Three important observations can be made at this point. The first is that the luminescence of the Λ enantiomer displays a similar trend in the high salt conditions as in the low salt. A maximum quenching is reached at a low P/D, and emission recovery is observed at higher P/D. Secondly the P/D at which both the enantiomers appear fully bound in the fluorescence spectra differs from that seen in the absorbance spectra, and finally, the emission data from the Δ enantiomer appears not to have a sharp change at its plateau

region in the high salt conditions, exhibiting an initial steep slope corresponding high rate of quenching to a P/D of 6.5, followed by a gradual slope indicative of a significantly reduced quenching rate to a P/D of 13.5.

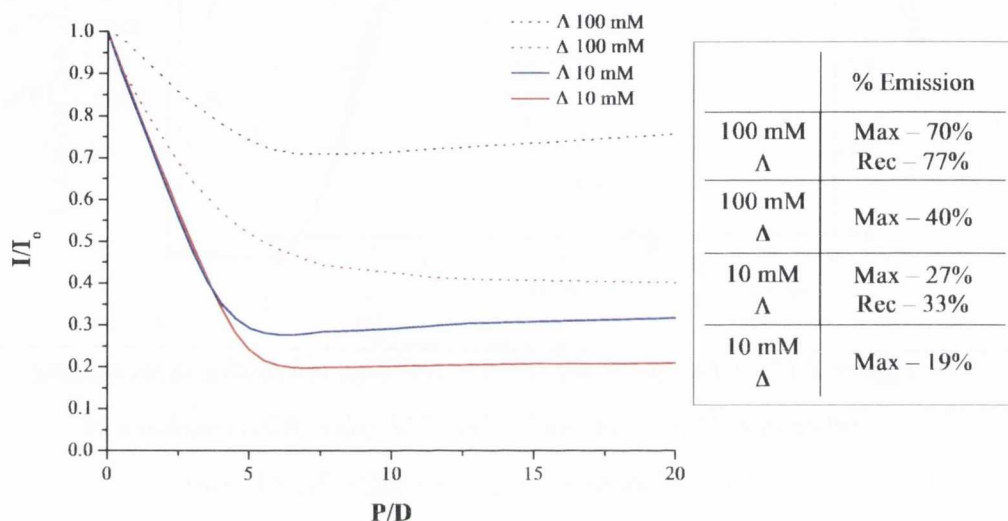


Figure 2.20 – Comparison of the changes in the relative emission intensities vs increasing ratios of P/D of both Λ and Δ enantiomers in 10 mM and 100 mM sodium phosphate buffer (λ_{ex} 435 nm)

The Λ enantiomer experiences a maximum quenching at a low P/D in both the high and low salt conditions, followed by a small recovery at higher P/D ratios. At 10 mM sodium phosphate concentrations, this effect may result from the ability of the complex to relocate to the increasingly available A-T binding sites as discussed previously. At 100 mM sodium phosphate concentrations this also occurs but to a lesser degree as the higher P/D of maximum binding allows the complex to initially bind to more of its preferred A-T sites. What is interesting, however, is that the recovery at low salt conditions, recorded as 6%, should be higher than that recorded at high salt conditions, but the degree of recovery is roughly equal at 7%. This discrepancy can be explained by the increased level of salt ions in the solution competing with the Λ complex for the various binding sites on the DNA. As the Λ complex has previously been shown to be unable to bind as tightly to the DNA, salt ions can displace the enantiomer leaving it free in solution where its luminescence is no longer quenched by the DNA, thus increasing its observed recovery. This salt displacement effect has been reported for previously studied polypyridyl

complexes⁵ and is the basis for ‘reverse titrations’ which determine the degree of influence electrostatic interactions have on the binding of compounds to oligonucleotides.^{44,61} This effect does not occur with the Δ enantiomer because it binds more tightly to the DNA and is not displaced at this 100 mM salt concentration, thus no recovery in fluorescence is observed.

The P/D ratios at which both the enantiomers display complete binding do not agree between the absorbance and fluorescence data at the higher salt conditions. This is most likely due to the interference of the higher ionic conditions on the absorbance spectra, which is sensitive to presence of high concentrations of ions. The fluorescence spectra, however, are not sensitive to most ions as they are non-emissive at excitation wavelengths of 435 nm. As such, the actual binding P/D ratios should be observed in the emission spectra, and are recorded as 6.5. This is an increase from 4.5 in the low ionic conditions indicating that the presence of more ions in solution does effect the binding ability of the two enantiomers, mirroring the conclusions drawn from the absorbance data.

Finally, the Δ enantiomer displays two distinct quenching slopes in its profile (Figure 2.19). A steep slope from a P/D of 0 to 6.5 followed by a much more gradual slope between the P/D ratios of 6.5 to 13.5 where it remains at a maximum quenching of 60.4%. This contrasts with the mono-phasic profile from the 10 mM sodium phosphate titration in which a steep slope is observed from P/D 0 to 4.5 at which point luminescence remains constant at this low level (Figures 2.13 and 2.20). The cause of this behaviour is related to the salt displacement effect. At low P/D ratios, the concentration of the Δ complex is such that it binds effectively to the DNA binding sites. However, at higher P/D ratios, where there are fewer free Δ complexes in solution, competition between the solvated ions and the enantiomers for the remaining binding sites dominates the binding, resulting in the reduced ability of enantiomer to bind and thus the more gradual slope to the maximum bound P/D ratio.

It is also noted that the difference between the quenching profiles of both enantiomers under the two salt conditions is significant. The increased ability of the Δ complex to bind to DNA compared to the Λ complex is highlighted, as expected. Also, neither enantiomer

exhibits the same degree of quenching in the high salt conditions as seen in the lower salt conditions. This is a result the combination of the competition for binding sites between the solvated cations and enantiomeric cations and the reduced binding site size caused by the higher ionic strength conditions.

In summary, the data collected from both the absorbance and fluorescence titrations at 100 mM sodium phosphate conditions compare well to the data from the 10 mM sodium phosphate titrations. The Δ enantiomer is still found to bind more effectively than the Λ enantiomer and the increased ionic concentration has the desired effect of reducing the binding ability of both enantiomeric complexes. The main differences between the two ionic conditions are that at lower salt concentrations, the relocation effect described in section 2.4.1.2, or lack thereof for the Δ , dominates the emission profiles and the non-covalent cross-linking interaction of the Λ complex is pivotal in understanding the absorbance spectra. At higher salt concentrations, it is the competition for binding sites that dominates the emission profile while the absorbance spectra can be explained by both this competition effect and the lack of non-covalent cross-linking interactions resulting from the lessened phosphate-phosphate repulsion within the DNA helix.

2.4.2.3 Thermal Denaturation Studies in High Salt Conditions – 100 mM sodium phosphate buffer

The melting temperature of DNA, while highly dependant on oligonucleotide sequence, also depends on the ionic strength of the surrounding solution. At higher ionic strengths, the DNA helices shorten in length as explained earlier. This has the effect of increasing the macromolecule's thermal stability because its surface-to-volume ratio is smaller resulting in more energy being required to break apart the two strands. The following formula is often used to theoretically calculate the T_m of oligonucleotides of various lengths in various solutions:

$$T_m = 81.5 + 16.6 \log M + 41(XG + XC) - \frac{500}{L} - 0.62F \quad (2.7)$$

Where M is the molar concentration of monovalent cations present, X_G and X_C are the mole fractions of G and C in the oligonucleotide, L is the length of the shortest strand in the duplex, and F is the molar concentration of any denaturing agents present. In a solution of 10 mM ionic strength, the temperature required to split the duplexes of salmon sperm DNA was 77°C whereas in a solution of 100 mM ionic strength the melting temperature increased to 81.5°C.

Thermal denaturation studies were performed between the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and salmon sperm DNA (150 μM) in 100 mM sodium phosphate buffer, pH 7.4, at a P/D ratio of 20, using degassed solutions. The absorbance at λ_{max} 260 nm was then monitored as the temperature was gradually increased from 20°C to 90°C, at a rate of 1°C per minute. The thermal denaturation curves are given below in Figure 2.21.

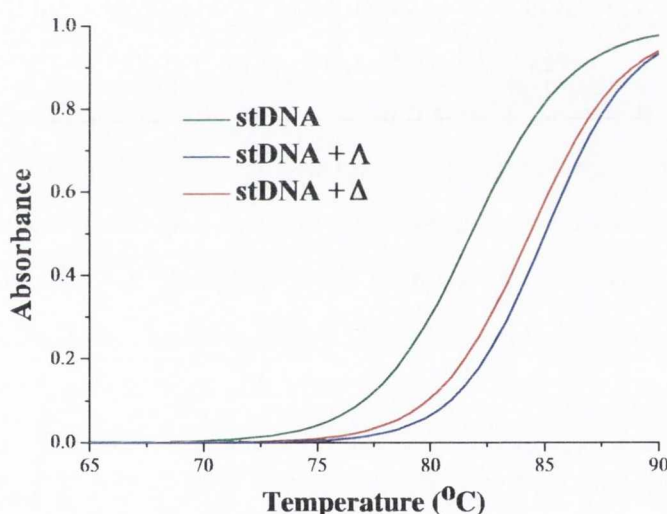


Figure 2.21 – Normalised thermal denaturation curves of stDNA (150 μM) in the absence of any complex (green), the presence of the Λ enantiomer (blue) and the presence of the Δ enantiomer (red) in 100 mM sodium phosphate buffer (P/D 20)

In the absence of metal complex, the T_m value for salmon sperm DNA was recorded as 81°C with significant changes noted in the presence of the Λ and Δ enantiomers. Both the enantiomers stabilise the DNA duplex, resulting in increased thermal stability. Binding of the Λ enantiomer increased the T_m temperature by 4°C to 85.5°C and the Δ enantiomer increased the T_m temperature by 3°C to 84.5°C (Table 2.6).

Complex	High Salt Conditions		Low Salt Conditions	
	T_m	ΔT_m	T_m	ΔT_m
stDNA	81°C	-	77°C	-
stDNA + Λ	85°C	+ 4°C	81°C	4°C
stDNA + Δ	84°C	+ 3°C	83°C	6°C

Table 2.6 – The T_m values from the melting curves in Figure 2.21 (P/D 20)

Increasing the ionic strength of the buffer, has the effect of conferring an innate thermal stability to the DNA helix as seen by its increased T_m temperature compared to the low ionic buffer experiments. As expected, the binding of both enantiomers imparts a thermal stability to the DNA duplex resulting in the higher T_m temperatures required to split the DNA strands. Rather unexpectedly, however, it seems that in the higher salt concentrations, the Λ enantiomer imparts more stability than the Δ enantiomer, which is contrary to expectations. The photophysical data supports the conclusion that the Δ complex is the more efficient binder and thus should increase the DNA thermal stability to a higher degree than the Λ . This seemingly erroneous result can be explained, however, by the very fact that the Δ enantiomer does bind more effectively than the Λ . As mentioned, the higher ionic concentration causes the duplex to shrink. From the emission studies, the Λ complex has been shown to bind to the DNA, but not as strongly as the Δ complex. The increased number of intercalated Δ enantiomers causes the DNA helix to unwind and lengthen to accommodate the complexes, thus reversing the shrinking effect of the higher ionic strength conditions. As such, while the Δ enantiomer does increase the thermal stability of the duplex, the loss of stability due to lengthening results in a T_m stabilisation value half of that of the DNA + Δ enantiomer in the low salt conditions. The Λ enantiomer also binds, imparting an added degree of stability, but due to its poorer ability to intercalate fully, the separation required to accommodate the complexes is smaller resulting in the degree of DNA lengthening being reduced compared to that of the Δ enantiomer. As such, in the presence of the Λ enantiomer, the DNA has the stability

from being both smaller in size and the binding effect of an intercalating complex, resulting in a T_m value higher than in the presence of the Δ enantiomer.

2.4.2.4 Binding Constant Calculations in High Salt Conditions – 100 mM sodium phosphate buffer

The binding constants for the interaction of the Λ and Δ enantiomers with salmon sperm DNA at 100 mM sodium phosphate buffer were determined using the McGhee/von Hippel method, as previously, applied to the fluorescence data. The data yielded a binding constant of $1.8 \times 10^5 \text{ M}^{-1}$ for the Λ enantiomer and $5.1 \times 10^5 \text{ M}^{-1}$ for the Δ enantiomer (Table 2.7). The accompanying plots are given in Appendix A.

Complex	Binding Constant $K_b \text{ (M}^{-1}\text{)}$	Binding Site Size n (base pairs)	R^2
Λ	$1.8 \times 10^5 \text{ } (\pm 0.1)$	1.7 (+ 0.1)	0.96
Δ	$5.1 \times 10^5 \text{ } (\pm 0.3)$	2.0 (+ 0.1)	0.93

Table 2.7 – *stDNA binding constants calculated from the fluorescence data in 100 mM sodium phosphate buffer utilising the McGhee/von Hippel model*

The increased salt conditions had the desired effect of reducing the DNA binding affinity of both the enantiomers to more realistically measureable values. From these calculated binding constants, we can confirm that the Δ enantiomer binds to DNA with a higher affinity than the Λ enantiomer.

From the data thus far reported, we can safely conclude that both the enantiomers of [Ru(TAP)₂dppz]Cl₂ display the ‘light-switch off’ effect when in the presence of DNA, both have an exceptionally high affinity for binding to DNA at physiological conditions and, due to the arrangement of its ancillary ligands, the Δ enantiomer has been shown to be a more effective binding agent than the Λ enantiomer. The main mode through which

both enantiomers bind to the DNA is intercalation of the dppz ligand between the base pairs, indicated by the hypochromic shifts in the absorbance spectra that are characteristic of the intercalative binding mode, however, due to the poorer binding observed at higher ionic strength buffer, the binding ability of the complexes may also be due to some electrostatic interactions. A reverse titration was conducted to determine the extent of the role these electrostatic interactions have in the binding of these complexes.

2.4.3 Determining the Roles of Intercalation vs Electrostatic Interactions on the DNA binding of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$

The 'reverse' salt titration consists of increasing the ionic strength of the DNA-ligand solution through the addition of NaCl. Subsequently, the competition for the binding sites between the ligand and salt cations increases results in the bound ligand eventually being displaced. This is detected in the UV spectra by a decrease in the DNA-ligand band. One application for this technique is to distinguish between binding modes (intercalation vs. groove binding) since groove binders are generally displaced more easily from DNA binding than intercalating molecules.

The reverse salt titration was conducted for each of the enantiomers at 10 mM sodium phosphate buffer conditions. Small aliquots of sodium chloride (4 M) were added to the buffered solutions containing the enantiomer in question and duplex DNA at a P/D ratio of 4, so that the complex was fully bound. The absorption spectrum of each addition was recorded. Figure 2.22(a) is the result from the reverse titration with the Λ enantiomer and Figure 2.22(b) is the result from the reverse titration with the Δ enantiomer.

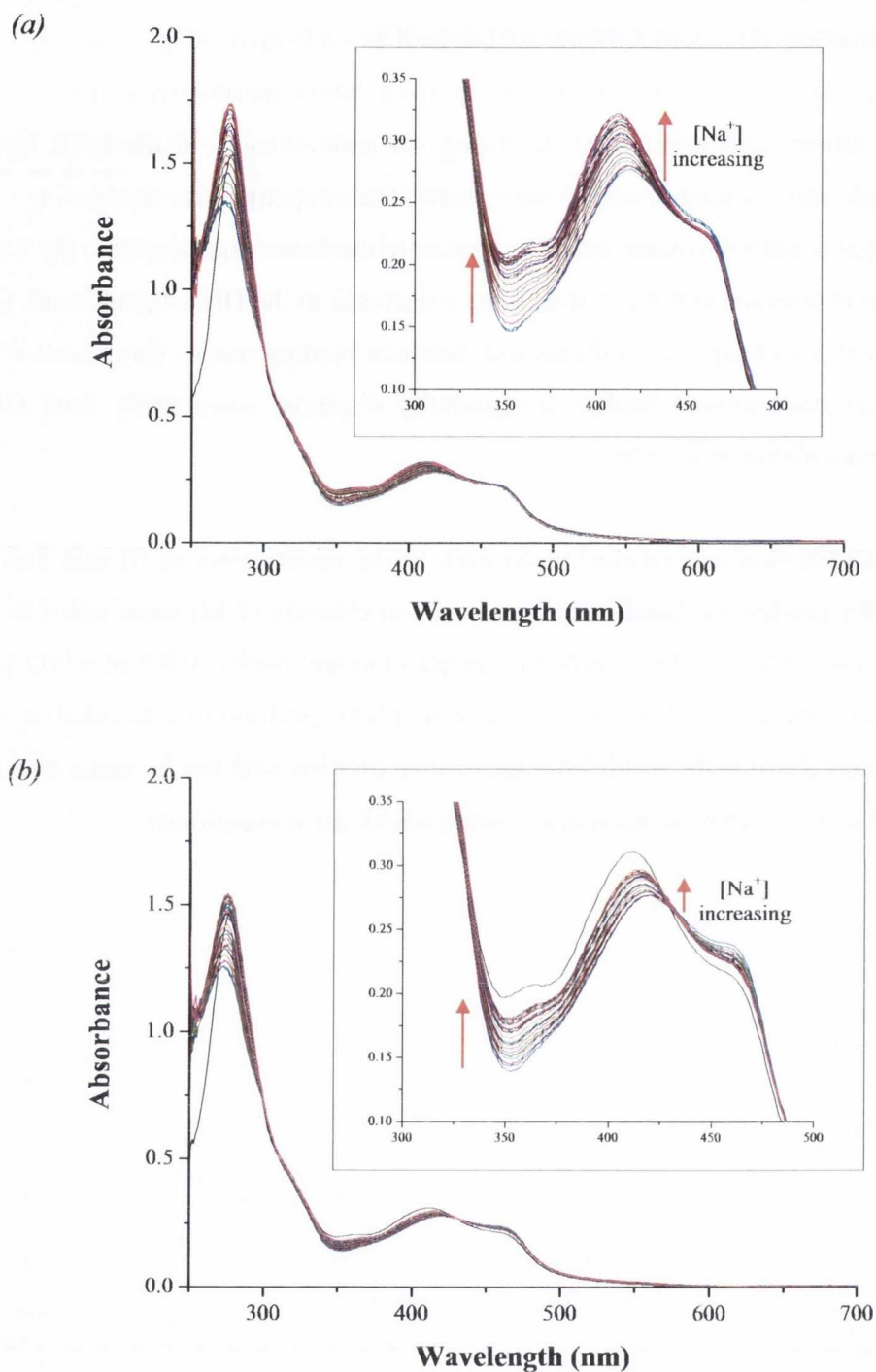


Figure 2.22 – UV/vis absorption spectrum for the reverse salt titration of (a) Λ -[Ru(TAP)₂dppz]Cl₂ (14.6 μM) and (b) Δ -[Ru(TAP)₂dppz]Cl₂ (14.5 μM)
 Note that the Δ enantiomer does not recover to absorbance of the free ligand (black trace) while the Λ enantiomer does

Since the binding constants, K , were previously evaluated from the absorbance and fluorescence titration experiments, it was possible to calculate the free energy term of each enantiomer from equation 2.8.

$$\Delta G^\circ = -RT \ln K \quad (2.8)$$

According to the polyelectrolyte theory,⁴⁴ the observed binding constant is a function of the charge on the cation and the fraction of the counterion associated to each DNA phosphate. The dependence for the binding of the enantiomers was investigated by plotting $\log K$ (calculated using equation 2.9 where A is the absorbance of the sample and A_0 is the absorbance of the unbound complex) against $\log[\text{Na}^+]$ and the slope of the graph (SK) was calculated according to equation 2.10 where Z is the charge on the ligand and ψ is the charge on the DNA phosphate (Figure 2.23):^{61,62}

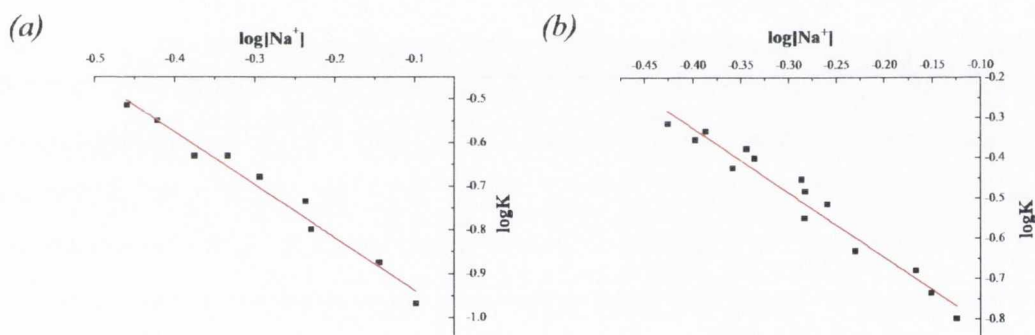


Figure 2.23 – Plot of the dependence of the binding constant on the salt concentration of (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$

$$K \propto \frac{A_0 - A}{A} \quad (2.9)$$

$$SK = \frac{\delta \log K}{\delta \log[\text{Na}^+]} = -Z\psi \quad (2.10)$$

The slope of the graph (SK) for the Λ enantiomer was found to be -1.21 and -1.61 for the Δ enantiomer. These values were used to partition the free energy term, ΔG° . This observed free energy (ΔG°) was calculated to be $-40.1 \text{ kJ mol}^{-1}$ for the Λ enantiomer and $-45.2 \text{ kJ mol}^{-1}$ for the Δ enantiomer and then split into the electrostatic (ΔG_{PE}) and the non-

electrostatic ($\Delta G_{\text{NON-PE}}$) terms by using equations 2.11 and 2.12. The corresponding results are shown in Table 2.8.

$$\Delta G_{PE} = SKRT\ln[Na^+] \tag{2.11}$$

$$\Delta G^{\circ} = \Delta G_{\text{NON-PE}} - \Delta G_{PE} \tag{2.12}$$

Complex	K ($\times 10^7 \text{ M}^{-1}$)	ΔG° (kJ mol^{-1})	ΔG_{PE} (kJ mol^{-1})	$\Delta G_{\text{NON-PE}}$ (kJ mol^{-1})
Λ	1.4	-40.1	-2.1	-38.0
Δ	11.7	-45.2	-3.0	-42.2

Table 2.8 – Calculated free energies from the reverse salt titration

The results in Table 2.8 show that the non-electrostatic term is significantly larger for both the enantiomers than the electrostatic value, thus indicating that binding via intercalation is much stronger a binding mode than the electrostatic interactions, solidifying the fact that the enantiomers bind via intercalation. Also, this reverse titration again shows that the Δ enantiomer is the more tightly bound of the two enantiomers.

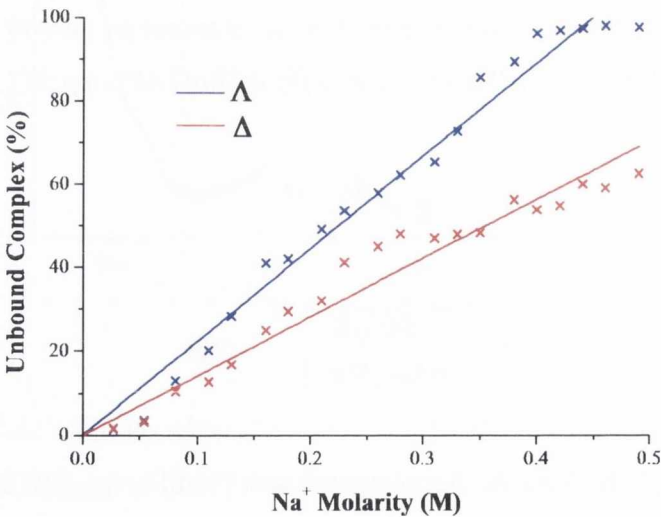


Figure 2.24 - Percentage of the enantiomeric complexes liberated with increasing [NaCl]

Comparison of the data in Figure 2.24 shows that the Λ complex has become completely unbound at a $[\text{Na}^+]$ of 0.44 M, whereas at this same end point, the absorbance of the Δ complex has not recovered to that of the free complex, indicating that more salt is required to fully dislodge the enantiomer. This is further proof of the Δ enantiomer's increased ability to bind more tightly to the DNA duplex than its Λ counterpart via increased penetration between the bases pairs of its intercalating dppz ligand.

2.4.4 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with the Synthetic Homopolymer Polynucleotide $[\text{Poly}(\text{dG-dC})]$

Previous studies conducted with $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ and *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ showed that while the complexes have a high binding affinity, their binding preference is for A-T sites over G-C sites. Thus the enantiomers in this work, Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$, were titrated, as before, with the double stranded synthetic polynucleotides $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$ in order to investigate their photophysical properties and determine if any sequence specific effects occur.

2.4.4.1 UV/visible Absorbance Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with $[\text{poly}(\text{dG-dC})]$

The interaction of the two enantiomers with the polynucleotide $[\text{poly}(\text{dG-dC})]$ in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4 was first investigated. The titrations were conducted as previously, adding small aliquots of $[\text{poly}(\text{dG-dC})]$ (1.6×10^{-3} M) to a buffered solution of the pure enantiomers (approx. 8×10^{-6} M) until a plateau in absorbance was reached. As previously, the absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific observation of the absorbance bands at 362 nm and 412 nm. The titrations resulted in significant changes in the absorption spectra of both enantiomeric complexes and are given in Figures 2.25(a) and 2.25(b) for the Λ and Δ complexes respectively. Figure 2.26 shows the changes in absorbance at the bands 362 nm and 412 nm for the Λ and Δ enantiomers, with Table 2.9 summarising the spectral shifts.

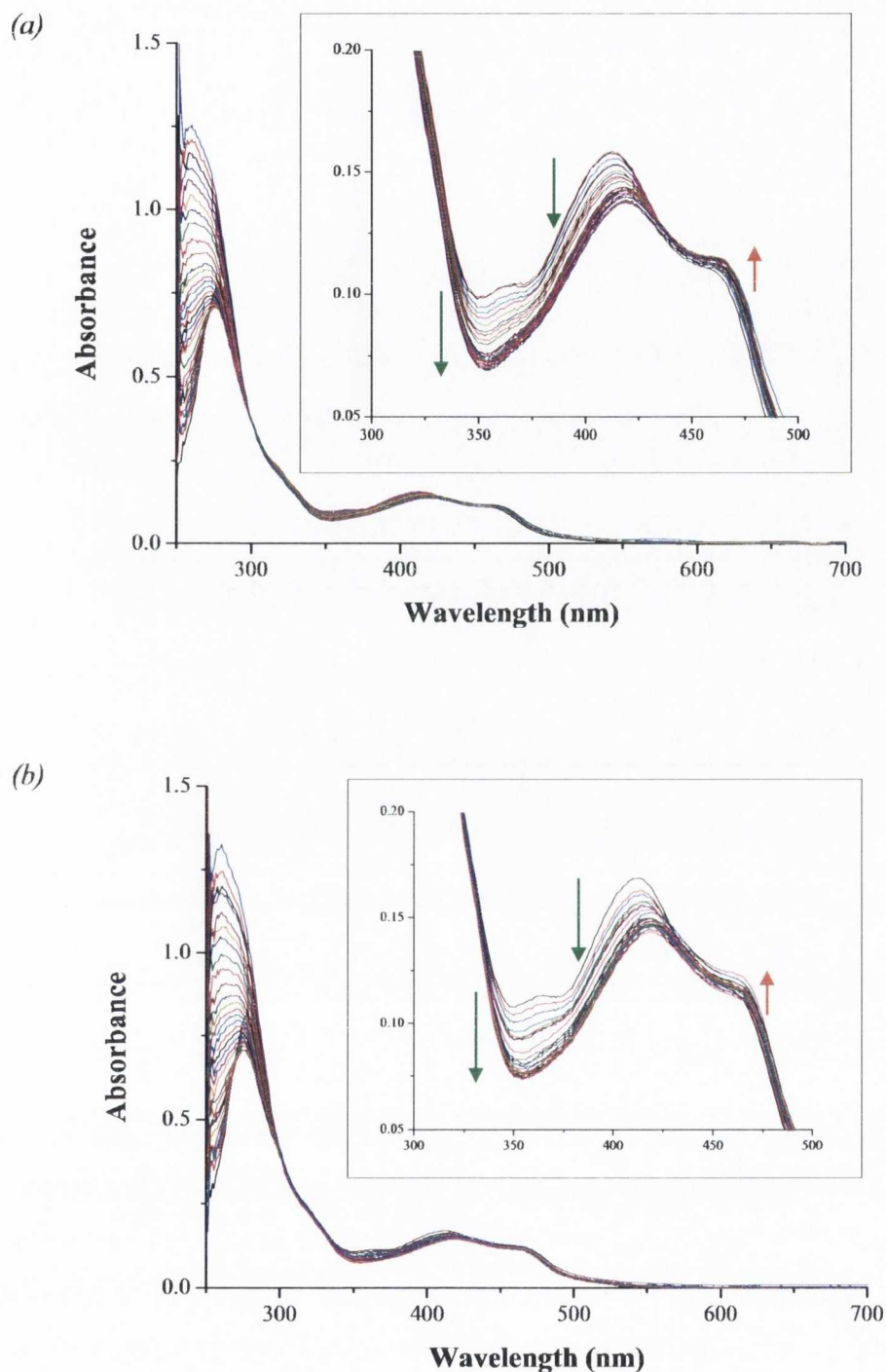


Figure 2.25 – UV/vis absorption spectrum for the titration of
 (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (7.7 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (7.9 μM)
 with $[\text{poly}(\text{dG-dC})]$ in 10 mM sodium phosphate buffer

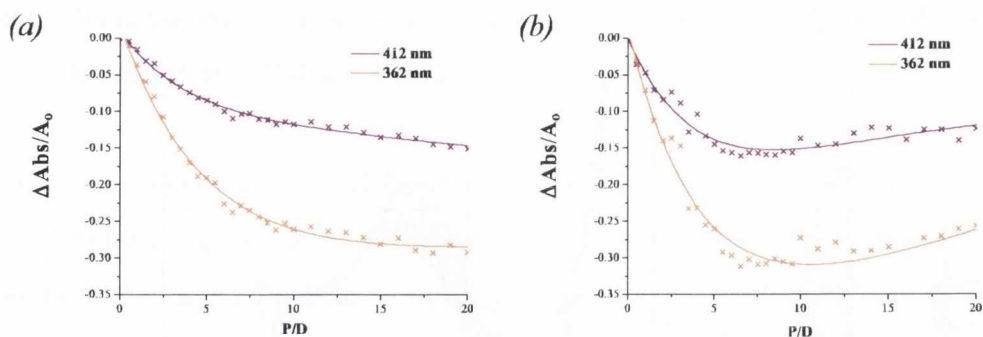


Figure 2.26 – Change in absorbance (ΔAbs) normalised by the absorbance without $[poly(dG-dC)]$ for (a) Λ - $[Ru(TAP)_2dppz]Cl_2$ and (b) Δ - $[Ru(TAP)_2dppz]Cl_2$ vs increasing ratios of P/D at different wavelengths in 10 mM sodium phosphate

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₆₂	Max - 29%	Max - 31% Rec - 29%	6 nm	8 nm
Abs ₄₁₂	Max - 13%	Max - 14% Rec - 12%	9 nm	10 nm

Table 2.9 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ - $[Ru(TAP)_2dppz]Cl_2$ with $[poly(dG-dC)]$

As with the previously conducted titrations, the band at 456 nm in both enantiomers does experiences a small increase in its strength of absorbance, indicative of the reversal of the hypochromic effect of aggregated metal complexes. It is interesting to note that unlike the previous titrations, the hyperchromic effect of this band is larger for the Δ enantiomer than the Λ , suggesting a difference in the binding of the enantiomers to $[poly(dG-dC)]$ as compared to stDNA.

In contrast, at the wavelengths of 362 nm and 412 nm, the two enantiomers show a dramatic difference. The absorption profile of the Δ enantiomer exhibits the expected biphasic behaviour, as observed in the previous titrations. Initially at low P/D ratios, significant hypochromic and bathochromic shifts occur linearly as the absorption

decreases until a P/D of 7. At this point the $[\text{poly}(\text{dG-dC})]$ duplex is saturated with the intercalating Δ enantiomers. The second phase, in which the absorption slightly increases at higher P/D ratios, is a consequence of the second electrostatic binding mode described previously. The increase in the saturation P/D of 7 compared to that of the stDNA (P/D 4.5) indicates that the complex does not bind to the $[\text{poly}(\text{dG-dC})]$ with the same affinity it has for the mixed sequence DNA. This may be the result of combination of factors. The $[\text{poly}(\text{dG-dC})]$ duplex is known to be quite a rigid macromolecule, owing to the presence of three hydrogen bonds between the base pairs. This increased rigidity may make the binding of complexes more difficult, especially one containing ancillary ligands that create sterical difficulties. The main reason for the reduced binding affinity may be in the sequence of pyrimidine and purines. In a $[\text{poly}(\text{dG-dC})]$ duplex, the single stranded sequence is $[\text{G-C}]_n$, ie. $[\text{purine-pyrimidine}]_n$. As such, there are no purine-purine steps in the duplexes and it is at these sites that the dppz moiety of the enantiomers has been shown have a preference for binding (discussed in Chapter 3). The absence of any purine-purine steps has the effect of reducing the enantiomer's binding affinity for the $[\text{poly}(\text{dG-dC})]$ as the size of the aromatic π surface the dppz ligand interacts with is lessened, and consequently the stabilization effect experienced during intercalation.

Unlike its Δ counterpart, the Λ enantiomer exhibits monophasic behaviour. The fact that the plots in Figure 2.26(a) do not plateau is indicative of the complex not being fully bound at the maximum P/D ratio measured of 20. The inability of the Λ enantiomer to achieve intercalation saturation of the $[\text{poly}(\text{dG-dC})]$ duplex implies that it has a significantly lower binding affinity than the Δ enantiomer. The absence of the second phase suggests the second binding mode does not occur, however, considering the profiles in Figure 2.26(a) are not linear but taper at higher P/D ratios would indicate that the electrostatic binding mode is present, and is beginning to reach an equilibrium with the intercalative binding mode. The implication of this observation is that the $[\text{poly}(\text{dG-dC})]$ does not experience intercalative saturation by a P/D of 20 in the presence of the Λ enantiomer, though this binding mode is more prevalent at lower P/D ratios. This again is a result of the increased rigidity of the $[\text{poly}(\text{dG-dC})]$, making intercalation significantly more difficult for a complex whose ancillary ligands actively sterically inhibit binding to right-handed duplexes and the absence of any purine-purine steps. The effect of both these occurrences is more pronounced with the Λ enantiomer than with the

Δ because of the inability of the Δ enantiomer to intercalate as deeply between base pairs as the Λ . The reduction of stability in the intercalation pocket has a much greater effect on the enantiomer that could not bind as fully, resulting in its inability to saturate the G–C duplexes via intercalation.

The conclusion drawn from the absorbance data of the titrations with the [poly(dG–dC)] polynucleotide is that a sequence specific effect is indeed observed. The binding affinity for both enantiomers is reduced for the polynucleotide compared with that of the stDNA. The Δ enantiomer remains the more tightly binding of the two enantiomers, and as such can be described as having a higher preference for binding to G–C sites than the Λ enantiomer. It is interesting to note that the Δ enantiomer has the ability to saturate the duplex via intercalation while the Λ enantiomer seemingly cannot at these P/D ratios. This may be attributed to the lack of purine-purine steps, however, instead of the lack of A–T steps. It would be interesting to repeat this titration in the presence of [poly(dG)][poly(dC)] to investigate this theory.

2.4.4.2 Fluorescence Titrations of Λ - and Δ - $[Ru(TAP)_2dppz]Cl_2$ with [poly(dG–dC)]

To complement the absorbance studies, fluorescence titrations between the two enantiomeric compounds and [poly(dG–dC)] were conducted in parallel. It is expected that the fluorescence data will mirror that of the absorbance by revealing similar G–C specific effects on both enantiomers. The titration was conducted by the addition of small volumes of [poly(dG–dC)] to a solution of each complex in the low salt conditions of 10 mM sodium phosphate buffer at pH 7.4. Each spectrum was examined at an excitation wavelength of 435 nm, which corresponds to the isosbestic point observed for the Δ enantiomer.

The titrations of both the Λ and Δ enantiomers with [poly(dG–dC)] (P/D 0 $\rightarrow$ 20) resulted in significant quenching of the emission spectrum compared with the free complexes. Figure 2.27 displays the fluorescence spectra of the Λ and Δ titration results, with Figure 2.28 comparing the relative emission intensities of both enantiomers at the various P/D ratios.

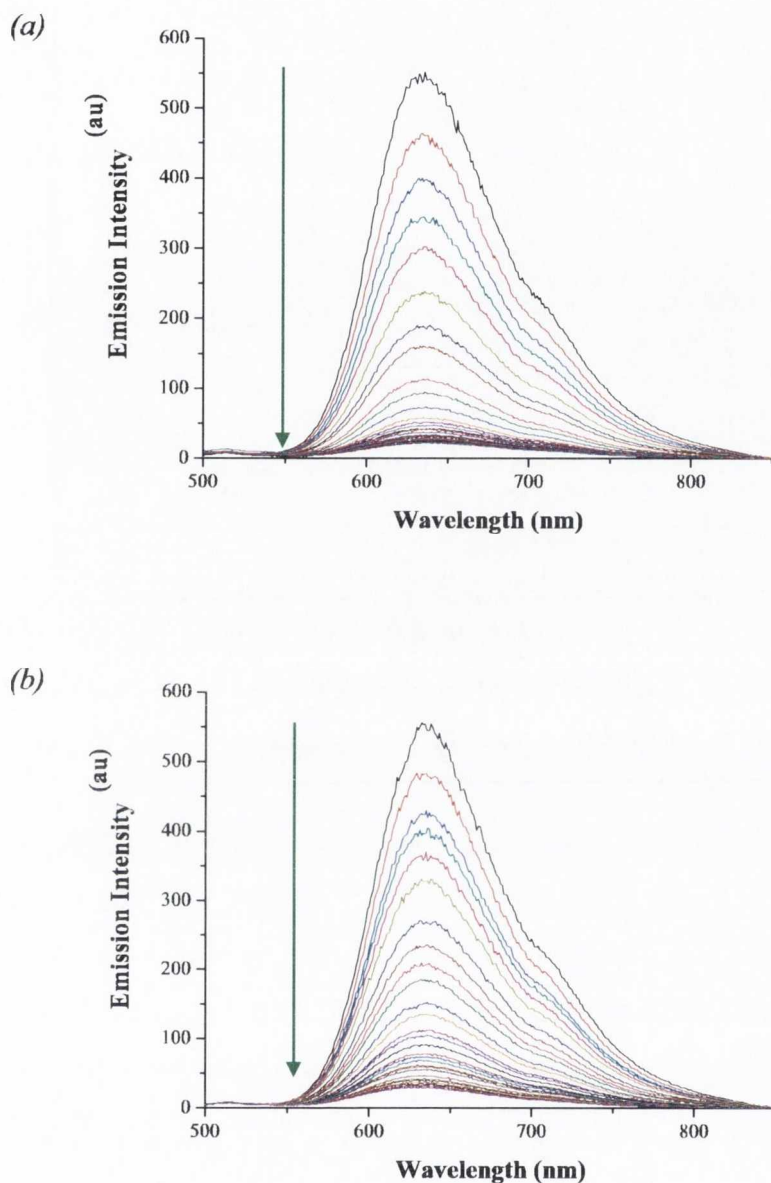


Figure 2.27 - Changes in the emission spectrum of (a) Λ -[Ru(TAP)₂dppz]Cl₂ (7.7 μM) and (b) Δ -[Ru(TAP)₂dppz]Cl₂ (7.9 μM) with increasing additions of [poly(dG-dC)] (λ_{ex} 435 nm)

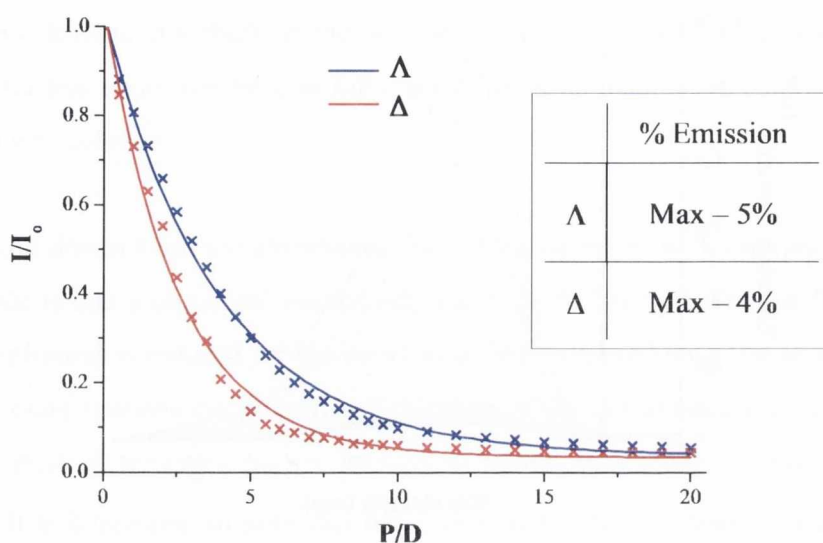


Figure 2.28 – Changes in the relative emission intensities vs increasing ratios of P/D of the Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ complexes in the presence of poly[dG–dC] in 10 mM sodium phosphate buffer

As is evident from the previous spectra, both enantiomers are highly quenched upon the addition of the polynucleotide [poly(dG–dC)]. The Λ complex was quenched by 95% with the maximum quenching occurring at a P/D of 20. The Δ complex was quenched by 96% with the maximum quenching occurring at a P/D of 10. In contrast to the results with stDNA, no recovery of luminescence was observed in either enantiomer because there are no A–T sites available for the enantiomers to relocate to and thus enhance luminescence.

The results from the fluorescence titrations do indeed mirror those from the absorbance experiments. The Δ enantiomer is quenched to a larger extent than the Λ enantiomer, indicating that it has an increased ability to bind to the G–C duplex. The Δ enantiomer also displays a maximum binding P/D ratio of 10 while the Λ enantiomer has not reached a plateau by a P/D ratio of 20. This again shows the increased affinity the Δ enantiomer has for the G–C duplex compared to its Λ counterpart. The slopes of the initial phases (P/D ratio 0 $\rightarrow$ 5) of the plots contained in Figure 2.28 further support this argument as it is much steeper for the Δ complex than the Λ , implying that the Δ enantiomer interacts better with the [poly(dG–dC)] at the lower P/D ratios, as observed by the intercalative

saturation of the $[\text{poly}(\text{dG-dC})]$ duplex by the Δ enantiomer in the absorbance titration, but not by the Λ enantiomer.

The conclusions drawn from the fluorescence data of the titrations with the $[\text{poly}(\text{dG-dC})]$ polynucleotide echo those drawn from the absorbance titrations in that the Δ enantiomer again is shown to be the more tightly binding of the two enantiomers, and has a higher preference for binding to G-C sites than the Λ enantiomer.

2.4.4.3 Binding Constant Calculations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with $[\text{poly}(\text{dG-dC})]$

Unfortunately, due to the nature of the Watson-Crick bonding between guanine and cytosine base pairs involving three hydrogen bonds, thermal denaturation studies to investigate the relative stability each enantiomer imparts to the duplex could not be performed. The presence of the three hydrogen bonds results in the T_m temperature for the lone $[\text{poly}(\text{dG-dC})]$ duplex exceeding 90°C which is the highest measurable temperature for such experiments. However, determination of the intrinsic binding constant, K_b , for the interaction of the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ allows for the evaluation and comparison of their binding affinities.

The binding constants for the interaction of the Λ and Δ enantiomers with $[\text{poly}(\text{dG-dC})]$ at 10 mM sodium phosphate buffer were determined using the McGhee/von Hippel method, as previously, applied to the fluorescence data. The figures produced yielded a binding constant of $1.2 \times 10^6 \text{ M}^{-1}$ for the Λ enantiomer and $3.6 \times 10^6 \text{ M}^{-1}$ for the Δ enantiomer (Table 2.10). These binding constants are much lower than those recorded for the stDNA at the same buffer concentrations (Table 2.4), showing that the affinity of both enantiomers for the $[\text{poly}(\text{dG-dC})]$ duplex is indeed much lower than that for the mixed sequence DNA duplex. As expected, the binding constant for the Δ enantiomer is higher than that of the Λ , confirming the qualitative conclusions drawn from the previous spectra that the Δ enantiomer has a higher affinity for $[\text{poly}(\text{dG-dC})]$. Both enantiomers exhibit a binding site size of 2 indicating that the nearest neighbour separation hypothesis applies to the binding.

Complex	Binding Constant K _b (M ⁻¹)	Binding Site Size <i>n</i> (base pairs)	R ²
Λ	1.2×10 ⁶ (+ 0.1)	2.2 (+ 0.1)	0.98
Δ	3.6×10 ⁶ (+ 0.2)	2.00 (+ 0.1)	0.95

Table 2.10 – [poly(dG–dC)] binding constants calculated from the fluorescence data utilising the McGhee/von Hippel model

2.4.5 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with the Synthetic Homopolymer Polynucleotide $[\text{Poly}(\text{dA}-\text{dT})]$

2.4.5.1 UV/visible Absorbance Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with $[\text{poly}(\text{dA}-\text{dT})]$

The interaction of the two enantiomers with the polynucleotide $[\text{poly}(\text{dA}-\text{dT})]$ in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4 was conducted following the procedure for the $[\text{poly}(\text{dG}-\text{dC})]$ experiments. The titrations were conducted as previously, adding small aliquots of $[\text{poly}(\text{dA}-\text{dT})]$ (1.7×10^{-3} M) to a buffered solution of the pure enantiomers (approx. 8×10^{-6} M) until plateau in absorbance was reached. As previously, the absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific observation of the absorbance bands at 362 nm, 412 nm and 456 nm. The titrations resulted in significant changes in the absorption spectra of both enantiomeric complexes and are given in Figures 2.29 (a) and (b). Figure 2.30 shows the changes in absorbance at the bands 362 nm and 412 nm for the Λ and Δ enantiomers, with Table 2.11 summarising the spectral shifts.

As with the previously conducted titrations, the band at 456 nm in both enantiomers does experiences a small increase in its strength of absorbance, indicative of the reversal of the hypochromic effect of aggregated metal complexes. It is interesting to note that unlike the previous titrations, the hyperchromic effect of this band is the same for both the Λ and Δ enantiomers, suggesting a further difference in the binding of the enantiomers to $[\text{poly}(\text{dA}-\text{dT})]$ as compared to stDNA and $[\text{poly}(\text{dG}-\text{dC})]$.

At the wavelengths of 362 nm and 412 nm, two phases of absorption are observed upon $[\text{poly}(\text{dA}-\text{dT})]$ addition, as with the previous titrations. During phase one, at low P/D ratios, significant hypochromic and bathochromic shifts occur as the absorption decreases linearly to P/D of 5. At this point the $[\text{poly}(\text{dA}-\text{dT})]$ duplex is saturated with intercalatively bound complex. At higher P/D ratios, a recovery in absorbance is observed, phase two. This second phase is a consequence of the second electrostatic binding mode described previously.

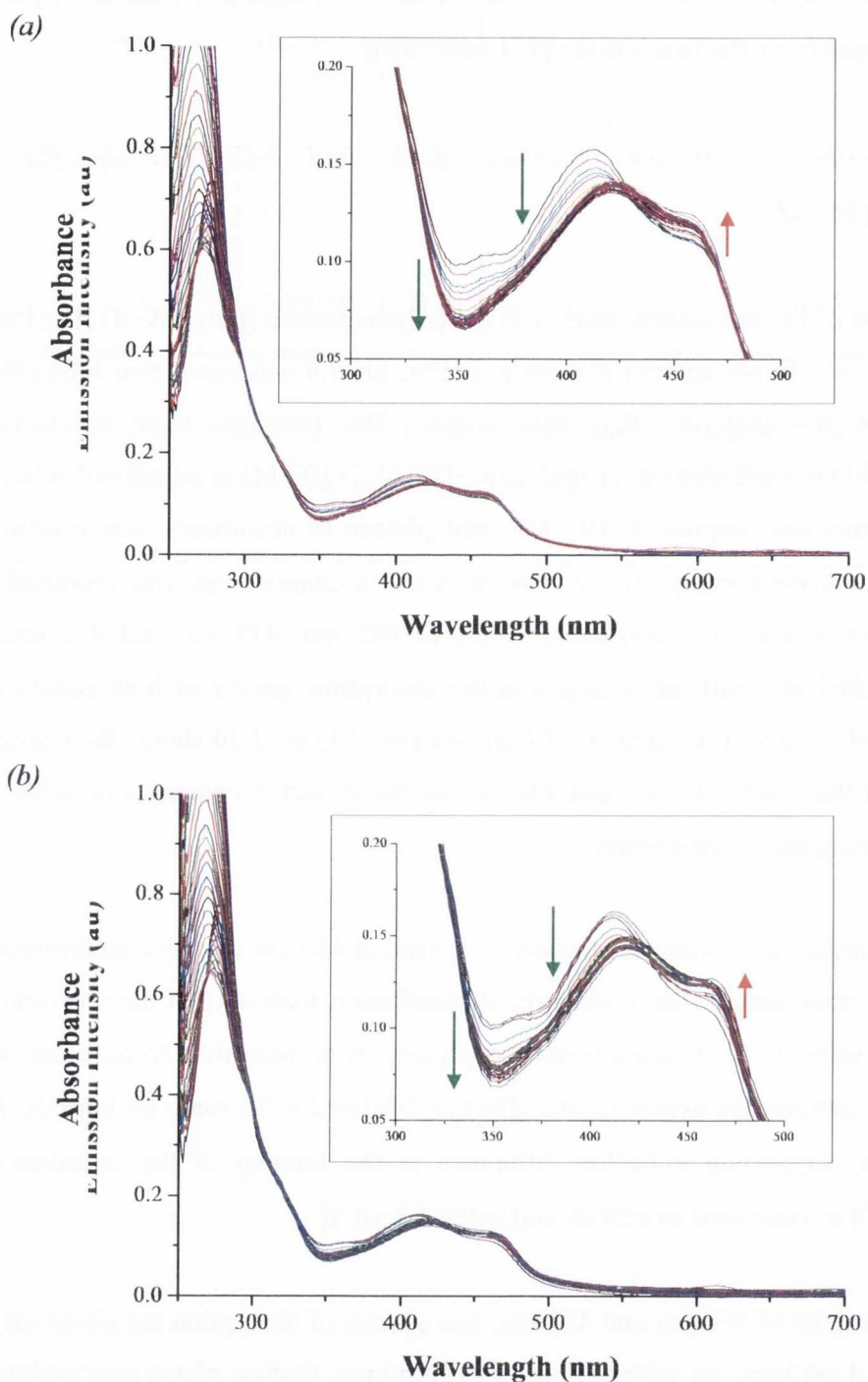


Figure 2.29 – UV/vis absorption spectrum for the titration of
 (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (7.9 μM) and (b) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (8.1 μM)
 with $[\text{poly}(\text{dA}-\text{dT})]$ at 10 mM sodium phosphate buffer

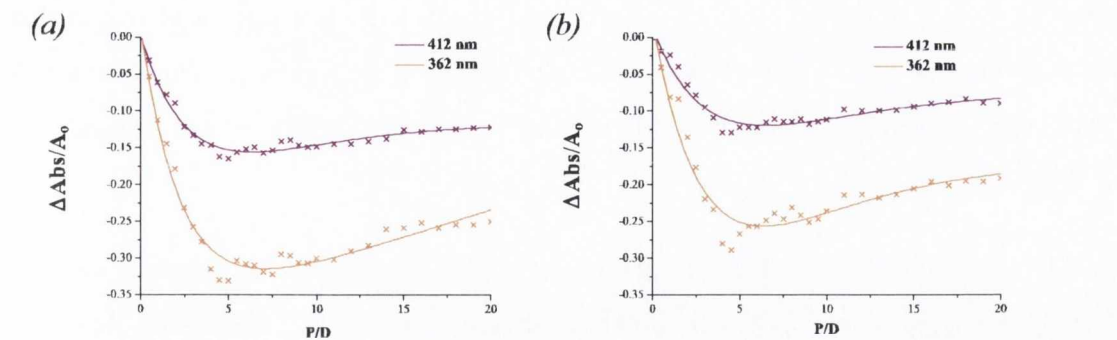


Figure 2.30 – Change in absorbance (ΔAbs) normalised by the absorbance without $[poly(dA-dT)]$ for (a) Λ -[Ru(TAP)₂dppz]Cl₂ and (b) Δ -[Ru(TAP)₂dppz]Cl₂ vs increasing ratios of P/D at different wavelengths

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₆₂	Max - 32% Rec - 25%	Max - 29% Rec - 20%	8 nm	9 nm
Abs ₄₁₂	Max - 16% Rec - 13%	Max - 13% Rec - 9%	9 nm	10 nm

Table 2.11 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ -[Ru(TAP)₂dppz]Cl₂ with $[poly(dA-dT)]$ at 10 mM sodium phosphate buffer

Both the Λ and the Δ enantiomers exhibit the same biphasic behaviour in their absorbance, unlike the results obtained from the $[poly(dG-dC)]$ titrations. This is the first indication that the Λ complex does indeed display a higher affinity for binding to A–T sites over G–C sites. The P/D ratio of 5, common to both enantiomers, at which the duplexes are intercalatively saturated indicates that the Δ enantiomer also has a higher binding affinity for A–T sites than G–C as the ratio of the fully bound material with $[poly(dG-dC)]$ occurs at the higher P/D ratio of 7.

Interestingly, however, the degree of hypochromicity experienced at the 363 nm and 412 nm wavelengths is higher for the Λ enantiomer than the Δ , a reversal of the trend thus far

observed in the stDNA and $[\text{poly}(\text{dG-dC})]$ experiments. The implication of this is that the Λ enantiomer has in fact a higher binding affinity for the $[\text{poly}(\text{dA-dT})]$ than the Δ enantiomer, though the marginal differences in the absorption values suggest the difference in affinity is equally small.

The absorbance of the titrations with the $[\text{poly}(\text{dA-dT})]$ polynucleotide imply a reversal in the binding affinities of the enantiomeric complexes as well as suggesting that both enantiomers have a preference for A-T sites over G-C sites. This agrees with literature reports of the enantiomers of the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ analogue exhibiting a preference for A-T binding sites.⁵ This phenomenon validates the proposed explanation for the recovery in luminescence observed in the previous stDNA experiments with the Λ enantiomer, that a preference for binding to A-T sites coupled with the poorer intercalative ability of the chiral complex allows the Λ enantiomers to relocate from their initial G-C binding sites to A-T sites upon the addition of further DNA aliquots.

2.4.5.2 Fluorescence Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with $[\text{poly}(\text{dA-dT})]$

As previously, fluorescence titrations between the two enantiomeric compounds and $[\text{poly}(\text{dA-dT})]$ were conducted in parallel with the absorption studies. The titrations were conducted from a P/D ratio of 0 to 20. The experiments resulted in a significant enhancement in the luminescence of the emission spectrum compared with the free complexes. Figure 2.31 (a) and (b) displays the fluorescence spectra of the Λ and Δ titration results, with Figure 2.32 comparing the relative emission intensities of both enantiomers at the various P/D ratios.

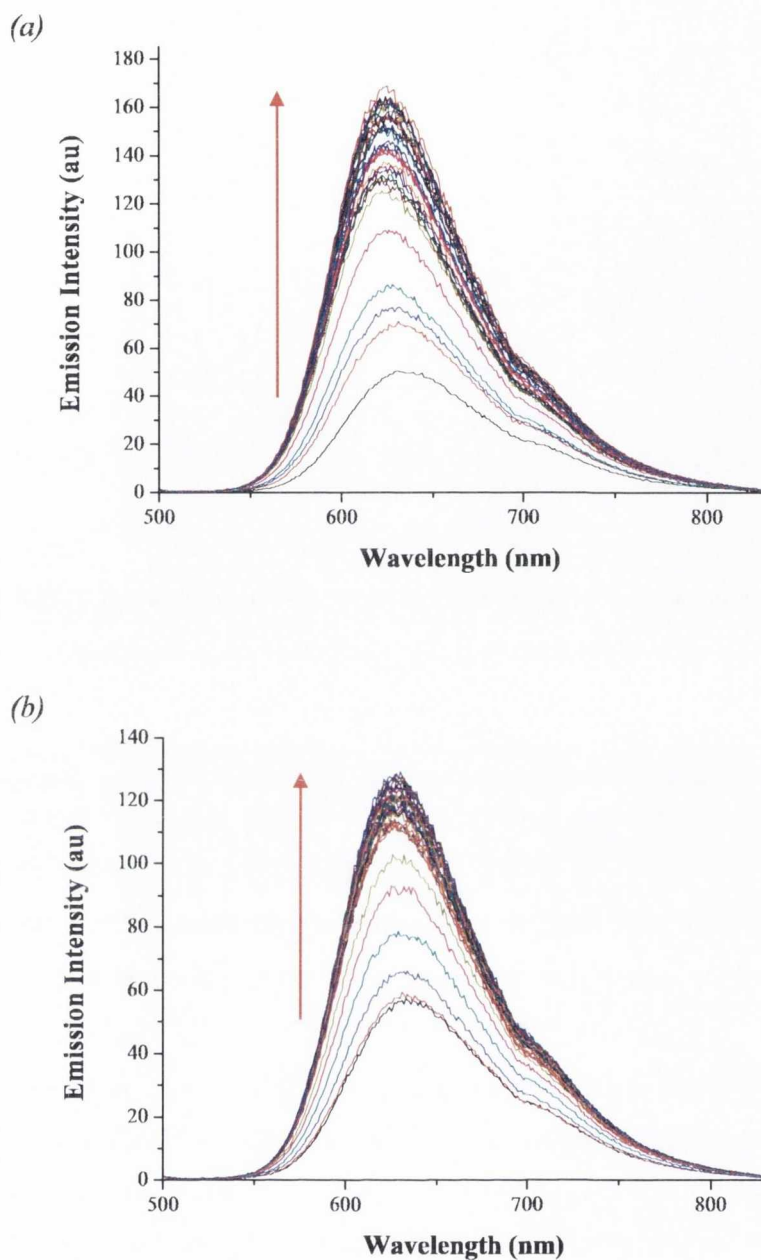


Figure 2.31 - Changes in the emission spectrum of (a) Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (7.9 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ (8.1 μM) with increasing additions of $[\text{poly}(\text{dA}-\text{dT})]$ at 10 mM sodium phosphate buffer (λ_{ex} 435 nm)

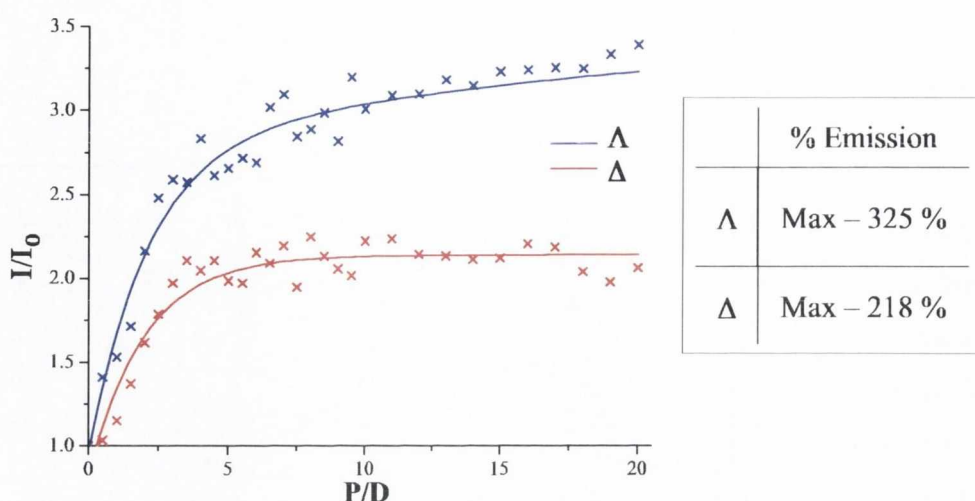


Figure 2.32 – Changes in the relative emission intensities vs increasing P/D ratios of poly[dA–dT] of the Λ- and Δ-[Ru(TAP)₂dppz]/Cl₂ complexes in 10 mM sodium phosphate buffer

As is evident from the previous spectra and above plot, the luminescence of both enantiomers is enhanced upon the addition of the polynucleotide [poly(dA–dT)]. The Λ complex was enhanced to 3.25 times its original value with the maximum enhancement occurring at a P/D of 15. The Δ complex was enhanced to 2.18 times its original value with the maximum enhancement occurring at a P/D of 4.5. The phenomenon of luminescence enhancement in the presence of [poly(dA–dT)] is a result of a decrease in the efficiency of the non-radiative deactivation process of the ³MLCT excited state of these complexes induced by the double helix microenvironment and protection from oxygen quenching. This increase also occurs in the mixed sequence duplexes, however it is masked by the quenching caused by the PET processes between the guanine nucleosides and Ru(II) polypyridyl centre.

The results from the fluorescence titrations do somewhat mirror the absorbance data. The luminescence of the Λ enantiomer is enhanced to a much greater degree than that of the Δ enantiomer, indicating that the [poly(dA–dT)] has a greater effect on the photophysics of the Λ enantiomer. The P/D ratio at which the complexes are fully bound differs between the two techniques. Both techniques report a P/D ratio of 5 at which the Δ enantiomer is fully bound, however while the absorption data yields a saturation P/D ratio of 5 for the Λ

enantiomer, the fluorescence data suggests the point of saturation has not yet been reached.

The reason for the significant enhancement of the Λ enantiomer's luminescence compared to the Δ enantiomer is perplexing. However, a theory may be suggested from the crystal structures discussed later in this thesis. The Λ enantiomer has been shown to form non-covalent cross-linking interactions between duplexes (see Chapter 3) via semi-intercalation of its ancillary TAP ligands. This would have the effect of further removing the solvent from close proximity to the complex, further reducing the quenching ability of solvated oxygen and thus enhancing the luminescence of the Λ complex to greater degree than that of the Δ , which is believed to be unable to form these non-covalent cross-linking interactions. This may also be the explanation for the apparent lack of binding saturation in the fluorescence titration of the Λ enantiomer. At lower P/D ratios, the luminescence increases dramatically until a P/D of 5 is reached, after which a much less pronounced increase in luminescence occurs. Perhaps at this P/D ratio of 5, the $[\text{poly}(\text{dA}-\text{dT})]$ duplex is saturated with regard to the intercalating Λ enantiomer and further increasing the concentration of $[\text{poly}(\text{dA}-\text{dT})]$ allows for the formation of more non-covalent cross-linking interactions. These interactions could further decrease the efficiency of the non-radiative deactivation process, resulting in the gradual increase in luminescence observed.

The conclusions drawn from both the absorbance and fluorescence data of the enantiomers with the polynucleotide $[\text{poly}(\text{dA}-\text{dT})]$ are that both have an extremely high binding affinity for the macromolecule, with the Λ enantiomer exhibiting a slightly increased binding ability compared to the Δ .

2.4.5.3 Thermal Denaturation Studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with $[\text{poly}(\text{dA}-\text{dT})]$

Unlike with the guanine-cytosine base pair, the Watson-Crick bonding between adenine and thymine base pairs involves only two hydrogen bonds. This results in a much lower T_m temperature for the lone $[\text{poly}(\text{dA}-\text{T})]$ duplex, recorded as 54°C , allowing the thermal denaturation studies to investigate the relative stability each enantiomer imparts to the duplex to be performed.

Thermal denaturation studies were conducted between the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{poly}(\text{dA}-\text{dT})]$ ($150\ \mu\text{M}$) in 10 mM sodium phosphate buffer, pH 7.4, at a P/D ratio of 20, using degassed solutions. The absorbance at λ_{max} 260 nm was then monitored as the temperature was gradually increased from 20°C to 90°C , at a rate of 1°C per minute. The thermal denaturation curves are given in Figure 2.33.

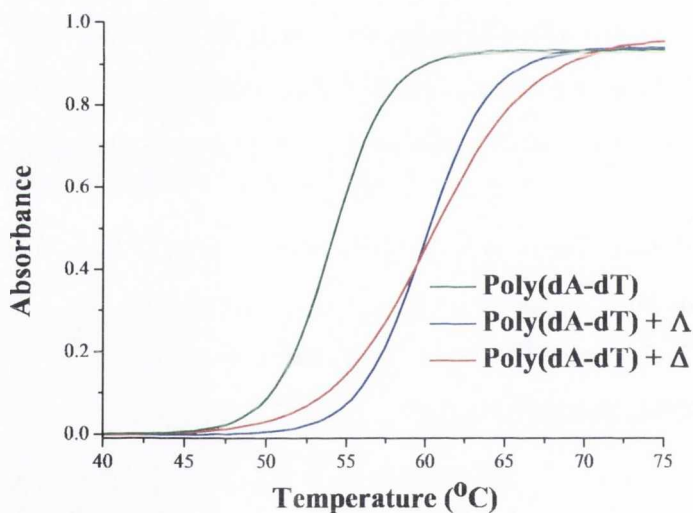


Figure 2.33 – Normalised thermal denaturation curves of $[\text{poly}(\text{dA}-\text{dT})]$ (150 mM) in the absence of any complex (green), the presence of the Λ enantiomer (blue) and the presence of the Δ enantiomer (red) in 10 mM sodium phosphate buffer (P/D 20)

In the absence of metal complex, the T_m value for $[\text{poly}(\text{dA}-\text{dT})]$ was recorded as 54°C with significant modulations being measured in the presence of the Λ and Δ enantiomers.

Both the enantiomers stabilise the DNA duplex, resulting in increased thermal stability. Binding of both the enantiomers increased the T_m temperature by 6°C to 60°C (Table 2.12). The fact that both enantiomers stabilise the [poly(dA–dT)] duplex to the same degree supports the conclusions drawn from the spectroscopic data that the two have a similar affinity for A–T binding sites. The increase in stability (6°C) is indicative of the complexes' increased preference for and ability to bind to A–T sites.

While the T_m temperature of both enantiomers is the same, it is clear from the plots in Figure 2.33 that the thermal stability imparted by the two are indeed different. The thermal transition of the [poly(dA–dT)] + Δ begins earlier with a less severe slope than the transition of the [poly(dA–dT)] + Λ . This is again a result of the increased binding ability of the Δ enantiomer compared to the initial binding and subsequent non-covalent cross-linking of the Λ enantiomer.

Complex	T_m	ΔT_m
[poly(dA–dT)]	54°C	-
[poly(dA–dT)] + Λ	60°C	+ 6°C
[poly(dA–dT)] + Δ	60°C	+ 6°C

Table 2.12 – The T_m values from the first derivative plots of the melting curves from Figure 2.33

As already explained, the Δ enantiomer is suspected to bind more deeply between the base pairs because its ancillary TAP ligands are in the correct orientation with the duplex groove to facilitate intercalation of the dppz ligand. As such, at the slightly lower temperature of 46°C the Δ enantiomers begin to become unbound, slowly allowing the duplex to unwind at the final temperature of 71°C.

The Λ enantiomer, which cannot intercalate completely because of its incorrect orientation of ancillary TAP ligands, does impart some stability via intercalation of its

dppz ligand, but also forms non-covalent cross-linking interactions between duplexes at higher P/D ratios. This increases the thermal stability of the [poly(dA-dT)] for two reasons. The first is the semi-intercalation interaction between the TAP ligand and polynucleotide duplex, while not very strong, is another association that must be broken before denaturation can occur. The second, and most important of the two, is that the non-covalent cross-linking interactions form a lattice (see Chapter 3 for full details). This has a similar effect as increasing the salt concentration in that the surface area-to-volume ratio dramatically reduces, resulting in the requirement of more energy to break the DNA strands apart. The consequence of this is that the temperature at which the polynucleotides begin to separate occurs later in the presence of the Λ enantiomer, 50°C, but once the lattices have split, the poorer ability of the enantiomer to intercalate results in dramatic thermal instability. Thus, the duplex unwinds rapidly at the final temperature of 68°C and the thermal plot is recorded as having a much steeper slope indicating a more rapid thermal transition than the Δ enantiomer.

2.4.5.4 Binding Constant Calculations of Λ - and Δ -[Ru(TAP)₂dppz]Cl₂ with [poly(dA-dT)]

Unfortunately, due to the very abrupt changes in the I/I_0 plot at low P/D ratios for the two enantiomers with [poly(dA-dT)], determining the binding constants via fitting according to the McGhee/von Hippel method was not possible. From this, however, we can conclude that the binding constants for these interactions are larger than those recorded previously with the stDNA.

2.4.6 Conclusions from the binding studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$

The results obtained from all the various titrations confirmed the trends reported for the enantiomers of such complexes in the literature. The Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ both display a particularly high affinity for binding to duplex DNA, with the Δ enantiomer being the slightly better binding agent of the two. This is a result of its chirality matching that of the duplex DNA, allowing for a better fit into the binding site, and thus a full intercalation of the dppz ligand. This trend was validated by a number of spectroscopic techniques, including absorbance, fluorescence and thermal denaturing studies.

Two methods of DNA binding were observed to occur in the presence of both enantiomers. The most prevalent binding mode is the intercalation of the dppz ligand between DNA base pairs. This method dominates at low P/D ratios. At higher P/D ratios, a second binding mode occurs, often after the DNA duplex has become saturated with intercalating complexes. This method of binding is believed to be electrostatically driven wherein the complexes bind to the grooves the DNA and the dppz ligands may partially intercalate between base pairs.⁴⁸

Increasing the ionic strength of the solution had the desired effect of reducing the binding affinity of the complexes, indicating that while intercalation is the main mode of binding (K_b remained at values of 10^5), electrostatic interactions also facilitate binding of the complexes. It also had the effect of reducing the effect observed for the second binding mode mentioned above, indicating that it is in fact an electrostatically driven interaction.

Investigation of the enantiomers' binding potential with the homopolymers $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$ revealed slight variations in the binding affinities. While both enantiomers bind to both polynucleotides, a trend in the K_b was noted in the binding ability for the two complexes as being $[\text{poly}(\text{dA-dT})] \geq \text{stDNA} \geq [\text{poly}(\text{dG-dC})]$. The Δ enantiomer was found to bind more effectively to the $[\text{poly}(\text{dG-dC})]$ duplex than the Λ enantiomer while in the presence of A-T duplexes, both enantiomers seemed to be of similar binding affinity. A possible reason for the A-T preference of both complexes may be derived from the structural

differences between the two homopolymers. While both are approximately B-DNA in structure, G-C duplexes are known to be the more rigid of the two due to the three hydrogen bonds connecting the base pairs. As such, the grooves in these duplexes are wider than those in an A-T rich duplex, however, the amino group on the guanine residue projects into the minor groove, causing steric hindrance to minor groove binders,⁶³ such as the enantiomers in this study. A-T rich duplexes, while having narrower grooves,^{64,65} are more flexible due to having only two hydrogen bonds between the bases. As such, assignment of the structure of A-T duplexes varies widely with even C-DNA and 'wrinkled D-DNA' structures being reported in the literature, the latter being the most favoured and having narrower than usual minor groove and alternating torsion angles between A-T and T-A steps.⁶⁶⁻⁶⁸ The narrower grooves of these A-T rich sequences have a higher negative electrostatic potential than their G-C counterparts, which, coupled with the fact that there is no amino group protruding into the groove, makes them more attractive to cationic binders, such as the enantiomers in this study.⁶⁴ This explains why both enantiomers have a binding preference for A-T sites over G-C in the mixed sequence DNA, and the flexibility inherent to the A-T rich sequence may allow a closer association of the Λ enantiomer to the helical backbone and thus a more complete intercalation of the dppz ligand compared to the mixed and G-C rich sequences. However, it must be noted that these are speculative explanations and further studies are required in order to gain a more complete understanding into the processes occurring in these systems.

2.4.7 Circular Dichroism Studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with duplex DNA

Circular dichroism (CD) is a spectroscopic technique based on the difference between the absorption of left- and right-circularly polarised light by a molecule.⁶⁹ CD has many applications, such as the study of chiral molecules, as each enantiomer will interact with different amounts of right- and left- circularly polarised light thus resulting in a CD spectrum.⁷⁰

Additionally, CD can be used to measure the interaction between small molecules and macromolecules such as proteins or DNA. Single stranded DNA is chiral due to the confirmation of the sugar residues, but produces only a very weak spectrum. However, when two strands come together to form duplex DNA, chirality is induced resulting from the rearrangement of the DNA bases within the helix. The CD spectrum of B-DNA contains two bands: a positive band at 275 nm and a negative band at 248 nm. The signals are a result of the right-handed chirality of the DNA and are linked by a positive Cotton effect.

The main uses of CD involving DNA are to probe DNA conformational changes and its interaction with small molecules.⁷¹ By adding small quantities of binding compounds, spectral changes indicative of structural modifications occurring to the double helix are monitored.

CD titrations are usually conducted in a manner reverse to the previously discussed experiments, wherein the DNA concentration is kept constant with the ligand concentration being altered. For the purpose of this study, however, two titrations were conducted with each enantiomer. One keeping the DNA concentration constant to monitor the effect, if any, the complexes have on the DNA, and the second keeping the ligand concentration constant to monitor the changes induced in the ligand spectrum upon increasing DNA concentrations.

The initial studies were conducted following the accepted procedure for CD titrations. The concentration of the stDNA was kept constant at 150 μM (OD = 1 at 260 nm) while

varying the concentrations of the Λ and Δ enantiomers. To avoid dilution effects, a range of solutions containing stDNA and each enantiomer in 10 mM sodium phosphate buffer were prepared at P/D ratios of 25, 20, 15, 10 and 5. The resulting spectra are shown in Figure 2.34 below.

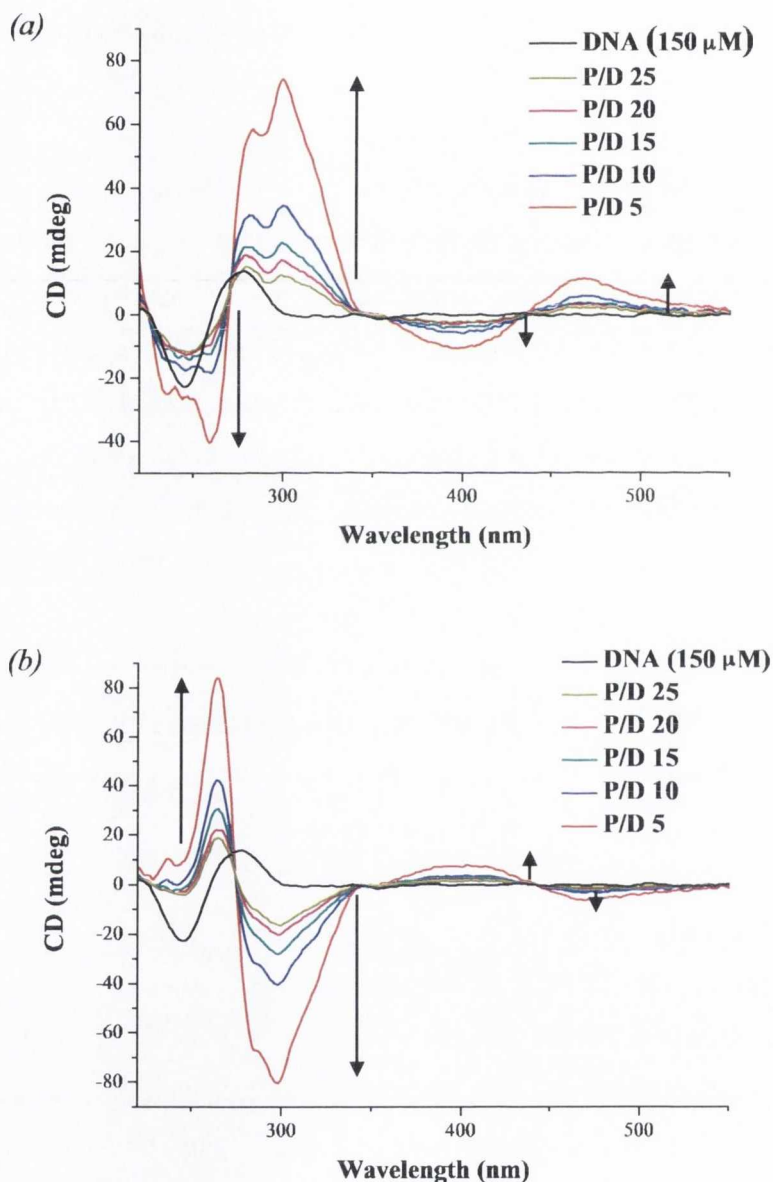


Figure 2.34 – CD spectra of a fixed concentration of stDNA (150 μM) in the presence and absence of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ at varying P/D ratios at 10 mM sodium phosphate buffer

The CD titrations show very little with regard to the changes induced in the spectrum of the DNA due to the spectrum of each enantiomer being strong enough, even at the high P/D of 25, to mask the signal of the DNA. Both enantiomers, between the wavelengths of 350 nm and 550 nm, show increasingly strong signals as the P/D ratio decreases. This region corresponds to the $\text{Ru(II)} \rightarrow \text{dppz}$ MLCT and is, correspondingly, a very weak signal. Naturally, the peaks in this region for the two enantiomers are mirror images of each other, and the increase in strength with decreasing P/D is a result of the increased concentration of the complexes in the sample.

Both enantiomers exhibit an enhancement at 300 nm (positive for the Λ , negative for the Δ) and 275 nm (negative for the Λ , positive for the Δ) with decreasing P/D ratios. Again, this is attributed to the increased concentration of the complexes in the sample. However, the spectra of both enantiomers in this region are not mirror images of each other. The overall chirality of Λ and Δ is maintained, but the spectrum shapes have altered between the enantiomers, indicating that the enantiomers are interacting with the DNA in different manners. This, of course, is expected as the spectroscopic titrations have shown that the Δ enantiomer binds more tightly to the DNA duplex, and the Λ enantiomer is known to cross-link DNA duplexes (Chapter 3). Unfortunately, because the signals from the complexes, even at high P/D ratios are so strong, it is impossible to comment on the changes they have induced on the DNA signal.

Following this first titration, a second titration was performed, opposite to that described above. The concentration of the enantiomers was kept constant ($2.1 \times 10^{-5} \text{ M}$) while aliquots of stDNA ($1.5 \times 10^{-2} \text{ M}$) were added to the buffered solutions. The spectra were recorded from P/D ratios of 0 to 25, however at a ratio of 19, the concentration of the stDNA became too high for accurate measurement at wavelengths below 300 nm. For clarity, the spectra of the lone enantiomers, and P/D ratios of 5, 10 and 15 are shown in Figure 2.35.

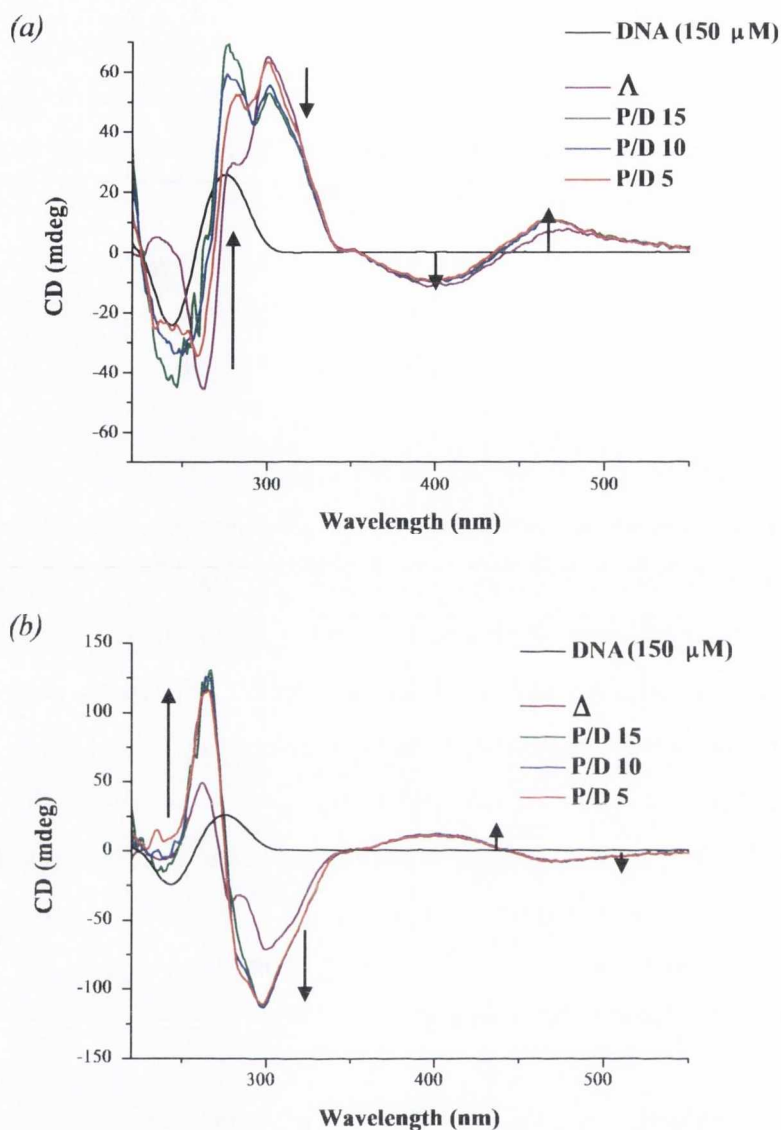


Figure 2.35 – CD spectra of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ (20.1 μM) and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ (19.8 μM) at varying P/D ratios of stDNA at 10 mM sodium phosphate buffer

The second CD titrations show significant changes induced in the spectra of both the Λ and Δ enantiomers upon the addition of stDNA. The presence of the DNA, regardless of concentration, causes a small enhancement of the signals between 350 nm and 500 nm for both enantiomers. As stated previously, this region corresponds to the $\text{Ru}(\text{II}) \rightarrow \text{dppz}$ MLCT and the enhancement in signal is consistent with intercalation. This is expected as it is predicted that the dppz ligand does indeed intercalate into the double helix.

At lower wavelengths, more significant changes are observed, and differences between the Λ and Δ enantiomers are noted though both maintain their overall chirality. The first contrast between the two is the extent to which their spectra are enhanced. The Λ enantiomer, while new peaks appear, does not experience any enhancement of its spectra. The Δ enantiomer, however, is enhanced dramatically. The signal at 300 nm is enhanced by 158% while the signal at 265 nm is enhanced by 261%. Peak changes of this magnitude are consistent with minor groove binding since the compound has more interaction with the chiral sugars of the DNA duplex. The Δ enantiomer experiences this enhancement while the Λ does not because of the difference in the distance between Ru centre and helical axis. The better ability of the Δ enantiomer to fit into the groove reduces this distance, bringing the ancillary ligands nearer the chiral sugars and increasing the interaction between the two. The magnitude of the enhancement also supports the argument that these complexes bind to DNA duplexes via the minor groove.

Interestingly, a new CD peak is formed in with Λ enantiomer at 275 nm, and increases with the increasing concentration of stDNA. While identification of this peak is almost impossible solely from the CD data, the fact that such a peak does not occur in the Δ spectra suggests that this may be caused by the non-covalent cross-linking interactions, believed to only occur in the presence of the Λ enantiomer.

A study of both the 'forward' and 'backward' CD titrations, considered with the other spectroscopic studies thus far presented, allow for various conclusions. It is clear from the data that both enantiomers bind to DNA via insertion of the dppz ligand between the DNA base pairs. The enhancement of the Δ spectrum implies that the complexes bind at the minor groove and that the Δ enantiomer is the more tightly bound of the two. Possible evidence for the non-covalent cross-linking interactions with the Λ enantiomer is observed with the formation of a new ICD peak, however this remains only speculation without further evidence. The use of linear dichroism will further characterise the orientation of the enantiomers within the helix.

2.4.8 Linear Dichroism Studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with duplex DNA

Linear Dichroism (LD) is a powerful method for determining the binding mode of small molecules to DNA. It is defined as the difference in the absorption of parallel polarised light (A_{parallel}) and perpendicular polarised light ($A_{\text{perpendicular}}$) to an orientation axis according to equation 2.13

$$LD = A_{\text{parallel}} - A_{\text{perpendicular}} \quad (2.13)$$

DNA duplexes, being long and thin, are ideally suited to linear dichroism as they are easy to align in flow.^{72,73} Once aligned, the direction of polarisation in a molecule can be assigned relative to its orientation (Figure 2.36).

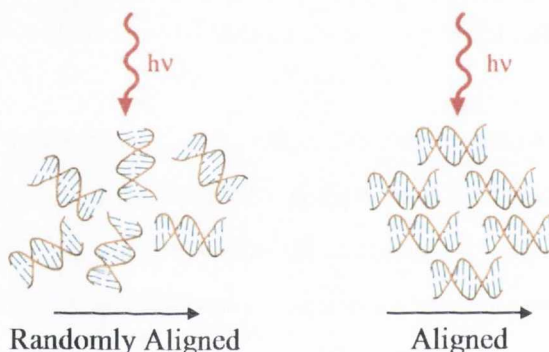


Figure 2.36 – Schematic representation of DNA molecules orientated in flow

The LD spectrum of DNA shows a strong negative signal at 260 nm, resulting from the absorbance of the DNA base pairs that are, of course, perpendicular to the helical axis. As such LD can provide important information about the nature of binding compounds quite easily. The appearance of an LD peak outside the region of DNA absorbance indicates that the compound is indeed interacting with the duplex and has become orientated. The nature and magnitude of the various signals provide more precise structural information regarding the DNA-ligand association. Generally, if an LD signal is > 0 , the transition is said to absorb light parallel to the helical axis. Conversely, if the LD signal is < 0 , the transition is said to absorb light perpendicular to the helical axis. Groove binding compounds are usually arranged parallel to the DNA axis and thus have a positive signal

in LD measurements, while intercalating molecules stack between the base pairs which are perpendicular to the helical axis and thus a negative LD signal is observed.

The LD studies of the enantiomers were conducted in a similar manner to the initial CD experiments wherein the concentration of the stDNA was kept constant (350 μM) with the concentration of the binding complexes being varied. A flaw to this technique is the very small sample volume (65 μL) used and consequently each solution was prepared individually. This often results in a wandering baseline between spectra. The LD spectra resulting from the addition of the enantiomers to stDNA are shown in Figure 2.37.

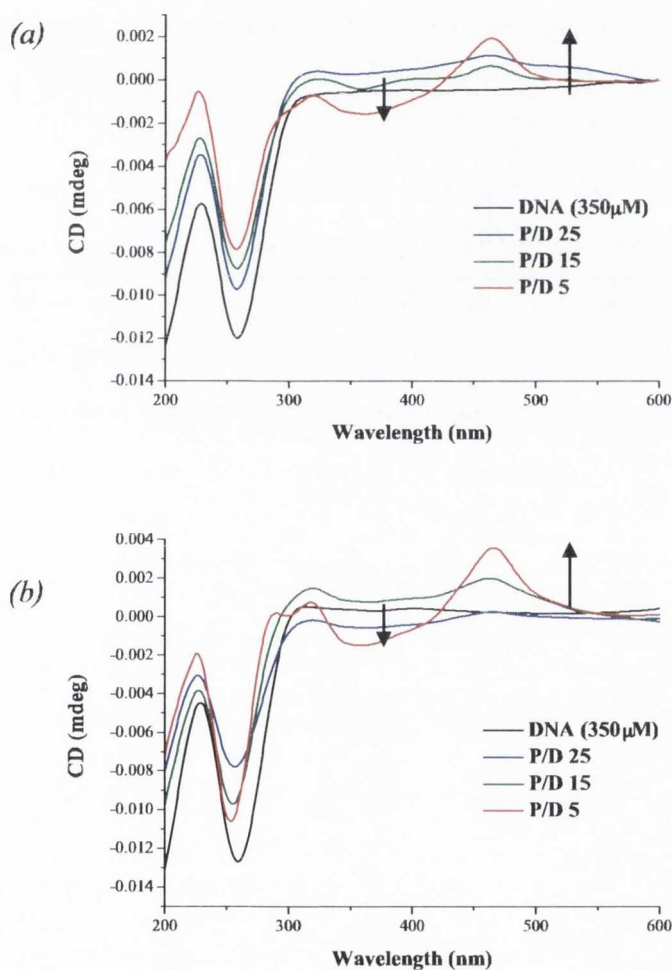


Figure 2.37 – LD spectra of stDNA (350 μM) in the presence and absence of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ at varying P/D ratios at 10 mM sodium phosphate buffer

It is clear from the changes observed that both enantiomers interact with DNA and have become orientated with the helical axis. A negative band at 350 nm in both spectra corresponds to the $\pi-\pi^*$ transitions within the dppz ligand. The fact that signal is negative implies absorption of perpendicular light, confirming the assumption that the dppz ligand intercalates between the base pairs. A stronger positive signal is noted at 475 nm indicating the absorbance of parallel light. This corresponds to the $\text{Ru(II)} \rightarrow \text{TAP}$ MLCT transition of the ancillary ligands, which naturally, are located in the groove of the DNA, parallel to the helical axis and again confirming the intercalative binding mode of the enantiomers. This signal is stronger than that of the dppz (in comparison to its $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ analogue) because the $^3\text{MLCT}$ state now resides on the TAP ligands. It is also clear that these signals in the spectra increase with increasing complex concentration.

Finally, comparison of the spectra generated from the binding of the Λ enantiomer with that of the Δ enantiomer shows that the signals produced with the latter are larger in magnitude. This again reflects the better ability of the Δ enantiomer to bind to duplex than the Λ , owing to the orientation of its ancillary ligands facilitating a more complete intercalation interaction. Figure 2.38 below compares the LD spectra of the Λ and Δ enantiomers at a P/D ratio of 5:1 to allow direct comparison of their signal magnitude.

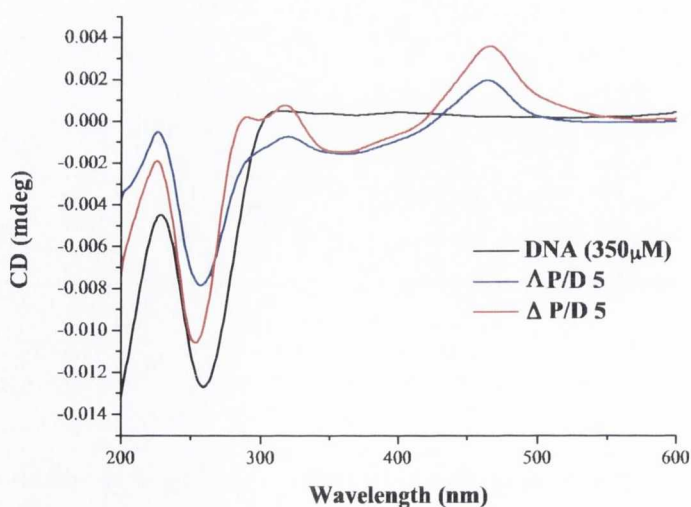


Figure 2.38 – LD spectra of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with stDNA (350 μM) at a P/D ratio of 5 at 10 mM sodium phosphate buffer

2.4.9 Summary of DNA Binding Studies

In this chapter, the binding affinities of the Λ and Δ enantiomers of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex for DNA have been explored in aqueous solutions using stDNA (salmon testes), $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$. The ground and excited state interactions of the enantiomers were firstly evaluated, yielding distinct spectral changes that enabled the determination of binding constants K_b of the order of 10^7 to 10^8 M^{-1} . Both enantiomers were shown to exhibit the ‘light switch off’ effect in the presence of DNA, a phenomenon resulting from the presence of the π -deficient electron accepting ligands TAP that facilitate the photoinduced electron transfer between the bound complex and the guanine residues within the DNA.

Two binding modes are observed upon the addition of both mixed sequence DNA and double stranded synthetic polynucleotides. The first, indicated by a dramatic hypochromicity at low P/D ratios in the absorbance spectra, is the non-specific binding via intercalation of the dppz ligands between base pairs. This interaction generally occurs until the duplex is saturated, at which point a second binding mode dominates. This is the electrostatically driven association of the remaining free complexes with the grooves and possible partial intercalation of the dppz ligands. This mode is observed by a small hyperchromicity in the absorbance spectra at higher P/D ratios. These trends have previously been reported with the DNA binding of a number of complexes including *rac*- $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, *rac*- $[\text{Os}(\text{phen})_2\text{dppz}]^{2+}$, and *rac*- $[\text{Ru}(\text{TAP})_2\text{PHEHAT}]^{2+}$.^{2,14,46-48}

Increasing the ionic strength of the buffer had the desired effect of reducing both the enantiomers’ ability to bind to the DNA, allowing the calculation of more reasonable and accurate binding constants (K_b of the order of 10^5 M^{-1}). It also serves to validate the theory of a second electrostatically driven binding mode at higher P/D ratios as the increased salt concentration reduces the electrostatic attraction between the cationic complexes and anionic duplexes.⁴⁸ Indeed, at the higher ionic strength of 100 mM sodium phosphate buffer, the hyperchromicity in the absorbance spectra at higher P/D ratios was decreased compared to the same experiment in 10 mM sodium phosphate conditions.

The reverse salt titration served to corroborate three hypotheses. The first is that the binding of such ruthenium polypyridyl complexes to duplex DNA is a dynamic process

and while the complexes exhibit an extremely high binding affinity for DNA, the process is ultimately reversible. Secondly, the dominating mode of binding is the intercalation of the dppz ligand between the base pairs. The ability of increased sodium concentrations to cause a once bound complex to be released from DNA serves to indicate the strength of binding of the complex. Groove binders are typically displaced at lower ionic concentrations ($\leq 0.001 \text{ M}$)⁷⁴ due to the interactions between compound and duplex being mostly electrostatic and hydrogen bonding. Intercalating complexes are generally more difficult to remove due to the nature of the stability imparted from the interaction of the π orbitals, often coupled with electrostatic interactions. As a result, much higher salt concentrations ($\leq 0.1 \text{ M}$) are required to cause adequate unbinding. Finally it once again demonstrated the higher binding ability of the Δ enantiomer compared to the Λ , as the Λ enantiomer was fully liberated from the duplex in the presence of 0.44 M NaCl while the Δ enantiomer was not fully displaced at this concentration.

Thermal denaturation studies were performed to further confirm the enantiomers' affinity for binding to DNA and demonstrated that the Δ enantiomer binds more tightly to the duplexes than the Λ enantiomer. This is undoubtedly a result of the orientation of the ancillary ligands in both enantiomers.

Studies in the presence of the homopolymers $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$ revealed modest specificity with both enantiomers exhibiting a preference for binding to $[\text{poly}(\text{dA-dT})]$. Further evidence for the photoinduced electron transfer process between the enantiomers was shown as the fluorescence emission was quenched by $>95\%$ in the presence of $[\text{poly}(\text{dG-dC})]$. The emission enhancement recorded in the presence of $[\text{poly}(\text{dA-dT})]$ is a result of a decrease in the efficiency of the non-radiative deactivation process of the $^3\text{MLCT}$ excited state of these complexes induced by the double helix microenvironment and protection from oxygen quenching.

Circular and linear dichroism investigations were conducted to confirm the suspected binding mode of intercalation of the dppz ligand.

The main objectives of this chapter were to separate and purify the two enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and perform a full investigation of their DNA binding abilities. The results show that even though the complexes contain π -deficient electron accepting

ligands, the trends of enantiomeric binding reported previously with their Λ - and Δ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ cousins remain consistent. The binding affinities of both complexes are extremely high but the Δ enantiomer is found to be the better able of the two to bind to DNA. Both display a preference for A–T binding sites and both infer a degree of thermal stability to the DNA macromolecule.^{2,3,5,75} The remainder of this chapter will deal with the final objective of the enantiomeric study; is there an enantiomeric effect on the forward and back-electron transfer pathways, observed with in racemic studies, utilising ultra-fast techniques?

2.5 Enantiomeric Effect on Electron-Transfer Reactions

The study of electron-transfer reactions in DNA is an important issue both from a fundamental materials science perspective⁷⁶⁻⁷⁸ and because of their biological consequences.⁷⁹⁻⁸¹ As such, electron transfer reactions are fast chemical processes that are usually initiated by the short laser-pulse excitation of sensitizers such as anthraquinones⁸² or metal complexes.^{24,83,84} Ruthenium polypyridyl complexes are excellent sensitizers for probing the various properties of DNA as their photophysical and electrochemical properties can be varied readily by exchange of the ancillary ligands. Complexes containing extended aromatic planar ligands are particularly attractive as their mode of binding is well understood as being intercalation of this ligand. A further requirement of these sensitising complexes is that they must have a redox chemistry sufficient to produce the guanine cation radical upon excitation. The incorporation of π -deficient electron accepting ligands imparts the required redox chemistry, and as such there have been previous investigations into the formation of transient species formed from $[\text{Ru}(\text{TAP})_3]^{2+}$, $[\text{Ru}(\text{TAP})_2\text{bpy}]^{2+}$ and *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$.^{18,19,24} To investigate the effect the various reactions between sensitizing compounds and DNA, highly specialised spectroscopic techniques are employed in which the sensitizing compounds are excited via a laser pulse (the pump) and the resulting changes to the DNA structure are observed via a second laser pulse (the probe).

The spectroscopic techniques required to perform these measurements on a picosecond and nanosecond timescale are highly specialised, involving powerful lasers and technical equipment. The two techniques used in this work are visible transient absorption spectroscopy (TA) and time-resolved infra-red spectroscopy (TRIR).

The nanosecond and picosecond results presented here are the product of a successful collaborative project with Prof. Michael George, Dr. Xue-Zhong Sun, Dr. Timothy Easun and Mr. Jonathan Pitchford of the University of Nottingham and Dr. Mike Towrie and Dr. Greg Greetham of the CCLRC Rutherford Appleton Laboratory, UK.

2.5.1 Ultrafast Techniques for Studying Excited States of DNA

The study of these processes within nucleic acids has been facilitated by recent advances in femtosecond spectroscopy. Two of the most important solution-phase methods are transient UV/vis absorption spectroscopy (TA) and time-resolved mid-infrared spectroscopy (TRIR). Both of these techniques are two-pulse processes in which a percentage of the sensitising complexes are promoted to an electronically excited state by means of an excitation (or pump) pulse. A weak probe pulse (i.e., a pulse that has such a low intensity that multiphoton/multistep processes are avoided during probing) is then sent through the sample with a delay τ with respect to the pump pulse. Depending on the wavelength of the probe pulse, information regarding the UV/vis or IR spectra can be obtained.

TA allows for the monitoring of both excited-state absorption and ground-state recovery of the nucleobases. It also offers some spectral information regarding the emissive and non-emissive excited states and other transient species.⁸⁵ TRIR offers all the features of TA plus far superior structural detail and information regarding specific vibrational modes, particularly the in-plane aromatic and carbonyl stretches between the region of 1400 cm^{-1} and 1750 cm^{-1} (Figure 2.39). The major disadvantage of TRIR is that due to the strong absorbance of water in this particular region, use of D_2O as the solvent is required.

The equipment used in the following studies was the Time-Resolved Picosecond apparatus at the University of Nottingham and the Ultrafast apparatus at the CCLRC Rutherford Appleton Laboratory.

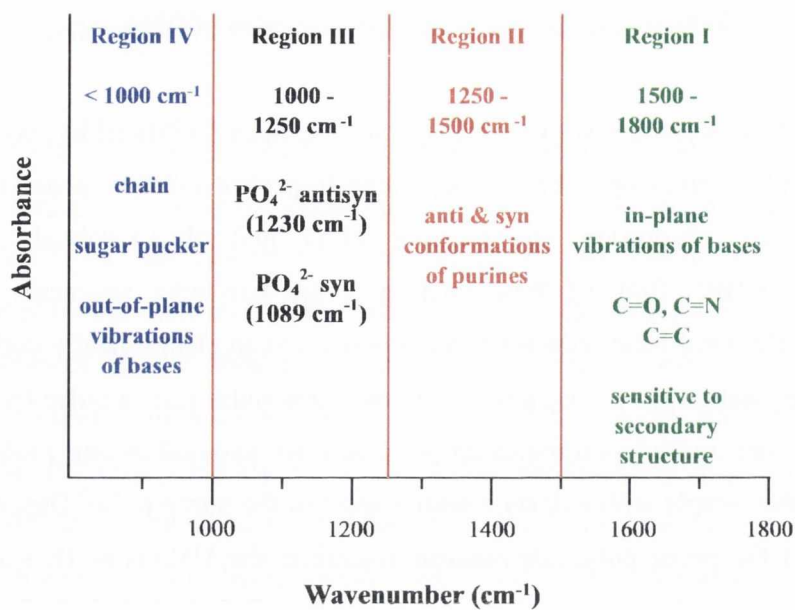
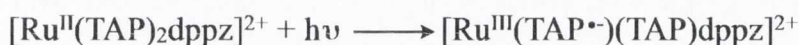


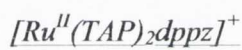
Figure 2.39 – Assignment of the signals in the infrared spectrum of the fingerprint regions of DNA

2.5.2 Studies with *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

A previous study was conducted on the transient species formed by the photoexcitation of the *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex in aqueous solution with the double-stranded polynucleotide [poly(dG-dC)].²⁴ The results from this investigation revealed that in the presence of a sufficiently strong reducing agent, such as guanine, excitation of *rac*- $[\text{Ru}^{\text{II}}(\text{TAP})_2\text{dppz}]^{2+}$ results in the formation of *rac*- $[\text{Ru}^{\text{II}}(\text{TAP})_2\text{dppz}]^+$ through the electron transfer process outlined in Scheme 2.3 below. This intermediate species strongly absorbs at between 500 nm and 550 nm and can be measured by TA. The subsequent changes induced in the guanine residue were observed via TRIR.



Scheme 2.3 – Electron transfer process resulting in the formation of the transient species



Interestingly it is at this point where covalent adducts have previously been shown to form between an amino group of a guanine base and methine group from one of the TAP ligands (see Figure 1.20) of $[\text{Ru}(\text{TAP})_3]^{2+}$.¹⁹ The formation of such an adduct has not thus far been reported for the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex.

This study, using picosecond TA and TRIR techniques, did show two processes occurring within the system upon excitation, forward and back electron transfer (though the lifetime of this latter process was estimated to be in the order of nanoseconds and so no significant data regarding it was obtained). The forward electron transfer process (lifetime of 506 ps) was found to be much slower compared with other sensitizers (lifetimes $\approx$ 200 fs).⁸⁶ This may be attributed to the fact that these other sensitizers are in close contact and strongly coupled to the polynucleotide base stack. With the $[\text{Ru}^{\text{III}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^{2+}$ complex, the ruthenium is located nearer the wall of the groove, thus the increase in distance slows the rate at which the electron from the guanine residue can effect the reduction of the excited state complex.

Once the ruthenium complex has been excited and its subsequent reduction coupled with the oxidation of the guanine residue has occurred (with a rate constant of $2.0 \times 10^9 \text{ s}^{-1}$), a second process was observed with the much longer lifetime (estimated at 8 ns) and slower rate constant of $1.1 \times 10^8 \text{ s}^{-1}$. This is attributed to the back electron transfer process whereby the oxidised guanine residue is reduced back to its ground state by the reduced ruthenium species also returning to its ground state.

Furthermore, a very short-lived species, with a lifetime of only 41 ps, was recorded during the psTA experiments. This species was recorded in both the presence and absence of [poly(dG-dC)] leading to the suggestion that is the initially formed excited state ($^1\text{MLCT}$) which is rapidly converted to the long-lived triplet Ru-TAP MLCT state (see Figure 1.14).

Curiously, a change in the rate constants of both the forward and back electron transfer reactions was noted upon deuteration of the solvent. Both processes were found to be slower in D_2O than H_2O yielding an isotope effect of k_H/k_D of approximately 1.3 for the forward reaction and 1.6 for the back reaction. This observation led to the assumption that both the oxidation and reduction processes involved may not be simple electron transfer but proton-coupled electron transfer. The most likely process occurring in the forward reaction involves a transfer of the imino proton from the guanine to yield the protonated cytosine, with the return of this proton to the guanine occurring to facilitate the back electron transfer (Scheme 2.4).

(a)



(b)



Scheme 2.4 – The proposed (a) proton-coupled forward electron transfer and (b) back electron transfer processes

The suggestion of the proton-coupled electron transfer processes allowed for the proposal of the formation of the guanine radical upon transfer of the proton to its partnered cytosine. Further studies are still required to corroborate this theory, however the work with the racemic complex demonstrated the forward and back electron transfer processes that are responsible for the 'light-switch off' effect of the complex's fluorescence upon the addition of DNA and introduced the isotope effect on said reaction rates. Following the success of both this work and the separation of the Λ and Δ enantiomers, an investigation into the differences the enantiomeric effect has on the rate of the back electron transfer processes was undertaken, a process not previously studied. The remainder of this chapter will discuss the results obtained.

2.5.3 Ultrafast Investigations of Λ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

The nanosecond transient absorption (ns-TA) and nanosecond time resolved infrared (ns-TRIR) measurements were conducted in collaboration with Jonathan Pitchford, Dr. Timonthy Easun, and Dr. Xue-Zhong Sun at the University of Nottingham and the Rutherford Appleton Laboratories under the supervision of Prof. Michael George (Nottingham) and Prof. John Kelly (Trinity). The results from both sites were very similar, indicating an acceptable reproducibility within the enantiomeric systems.

Samples for both the ns-TA and ns-TRIR were prepared to 2.5×10^{-4} M [ruthenium enantiomer] at a P/D of 20:1 in a Harrick cell (CaF_2 plates) and excited at a pump wavelength of 400 nm. UV/visible absorption spectra were taken both before and after each experiment and showed the sample decomposition to be negligible. A high P/D ratio was used in an attempt to maximise the amount of complex bound and to minimise any aggregation effects.

2.5.3.1 ns-TA of Λ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ in D_2O

ns-TA experiments were initially conducted with both the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ in deuterium oxide. Each resulting spectrum displayed similar trends, a strong bleach between 400 and 500 nm with a significant positive absorbance between 500 and 550 nm, shown in Figure 2.40. This absorbance results from the ruthenium complex only because the oxidized guanine, whether it is the radical cation ($\text{G}^{\bullet+}$) or the deprotonated radical ($\text{G}^\bullet$), absorbs very weakly above 500 nm and thus will not contribute significantly to the signal.²⁴

The TA spectra were recorded between 0 and 500 ns after excitation of the sample with 400 nm irradiation. The absorption changes were fitted by means of multi-Lorentzian curve fitting functions within the OriginPro 8.0 data processing software.

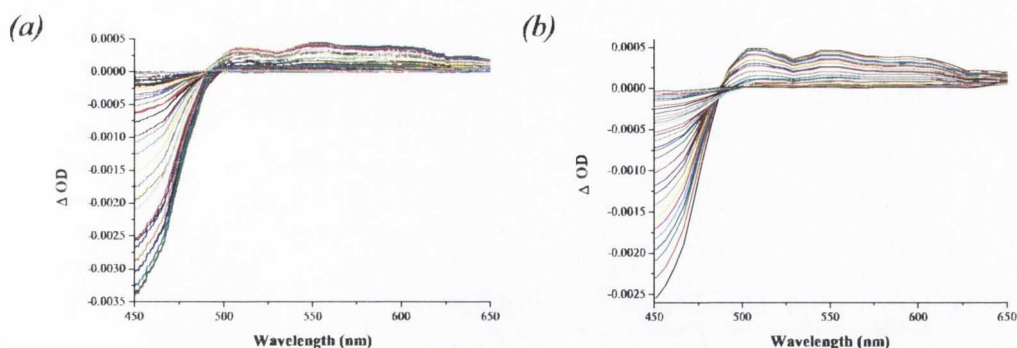


Figure 2.40 – ns Transient Absorption spectra of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ in 10 mM sodium phosphate buffer D_2O

After excitation the complexes are expected to initially populate the dppz centred $\pi\text{-}\pi^*$, the Ru-TAP $^1\text{MLCT}$ and possibly the Ru-dppz $^1\text{MLCT}$ excited states. The intersystem crossing is expected to occur rapidly ($< \text{ns}$) to yield the longer lived Ru-TAP $^3\text{MLCT}$ state, formally represented as $[\text{Ru}^{\text{III}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^{2+}$. This complex is then presumed to oxidise the guanine residue via PCET and reduce to yield the $[\text{Ru}^{\text{II}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^+$ excited state (Scheme 2.3 (a) previously). BET is then expected to occur reducing the guanine radical and oxidising the ruthenium complex to the ground state $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ (Scheme 2.3 (b) previously).

The kinetics obtained (Figure 2.41) from the band fitting at 510 nm (the maxima of the absorbance signal) with both the Λ and Δ enantiomers supports the observations made from the spectra. The kinetics were best fitted to a double exponential decay, with the first lifetime being sub-nanosecond and thus cannot be accurately recorded from these experiments. This short-lived species is presumed to be the excited MLCT state ($[\text{Ru}^{\text{III}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^{2+}$) which undergoes PCET to oxidise the guanine residue and form the reduced $[\text{Ru}^{\text{II}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^+$ complex in the picosecond studies performed with the racemic complex.^{24,87} The second lifetime is assigned to the reduced complex as it is oxidised to the ground state via back electron transfer. The data from these experiments show that the process of BET occurs more slowly for the Λ enantiomer than with the Δ enantiomer in D_2O as shown in the Table 2.13.

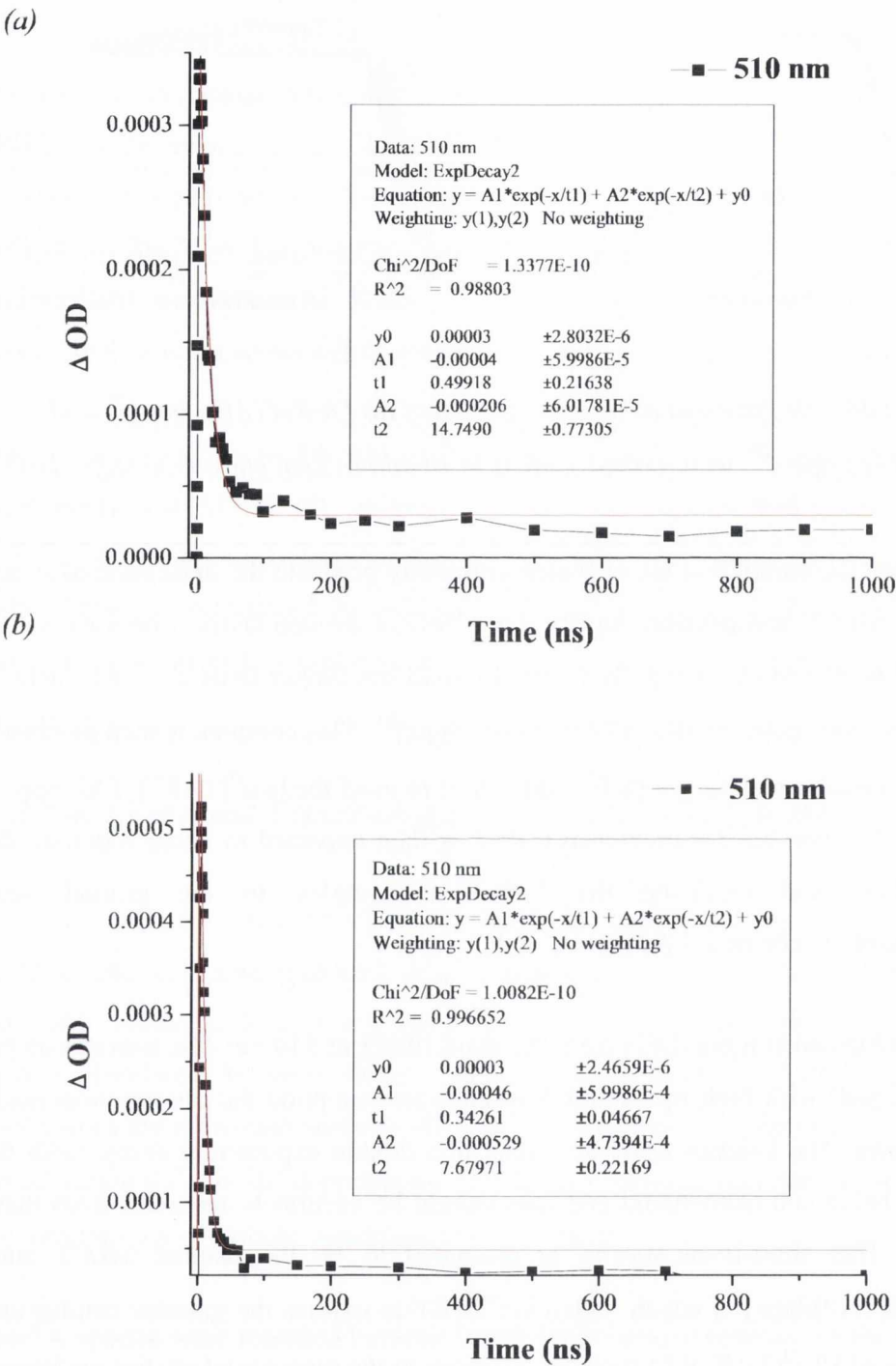


Figure 2.41 – Kinetic plots and data of the nsTA data collected from (a) Λ - and (b) Δ - $[Ru(TAP)_2dppz]^{2+}$ binding to $[poly(dG-dC)]$ at 510 nm in D_2O buffer

racemic	Λ	Δ
$\tau_1 - 0.7 \text{ ns}^{24}$ $\tau_2 - 14 \text{ ns}^{24}$	$\tau_1 - 0.5 \text{ ns}$ $\tau_2 - 14.7 \text{ ns}$	$\tau_1 - 0.3 \text{ ns}$ $\tau_2 - 7.7 \text{ ns}$
$k_2 - 1.3 \times 10^7 \text{ s}^{-1}$	$k_2 - 1.2 \times 10^7 \text{ s}^{-1}$	$k_2 - 2.1 \times 10^7 \text{ s}^{-1}$

Table 2.13 – The lifetimes of the $[\text{Ru}^{\text{II}}(\text{TAP}^)(\text{TAP})\text{dppz}]^+$ species in the racemic, Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ from the ns-TA experiments with $[\text{poly}(\text{dG}-\text{dC})]$ in D_2O buffer*

These results show a dramatic difference between the enantiomers. The lifetime of the reduced ruthenium intermediate for the Λ enantiomer is almost twice that of the Δ so that the rate constant of the BET process for the Δ enantiomer is almost twice that of the Λ enantiomer. This significant effect may again be rationalised by the degree of the dppz intercalation experienced by each enantiomer.

As the previous binding studies have eluded to, the orientation of the ancillary TAP ligands of the Δ enantiomer allows the dppz ligand to intercalate more completely between the base pairs. This has the effect of shortening the distance between the ruthenium centre and the DNA helical axis. As a result, the ruthenium ion of the Δ enantiomer will be significantly closer to the guanine residue than its Λ counterpart. The process of back electron transfer, following PCET, is proposed to involve the exchange of a hydrogen atom from the now protonated cytosine residue back to the guanine radical, coupled with the donation of an electron from the reduced $[\text{Ru}^{\text{II}}(\text{TAP}^*)(\text{TAP})\text{dppz}]^+$ species to reform the ground state $[\text{Ru}(\text{TAP})_2\text{dppz}]_{2+}$ and $\text{poly}[\text{dG}-\text{dC}]$, as shown in Figure 2.42. The increased ruthenium – guanine distance with the Λ enantiomer increases the distance over which the electron has to relocate and also reduces the oxidative power of the $\text{GH}^{\bullet+}$ species. This has the effect of reducing the BET rate constant and yielding a longer lifetime for the reduced ruthenium intermediate compared to that of the Δ .

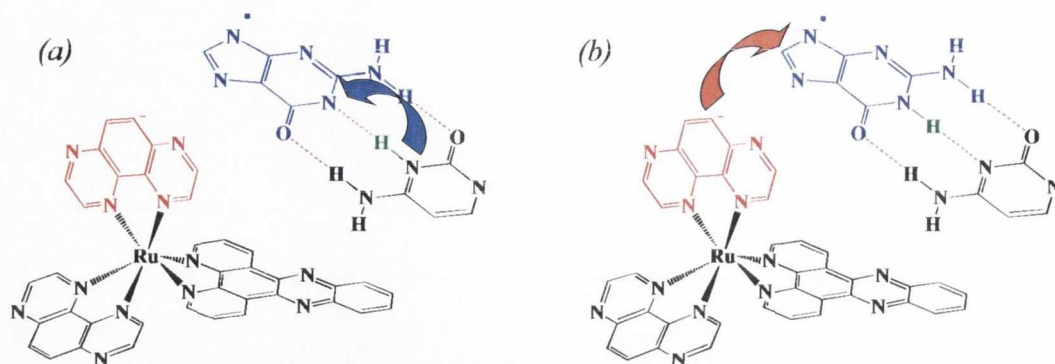


Figure 2.42 – A representation of the back electron transfer process between $[\text{Ru}^{\text{II}}(\text{TAP}^{\bullet})(\text{TAP})\text{dppz}]^+$ and the guanine radical. (a) The proton exchange between CH^+ and $\text{G}^{\bullet}$. (b) The electron relocation from $\text{TAP}^{\bullet}$ to $\text{GH}^{\bullet+}$

Note! These two processes are simultaneous

2.5.3.2 ns-TA of Λ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG}-\text{dC})]$ in H_2O

The same ns-TA experiments were repeated with both the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG}-\text{dC})]$ but in water. The TA spectra were recorded between 0 and 500 ns after excitation of the sample with 400 nm irradiation. The absorption changes were fitted by means of multi-Lorentzian curve fitting functions within the OriginPro 8.0 data processing software. Figure 2.43 shows the spectra recorded from the experiments, with Figure 2.44 detailing the kinetics plots.

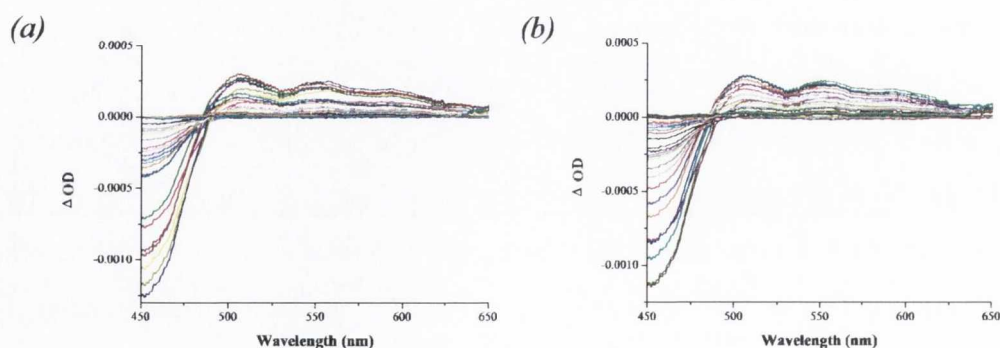


Figure 2.43 – ns Transient Absorption spectra of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG}-\text{dC})]$ in 10 mM sodium phosphate buffer H_2O

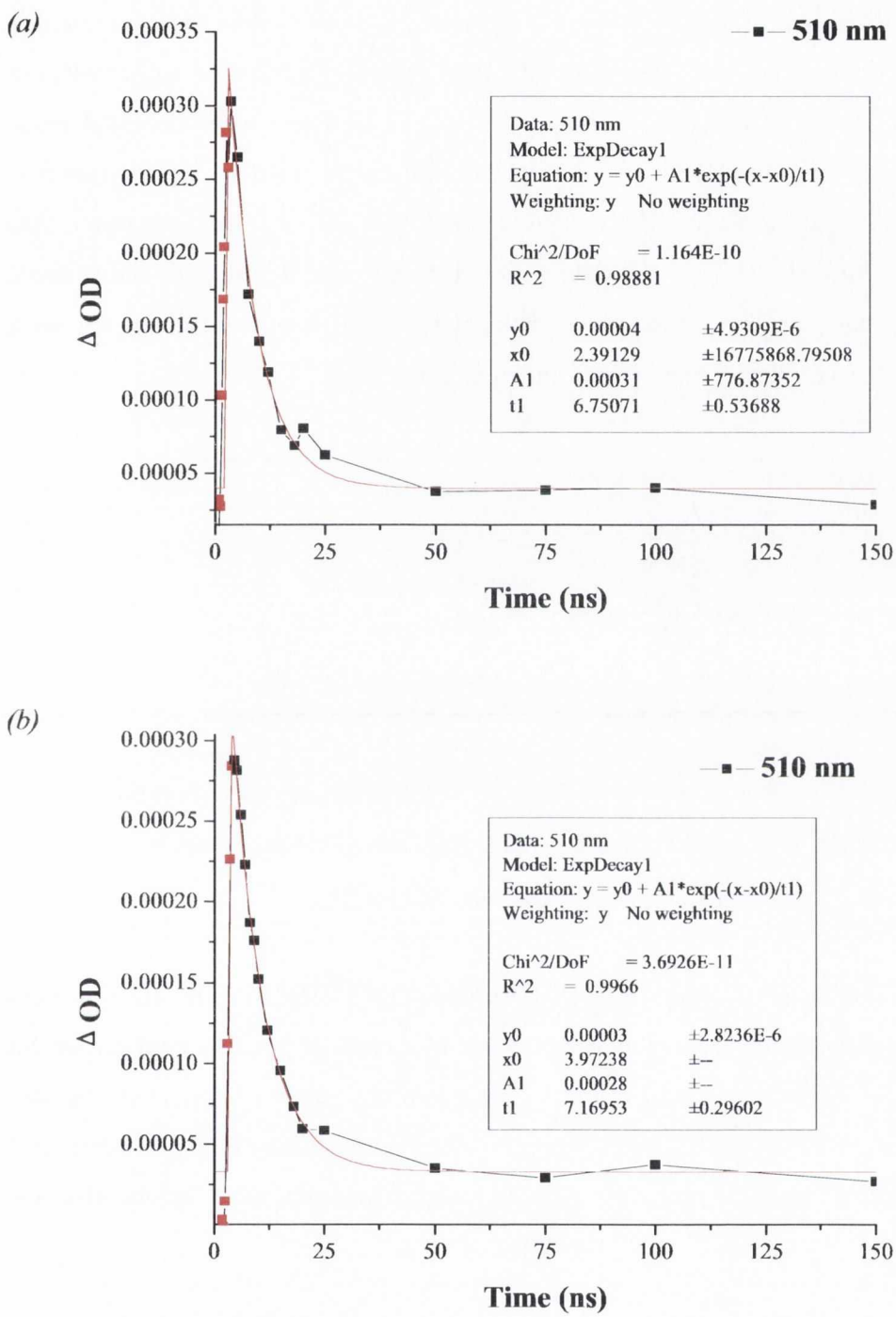


Figure 2.44 – Kinetic plots and data of the nsTA data collected from (a) Λ - and (b) Δ - $[Ru(TAP)_2dppz]^{2+}$ binding to $[poly(dG-dC)]$ at 510 nm in H_2O buffer

The spectra and kinetics obtained (from the band fitting at 510 nm) of the two enantiomers in water are quite different to the results in D_2O . Where the enantiomeric effect on the BET process was evident in D_2O , there seems to be no such effect when in H_2O . It was shown previously that both the PCET and BET processes occurred much more slowly in D_2O than in H_2O . As a result, the lifetimes detected for the intermediate species from the PCET in the initial TA experiments (D_2O) are not detected in water, thus the kinetics are best fitted to a mono-exponential decay. The lifetimes of the reduced complexes oxidising to the ground state recorded for both enantiomers in water were approximately 7 ns (within error) and are shown in Table 2.14.

racemic	Λ	Δ
$\tau_1 - 8.5 \text{ ns}^{24}$ <i>derived value</i>	$\tau_1 - 6.8 \text{ ns}$ (± 0.5)	$\tau_1 - 7.2 \text{ ns}$ (± 0.3)
$k_2 - 1.0 \times 10^7 \text{ s}^{-1}$	$k_2 - 1.3 \times 10^7 \text{ s}^{-1}$	$k_2 - 1.2 \times 10^7 \text{ s}^{-1}$

Table 2.14 – The lifetimes of the $[\text{Ru}^{\text{II}}(\text{TAP}^)(\text{TAP})\text{dppz}]^+$ species in the racemic, Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ from the ns-TA experiments with $[\text{poly}(\text{dG}-\text{dC})]$ in H_2O buffer*

These results indicate that when in D_2O , an enantiomeric effect on the BET process occurs. When in water however, this effect is not observed. A possible explanation for this could be the difference in atomic size as deuterium represents a 100% mass increase from hydrogen. Whereas in D_2O , the relative distance between the ruthenium centre and oxidised guanine dictated the rate of reaction, in water, it is unsure what factors affect the rate of BET, however clearly distance is not one of them.

2.5.3.3 ns-TRIR of Λ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG}-\text{dC})]$ in D_2O

Experiments on the nanosecond timescale using transient infrared absorption spectroscopy (TRIR) of the two enantiomers in the presence of $[\text{poly}(\text{dG}-\text{dC})]$ were conducted in parallel to the ns-TA. The same samples were used for both techniques, again with the UV/visible absorption spectrum taken before and after each experiment to

check for sample degradation. Where transient absorption spectroscopy yields information pertaining to the nature of the excited states of the ruthenium complexes, TRIR spectroscopy probes the Watson-Crick hydrogen bonds of the duplex, allowing the monitoring of any changes induced in the polynucleotide. Unlike the nsTA experiments, which were conducted in both H_2O and D_2O , the nsTRIR experiments can only be conducted in D_2O due to water absorbing too strongly in this region of infrared.

The $1572 - 1725 \text{ cm}^{-1}$ region of the fourier transform infrared spectroscopy (FTIR) spectrum of $[\text{poly}(\text{dG-dC})]$ contains strong absorptions assigned primarily as the carbonyl stretches of guanine (at 1690 cm^{-1}) and cytosine (at 1659 cm^{-1}).^{88,89} The TRIR spectrum obtained by direct excitation of the polynucleotide (267 nm) is shown in Figure 2.45 and is dominated by the transient bleaching of these two bands.⁹⁰

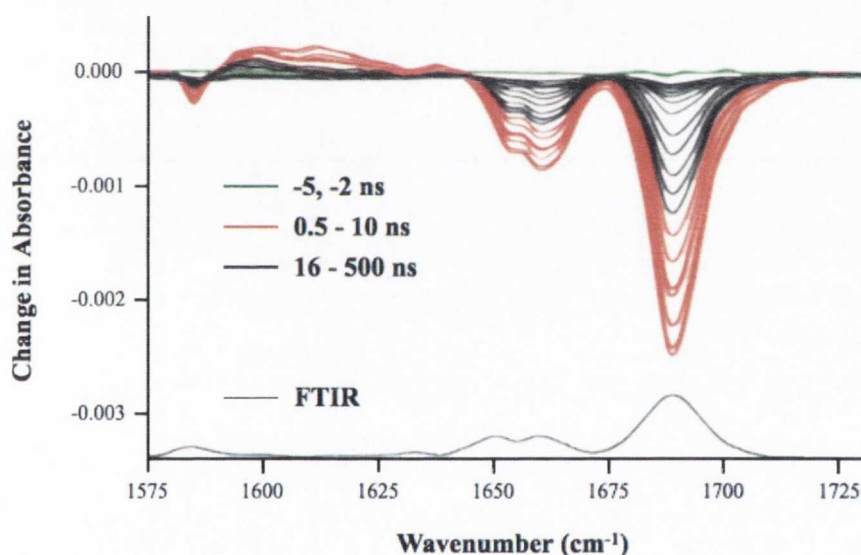


Figure 2.45 – nsTRIR of B-form $[\text{poly}(\text{dG-dC})]$ (10 mM) in 10 mM sodium phosphate D_2O buffer with the FTIR spectrum below⁹⁰

The TRIR experiments in this work were conducted with a λ_{ex} of 400 nm at which the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ absorbs but not the polynucleotide. As a result, the TRIR transitions observed in Figure 2.46 are a result of guanine oxidation by the excited state of the ruthenium complex. The TRIR spectra were recorded for each enantiomer between 0.5 and 500 ns after excitation of the sample with 400 nm irradiation.

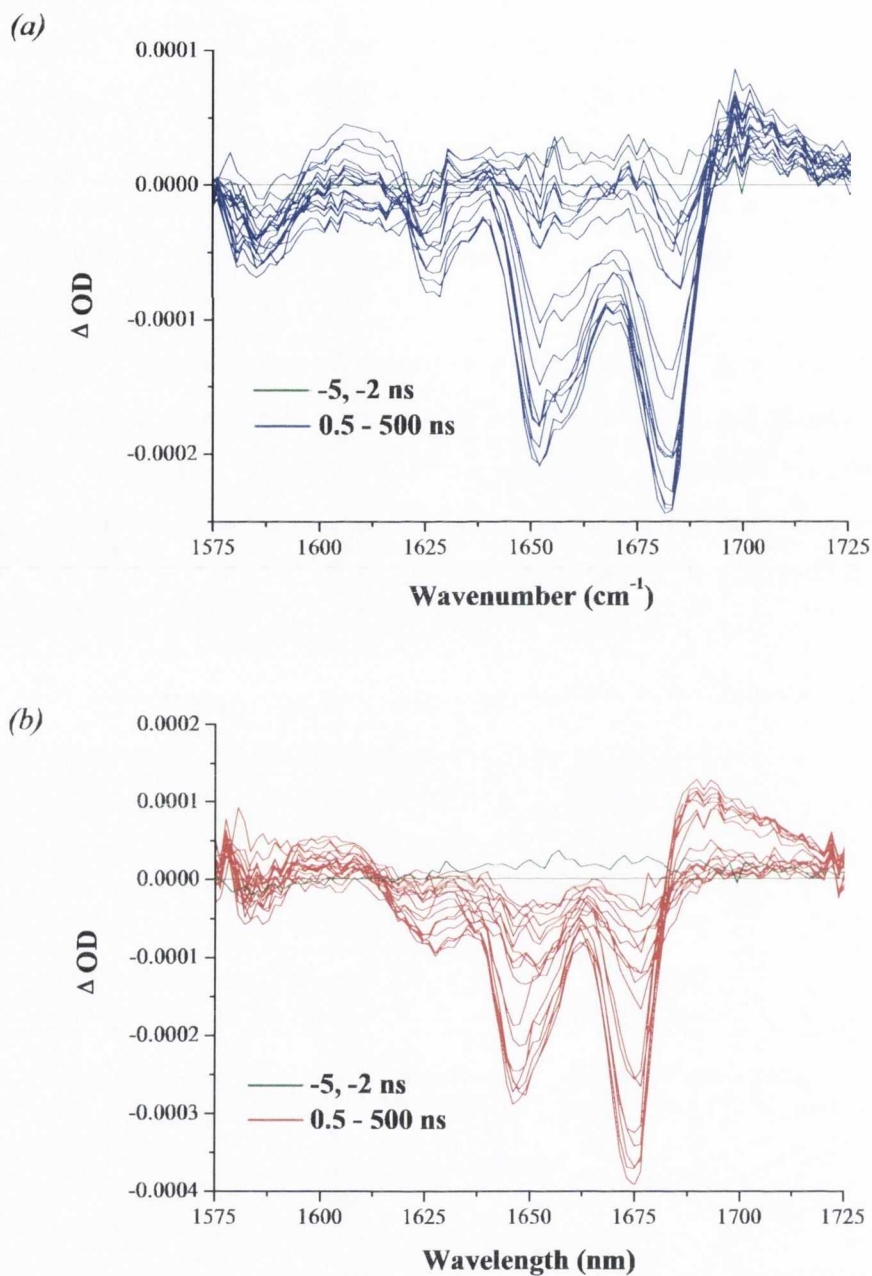


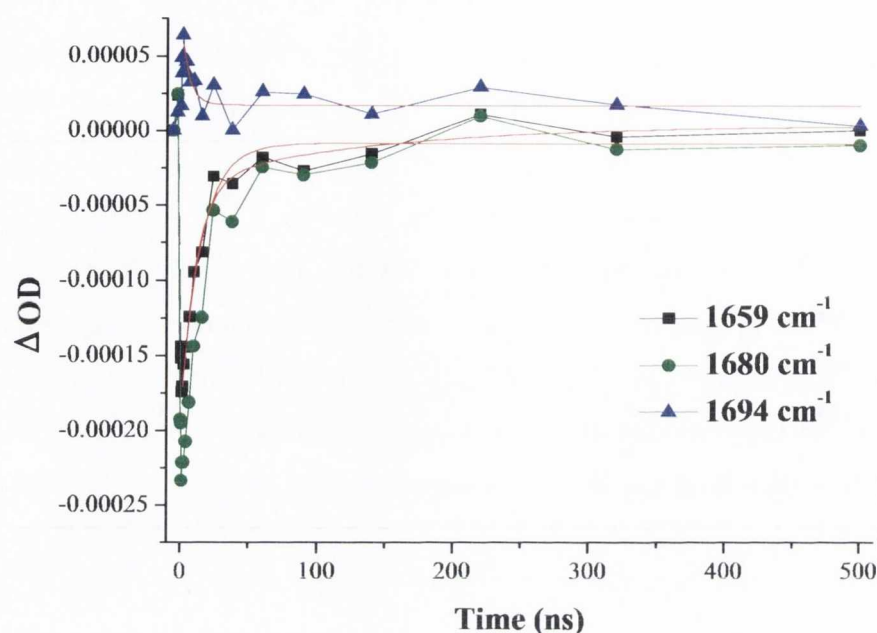
Figure 2.46 – ns TRIR spectra of (a) $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and (b) $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ in 10 mM sodium phosphate D_2O buffer

Note the difference in the scales of the Y axis

In both spectra there is clear evidence of bleaching of both the 1656 cm^{-1} (cytosine carbonyl stretch) and 1680 cm^{-1} (guanine carbonyl stretch) bands, indicating that the excitation of the ruthenium enantiomers have induced changes in the base-pairing of the

nucleotides. These strong bleaches are predominately localised to the guanine and cytosine vibrations. In conjunction with the bleaching of the bands, there is a simultaneous production of two transient absorption bands at 1590 cm^{-1} - 1620 cm^{-1} and 1685 cm^{-1} - 1720 cm^{-1} . Both the bleaching bands have been recorded with the direct excitation of $[\text{poly}(\text{dG-dC})]$, Figure 2.44, indicating that the occurrence of these signals in the presence of the enantiomers is a result of guanine oxidation by the excited state of the complex.^{24,89} The two transient absorbance bands are not observed following direct excitation of $[\text{poly}(\text{dG-dC})]$, thus must be a product of the ruthenium complexes. While the nature of the former transient has not yet been elucidated, latter transient absorbance, at approx. 1700 cm^{-1} has been reported for oxidised guanine formed by photoionisation of both 5'-GMP and $[\text{poly}(\text{dG-dC})]$ and has been attributed to the presence of the radical cation.^{89,91}

As with the ns-TA study, the data collected from the ns-TRIR experiments allowed the calculation of the lifetimes of the excited state species. In this latter case, it is the lifetime of the oxidised guanine that is revealed, again indicating the lifetime of the BET process. The kinetics obtained from the band fitting at 1659 cm^{-1} and 1680 cm^{-1} nm (the maxima of the bleached signals) with both the Λ and Δ enantiomers were best fitted to a double exponential decay (Figure 2.47 – Λ , Figure 2.48 – Δ and Table 2.15). Again the first lifetime is sub-nanosecond in nature and thus cannot be accurately recorded from these experiments. Picosecond TRIR experiments conducted with the racemic complex have ascribed this short lifetime to the guanine oxidation by the excited state of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ resulting from the proton-coupled electron transfer process. The second lifetime, significantly longer in duration is that of the oxidised guanine moiety returning to its ground state via the back electron transfer pathway surmised previously in Figure 2.42. The lifetimes from the ns-TRIR data mirror those found in the ns-TA experiments and also show that the process of BET occurs more slowly in the presence of the Λ enantiomer than with the Δ enantiomer in D_2O .

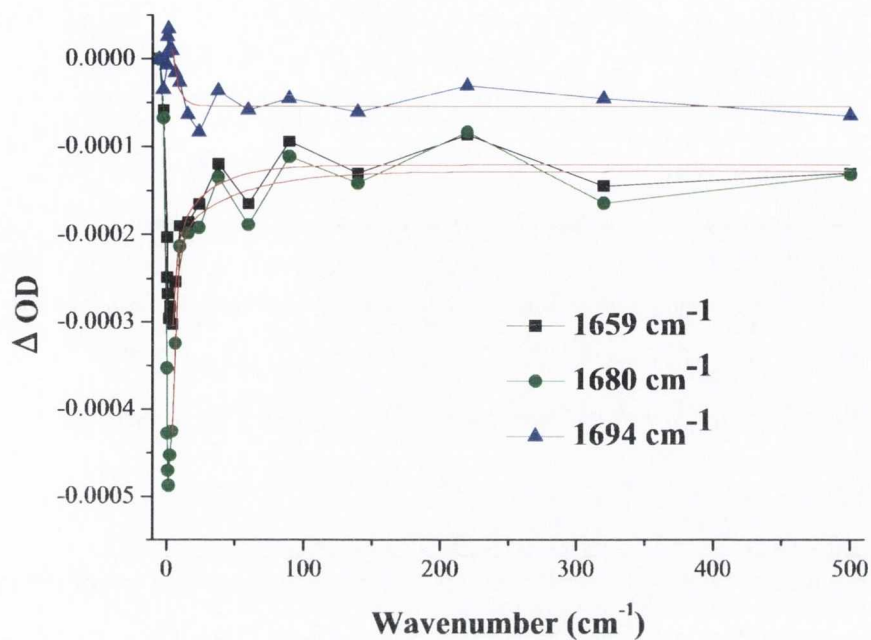


Data: Kinetics 1694 cm^{-1} , 1680 cm^{-1} , 1659 cm^{-1}
 Model: ExpDec2
 Equation: $y = A1 \cdot \exp(-x/t1) + A2 \cdot \exp(-x/t2) + y0$
 Weighting: y(1), y(2), y(3) No weighting

Chi²/DoF = 1.7503E-10
 R² = 0.97608

y0	-0.000006	$\pm 1.1871\text{E-}5$
A1	-0.00004	$\pm 5.9986\text{E-}5$
t1	0.51118	± 0.21638
A2	-0.000206	$\pm 6.01781\text{E-}5$
t2	14.86582	± 1.6111

Figure 2.47 – Kinetic plot and data of the nsTRIR data collected for Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ binding to $[\text{poly}(\text{dG}-\text{dC})]$ in 10 mM sodium phosphate buffer at in D_2O



Data: Kinetics 1694 cm^{-1} , 1680 cm^{-1} , 1659 cm^{-1}
 Model: ExpDec2
 Equation: $y = A1 \cdot \exp(-x/t1) + A2 \cdot \exp(-x/t2) + y0$
 Weighting: y(1),y(2), y(3) No weighting

Chi²/DoF = 1.92536E-10

R² = 0.94738

y0 -0.0002 ±1.2283E-5

A1 -0.0006 ±1.7412E-4

t1 0.34261 ±0.05638

A2 -0.00584 ±4.9246E-4

t2 6.52128 ±1.58983

Figure 2.48 – Kinetic plot and data of the nsTRIR data collected for Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ binding to $[\text{poly}(\text{dG}-\text{dC})]$ in 10 mM sodium phosphate buffer at in D_2O

	Λ	Δ
ns-TA	$\tau_1 - 0.5$ ns $\tau_2 - 14.7$ ns	$\tau_1 - 0.3$ ns $\tau_2 - 7.7$ ns
ns-TRIR	$\tau_1 - 0.5$ ns $\tau_2 - 14.9$ ns	$\tau_1 - 0.3$ ns $\tau_2 - 6.5$ ns

Table 2.15 – Comparison of the lifetimes of the oxidised guanine species in the Λ - and Δ - $[Ru(TAP)_2dppz]^{2+}$ from the ns-TRIR experiments and the $[Ru^{II}(TAP^{\bullet})(TAP)dppz]^+$ species from the ns-TA experiments with $[poly(dG-dC)]$ in D_2O buffer

These results support the findings from the ns-TA experiment showing a major difference between the enantiomers. The lifetime of the oxidised guanine intermediate in the presence of the Λ enantiomer is almost twice that of the Δ enantiomer. As before, this difference may be ascribed to the degree of the dppz intercalation experienced by each enantiomer.

2.5.4 Conclusions from the Ultrafast Investigations of Λ and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$

The nanosecond transient absorption and time-resolved infrared spectroscopy experiments were conducted with a view to investigating if there is an enantiomeric effect upon the rate constants of the proton-coupled electron transfer and back electron transfer processes that occur upon the excitation of such a ruthenium polypyridyl complex in the presence of [poly(dG-dC)]. The results from both these experiments indicate that there is a significant difference upon the rate of reaction between the two enantiomers.

All the data was fitted bi-exponential decay kinetics, which is indicative of the occurrence of two processes, PCET and BET. However due to the exceedingly fast nature of the PCET process (approx. 700 ps)²⁴, the first lifetime of each of the experiments cannot be considered accurate. As a result, these nanosecond experiments only yield reliable data regarding the latter of the two processes, the back electron transfer.

The TA data showed that in the presence of the Λ enantiomer, the lifetime of the reduced species $[\text{Ru}^{\text{II}}(\text{TAP}^{\bullet-})(\text{TAP})\text{dppz}]^+$ was relatively long-lived at 14 ns. This result compares favourably to the prediction of a lifetime of 14 ns extrapolated from a picosecond study with the racemic complex.²⁴ In the presence of the Δ enantiomer, the same experiment resulted in the much shorter lifetime of only 7.7 ns for the triplet excited state species. This of course would have occurred in the racemic studies, but would have been masked by the lifetime of the Λ -excited state. The significant difference between the lifetimes of the enantiomeric intermediates is attributed to the distance between the ancillary ligands of the ruthenium complex and the oxidised guanine moiety. The Δ enantiomer, being of such an orientation to allow for a deeper penetration of the dppz ligand, has a shorter distance between the ruthenium centre and DNA axis (guessed to be in the order of 2 Å which equates to distance shortening of approximately 50 % – see Chapter 3) compared to that of the Λ enantiomer. This allows for the more rapid exchange of the electron from the ruthenium radical to the guanine radical resulting in a return to the ground state for both species. These experiments were conducted in both H_2O and D_2O and interestingly the enantiomeric effect was only observed in D_2O , see Figure 2.49 for a comparison of the kinetics.

ns-TRIR experiments were conducted in parallel to the ns-TA studies and the results from this spectroscopic technique supported those of the transient absorption. This technique allowed for the probing of the Watson-Crick hydrogen bonding within the double helix, thus revealing information regarding the excited states of the guanine-cytosine base-pair. Any changes in the spectrum will be as a result of interaction with the excited state ruthenium complex because the polynucleotide does not absorb at the wavelength used, 400 nm. The bleaching of two bands and the appearance of two transient bands are observed upon excitation of the ruthenium complex. This is believed to correspond to the formation of the deprotonated guanine radical and protonated cytosine compound via PCET, as depicted in Figure 2.42. The TRIR data thus shows the lifetime of these excited state species as they return to the ground state via BET. In the presence of the Λ enantiomer, the oxidised guanine was shown to have a lifetime of 14 ns, while in the presence of the Δ enantiomer the lifetime of this intermediate was only 6.5 ns. These lifetimes mirror those determined from the ns-TA experiments and corroborate the conclusion that there is indeed an enantiomeric effect on the rate at which the process of back electron transfer can occur between duplex DNA and ruthenium polypyridyl intercalating complexes. The kinetic data for the conducted experiments are summarised in Figure 2.49.

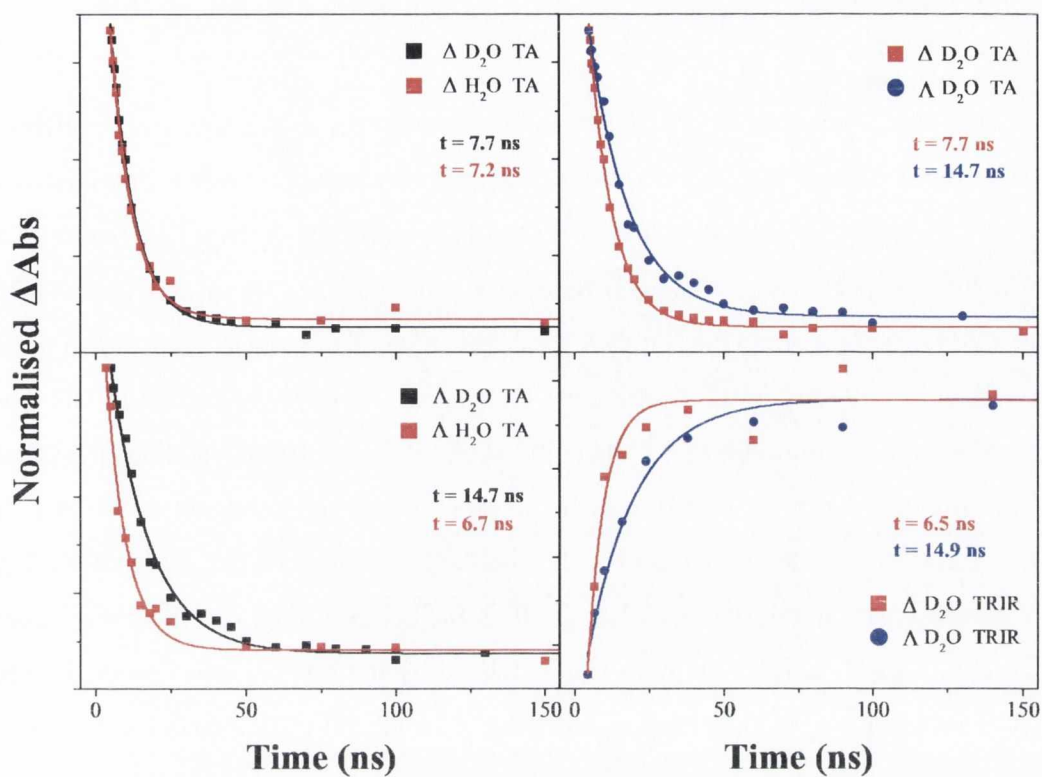


Figure 2.49 – Comparison of the ns-TA and ns-TRIR spectra of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $[\text{poly}(\text{dG-dC})]$ in D_2O and H_2O 10 mM sodium phosphate buffer

2.6 Conclusions

It is clear from the work presented in this chapter that there is a significant difference between the interactions of two enantiomers of the same complex with a chiral substrate, such as DNA. The separation of the enantiomeric complexes $\Lambda\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $\Delta\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and subsequent binding studies between them and various DNA sequences have highlighted these differences. Both enantiomers have been shown to have a preference for binding to A–T sites over G–C sites, and while both bind to DNA with extraordinary strength (binding constants in excess of 10^7 in 10 mM sodium phosphate), the Δ enantiomer has been shown to be the more effective binding agent of the two. This has been attributed to the orientation of the ancillary TAP ligands that allows for a better fit of the Δ enantiomer to the DNA helix, which facilitates a more complete intercalation of the dppz ligand. This assumption was validated by the reverse salt titration in which the Λ enantiomer was found to unbind from the DNA at lower salt concentrations than its Δ counterpart, indicating that the Δ enantiomer does indeed have a tighter binding interaction.

Both enantiomers were found to impart a degree of thermal stability both to the mixed sequence DNA helix and the [poly(dA–dT)] duplex. Interestingly, while the Δ enantiomer provided an increased level of stability to the mixed sequence DNA, both the enantiomers had the same stabilising affect on the [poly(dA–dT)]. This would lead to the speculation that the Λ enantiomer has a similar degree of dppz penetration as the Δ when bound to A–T sites. This may be as a result of the increased flexibility inherent to A–T rich DNA sequences due to the presence of only two hydrogen bonds between the bases.

Investigation of the binding modes of both enantiomers via circular and linear dichroism showed that both enantiomers bind mainly through intercalation, however, a second binding mode, which is predominately electrostatic in nature, may dominate at higher P/D ratios.

Finally, ultrafast experiments on the nanosecond scale were performed with a view to investigating if the enantiomeric nature of the complexes would have an effect on the proton-coupled electron transfer and back electron transfer processes postulated to occur

upon excitation of the ruthenium complex in the presence of DNA. Transient absorption and time-resolved infrared spectroscopic techniques were employed to study these systems and proved that indeed the nature of the enantiomer does have an effect on the rate at which the electronic process can occur. Lifetimes of the excited state intermediates in the presence of the Λ enantiomer were calculated as 14 ns, with the Δ equivalents having lifetimes of only 7 ns. As only data regarding the BET could be reliably gleaned from these results, we can conclude that the process of back electron transfer between the oxidised guanine moiety of DNA and the excited state of the Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex can occur at a rate approximately twice that of the Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex.

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Chapter 3

Crystallisation of Ruthenium Complexes

3.1 Aims and Acknowledgements

The purpose of this work was to attempt to grow crystals of the Λ and Δ enantiomers, of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ in the presence of an oligonucleotide with the intention of investigating the binding modes present. Such a study of ruthenium complexes has not yet been reported in the literature due to the extreme difficulties in obtaining such crystals of adequate diffraction potential, as explained in the following pages.

The work presented in this chapter is the result of a highly successful collaboration with the HeliX Research Group at the University of Reading and the Diamond Light Source, located in Oxfordshire. The crystals used in the following study were prepared and grown by myself at the University of Reading utilizing their specialist crystallography equipment. The initial crystallographic studies were conducted using the 'in-house' Gemini-S-Ultra Diffractometer at the University, with the atomic resolution data collected on the I02 beamline at the Diamond facility.

Presented in this chapter is the crystallographic work conducted on the crystals that were formed in solutions containing the Λ enantiomer of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and the oligonucleotide TCGGCGCCGA. However, before launching into the results, a brief introduction into the art form that is crystallography is required.

3.2 Introduction to X-ray Crystallography

X-ray crystallography is the most powerful method of studying the three dimensional conformation of a molecule. While other methods provide only partial or indirect information, crystallography allows for the visualisation of a molecular structure in atomic detail. It is a wonderfully elegant science, perfectly combining mathematics and physics and without it, chemistry would not be as advanced as it is today. The subject has attracted several Nobel Prizes; famous Laureates include Lawrence Bragg, who was the youngest ever to receive the prize and still holds this record, and Dorothy Crowfoot Hodgkin who is one of the few female Nobel Prize winners.

The key facet to crystallography is the use of X-rays. X-rays are found at the high-energy end of the electromagnetic spectrum (Figure 3.1) and have a typical wavelength ranging from 10 Å to 0.5 Å. This is comparable in size to the bonds between atoms in a molecule, for example, the C-C bond length is approximately 1.5 Å and the C-S bond is 1.8 Å.

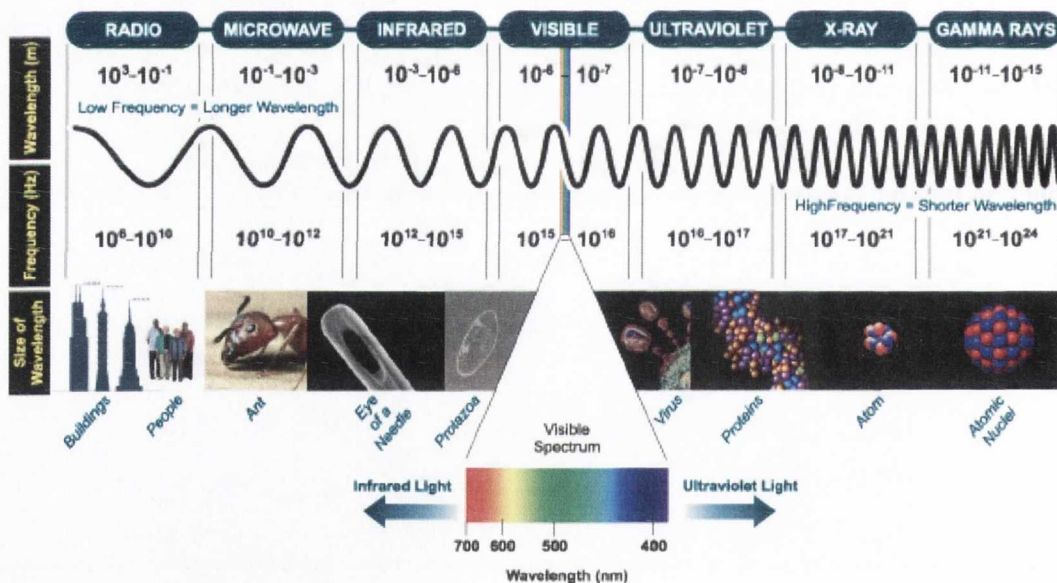


Figure 3.1 – The Electromagnetic Spectrum

When a crystal is exposed to an X-ray beam, the incident X-ray photons interact with electrons around the atomic nuclei, causing the electrons to vibrate and emit photons with the original energy and wavelength. These diffracted photons can be counted using a

detector with characteristic patterns reflecting the distribution of electrons. These diffraction patterns can be deconvoluted using inverse Fourier transformations to give an electron density map representing the distribution of electrons in the molecules as they are contained within the crystal. From these electron density maps we can decipher the positioning of complexes, molecules and macromolecules within a crystal and obtain the beautiful crystal structures we find available in databases today.

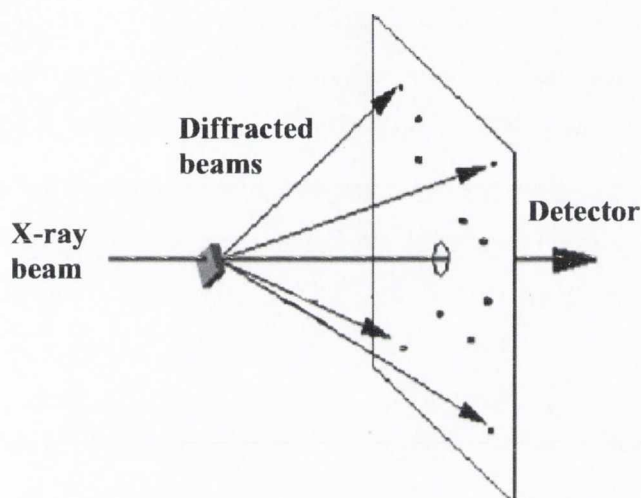


Figure 3.2 - A schematic representation of a crystal (shown in grey) mounted between an X-ray source and detector to record the diffraction pattern.

3.2.1 Crystals

The word crystal is derived from the Greek word 'Krustallos' which means 'clear ice'.¹ Like ice, crystals are chemically well defined with many being transparent and glittering in appearance. Crystallinity, even in the figurative meaning, is taken as a symbol of perfection and purity because crystals are often beautiful geometrical solids with regular faces and sharp edges.²

Physically, a macromolecular crystal is a regular repeating array of ions, atoms or molecules extending in three dimensions. While ideal crystals can be considered as infinite, perfect arrays, experimental crystals have finite dimensions that are grown when the material precipitates from a solution. As a result the molecules attempt to reach the lowest free energy state, often caused by packing in a regular format, resulting in a crystal.

The smallest group of atoms or molecules in a crystal which, if repeated in three dimensions generate the whole crystal, is called the unit cell. This is the basic 'building block' of the crystal. Once the contents of the unit cell are known, the whole crystal can be imagined as an efficiently packed array of many unit cells stacked three dimensionally.³ The size of the unit cell is measured by its lattice parameters: the lengths of three edges a , b and c and three angles α , β and γ as shown in Figure 3.3.

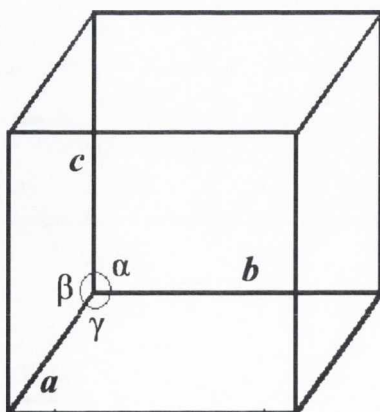


Figure 3.3 – The unit cell dimensions of a crystal. The six parameters used to define the unit cell are shown in the figure; three lengths a , b , c and three angles α , β , γ

The smallest unit of a crystal is known as the asymmetric unit. This unit, when operated on by the symmetry operations of the space group, generates the unit cell.

Crystallography has developed a descriptive terminology that is applied to crystals and crystal features in order to describe their structure, symmetry and shape. This terminology defines the crystal lattice that provides a crystal with its ordered internal structure, as well as describing the various types of symmetry that exist within the crystal. By considering the type of symmetry a crystal possesses, it may be categorized as a member of one of seven crystal systems and one of thirty-two crystal classes (or point groups).

The concept of symmetry describes the periodic repetition of structural features. Two general types of symmetry exist; *translational symmetry*, describing the periodic repetition of a motif across a length or through an area or volume, and *point symmetry*, describing the periodic repetition of a motif around a point. Reflection, rotation, inversion and rotoinversion are all point symmetry operations. Translational symmetry operations produce a lattice, which is an array of points defining a repeated spatial entity. The smallest unit of this lattice is the aforementioned unit cell. The French crystallographer Auguste Bravais established that in three-dimensional space, only fourteen different lattices may be constructed. These have been coined the Bravais lattices.

The seven crystal systems can be defined as cubic, hexagonal, rhombohedral, tetragonal, orthorhombic, monoclinic and triclinic. Every crystal of a certain crystal system will share a characteristic point group and these systems may sometimes be determined visually by examining a particularly well-formed crystal.

The point symmetry operations, reflection, rotation, inversion and rotoinversion, may be combined in a variety of different ways to form a crystal class. There are thirty-two possible unique combinations of these operations, thus thirty-two crystal classes (or point groups) which are, in turn, grouped into the seven crystal systems according to the characteristic symmetry operation they possess. These crystal systems, crystal classes and associated Bravais Lattices are detailed in Table 3.2. The fourteen Bravais lattices combine with the thirty-two point groups and give rise to 230 possible space groups representing all the symmetry possibilities that may be adopted by a crystalline lattice.

All space groups apart from P1 include three lattice translations and other operations that relate to the unit cell. However, protein and DNA structures are restricted to non-mirror and non-inversion symmetry operations due to the chirality of their molecules. This feature effectively reduces the number of space groups to sixty-five for protein and DNA crystals (Table 3.1).

Cubic	P23, F23, I23, P2 ₁ 3, I2 ₁ 3, P432, P4 ₁ 32, P4 ₃ 32, P4 ₂ 32, F432, F4 ₁ 32, I432, I4 ₁ 32
Tetragonal	P4, P4 ₁ , P4 ₂ , P4 ₃ , I4, I4 ₁ , P422, P42 ₁ 2, P4 ₁ 22, P4 ₁ 2 ₁ 2, P4 ₂ 22, P4 ₂ 2 ₁ 2, P4 ₃ 2 ₁ 2, P4 ₃ 22, I4 ₁ 22, I422
Orthorhombic	P222, P2 ₁ 2 ₁ 2 ₁ , P2 ₁ 2 ₁ 2, P222 ₁ , C222, C222 ₁ , F222, I222, I2 ₁ 2 ₁ 2 ₁
Rhombohedral	P3, P3 ₁ , P3 ₂ , P312, P321, P3 ₁ 21, P3 ₁ 12, P3 ₂ 21, P3 ₂ 12, R3, R32
Hexagonal	P6, P6 ₁ , P6 ₂ , P6 ₃ , P6 ₄ , P6 ₅ , P622, P6 ₁ 22, P6 ₂ 22, P6 ₃ 22, P6 ₄ 22, P6 ₅ 22
Monoclinic	P2, P2 ₁ , C2
Triclinic	P1

Table 3.1 – Sixty-five possible chiral space groups for protein and DNA crystals

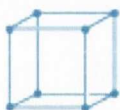
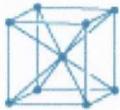
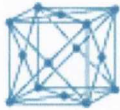
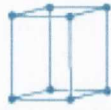
Crystal System	Crystal Class	Bravais Lattice
Cubic	hexoctahedron gyroid hextetrahedron diploid tetartoid	   Cubic P Cubic I Cubic F
Hexagonal	dihexagonal dipyramid hexagonal trapezohedron dihexagonal pyramid ditrigonal dipyramid hexagonal dipyramid hexagonal pyramid trigonal dipyramid	 Trigonal and hexagonal C (or P)
Rhombohedral	hexagonal scalenohedron trigonal trapezohedron ditrigonal pyramid rhombohedral	 Trigonal R (rhombohedral)
Tetragonal	ditetragonal dipyramid tetragonal trapezohedron ditetragonal pyramid tetragonal scalenohedron tetragonal dipyramid tetragonal pyramid tetragonal disphenoid	   Tetragonal P Tetragonal I
Orthorhombic	rhombic dipyramid rhombic disphenoid rhombic pyramid	    P C I F
Monoclinic	prism spenoid dome	  Monoclinic P Monoclinic C
Triclinic	parallelohedron monohedron	 Triclinic P

Table 3.2 – The seven crystal systems with their associated crystal classes

and Bravais lattices. Adapted from www.answers.com/topic/crystallography

3.2.2 Crystals in Biochemistry

Crystallography is a wonderful tool in the chemistry arsenal to empirically view the arrangement of atoms within given structures. This technique allows for unequivocal evidence as to the bonding occurring within macromolecules and is the ‘holy grail’ when investigating covalent systems of any nature, but particularly proteins and DNA. Macromolecular crystals are very small (usually requiring a microscope for examination) and are extremely fragile. This is due to the weak interactions between macromolecules in crystal lattices and the high solvent content (from 20% to more than 85%). A consequence of this is that macromolecular crystals must be kept in a solvent-saturated environment to prevent dehydration which leads to cracking and the destruction of the crystals.²

The crystallography of macromolecules is often considered to be more of an ‘art’ than a science due to the difficulty involved in obtaining a crystal. Crystallographers may spend many months painstakingly growing crystals only to find when they eventually obtain crystals of good size and shape, they may not diffract. Despite this, crystallography in biology has become commonplace, particularly in the field of protein research. There is an abundance of methodology supporting the various techniques for the crystallisation of proteins, and a Protein Data Bank (PDB) containing over 68,000 solved crystal structures (PDB, 2010) of many different proteins. An example of such a structure is that of pepsin, the first globular protein crystal successfully used in X-ray diffraction, in 1934.



Figure 3.4 – Three-dimensional structure of porcine pepsin (PDB ID: 3PSG)

The subject of this thesis, however, is DNA and there are far fewer resources available to the DNA crystallographer than the protein crystallographer. Previous work conducted in the HeliX Group at the University of Reading observed that DNA crystals are more robust than protein crystals due to their ability to withstand transfer from a cold room (4°C) to room temperature. Some DNA crystals can survive dehydration, often forming a slightly more compact version of the original crystal, and they can be frozen in liquid nitrogen for storage and use at a later date. This robustness may be attributed to the stacking of DNA helices in the crystal form, allowing the formation of solvent channels that aid in structure stability. A typical oligonucleotide crystal is chemically stable, resists biological attack and does not decompose in the x-ray beam.

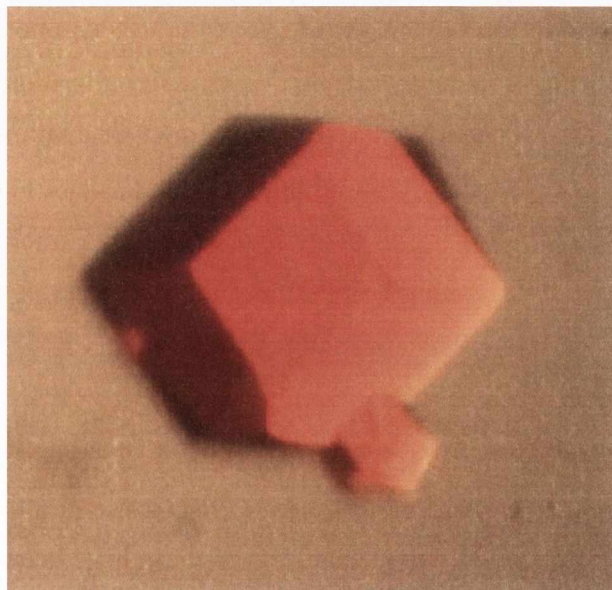


Figure 3.5 – One of the crystals obtained in this work

$[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with $d(\text{TCGGCGCCGA})$. Approx. size of 0.2 mm

3.2.3 Crystallisation

The crystallisation process of compounds of any description from their solution represents a reversible equilibrium phenomenon, driven by the minimisation of the free energy of the system. A solution, in which the molecules are fully solvated, corresponds to the system at equilibrium; in other words its free energy is minimised. If more molecules are added to the solution, the system undergoes internal changes until the point is reached where there is insufficient solvent to maintain full hydration of the molecules. Under these conditions (the so-called '*supersaturated state*') the system is no longer at equilibrium. Therefore, it will be thermodynamically driven toward a new equilibrium state with a corresponding new free energy minimum. Individual molecules lose rotational and translational freedom by forming many new stable non-covalent chemical bonds, thus minimising the free energy of the system. Crystallisation is known to lower the free energy of solution by approximately $3 - 6 \text{ kJmol}^{-1}$ relative to the dissolved state in solution. This aggregation results in partitioning of the molecules between soluble and solid phases. The solid phase can appear in the state of either an amorphous precipitate or crystal nuclei. Amorphous precipitates are usually more favourable kinetically, and so they tend to dominate the solid phase and inhibit crystal formation.

The principles of crystal growth have been the subject of intense investigation for a number of years. Theoretical and practical aspects of the crystallisation of small organic compounds are, as a result, well established. These aspects have been shown to similarly apply to the crystallisation of macromolecules such as DNA and RNA. Three events occur during the crystallisation process to allow the formation of a crystal: nucleation, growth and cessation of growth. These stages are common to all systems.

3.2.3.1 Nucleation

This is the process where free molecules in solution come together and produce a thermodynamically stable aggregate with a repeating lattice, by which the free energy is lowered compared to the solution state. Figure 3.6 shows an oligonucleotide overcoming an energy barrier, analogous to that of a conventional chemical reaction, to form a bigger crystal. The formation of crystalline aggregates must exceed a specific size (the critical

size) defined by the competition of the surface area ratio of the aggregate to its volume, following which the aggregate becomes a supercritical nucleus ready for further growth. Conversely, if the size of the nucleus is smaller than the critical size, spontaneous dissolution will occur. The degree of nucleation is closely associated with the degree of supersaturation, which is determined by the overall solubility of the crystallising material. Higher solubility results in a higher chance of molecular diffusion collisions, thus a higher degree of supersaturation is more likely to produce more stable nuclei.

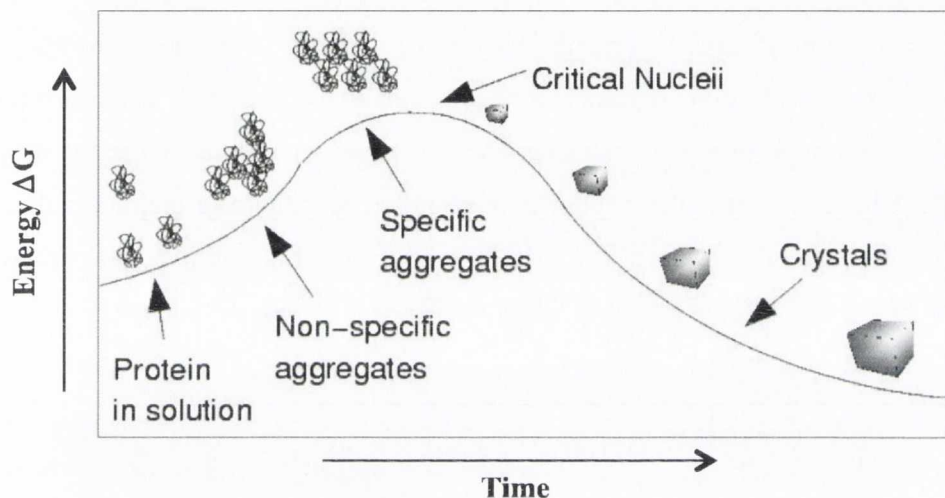


Figure 3.6 – An energy diagram for the crystallisation of a protein⁴

3.2.3.2 Growth

Once the energy barrier has been overcome, crystal growth begins. The driving force is a combination of the natural properties of the growing crystal surface and the diffusion rate, as it is easier to add further molecules to a rough surface than a smooth surface where surface nucleation is required. Generally, crystals should grow slowly to reach a maximum degree of order in their structure.

There are a number of different methods to bring the crystallisation solution to the supersaturated state with vapour diffusion being the technique most popular for the crystallisation of macromolecules, as the equipment is commercially available and it allows for the rapid screening of many conditions. The method utilises the evaporation and diffusion of water between solutions of differing concentrations as a means of

approaching supersaturation. Typically the reservoir contains a bulk amount of crystallisation solution (without the DNA/protein present) in which the concentration of the precipitating agent is much higher than that within the drops. In all the crystallisation experiments described in this work, a higher concentration of the precipitating agent (2-methyl-2,4-pentanediol) was used as a reservoir in a well outside the drop, whilst inside the drop, DNA and the other precipitating agents present in the solution were at lower concentrations. This concentration difference was the major driving force causing water to evaporate from the drop until all concentrations equalled. Figure 3.7 illustrates the phase transition of crystal growth during the vapour diffusion technique. The oligonucleotide concentration increases from an unsaturated point A towards point B where the concentration of the crystallising agents is twice as high as that in the drop. At point B, the nucleation zone, the oligonucleotide molecules nucleate and crystallise out, resulting in the solution concentration falling back onto the solubility curve where the solid and solution exchange is equilibrated (point C).^{2,5}

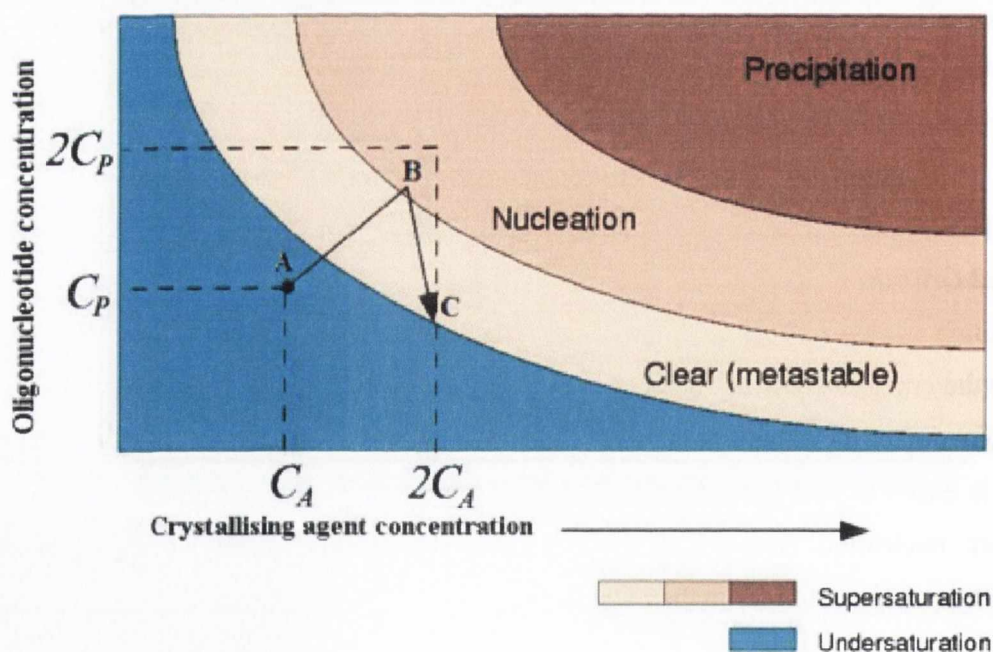


Figure 3.7 – Phase diagram for the vapour diffusion experiment⁶

3.2.3.3 Cessation

The reasons for cessation of crystal growth are multiple, the most obvious being that the concentration of the crystallising solute decreases to the particular point at which the solid and solution phase reach an exchange equilibrium. Lattice strain also limits the crystal growth by effectively preventing addition of molecules to the surface once a certain critical volume is reached. In addition, when foreign or damaged molecules are incorporated into the growing crystal face, this can result in successive defects, which interrupt the crystal lattice, creating surface poisoned crystals, and crystals will stop growing.⁷⁻¹⁰

3.2.4 Factors influencing the process of crystallisation

The variability in the crystallisation of macromolecules is extremely complex, usually rendering attempts to predict crystallisation behaviour by systematic calculations futile. However, some knowledge of the factors strongly associated with the production of crystals can aid in increasing the probability of crystal formation. Generally, the main classes of crystallisation factors for crystallising DNA can be divided into six categories.¹⁰⁻¹⁵

- **Purity**

The purity of crystallisation agents will decide if crystal formation occurs. Although crystals can form from impure solutions, they frequently give poor diffraction data. The purity of oligonucleotides is ensured by High Performance Liquid Chromatography.

- **Ultra-pure Demineralised Water**

The use of normal demineralised water, as found in many chemical laboratories, often results in poor quality crystals. A demineralised, ultra-filtered water source is essential in crystallisation trials.

- **Temperature**

Due to the complicated thermodynamics involved, temperature is perhaps the most important factor involved in the crystallisation of DNA. At higher temperatures (approximately 70°C), the hydrogen bonds between the DNA bases are broken resulting in the separation of complementary strands. At lower temperatures, the rate of equilibrium slows, allowing the molecules to properly align and usually yield better crystals. In the work presented in this document, crystals were grown at 4°C.

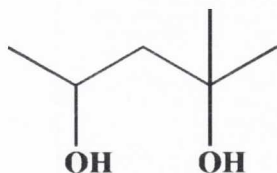
- **pH value**

The solution pH appears to play a smaller role in nucleic acid than in protein crystallisation where the overall charge of a protein, and thus its capacity of packing in a certain way, may be tuned through pH variations. The buffer is used

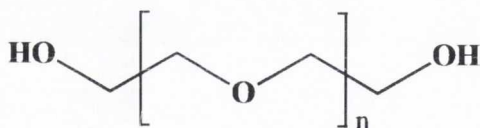
to maintain the pH over a large temperature range and prevents bacterial growth. The buffer (pH range 6-7) which is often used is sodium cacodylate [$\text{Na}^+[(\text{CH}_3)_2\text{AsO}_2]^-$]. Cacodylate ions are sometimes found in the resulting crystal structure. This buffer is used in all the experiments described in this work.

- **Precipitants**

The function of the different precipitants in the crystallisation drop is to alter the macromolecule-solvent contact so that the macromolecules precipitate out of solution, preferably as ordered crystals and not as disordered aggregates. Historically, salts have shown to be the most effective precipitants. Though the nature of how the salts act on macromolecules is poorly understood, the ionic strength of a particular salt greatly affects the solubility of the DNA. The most widely used precipitants in general are alcohols, and especially methyl-2,2-pentane diol (MPD) which is not volatile and therefore easy to handle. It is used in the range of 30%-40% (v/v) in the case of oligonucleotides. Polyethylene glycol (PEG) is also used in some cases ($M_r = 4000-8000$). These compounds are added in order to induce and improve crystallisation. They also act as cryo-protectants, preventing the formation of ice crystals on rapid freezing.



2-Methyl-2,4-pentanediol (MPD)



Polyethylene Glycol (PEG)

Figure 3.8 – The structural formulae of MDP and PEG

- **Other crystallisation agents**

Nucleic acids are polyelectrolytes and therefore the counterions are important additives for crystallisation. Cations and polyamines like spermine and cobalt hexammine are generally used; spermine is the polyamine used in this work. Polyamines and cations are involved in the stabilisation of nucleic acid structures.¹⁶ Spermine is a member of the family of aliphatic, polycationic small

molecules present in prokaryotic and eukaryotic cells that interact with anionic cellular components such as membranes^{17,18} and nucleic acids. Through their interactions with DNA, polyamines play an important role in the diverse array of fundamental biological processes.¹⁹ Spermines have been reported in crystal structures ranging in resolution from 2.7 Å for tRNA,²⁰ to 1.0 Å for Z-DNA²¹ and to 2.0 Å for B-DNA¹⁹. For example, the spermine molecule shown in Figure 3.9²² was found in the crystal structure of the Holliday junction bound with a bisacridine derivative.

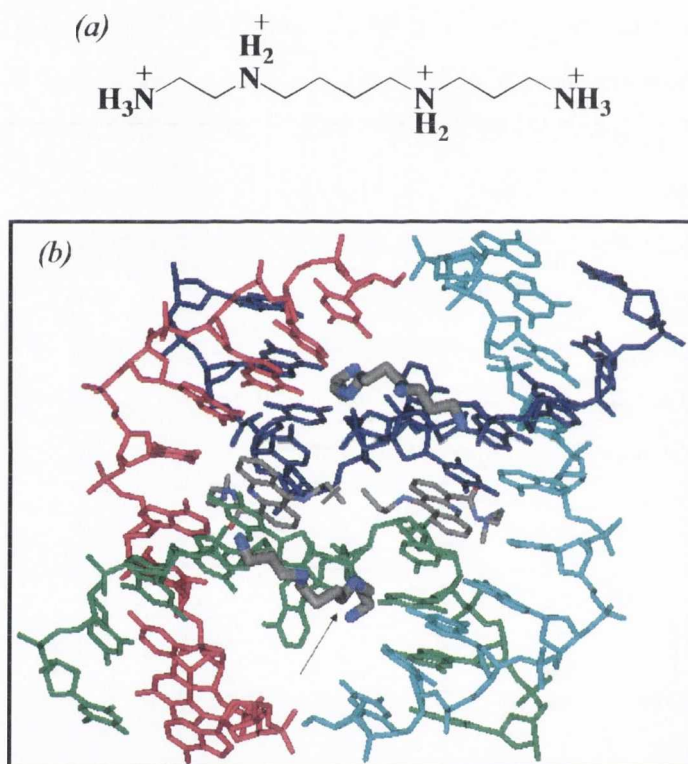


Figure 3.9 – (a) Structural formula of spermine. (b) Stacked-X Holliday junction structure solved with bisacridine derivative.²² The bisacridine ligand can be seen bound to the centre of the junction together with spermine shown with an arrow

3.2.5 Methods of Crystallisation

A number of techniques have been developed for the growth of diffraction-quality crystals. These methods are batch techniques, vapour-diffusion, dialysis and free interface diffusion.

3.2.5.1 Vapour-diffusion method

As mentioned previously, vapour diffusion is a method that uses evaporation and diffusion of water between solutions of different concentrations as a means of approaching and achieving supersaturation of macromolecules. It is well suited for smaller volumes (10 μl or less), however, it tends to produce smaller crystals than other methods. A droplet containing the macromolecule to be crystallised, together with buffer, crystallising agent, and additives, is equilibrated against a reservoir containing a solution of crystallising agent at a higher concentration than the droplet. Equilibration proceeds by diffusion of the volatile species (water or organic solvent) until the vapour pressure in the droplet equals that of the reservoir. There are variations in the performance of this technique:

- Hanging drop method
- Sandwich drop method
- Sitting drop method

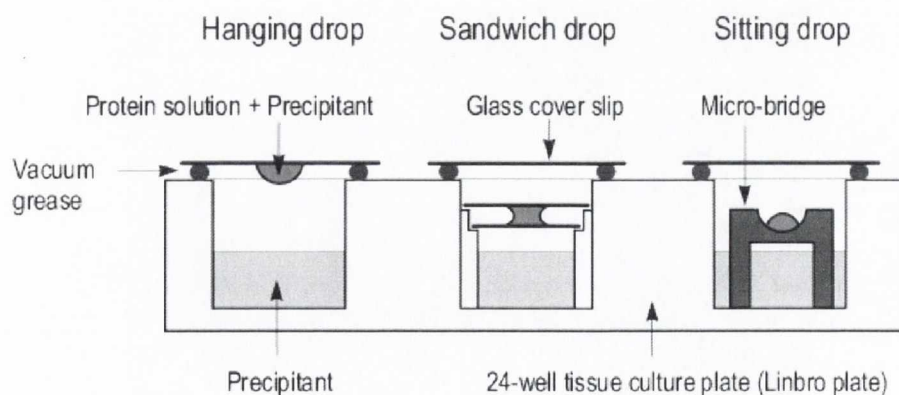


Figure 3.10 - Schematic representation of hanging, sandwich and sitting drop vapour-diffusion crystallisation methods

- **Hanging Drop Method**

The volume of the hanging drop can vary from a few μl to 20 μl . The shape of the droplet is important because it influences the number of nucleation sites and consequently crystal size. If the solutions do not contain detergents or organic solvents, well structured, spherical droplets can be readily formed, by hanging from a siliconised glass cover slip.

- **Sandwich drops**

Drops can be sandwiched between two cover slips. This technique is rarely used, but provides a method of reducing the area of the drop that is exposed to the air. Consequently the rate of diffusion is slower than in hanging and sitting drops.

- **Sitting drops**

The sitting drop vapour diffusion method is very popular due to its ease of use and has been used in all the experiments reported here. This method avoids greasing plates and is more effective on both cost and time; moreover drops can be positioned in a stable sitting position. In this work, the 24 well Cryschem plates (Figure 3.11) manufactured from clear polystyrene by Hampton Research have been used. Each well contains a plastic 'altar' in the centre to hold the macromolecule sitting drop. The reservoir solution is held within the narrow 'moat' surrounding the support post. The plates are sealed with an adhesive tape, supplied by the manufacturer.

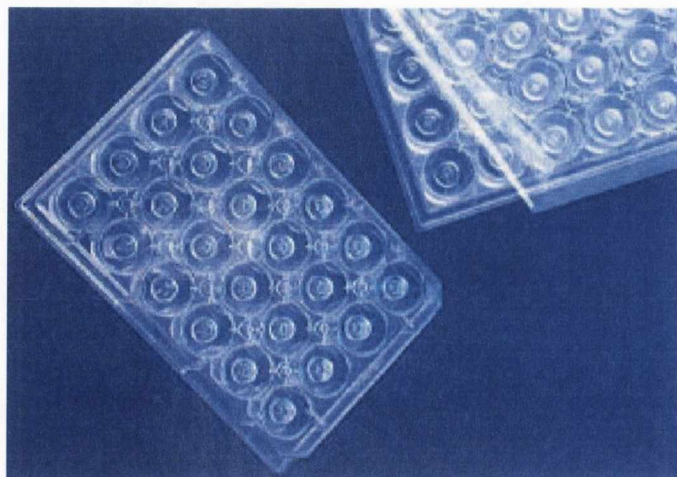


Figure 3.11 – Cryschem plates used in the vapour diffusion technique

3.2.6 X-ray Sources

Two X-ray sources were used in the collection of data sets used in determining the crystal structures detailed in this work; the 'in-house' source, a weaker X-ray source, and synchrotron radiation, the brightest and most useful X-rays sources available to date.

3.2.6.1 'In-house' Data Collection

X-rays are produced in the laboratory by accelerating a beam of electrons into an anode, the nature of which dictates the wavelength of the resulting X-rays. Commonly copper or molybdenum is used. The metal anode is bombarded with electrons produced by a heated filament and accelerated by an electric field. A high-energy electron collides with and displaces an electron from a low-lying orbital in a target metal atom, allowing an electron from a higher orbital to drop into the resulting vacancy, emitting its excess energy as an X-ray photon. For most work with proteins and DNA, this target is copper and the characteristic wavelength of the radiation is 1.54 Å.²³

The 'in-house' data was collected on the Oxford Diffraction Gemini-S-Ultra Diffractometer in the HeliX Group at the University of Reading. A copper sealed tube is used as the X-ray source and is fitted with graded multilayer optics. The quality of the data collected from this machine of the crystals obtained in this study was remarkably high with a resolution of 1.72 Å.

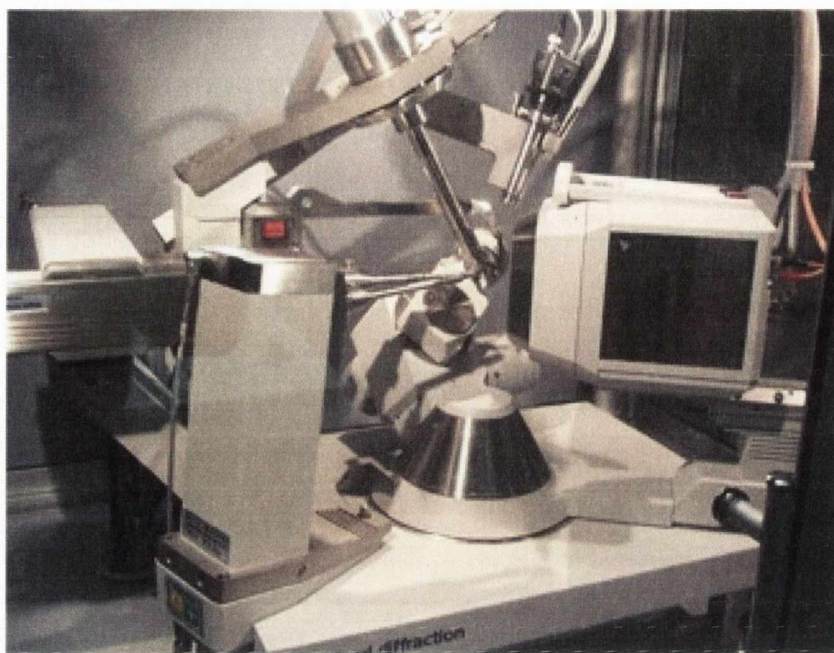


Figure 3.12 – Image of the Gemini-S-Ultra X-ray diffractometer installed at the University of Reading

3.2.6.2 Synchrotron radiation Data Collection

Synchrotrons are particle accelerators used by physicists to study subatomic particles as they produce the most powerful X-rays. They are devices for circulating electrically charged particles at nearly the speed of light. The particles are injected into the storage ring directly from a linear accelerator or through a booster synchrotron, driven by energy from radio-frequency transmitters, and maintained in circular motion by powerful magnets. A charged body like an electron emits energy (synchrotron radiation) when forced into curved motion, and in accelerators, the energy is emitted as X-rays or synchrotron radiation. These radiations are channelled off the main circular route of accelerated particles and onto the research beam lines, positioned all around the main storage ring (Figure 3.13), where they can be used for conducting experiments.

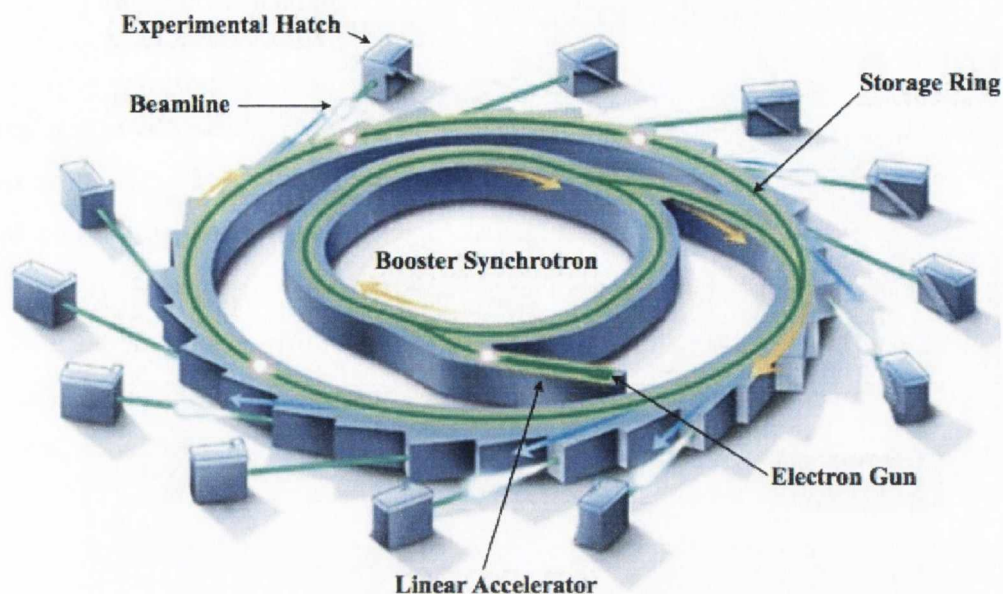


Figure 3.13 – Schematic of a synchrotron facility

The synchrotron radiation source used for this work is located at the Diamond Light Source (DLS), near Reading. The data was collected on the macromolecular beam line I02 complete with an ADSC Q315 CCD detector, allowing for refinement of a crystal structure to $1.10 \text{ \AA}$, atomic resolution.



Figure 3.14 – A view of the Diamond Synchrotron Radiation Source

3.3 Ruthenium Complexes and DNA

As shown in the previous chapter, polypyridyl ruthenium complexes have been studied using many spectroscopic and hydrodynamic techniques. However, to date there are no examples of a full structural study of such complexes with nucleic acids due to the difficulty in obtaining crystals of adequate size and diffraction potential. As such, the spectroscopic data has lead to many theories as to the nature of the binding modes of polypyridyl ruthenium complexes, such as the interesting concept of semi-intercalation of ligands into the double helix,^{24,25} but without a crystal structure these theories cannot be unequivocally proven.

There are three modes through which ruthenium complexes are known to associate non-covalently with DNA; external binding, groove binding and intercalation (Figure 3.15).²⁶

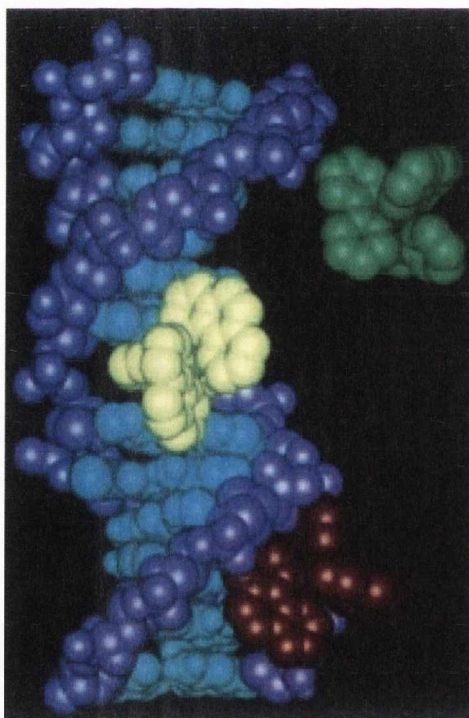


Figure 3.15 – Model of the three binding modes of ruthenium complexes to duplex DNA. External binding in green, Intercalation in yellow and Groove Binding in red²⁶

3.3.1 The Major/Minor Groove Debate

Of these three modes of binding found with ruthenium (II) complexes, groove binding and intercalation are by far the most common, with intercalation being the most prevalent method due to the majority of Ru (II) complexes containing aromatic ligands. Unfortunately there are very few examples of ruthenium complexes in the crystallographic databases, and no record of a ruthenium complex interacting with DNA. This has lead to our understanding of the binding modes of such complexes being dependent on spectroscopic techniques such as UV/visible, circular dichroism, linear dichroism and viscosity.²⁷⁻³⁰ While these techniques provide a wealth of information, they cannot unequivocally prove the exact nature of the binding of such a complex to DNA. With $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ considered a model binder for this important family of ruthenium compounds, there has been much interest into the binding of this complex to DNA. Despite universal support for the intercalative binding mode of dppz-based complexes, the groove from which the complexes intercalate has become a point of contention. While present understanding is that the complexes bind from the minor groove, spectroscopic evidence for binding from both the major³¹ and minor²⁸ grooves has been reported, but again, without crystallographic data, current understanding remains an educated guess.

The work presented here is the first example of a ruthenium coordination complex to be crystallised with an oligonucleotide, unequivocally demonstrating the mode and direction of binding of the complex.

3.4 The Oligonucleotide

The decamer d(TCGGCGCCGA) is the oligonucleotide used in this study. There are two main reasons for choosing this particular sequence. Firstly the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex has been shown to cause the direct oxidation of a guanine moiety³² and form adducts via covalent bonding with nucleic acids.³² Thus despite the A-T preference displayed by its analogue $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$, we expected the TAP complex to show binding to G-C, thus an oligonucleotide with a G-C content was preferred.

Secondly, this particular sequence is known to form both B-DNA and Holliday junctions. A Holliday junction, proposed first in 1964 by Robin Holliday,³³ is a four stranded DNA structure. Holliday suggested that this form of structure must be formed as an intermediate during the process of homologous recombination, and since then his proposed topology had been recognised as a central recombination intermediate.³⁴ Also, ruthenium(II) dppz complexes have recently been shown to bind to higher order DNA structures such as quadruplex DNA and i-motif conformations.^{35,36} As such, we were curious as to whether ruthenium(II) dppz complexes would interact with a Holliday junction structure.

A further advantage of using this particular oligonucleotide is the extensive experience using this sequence within the HeliX Group at the University of Reading. It was the subject of the thesis completed by Dr. Abeer Naseer, who investigated its junction formation with various counterions (Sr^{2+} , Ba^{2+} , Ca^{2+}) and the binding of bisacridine compounds. The structure of the Holliday junction formed with this particular oligonucleotide is shown in Figure 3.16, with a graphical schematic in Figure 3.17.

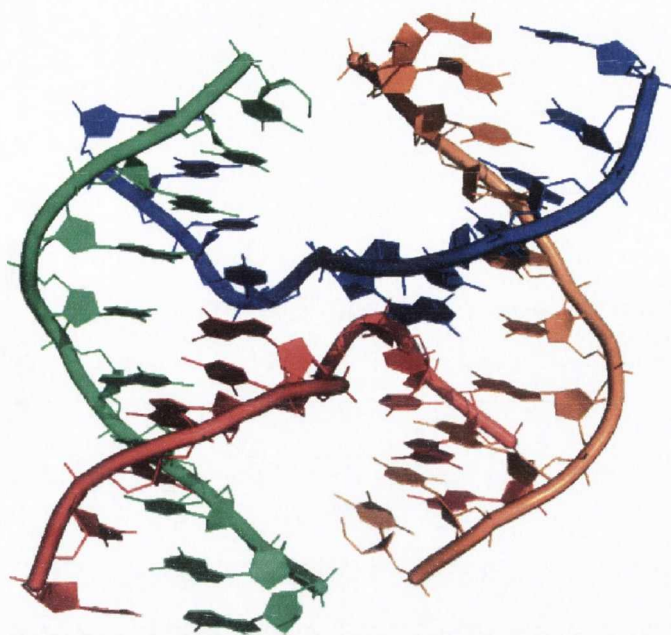


Figure 3.16 – A view of the stacked-X structure of the Holliday junction formed by the d(TCGGCGCCGA) oligonucleotide sequence (PDB ID: 3GOM)

		5'	3'
		T31	- A30
		C32	- G29
		G33	- C28
		G34	- C27
		C35	- G26
		G36	- C25
		C17	- G24
		C18	- G23
		G19	- C22
		A20	- T21
		3'	5'
5'	3'		
T1	- A40		
C2	- G39		
G3	- C38		
G4	- C37	=	
C5	- G16	=	
G6	- C15		
C7	- G14		
C8	- G13		
G9	- C12		
A10	- T11		
3'	5'		

Figure 3.17 – A schematic representation of the four strands forming the Holliday junction. Strand A is in green, Strand B in red, Strand C in orange and Strand D in blue. G16 and C17 of Strand B and C37 and G36 of Strand D forms the crossover junction in the stacked-X structure

3.5 Material and Methods

3.5.1 Crystallisation

The self-complementary DNA decamer sequence d(TCGGCGCCGA) was purchased as a HPLC doubly purified solid from ATDBio. The white solid was dissolved in water purified by reverse osmosis to make 6 mM (single stranded) stock solution which was then diluted to give a final concentration of 1 mM (single stranded). Crystallisations were conducted using the vapour diffusion method from sitting drops at 277K. The initial experiment was set up using the commercially available kit ‘Nucleic Acid Mini Screen’ (NAMS), purchased from Hampton Research USA (see Appendix C for the constituents of the 24 NAMS solutions). Both $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ were set up with the 24 NAMS conditions in two separate trays. Four drops from the Λ -tray produced crystals after only seven days. Table 3.3 below details the components of these crystallising solutions. To date, there have been no crystals formed with the Δ -enantiomer. All drops were equilibrated against a 1 ml reservoir of 35% (v/v) MPD.

Enantiomer	Λ	Λ	Λ	Λ
Enantiomer Conc.	1 mM	1 mM	1 mM	1 mM
Oligonucleotide	TCGGCGCCGA	TCGGCGCCGA	TCGGCGCCGA	TCGGCGCCGA
Oligonucleotide Conc.	1 mM	1 mM	1 mM	1 mM
Precipitant	10% v/v MPD	10% v/v MPD	10% v/v MPD	10% v/v MPD
Buffer	40 mM Na Cacodylate pH 6.0	40 mM Na Cacodylate pH 6.0	40 mM Na Cacodylate pH 7.0	40 mM Na Cacodylate pH 7.0
Polyamine	12 mM Spermine tetra-HCl	12 mM Spermine tetra-HCl	12 mM Spermine tetra-HCl	12 mM Spermine tetra-HCl
Monovalent Ion	80 mM Sodium Chloride	80 mM Potassium Chloride	80 mM Sodium Chloride	80 mM Potassium Chloride
Divalent Ion	20 mM Barium Chloride	20 mM Barium Chloride	20 mM Barium Chloride	20 mM Barium Chloride

Table 3.3 – Components of the four drops that produced crystals

In the absence of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ the crystals formed of the Holliday junction are colourless and display characteristic flat ‘diamond’ morphology. They are of approximate size $0.4 \times 0.3 \times 0.01$ mm. In the presence of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ the crystals achieved in this study were small, orange and rhombohedral in shape. They are of average size $0.1 \times 0.1 \times 0.1$ mm with the biggest crystal found to be $0.2 \times 0.2 \times 0.2$ mm.

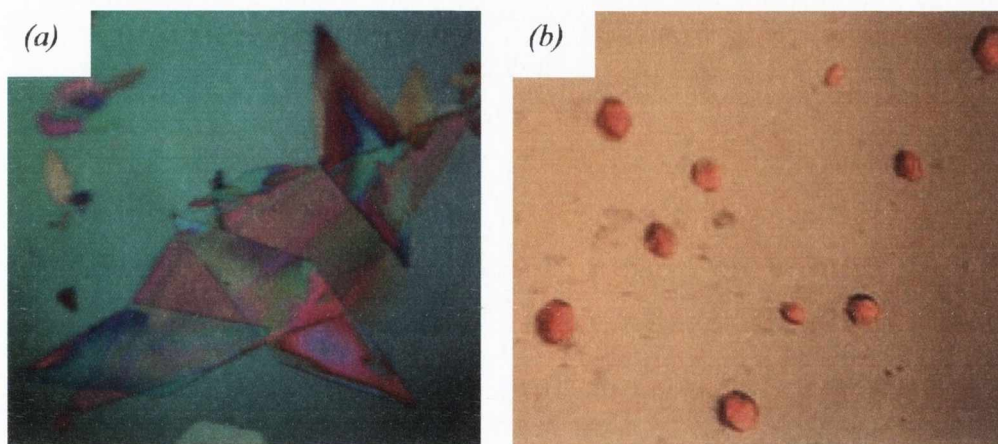


Figure 3.18 – (a) The crystals formed by *d(TCGGCGCCGA)* only (b) The crystals formed by *d(TCGGCGCCGA)* with $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$

3.5.2 Data Collection – ‘In-House’

The first data set was collected on the Gemini-S-Ultra Diffractometer in the Chemistry Department at the University of Reading. The crystal was mounted in a loop and flash cooled to 180 K under a nitrogen stream. The oscillation angle was 0.5° and the exposure time was 60 seconds per image.

The crystal diffracted to a resolution of 1.7 Å. As there are no examples of ruthenium complexes crystallising with nucleic acids in the literature, there were no models on which to base our structural interpretations. Serendipitously, the presence of the barium ions in the solution as heavy atoms provided sufficient anomalous scattering behaviour (Figure 3.19) to allow for their identification in the resulting electron density maps, thus providing a standard by which the remaining densities in the map could be compared and identified.

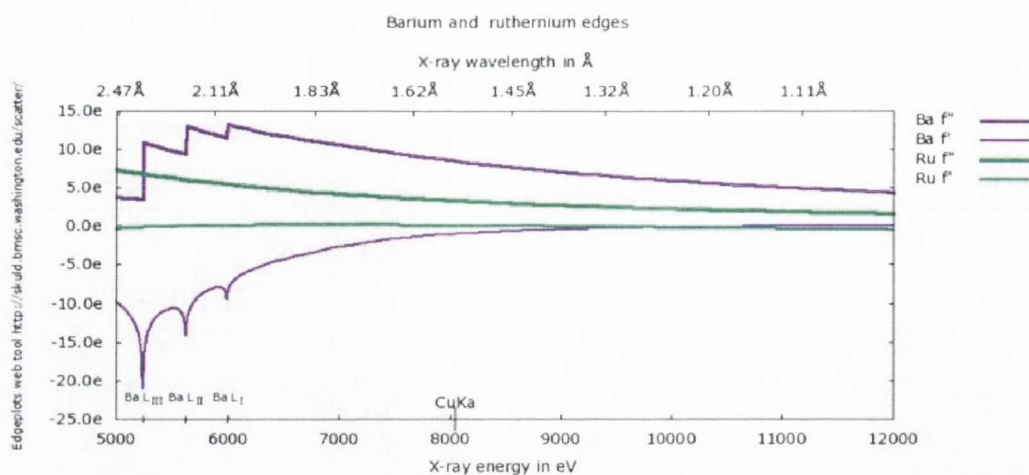


Figure 3.19 - Calculated anomalous scattering behaviour of barium and ruthenium ions as a function of X-ray wavelength

The structure crystallised in the tetragonal crystal system with a space group of $P4_32_12$. The unit cell dimensions were found to be $a = 42.2 \text{ \AA}$, $b = 42.2 \text{ \AA}$, $c = 39.4 \text{ \AA}$, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 90^\circ$. A total of 87 water molecules, one barium molecule and one $\Lambda\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ were modelled into the electron density and the final R-factor and R-free values obtained after the complete structural refinement were 18.21 and 23.31 respectively (as detailed in Table 3.3).

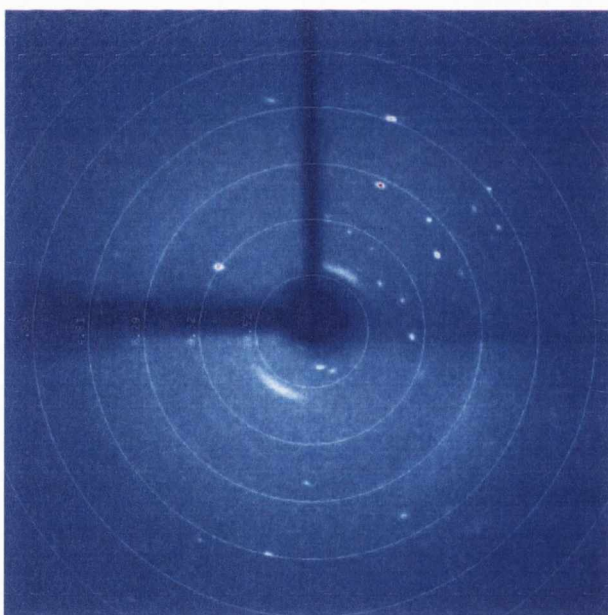


Figure 3.20 – Diffraction pattern obtained from the crystal of $d(\text{TCGGCGCCGA})$ and $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$

The final refined structure consists of one strand of DNA with a two-fold symmetry axis to generate the complete double stranded B-DNA structure. This structure has been deposited in the database; PDB ID – 3QF8.

	Medium Resolution Data Collection
Data collection	
Space Group	P4 ₃ 2 ₁ 2
Cell Dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	42.2, 42.2, 39.4
α , β , γ (°)	90.0, 90.0, 90.0
Resolution (Å)	29.87-1.72 (1.82-1.72)*
<i>R</i> _{sym} or <i>R</i> _{merge}	0.128 (0.661)
<i>I</i> / σ <i>I</i>	8.7 (0.5)
Completeness (%)	83.7 (38.6)
Redundancy	6.6 (1.4)
Refinement	
Resolution (Å)	26.87-1.734
No. of Reflections	2497
<i>R</i> _{work} / <i>R</i> _{free}	18.21/23.31
No. of Atoms	
DNA	202
Ligand	51
Water	87
B-factors	
DNA	20.62
Ligand	15.89
Water	27.62
R.m.s deviations	
Bond Lengths (Å)	0.0209
Bond Angles (°)	2.7222
One crystal used	

*Highest resolution shell is shown in parenthesis

Table 3.4 – Data collection, phasing and refinement statistics from the 'In-House' data

3.5.3 Data Collection – Synchrotron Radiation

A second set of data was collected at the Diamond synchrotron (DLS) on the macromolecular I02 beam line using the ADSC Q315 CCD detector. The crystal was mounted in a loop and flash cooled to 100 K under a nitrogen stream. The oscillation angle was 0.5° with an exposure time of 0.5 seconds per image. The total number of images collected was 415. Note a different crystal was used at DLS than with the Gemini-S-Ultra Diffractometer.

The crystal diffracted to a resolution of 1.1 Å. Resolution to this higher degree allows for the observation of the structure at atomic detail and while this is the accepted standard for small molecules, it is quite rare to obtain such high quality data for macromolecules. The power of the beam line was only at 7% which prevented the degradation of the crystal used, another uncommon trait with macromolecular crystals which are universally accepted as being very delicate.

The structure again crystallised in the tetragonal crystal system with a space group of $P4_32_12$. The cell dimensions were found to be $a = 42.3$ Å, $b = 42.3$ Å, $c = 39.9$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 90^\circ$. A total of 64 water molecules, one barium ion and one $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ were modelled

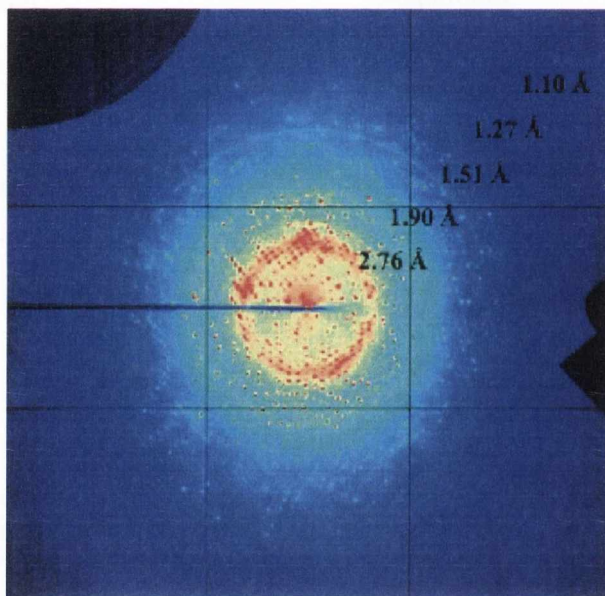


Figure 3.21 – Diffraction pattern obtained from the crystal of $d(\text{TCGGCGCCGA})$ and $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with synchrotron radiation

from the electron density and the final R-factor and R-free values obtained after the complete structural refinement were 0.108 and 0.124 respectively (as detailed in Table 3.5).

	High Resolution Data Collection
Data collection	
Space Group	P4 ₃ 2 ₁ 2
Cell Dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	42.3, 42.3, 39.9
α , β , γ (°)	90.0, 90.0, 90.0
Resolution (Å)	21.16-1.10 (1.16-1.10)*
<i>R</i> _{sym} or <i>R</i> _{merge}	0.066 (0.452)
<i>I</i> / σ <i>I</i>	24.6 (6.1)
Completeness (%)	99.6 (100)
Redundancy	15.8 (16)
Refinement	
Resolution (Å)	18.93-1.10
No. of Reflections	13340
<i>R</i> _{work} / <i>R</i> _{free}	0.108/0.124
No. of Atoms	
DNA	202
Ligand	51
Water	64
B-factors	
DNA	14.8
Ligand	11.96
Water	24.03
R.m.s deviations	
Bond Lengths (Å)	0.0273
Bond Angles (°)	3.044
One crystal used	
*Highest resolution shell is shown in parenthesis	

Table 3.5 – Data collection, phasing and refinement statistics from the synchrotron radiation data at DLS

3.6 Results and Discussion

The DNA structure described here was formed by the binding of the complex $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to a TCGGCGCCGA oligonucleotide sequence with Ba^{2+} ions. This is the first time a crystal structure of a polypyridyl ruthenium complex bound to a nucleic acid has been obtained, solved and refined. In this structure, the biological unit of DNA duplex and $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ are generated through symmetry operations from the asymmetric unit consisting of a single DNA strand, one barium ion, one metal complex and sixty-four water molecules. Interestingly, the presence of the ruthenium complex resulted in the oligonucleotide adopting a B-DNA confirmation and not forming the Holliday junction structure that has been reported previously with identical crystallisation parameters only without the Λ -complex.³⁷

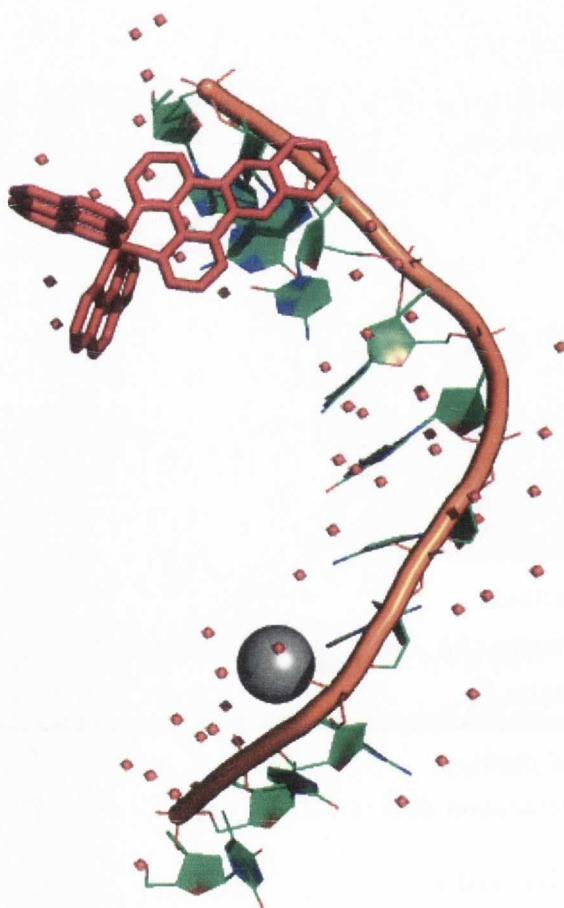


Figure 3.22 – The asymmetric unit cell, $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ is shown in red, Ba^{2+} ion is the grey sphere, sixty-four water molecules (red dots)

The structural analysis revealed a symmetrical duplex containing a twofold axis running through the mid-point (Figure 3.23). In each of the two strands of the duplex, two different binding modes of the Λ -enantiomer can be seen;

- the intercalation of the dppz ligand at the terminal G9-C2/A10-T1 step
- the semi-intercalation of a TAP ligand at the G3-C8/G4-C7 step

The DNA duplex, while mostly adhering to the B-type conformation, does exhibit some A-DNA properties. It is unwound and lengthened due to the intercalation, and is dramatically kinked at the site of semi-intercalation. A further interesting aspect of this crystal structure is that the intercalation at the oligonucleotide terminus results in the ‘flipping out’ of the adenine to facilitate the binding of the dppz moiety. This allows for the cross-linking of DNA duplexes, yielding a beautiful lattice arrangement containing hydration channels of two distinct sizes. These findings are discussed in full in the following sections.

From a chemistry perspective, this crystal is a very exciting step forward in our understanding of the binding nature of ruthenium complexes to DNA. However, two surprising features that occur to the oligonucleotide as a consequence of the interaction reveals that this ruthenium complex is highly relevant in biological terms. These features are the flipping out of an adenine residue as a consequence of intercalation and the dramatic kinking of the DNA caused by semi-intercalation. Both these phenomena are commonly found in biology, particularly during the study of the cellular transcription and replication.

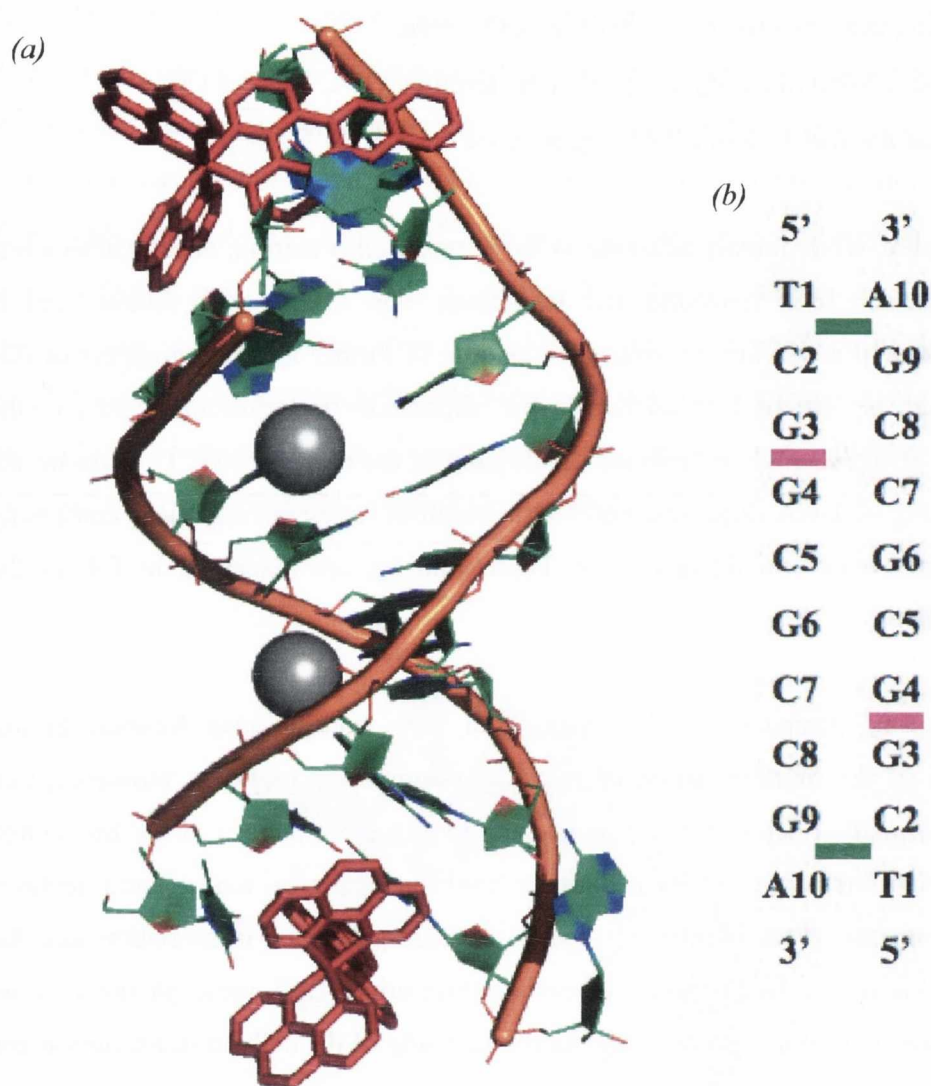


Figure 3.23 – (a) Double stranded B-DNA conformation of the resolved crystal structure.

$[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes are shown in red, Ba^{2+} ions are the grey spheres (b) Residue numbering for the oligonucleotide with the dppz binding sites in green and TAP binding sites in magenta

3.6.1 Binding Modes of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to d(TCGGCGCCGA)

Refinement of the crystal structure revealed two distinct binding modes of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex; intercalation of the dppz ligand and semi-intercalation of a TAP ancillary ligand. Intercalation in DNA, as explained previously, is the insertion of the planar chromophore of a molecule between adjacent bases pairs of a DNA. This feature of DNA binding has long been suspected as the major mode by which ruthenium dppz complexes associate with DNA as the resultant distortion caused to the duplex can be seen as a hypochromic and bathochromic shifts in UV/vis spectroscopy. The discovery of a semi-intercalative binding mode was somewhat unexpected. Though proposed as a possible method of binding for $[\text{Ru}(\text{phen})_3]\text{Cl}_2$ ²⁵ and $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ ³⁸ this method of binding is very difficult to prove. In contrast to classical intercalation, semi-intercalation is defined as a ligand of a molecule wedging an opening between adjacent base pairs, as shown diagrammatically in Figure 3.24.

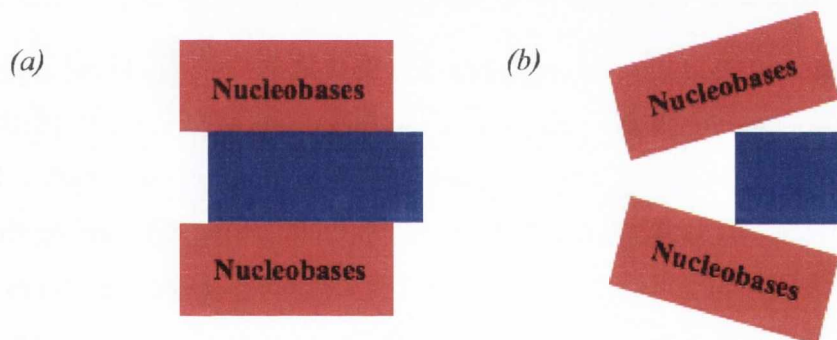


Figure 3.24 – Diagrammatic representation of (a) Classical intercalation

(b) Semi-intercalation. The red blocks represent adjacent base pairs in a DNA helix, the blue blocks represent the intercalating ligand

Semi-intercalation is usually attributed to ligands of an aromatic nature which are truncated (relative to a known intercalator such as dppz) such that they are unable to undergo classical intercalation into the base stack due to steric hindrance from the bulk of the metal complex.

3.6.1.1 Intercalation of the dppz ligand

It has been well documented that intercalating ligands contain an extended aromatic chromophore. While many such ligands have been investigated with regard to nucleic acid interactions, the success of the dppz ligand in fulfilling this role is unparalleled in ruthenium chemistry. Throughout the literature, ruthenium-dppz complexes have been well studied because of their binding affinity for DNA and their ability to cause photo-oxidative damage to the nucleic acids once bound. This mode of binding can be detected quite easily by more conventional techniques as an intercalated molecule not only induces change in the UV/vis and linear dichroism spectra, but also an increase in viscosity as the accommodation of the intercalator results in a lengthening and stiffening of the base stack. The mode of binding has been seen in the crystal structures of many compounds, but as yet not with ruthenium complexes. As such, upon obtaining crystals from the binding of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex with the decamer $\text{d}(\text{TCGGCGCCGA})$, intercalation was the primary mode of binding expected.

The dppz complex was indeed found to intercalate into the oligonucleotide in the crystal structure. The intercalation occurs from the side of the minor groove, i.e. the $\text{Ru}(\text{TAP})_2$ moiety resides in the minor groove, and causes an unwinding of the duplex by approximately 15° . Interestingly the Λ -complex shows a preference for A-T binding sites over G-C, hence the intercalation occurs at the termini of the duplex. From the images in Figure 3.25 it is clear that the dppz ligand interacts almost exclusively with the purine bases, guanine and adenine. There is minimal overlap between the aromatic rings of the dppz and the pyrimidine bases. The interaction occurs with the long axis of the dppz ligand at an angle of approximately 45° to the long axis across the G-C and A-T base pairs on either side. The principal stacking interaction is between the pyrazine ring of the dppz ligand and the six-membered purine rings of G9 and A10, so that the ruthenium atom is approximately 6.5 Å from the helical axis of the duplex and the ligand does not directly contact any of the surrounding water molecules.

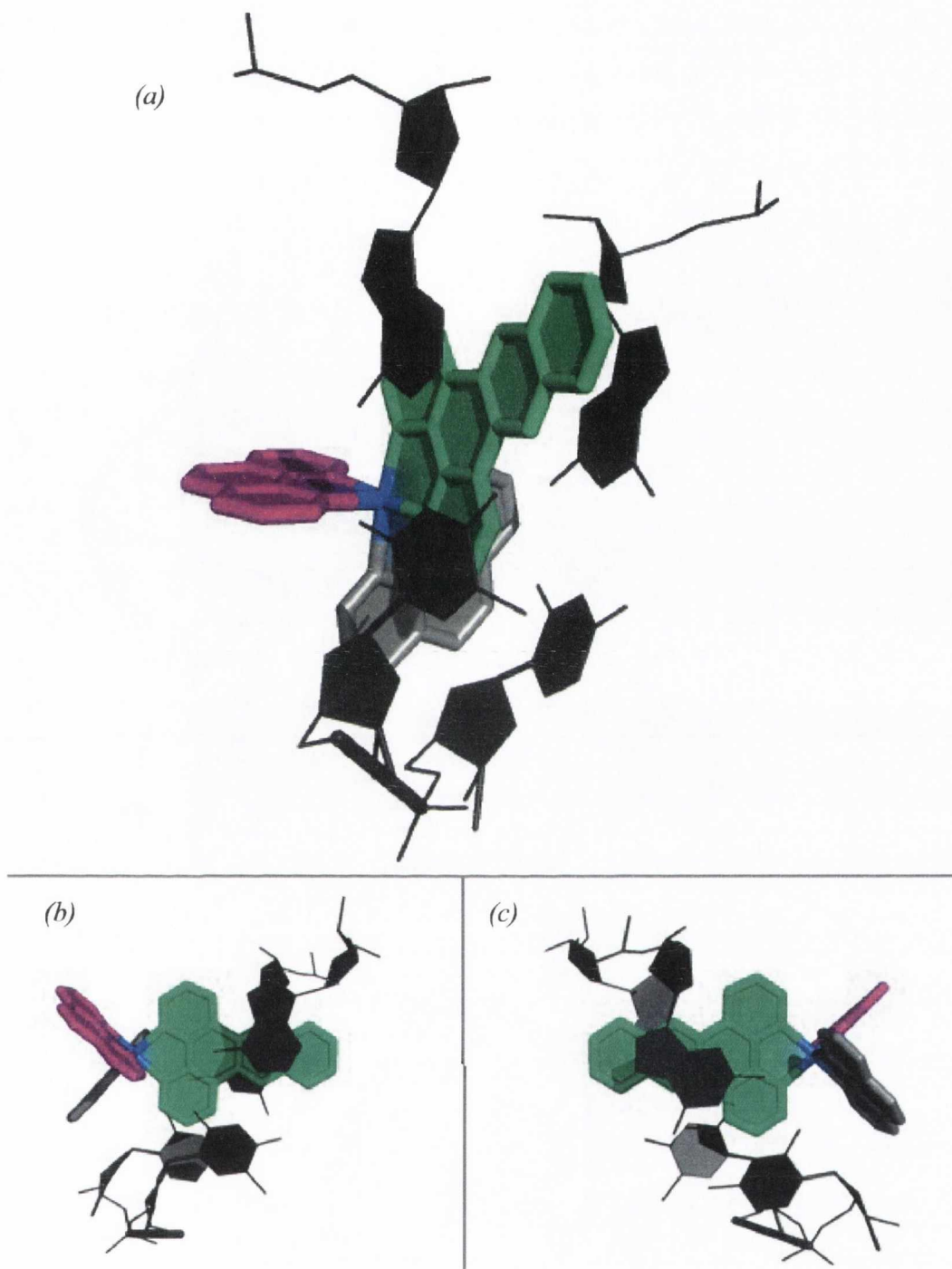


Figure 3.25 – (a) Intercalation of the dppz ligand of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ at the terminus of the DNA duplex. The dppz ligand is in green, the adenine, guanine, thymidine and cytosine bases are in black. (b) The overlap of the dppz intercalation viewed from the adenine side. (c) The overlap of the dppz intercalation viewed from the guanine side

Upon first examination, classical intercalation was presumed, however, further refinement of the high-resolution data showed this was not the case. The terminal adenine of each oligonucleotide strand is, in fact, flipped out.

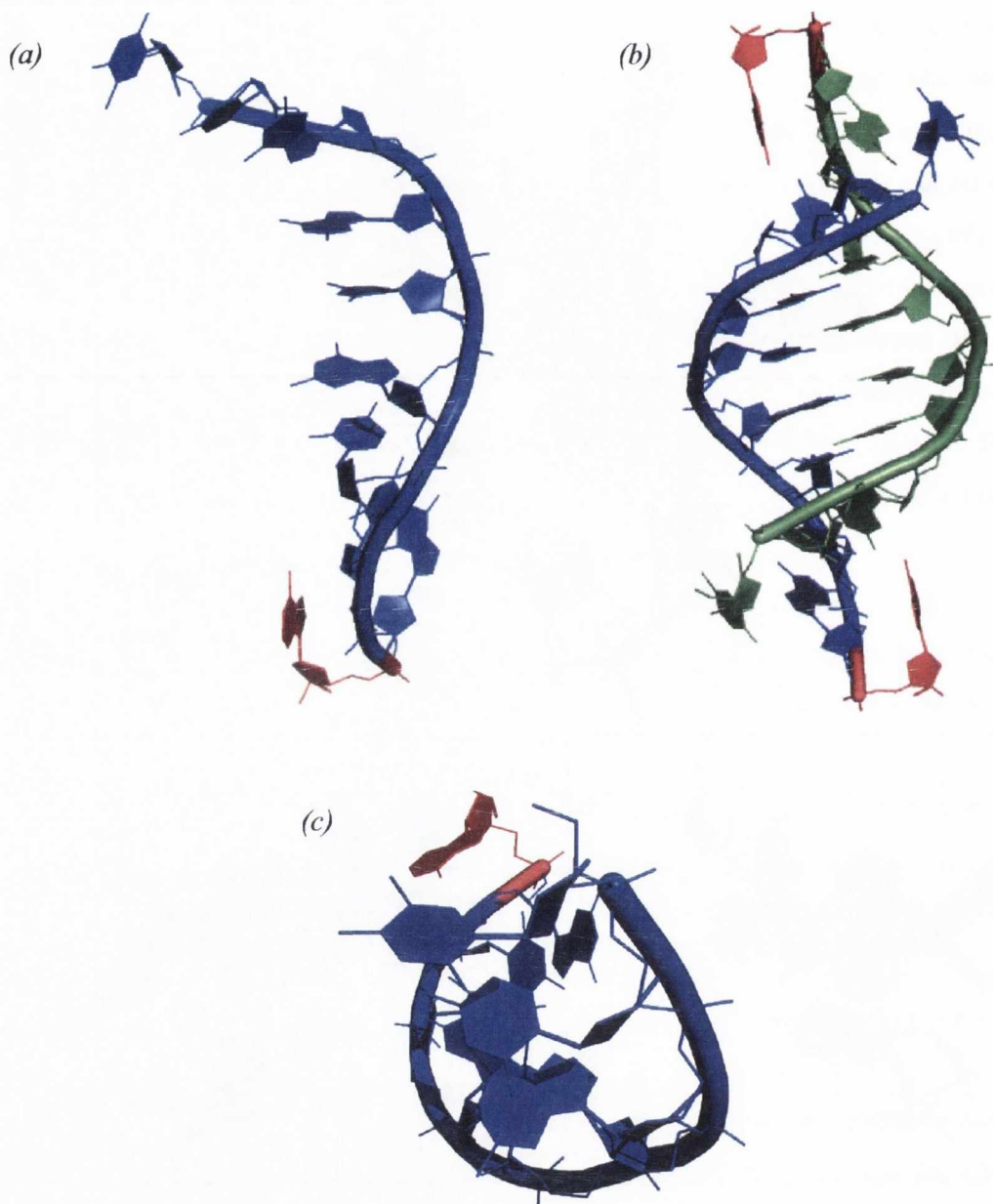


Figure 3.26 – (a) Single stranded (TCGGCGCCGA) with the flipped-out adenine in red (b) Double stranded helix with the two flipped-out adenines in red (c) Single stranded (TCGGCGCCGA) with the flipped-out adenine in red viewed from the top

First confirmed in bacteria, DNA base flipping occurs in all organisms, from plants to people. It has been described as a simple structural change that may be the first step in the replication and transcription of DNA. While many studies have shown the structure of flipped out DNA bases (commonly adenine,³⁹ cytosine⁴⁰ and thymine⁴¹) the process through which the flipping out occurs is as yet not fully understood. Base flipping has been shown to be essential for a number of processes in which enzymes interact with DNA, for example DNA methylation,⁴⁰ various repair mechanisms⁴² and possibly early events in the opening of DNA as required for transcription.^{43,44} Enzymatic action requires flipped out bases to facilitate insertion into their active site. The ensuing chemical modifications of certain bases are involved in the regulation of the expression and transcription of DNA. A central question, key to understanding this process, is how big a role do enzymes play in base flipping. Do they incite the base to flip out, or do they simply bind after the fact? As yet we do not know the answer but current understanding is that the enzymes require a flipped base before binding to the active site can occur.

A further question relevant to the flipping out of bases concerns the major and minor grooves in DNA. Through which of these grooves do the bases turn as they flip outward? Structural evidence to date suggests the minor groove,⁴⁵ but some experimental evidence alludes to the major groove.⁴⁶

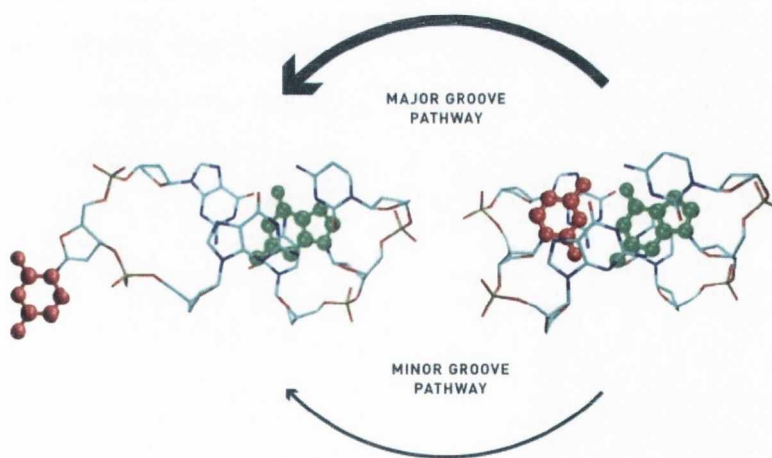


Figure 3.27 – Example of the flipping of a cytosine (red) from the DNA double-helical conformation (right) to a flipped conformation (left) via the major or minor grooves. The orphaned guanine is shown in green⁴⁰

The flipping out of the adenine residue caused by the intercalation of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex proceeding via the minor groove of the double helix. In contrast to many previous studies, the adenine is not only flipped out, but also reversed in its orientation to allow reverse Watson-Crick bonding between it and an orphaned thymine residue of another duplex. This is the first of three interactions found within this crystal structure that cause the non-covalent cross-linking of duplexes.

The second, and probably most significant non-covalent cross-linking interaction also comes as a result of the flipping out of adenine. The main intercalative interaction occurs between the dppz ligand and the guanine residue G9 as seen in the optimal π stacking between the two aromatic systems (Figure 3.25). To facilitate this interaction the terminal adenine (A10) flips out, creating a cavity into which the dppz ligand can insert and associate with the G9 residue (Figure 3.28). Once the ligand is bound however, the adenine cannot regain its original positioning and remains flipped out. This allows a flipped out adenine from a second duplex (Duplex 2) to interact with the top face of the dppz π system and stabilise the intercalation. To complete the interaction, this second adenine forms a reverse Watson-Crick bonding arrangement with the orphaned thymine (T1) from the first duplex (Figure 3.29). The result is the non-covalent cross-linking of Duplex 1 and Duplex 2 at their termini in an almost perpendicular arrangement (Figure 3.30). The flipped out adenine from Duplex 1 is thus free to interact with the π system of a dppz ligand bound to the G9 residue from a third duplex (Duplex 3) and reverse Watson-Crick bind to the orphaned thymine from that duplex (Figure 3.31). This creates an extensive non-covalently cross-linked lattice structure that is undoubtedly the reason for the wonderful diffraction data obtained from this system (Figure 3.45).

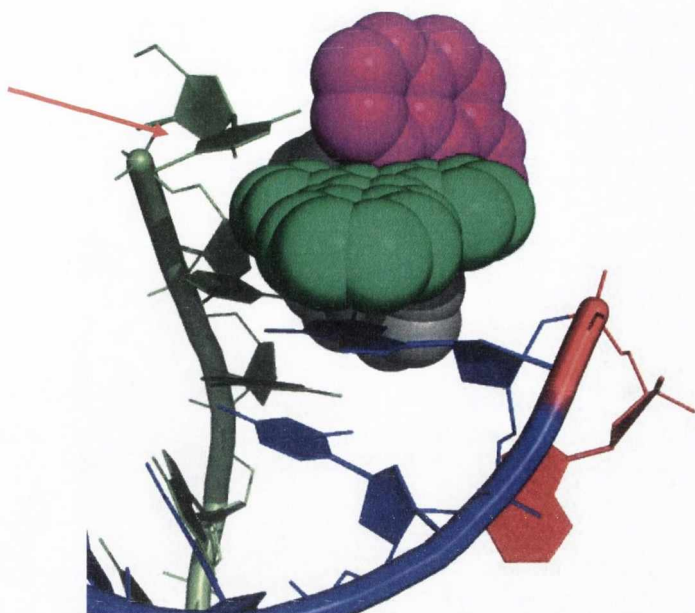


Figure 3.28 – The cavity formed by the flipping out of A10 (red), allowing the intercalation of the dppz ligand (green). G9 (blue) is beneath the dppz. The orphaned T1 (green) is indicated by the arrow

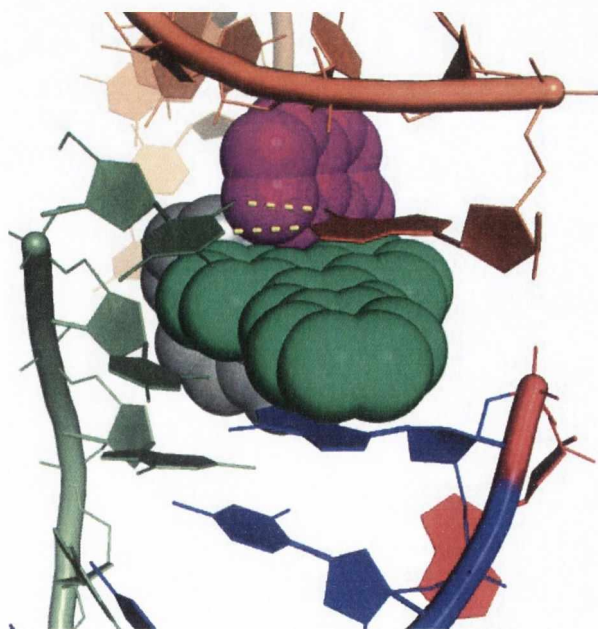


Figure 3.29 – Stabilisation of the intercalation pocket by a flipped out adenine from Duplex 2 (orange). The reverse Watson-Crick bonding is shown (dotted yellow lines). The dppz ligand is in green with the flipped out A10 of Duplex 1 in red



Figure 3.30 – The almost perpendicular arrangement of Duplex 1 (blue) and

Duplex 2 (orange) as a result of non-covalent cross-linking



Figure 3.31 – The association of the flipped out adenine (indicated by the arrow) from Duplex 1 (blue) with Duplex 3 (purple). Note a second $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex (flat) in close proximity to the initial complex (spheres).*

Duplex 2 is in orange

**Close proximity of ruthenium complexes is discussed later in the chapter*

3.6.1.2 Semi-Intercalation of a TAP ligand

First postulated in 1975,⁴⁷ partial-intercalation was evidenced by the shielding of specific proton resonances in NMR experiments and a decrease in the electrophoretic mobility of DNA. This behaviour is contrary to classical intercalation, which increases viscosity, because it causes the wedging apart of base pairs rather than a formal rise between adjacent base pairs; which in turn induces kinking, a decrease in the effective length of the duplex, and consequently, a decrease in viscosity. Initially thought to be difficult to prove, considerable evidence for a partially-intercalative binding mode has emerged from the results of linear dichroism experiments,²⁵ viscosity measurements,²⁴ NMR experiments,⁴⁸ electrochemical studies,⁴⁹ and molecular modelling investigations.⁵⁰ The data obtained for partially intercalating compounds indicate that there are two differing modes of partial intercalation; semi-intercalation and quasi-intercalation.

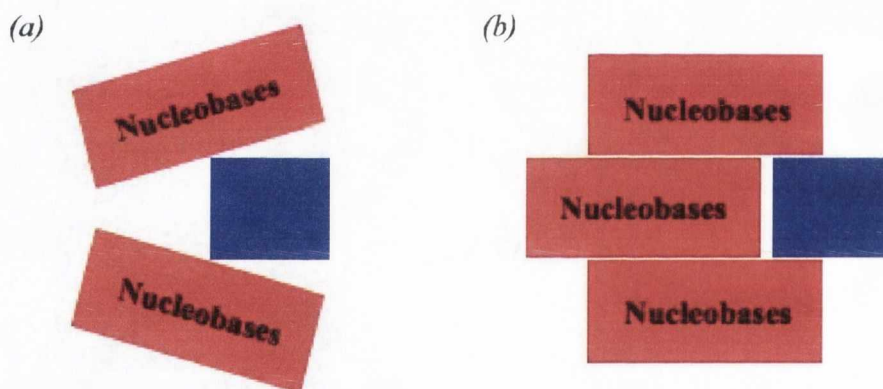


Figure 3.32 – Schematic representation of (a) semi-intercalation and (b) quasi-intercalation. The red blocks represent adjacent base pairs in a DNA helix, the blue blocks represent the intercalating ligand

One of the surprising discoveries of this crystal structure was the observation of the semi-intercalative binding mode into duplex DNA. This occurs as a minor groove-based interaction between a TAP ligand and the G3-C8/G4-C7 step of the duplex (See Figure 3.33). The truncated nature of the ligand is such that it is unable to undergo classical intercalation. Instead, the distal ring of the TAP ligand is partially inserted between the G3 and G4 residues, wedging these bases apart by some 51°. The net result is a large

positive roll, opening those base pairs towards the minor groove, and creating an approximately right-angled bend to the overall base stack. The insertion of the TAP ligand also increases the distances between the G4 and G5 residues as shown in Table 3.6

	C2-G9	G3-C8	G4-C7	C5-G6	G6-C5	C7-G4	C8-G3	G9-C2
Shear	0.11	-0.22	-0.20	0.21	-0.21	0.20	0.22	-0.11
Stretch	-0.09	-0.04	-0.15	-0.08	-0.08	-0.15	-0.04	-0.09
Stagger	0.27	0.12	-0.07	0.24	0.24	-0.07	0.12	0.27
Buckle	-11.92	23.55	-9.43	-4.05	4.05	9.43	-23.55	11.92
Prop-Tw	7.15	-5.23	1.45	-5.46	-5.46	1.45	-5.23	7.15
Opening	1.45	0.08	-1.48	0.35	0.35	-1.48	0.08	1.45
Shift	0.00	-0.53	-0.13	-0.83	0.00	0.83	0.13	0.53
Slide	0.00	1.70	0.86	0.13	0.78	0.13	0.86	1.70
Rise	0.00	2.60	5.21	3.22	3.33	3.22	5.21	2.60
Tilt	0.00	2.63	-2.94	-2.95	0.00	2.95	2.94	-2.63
Roll	0.00	3.74	50.86	-2.99	22.84	-2.99	50.86	3.74
Twist	0.00	19.40	15.13	38.66	27.80	38.66	15.13	19.40

Table 3.6 – Local base pair and step parameters, calculated with 3DNA v.1.5.
The roll angles caused by the semi-intercalation of the TAP ligand are highlighted in red.
The rise in the G3-G4 distances are highlighted in yellows

Interestingly, the presence of a semi-intercalative binding mode results in distinct roles for the two chemically identical TAP ligands (distinguished as TAP1 and TAP2). The TAP1 ligand is the semi-intercalating moiety at the G3-C8/G4-C7 step. TAP2 lies in the minor groove and remains inert in terms of the supramolecular structure. For clarity, the TAP1 ligand is coloured pink and the TAP2 ligand is grey (Figure 3.33).

A serendipitous discovery in this work, arising from the binding of the dppz and TAP1 ligands, is the importance of adjacent purines in the sequence. Writing the oligonucleotide sequence as py1-py2-**pu3-pu4**-py5-pu6-py7-py8-**pu9-pu10**, highlighting the binding sites, shows that interaction of the Λ -complex with the duplex only occurs at the sites of adjacent purines, implying a preference for purine-purine steps as opposed to the base specific steps.

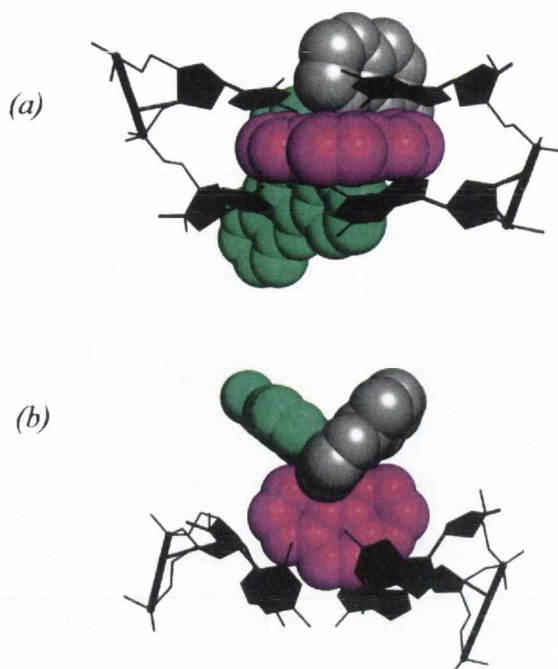


Figure 3.33 – Semi-intercalation of TAP1 (pink) between G3-C8 and G4-C7 of the duplex. (a) Side on view, looking down the TAP Axis, (b) Top-down view, looking down the helical axis

Inspection of the planes shows that the alignment here is not symmetrical, the G3-TAP1 plane angle being 37.5° , with the G4-TAP1 plane angle much smaller at 18.9° (Figure 3.37) The net effect of this asymmetry is that there is stronger stacking between residue G4 and TAP1.

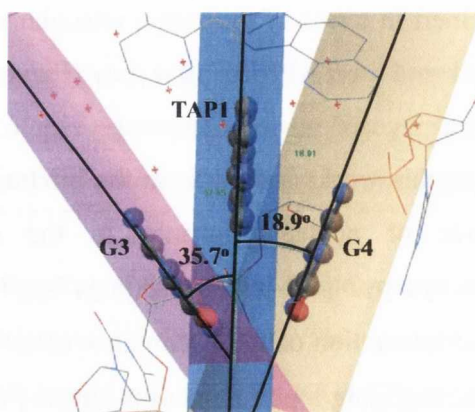


Figure 3.34 – Angles of the planes between G3 (pink), G4 (yellow) and TAP1 (blue) showing a greater stacking between G4 and TAP1

The semi-intercalation serves to reinforce the non-covalent cross-linking that occurs between duplexes within this crystal structure, and is the third and final non-covalent cross-linking association. While the dppz ligand fully intercalates between the G9-C2/T1 residues of Duplex 1 and flipped out A10 of Duplex 2 creating the initial non-covalent cross-linking, the TAP1 ligand semi-intercalates between the G3/G4 step of Duplex 2, resulting in a solidifying of the arrangement of the two duplexes through two separate non-covalent interactions. This also leads to the formation of the beautiful, yet complex lattice structure discussed later in the chapter.

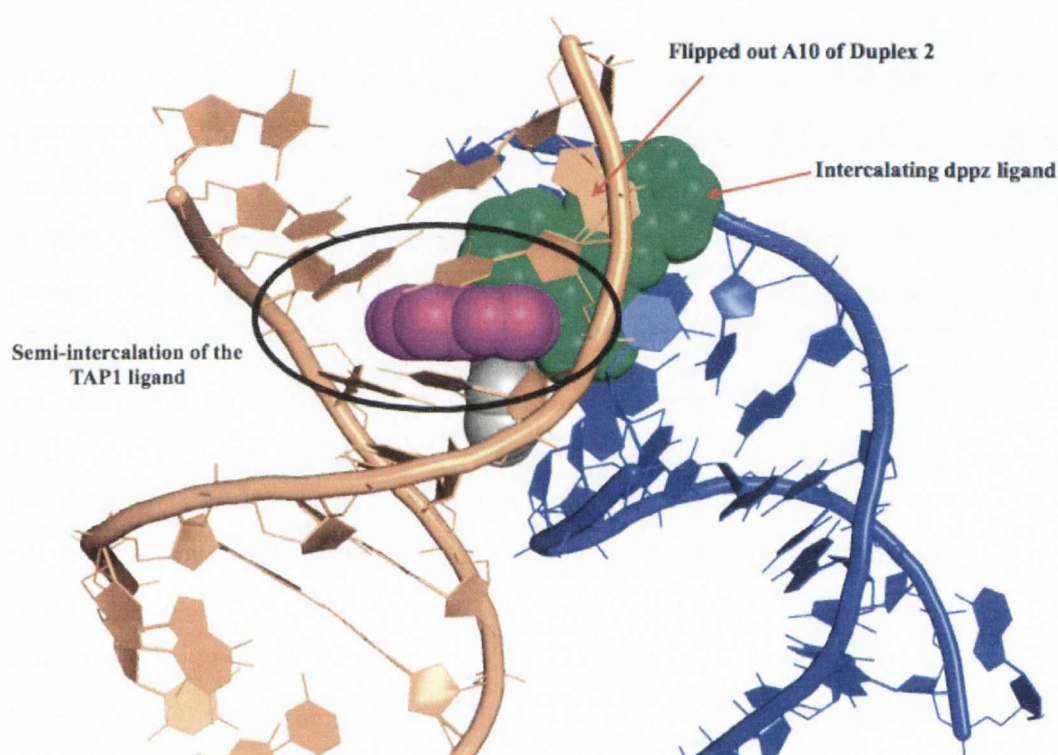


Figure 3.35 – Semi-intercalation of the TAP1 ligand (pink) reinforcing the non-covalent cross-linking of Duplex 1 (blue) and Duplex 2 (orange)

In terms of photochemical behaviour, the TAP ligand and the more extensively studied phen ligand are very different, however the two are approximately isosteric and therefore may not exhibit any structural differences at this level. Interestingly, the TAP1 ligand does not form any specific hydrogen-bonded interactions with the guanine bases, suggesting that this particular mode of binding might not be specific to the TAP ligand. A variety of other ligands with polyaromatic surfaces, such as dpq,⁵¹ ppz,⁵² and imidazo-

phenyl derivatives of the phen ligand,⁵³⁻⁵⁵ are also believed to interact in this matter, though none have yet to be crystallized.

Intercalation interactions such as these are reminiscent of those observed in biological systems. DNA-binding proteins commonly interact with specific sites on a DNA duplex via partial intercalation of aromatic amino acid side chain residues (such as phenylalanine or tryptophan) between base pairs.⁵⁶ As with the present complex, and many ascribed this binding mode, the result of the partial insertion is a wedging apart of the base pairs and a subsequent kinking of the DNA helix.

Kinking in a DNA helix occurs when stacked base pairs become unstacked and allow the now strained DNA to make a sharp turn.⁵⁷ It is a phenomenon almost exclusively observed with protein-DNA interactions^{58,59} which are known to bind sequence-specifically to DNA causing local bending or kinking. Biological processes involved in the control and regulation of transcription are dependent on this protein-induced distortion in the DNA structure⁶⁰ to enhance the recruitment of proteins to their specific DNA targets. An excellent example of the phenomenon occurs upon the binding of the sequence-selective TATA box Binding Protein (TBP) to the TATA box regulatory DNA sequence in order to initiate the assembly of a transcription complex. TBP binds via partial intercalation of phenylalanine residues via the minor groove either side of the recognition site, inducing significant roll angles at these locations and causing an overall dramatic kink (approximately 47°) in the DNA.^{56,58,61} Similarly, proteins possessed of a high-mobility group (HMG) domain bind to DNA via partial intercalation of a phenylalanine residue via the minor groove, with concomitant bending of the DNA.⁵⁶ While the TATA box system regulates transcription within a cell, the HMG domains exhibit a strong binding preference for regions that are already distorted from canonical B-DNA and hinder the DNA repair process.^{62,63} This induces cell apoptosis and is a pathway utilised in the treatment of many cancers, particularly through the use of cis-platin.

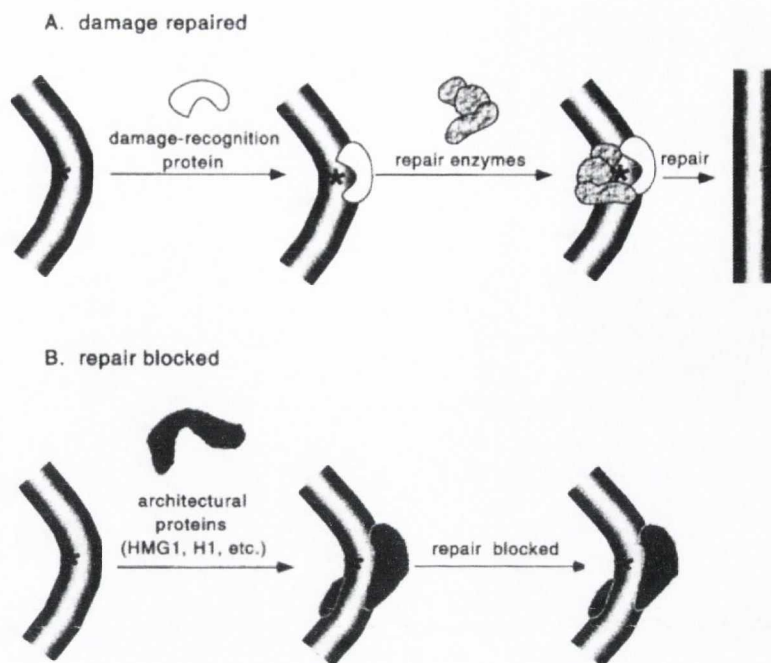


Figure 3.36 – Schematic representation of (a) the repair mechanism of DNA and (b) HMG proteins preventing damage recognition and thus repair, leading to apoptosis⁶⁴

Cis-platin is one of the very few small molecules reported to induce kinking in DNA helices. It is a potent anticancer drug⁶⁵⁻⁶⁷ whose biological activity is a consequence of the formation of covalent adducts between the platinum atom and specific base sequences in DNA. Interestingly, only the *cis* isomer of the compound is therapeutically active. The *trans* isomer (commonly called trans-platin) is inert in terms of its medical efficacy.

Both the *cis*- and *trans*-isomers covalently bind to DNA with a preference for the purine bases. Cis-platin can cause intra-GG interactions and cross-link the duplex via inter-(G-C)/(G-C) bonding while trans-platin can only cross-link the two strands via inter-(G-C)X(G-C) bonding. The binding of both compounds inhibits replication, however only the *cis* isomer is cytotoxic. This is as a result of the intra-strand link formed by cis-platin, causing a kink in the DNA helix by between 30° and 50°. The induced kink promotes the binding of the architectural HMG proteins that prevent the removal of the platinating compound and thus hinder the repair of the DNA helix, preventing transcription from occurring and causing cell death through apoptosis.

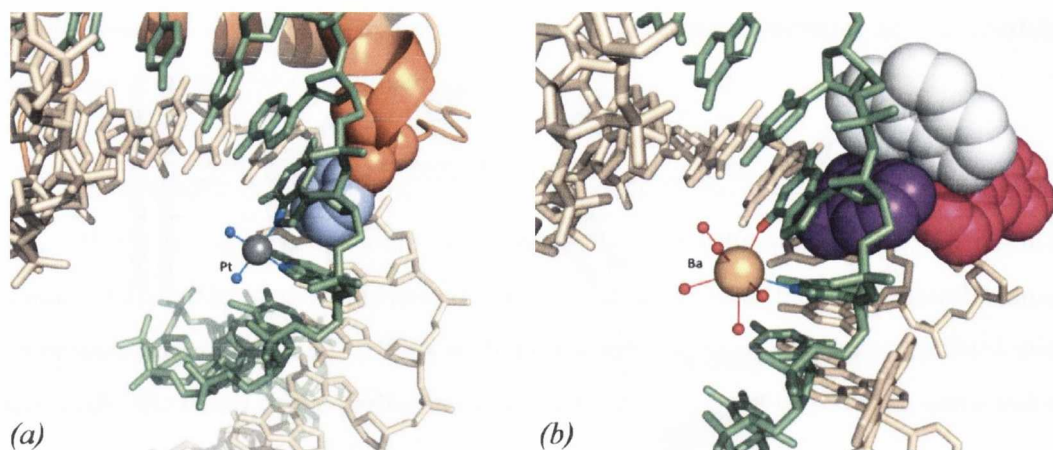


Figure 3.37 – Crystal structure of the DNA binding of (a) cis-platin with the a HMG protein and (b) Λ -[Ru(TAP)₂dppz]²⁺

The binding and DNA kinking by cis-platin, a small molecule, allows us to compare and contrast the kinking induced by the semi-intercalation of the TAP1 ligand of the $[\Lambda\text{-Ru(TAP)}_2\text{dppz}]^{2+}$ complex, another small molecule, observed in this work. It is interesting to note that both complexes have similar sequence specificity. Cis-platin binds to two adjacent purine residues within a single strand,⁶⁴ and in this study the TAP1 ligand also associates between two adjacent purine residues on a single strand. They also both induce a kink in the DNA double helix with a roll angle of approximately 50° when interacting with adjacent guanine bases. The methods through which they associate with DNA are, however, very different.

Cis-platin forms two covalent bonds between the central Pt²⁺ ion of the compound and the N7 of guanine and either N1 or N7 of adenine via platination. This results in the cross-linking of neighbouring purines and the kinking of DNA when such cross-links are formed within the same strand. Once bound, the compound is very difficult to remove due to the strong Pt-N bonds formed and the kinetic inertness of the compound. Association of the TAP1 ligand in the $[\Lambda\text{-Ru(TAP)}_2\text{dppz}]^{2+}$ complex occurs non-covalently via semi-intercalation. This process is driven by the stabilisation that results from hydrophobic interactions and the overlapping π electron systems of the two guanine residues and the aromatic TAP1 ligand. Due to the truncated nature of this ligand, the TAP1 can only partially insert between the purine residues, resulting in the formation of a wedge in the DNA helix and a kink in the backbone. This method of binding is much

weaker than the covalent platination of the cis-platin and thus is thought to be a reversible process. Furthermore, the binding locations of both compounds are very different. Cis-platin binds from within the DNA helix at the major groove, i.e. compound associates to the purine residues from within the centre of DNA molecule. This results in the kinking of the backbone occurring from the inside out and the DNA bending in on itself over the cis-platin. The TAP1 ligand, however, semi-intercalates to the exterior of the DNA molecule, at a minor groove. This results in the DNA helix also bending back on itself, but the bend occurring away from the associating molecule. A small pocket is formed at the major groove into which smaller molecules may also associate with the DNA to stabilise the structure. An example of this is the barium ion, Ba^{2+} , which will be discussed shortly in this chapter. Table 3.7 summaries the similarities and differences between the two complexes.

	Cis-platin	$[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$
Sequence Specificity	G-G	G-G
Roll Angle	$30^\circ\text{-}50^\circ$	51°
Binding Mode	Covalent	Non-covalent
Binding Location	Within the Duplex	Outside the Duplex
Binding Ligand	Pt^{2+}	TAP
Groove	Major	Minor

Table 3.7 – Comparing and contrasting the binding of cis-platin and $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to DNA

The cytotoxicity of the cis-platin compound is specific to the formation of intra-strand links, and thus a kink of $30^\circ\text{-}50^\circ$. Ruthenium(II) complexes have recently been shown to cross the nuclear membrane and enter the cell nucleus.⁶⁸⁻⁷¹ These findings also show that the ruthenium complexes have a cytotoxic affect on cellular activity. Speculations as to the reasons for cellular apoptosis have been made, but to date the mode of nuclear binding has not been identified. The similarity in the overall effect to the DNA helix

caused by the semi-intercalation of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex, coupled with the fact that semi-intercalation is thought to occur with many truncated ligand systems could possibly explain the cytotoxicity of aromatic ruthenium(II) compounds reported in various cell cultures.

3.6.1.3 The $A \leftrightarrow B$ Transition

Another interesting facet to emerge from the study of this crystal structure is the transition from the B-DNA form to the A-DNA form within the decamer. The overall conformation of the double helix was assumed to be B-DNA but investigation of the torsion angles, prompted by the unusual size of the minor and major grooves compared to B-DNA norms (Figure 3.38), revealed the sugar puckers in the phosphate backbone oscillate from C3'-*endo* (A-DNA) to C2'-*endo* (B-DNA).

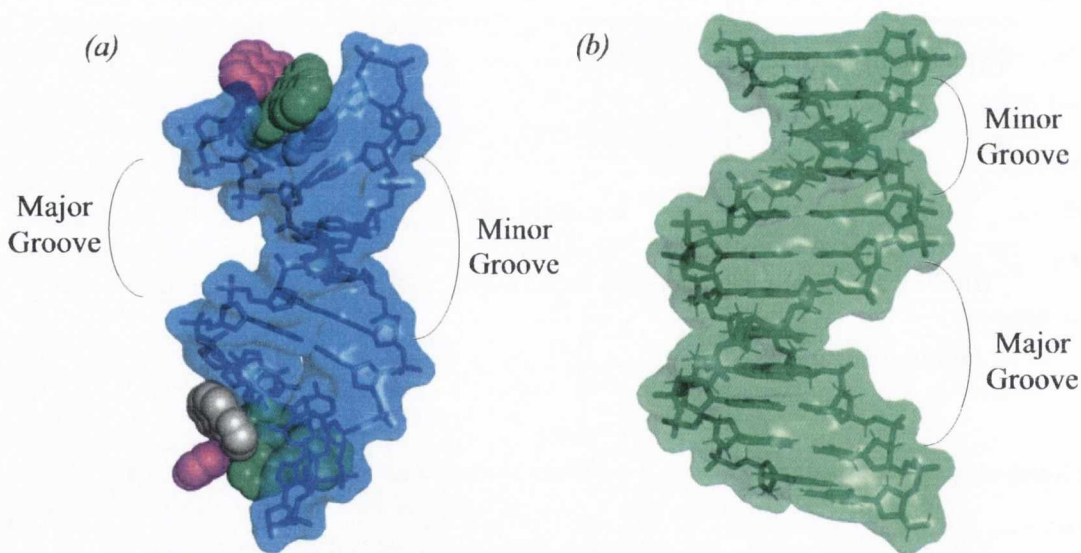


Figure 3.38 – Major and minor grooves of (a) the A/B-DNA hybrid obtained in this work and (b) B-DNA

Sugar puckers within DNA arise from the fact that the five-membered deoxyribose sugar ring in DNA is inherently non-planar in order to minimise non-bonded interactions between their substituents and minimise the steric strain energy within the ring. The nature of the sugar pucker determines the shape of the DNA helix, and also whether the

DNA molecule adopts the A or the B conformation. There are two commonly found conformations in the sugar pucker known as *endo*-puckers, C2'-*endo* and C3'-*endo*. In the A-DNA form, the C3' atom lies above the plane of the sugar ring with the C2' atom lying below it and, as such, the A form is described as C3'-*endo*. Similarly in the B-DNA form, the C2' atom sits above the plane of the sugar ring with the C3' atom below the plane and as such is named C2'-*endo* (Figure 3.39).

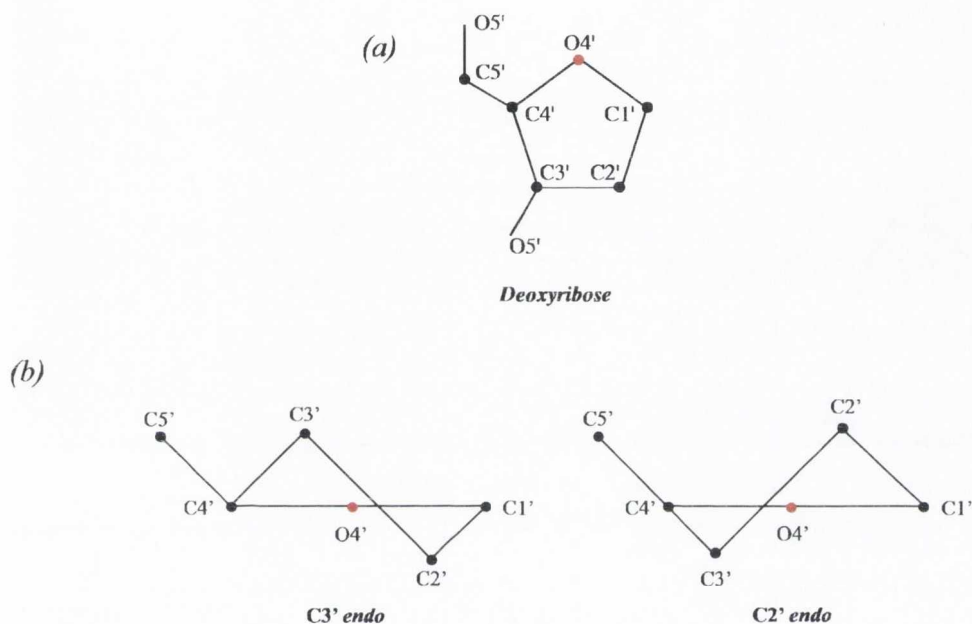


Figure 3.39 – (a) Labelled structural formula of deoxyribose sugar (b) The C3' and C2' endo conformations of the pentose sugar ring

The differing sugar pucker conformations are caused by the various rotation angles that characterise the nucleotide chain. As Figure 3.40 shows, there are six independent angles; α (rotation around P-O5'), β (around O5'-C5'), γ (around C5'-C4'), δ (around C4'-C3'), ϵ (around C3'-O3') and ζ (around O3'-P).

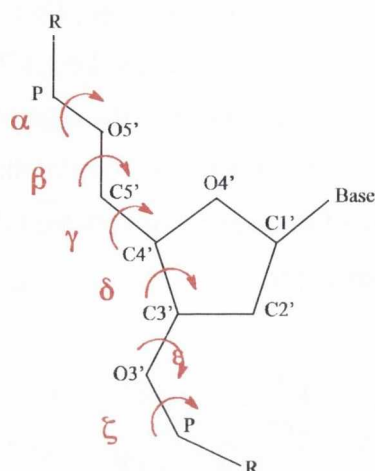


Figure 3.40 – The backbone torsion angles in a unit nucleotide. Each rotatable bond is indicated by a curved arrow

The values for each of these angles were obtained for the decamer sequence in this study and are shown in Table 3.8.

Nucleotide	α	β	γ	δ	ϵ	ζ
T1			55.871	81.428	-175.230	-76.924
C2	-65.895	-176.377	61.741	86.563	-175.527	-83.207
G3	-68.468	165.309	55.850	90.915	-147.337	-65.376
G4	-71.584	-174.537	53.395	146.898	-165.237	-138.248
C5	-66.869	165.994	45.120	93.119	-160.776	-64.500
G6	-65.176	-170.490	50.620	140.718	-174.028	-93.824
C7	-68.175	167.137	44.999	82.085	-142.256	-71.605
C8	-65.405	178.229	53.380	108.355	-161.049	-84.327
G9	-64.622	172.083	46.638	140.782	-111.206	48.954
A10	94.147	-171.535	-177.496	139.348		

Table 3.8 – Torsion angles of TCGGCGCCGA

In particular the torsion angle δ adopts values that relate to the pucker of the sugar ring. Ideally these values are approximately 75° for C3'-endo and 150° for C2'-endo

conformations. It is interesting to note the positive value of the A10 α and negative value of A10 γ as being the opposite signs to the other nucleobases. This is due to the flipping out the adenine residue.

Nucleotide	δ	Sugar Pucker	Conformation
T1	81.428	C3'-endo	A-DNA
C2	86.563	C3'-endo	A-DNA
G3	90.915	C3'-endo	A-DNA
G4	146.898	C2'-endo	B-DNA
C5	93.119	C3'-endo	A-DNA
G6	140.718	C2'-endo	B-DNA
C7	82.085	C3'-endo	A-DNA
C8	108.355	C2'-endo	B-DNA
G9	140.782	C2'-endo	B-DNA
A10	139.348	C2'-endo	B-DNA

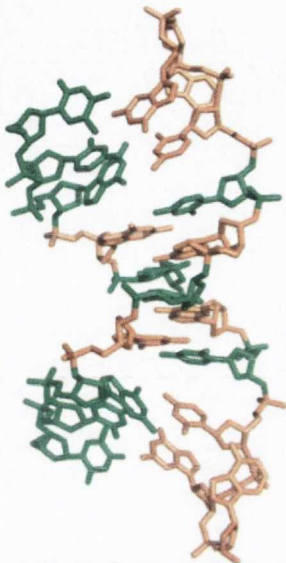


Table 3.9 – δ torsion angles and sugar puckers in TCGGCGCCGA

The transition from A- to B-DNA and vice versa is not uncommon in the study of DNA.^{72,73} In crystallographic studies, the reasons for these transitions are unclear though many believe it may be either as a consequence of the influence of crystal-packing forces or the slow dehydration of the solution to form the crystal. Within cellular DNA however, the transition from B- to A-DNA is essential for the processing of genetic information, particularly during transcription,^{74,75} and like kinking it is a feature often induced by the association of a protein. In terms of the present study, however, the B- to A-DNA transition was an unexpected discovery. The previously crystallised decamer sequence without any binding compounds yielded a Holliday junction structure, with all four DNA strands conforming to B-DNA torsion angles. Due to the substantial differences in the structure between the two DNA polymorphs, the junction between A- to B- DNA is known to cause a bend of approximately 20° in the helix. This is found to occur in the crystal structure presented here between C5 and G6 of the helix, with a value of 22.84°. This third kink in the DNA backbone, coupled with those induced by the semi-

intercalation of the TAP1 ligand results in an overall distortion of the B-DNA structure by over 115° .

	Shear	Stretch	Stagger	Buckle	Prop-Tw	Opening	Shift	Slide	Rise	Tilt	Roll	Twist
G6-C5	-0.21	-0.08	0.24	4.05	-5.46	0.35	0.00	0.78	3.33	0.00	22.84	27.80

Table 3.10 – Extract from Table 3.6 detailing the C5-G6 values.

The roll angle is highlighted in red

While a definitive explanation for the hybrid nature of the macromolecules remains elusive, our current understanding is that due to the dramatic changes forced upon the DNA backbone as a result of the various binding sites of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$, the macromolecule has to adopt the hybrid configuration in order to optimise its overall stability.

3.6.2 The Role of the Barium Ion

The crystal structure contains one barium ion per half duplex bound to the G3 and G4 in the major groove. This metal ion has proven vital to the formation of crystals as a various selection of Group I and Group II ions were screened (see Table 3.11) with only the solutions containing barium ions forming crystals. Interestingly, all of the solutions containing barium ions crystallised, implying that the nature of the Group I ion (i.e. sodium or potassium), or the pH (6.0 or 7.0) do not effect the formation of crystals with this particular complex and decamer.

Group I Ion	Group II Ion	Result
Sodium	Magnesium	X
Potassium	Magnesium	X
Lithium	Magnesium	X
Sodium	Barium	✓
Potassium	Barium	✓
Sodium	Strontium	X
Potassium	Strontium	X
Lithium	Strontium	X

Table 3.11 – Combinations of Group I and Group II ions attempted during crystallisation. For a more complete list see Experimental Chapter Table X.X

Two questions were paramount after the discovery of the crystal structure in this work;

- Is the conformation of the DNA a result of the ruthenium complex or the barium ions?
- Is the ruthenium complex binding first followed by the barium ion or vice versa?

This latter question is very pertinent to the field of ruthenium(II) DNA binders due to the argument as to the major/minor groove nature of the metal complex binding.

The work conducted previously by Dr. Naseer obtained crystals of this sequence as the Holliday junction using calcium ions, strontium ions and barium ions. This is the initial evidence that suggests the presence of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ is the main factor in the formation of the A/B-DNA hybrid duplex. In the absence of the Λ -complex but presence of barium ions, under identical crystallising conditions, TCGGCGCCGA forms the Holliday junction structure containing only C2'-*endo* torsion angles (see Figure 3.16). Thus the Λ -complex causes the DNA to adopt the A/B hybrid conformation, answering the first question.

It was also discovered that in the absence of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ the asymmetric unit of the Holliday junction (i.e. half the junction) contained two barium ions that may bind to any of five barium binding sites, Ba1 – Ba5, shown below. The occupancy of each of the barium sites is calculated at 0.3, resulting in the assumption of two ions with five possible binding sites. The binding of the barium ions is dynamic, allowing the ions to move from site to site.

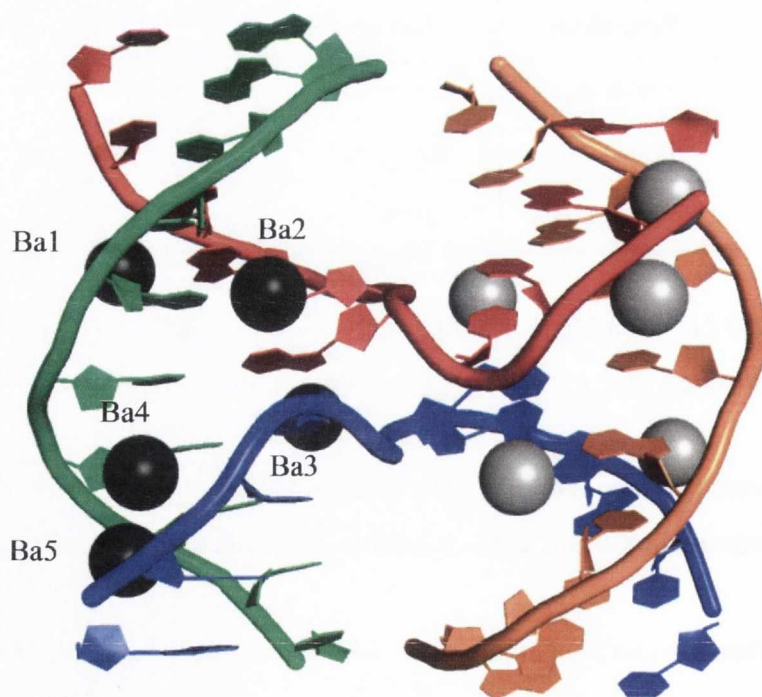


Figure 3.41 - A View of the stacked-X structure of d(TCGGCGCCGA) formed in the presence of BaCl₂. The four strands are shown in different colours. The Ba²⁺ binding sites are shown in black and grey. (PDB ID: 3GOM)

The first Ba^{2+} site is found in the minor groove of the DNA structure and occupies a site between the G3-C18 and G4-C17 base pairs. The second site, Ba2, is located closer to the crossover region but the barium ion was not found to be interacting directly with any of the DNA residues. The third site is located in the major groove, between the steps of C15-G6 and G14-C27. Ba4 occupies between the G14-C27 and G13-C28 sites in the minor groove and finally Ba5 is also located in the minor groove of the structure between the steps G13-C28 and C12-G29.⁷⁶ Four of the five barium binding sites are located in the minor grooves with the other occupying a single major groove. The presumption from this data is that the barium ions, when both the minor and major grooves are accessible, show a preference for binding to the minor groove.

The crystal structure of TCGGCGCCGA in the presence of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ contains only one barium ion per asymmetric unit, giving a total of two Ba^{2+} ions per duplex (see Figure 3.26). These two ions are bound to the G3 and G4 residues in the duplex (with an occupancy of 0.99), and most notably are located in the major groove of the structure. While this is not definitive proof of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex binding first, and thus ordering the macromolecule, it allows for the presumption of this fact as in the absence of such a complex, the barium occupies the minor grooves. This gives further weight to argument that such metal complexes preferentially bind from the direction of the minor groove.

The main role of the barium ion in this crystal structure is to stabilise the drastically kinked hybrid duplex. The semi-intercalation of the TAP1 ligand creates a binding site for a barium ion quite unlike that seen in the Holliday junction. The kink induced by TAP1 allows the barium to directly coordinate to the N7 position of G3 and O6 of G4, allowing it to act as an anchor to stabilize the semi-intercalation interaction by maintaining the two guanine residues in their kinked formation (Figure 3.42). The barium ion is fully coordinated by six ordered water molecules in the major groove, all at Ba-O distances of approximately 2.8 Å, and the two barium ions are separated by a distance of 9.3 Å.

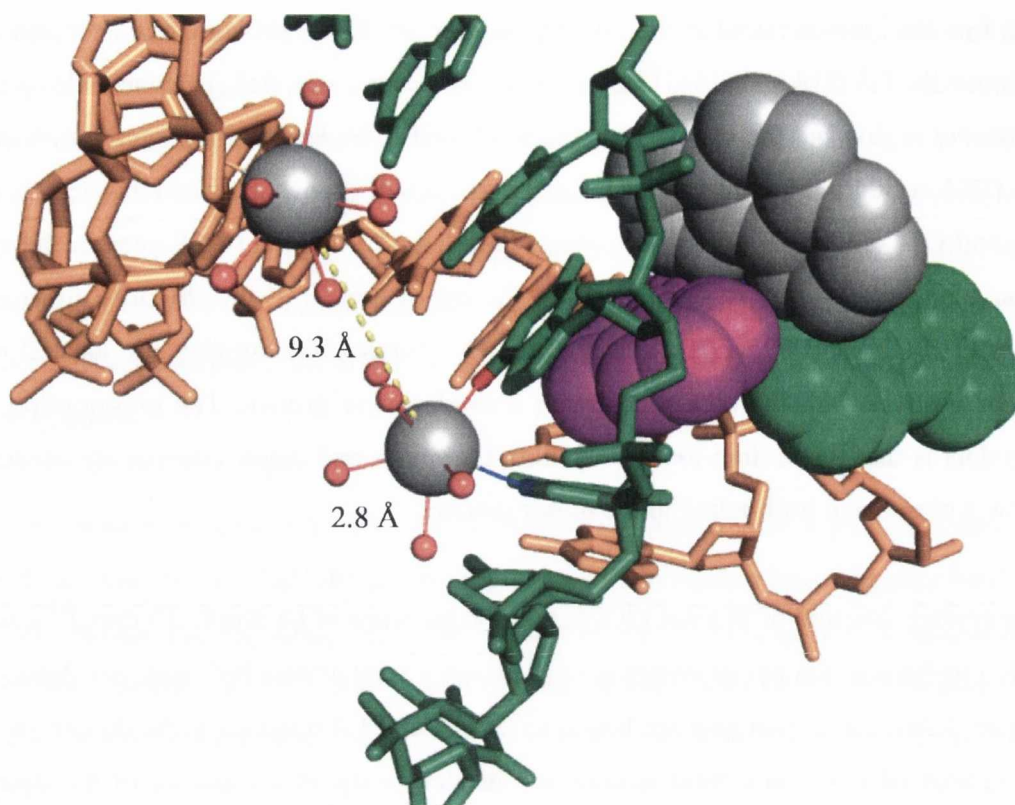


Figure 3.42 – The binding ‘pocket’ of the barium ion (grey)

While the reason for the success of the barium ion compared to the calcium and strontium ions is difficult to unequivocally prove, we suggest the size of the ion as the main factor. Initially we suspected a difference in the atomic radii of the metals, and indeed calcium has a smaller radii at 1.8 Å than both strontium and barium, who surprisingly have the same atomic radius of 2.15 Å. However, the ionic radius of the Sr^{2+} ion versus that of the Ba^{2+} highlights the difference, with a value of 1.32 Å for the former and 1.49 Å for the latter. Both Ca^{2+} and Sr^{2+} are too small to form coordinate bonds with both the N7 of G3 and O6 of G4, and thus cannot stabilise the semi-intercalative binding of the TAP1 ligand. We surmise that the binding of these ions result in a dynamic semi-intercalation that would not allow for the extended lattice arrangement required for the formation of the crystals.

3.6.3 The Extended Lattice and Hydration of the Duplex

The intercalation of the dppz ligand into one duplex, semi-intercalation of the TAP1 ligand into a second duplex, flipping out of the terminal adenine to pair with an orphaned thymine of a third duplex, all stabilized by the coordination of a barium ion has led to intensive cross-linking within the crystals obtained in this study. This, in turn, leads to an extensive lattice network that is responsible for the formation of the crystals we see. As can be seen in Figure 3.44, and previously in Figure 3.31, the ruthenium complexes at the termini of the duplexes are in very close proximity to each other. As these complexes are known to aggregate in solution and stack upon each other, it was feared that the lattice observed was formed as a result of the dppz ligands stacking with each other as opposed to the DNA molecules ordering the lattice. Up to four ruthenium complexes are found very close to each other. However, further examination of the orientation of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes reveal that the dppz moieties are arranged in such a fashion that they cannot overlap (Figure 3.43 a), thus the lattice formation is indeed dictated by the oligonucleotides. Also, the distances between the ruthenium complexes, while short, are not sufficiently short to allow for the π interactions of the aromatic moieties. The distance between the complexes (measured between the Ru^{II} centres) is 13.3 Å (Figure 3.43 b). Typically, aromatic π interactions resulting in stacking occur over distances of less than 5 Å.

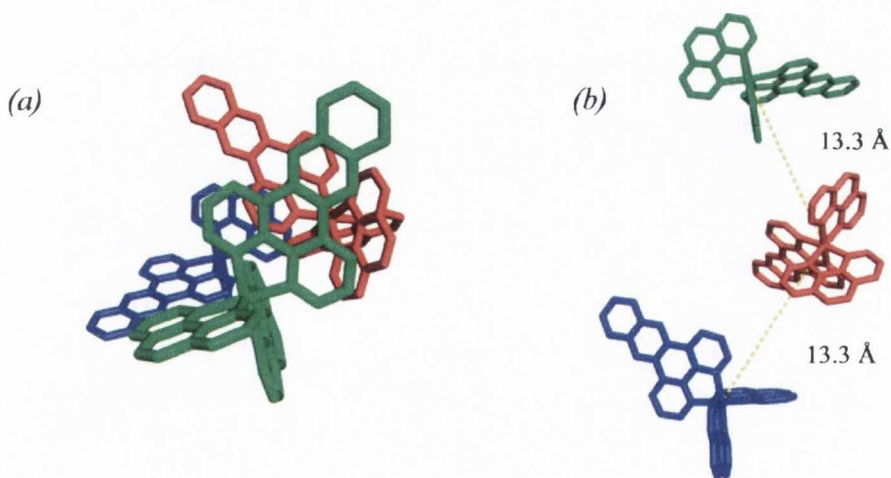


Figure 3.43 – The arrangement of three $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes in close proximity in the lattice network. The oligonucleotide has been omitted for clarity. (a) A view from the top-down through the complexes, (b) A side-on view of the complexes

Increasing the number of symmetry mates within the crystallisation program yields the extended lattice formation seen in Figure 3.45. Two distinct channels are clearly visible; a larger channel between four oligonucleotide chains and a smaller channel between four ruthenium complexes at the termini of the helices (Figure 3.44). These are thought to be hydration channels, with the large channel having the approximate dimensions 38.5 Å x 21.7 Å and the smaller channel having approximate dimensions of 20.6 Å x 20.6 Å.

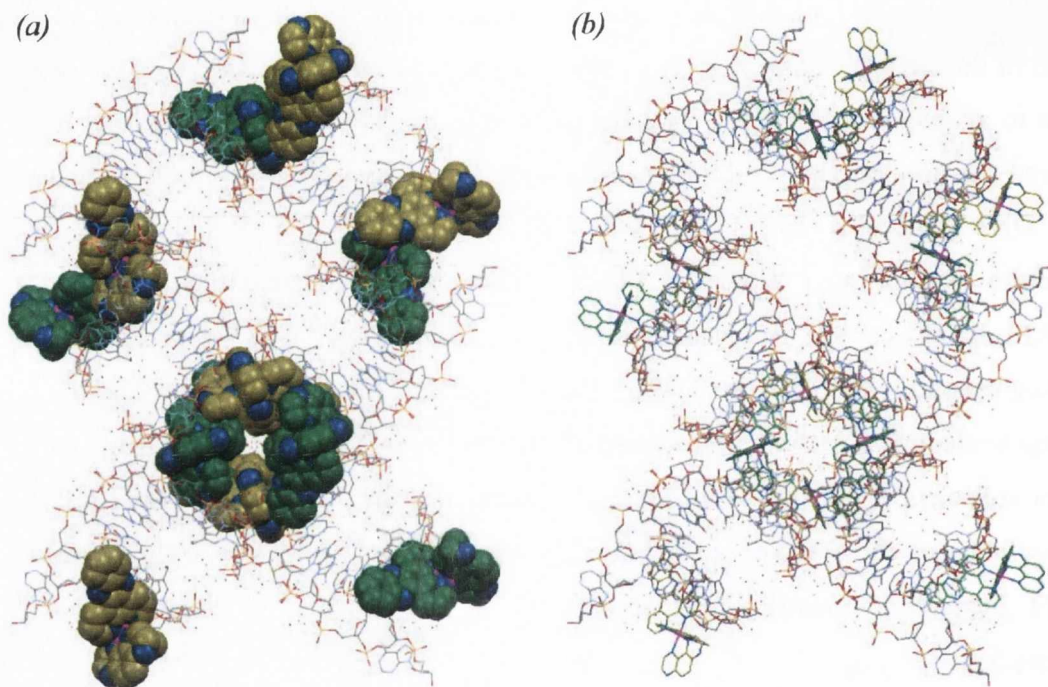


Figure 3.44 – Packing diagram of TCGGCGCCGA with $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$

(a) Showing the arrangement of the Λ -complexes within the lattice structure

(b) Showing the large and small channels in the lattice

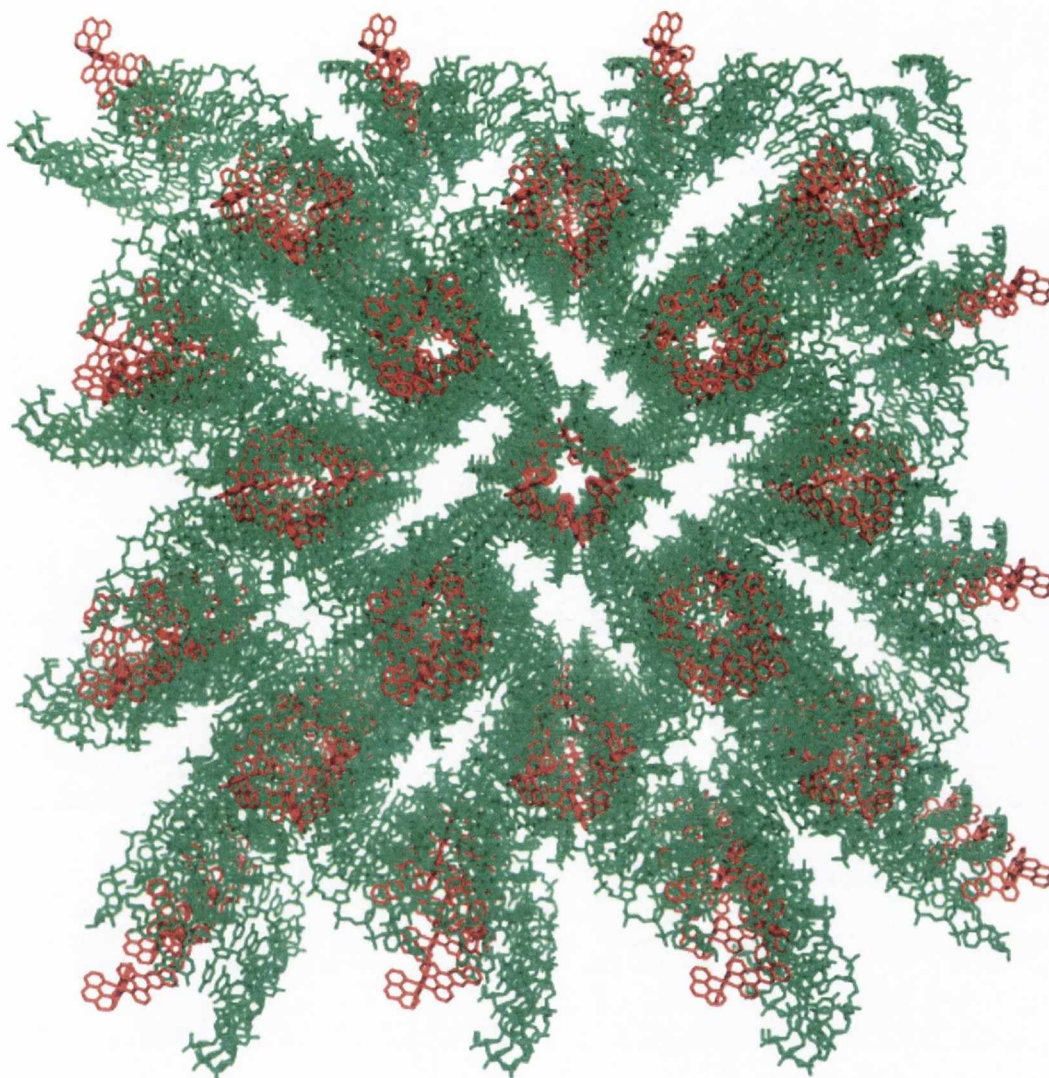


Figure 3.45 – The extended lattice of TCGGCGCCGA with $[\Lambda\text{-Ru(TAP)}_2\text{dppz}]^{2+}$

The oligonucleotide is in green with the Λ -Complex coloured red

The refined structure obtained gave very well ordered packing, with extensive networks of water molecules surrounding the duplex. Sixty-four water molecules are clearly seen within the structure, but these have thus far been omitted from most of structures for clarity, however, the positioning of these molecules are also interesting in terms of understanding the complete structure. Inclusion of these water molecules into the extended lattice structure confirms that the channels observed are indeed hydration channels (Figure 3.46).

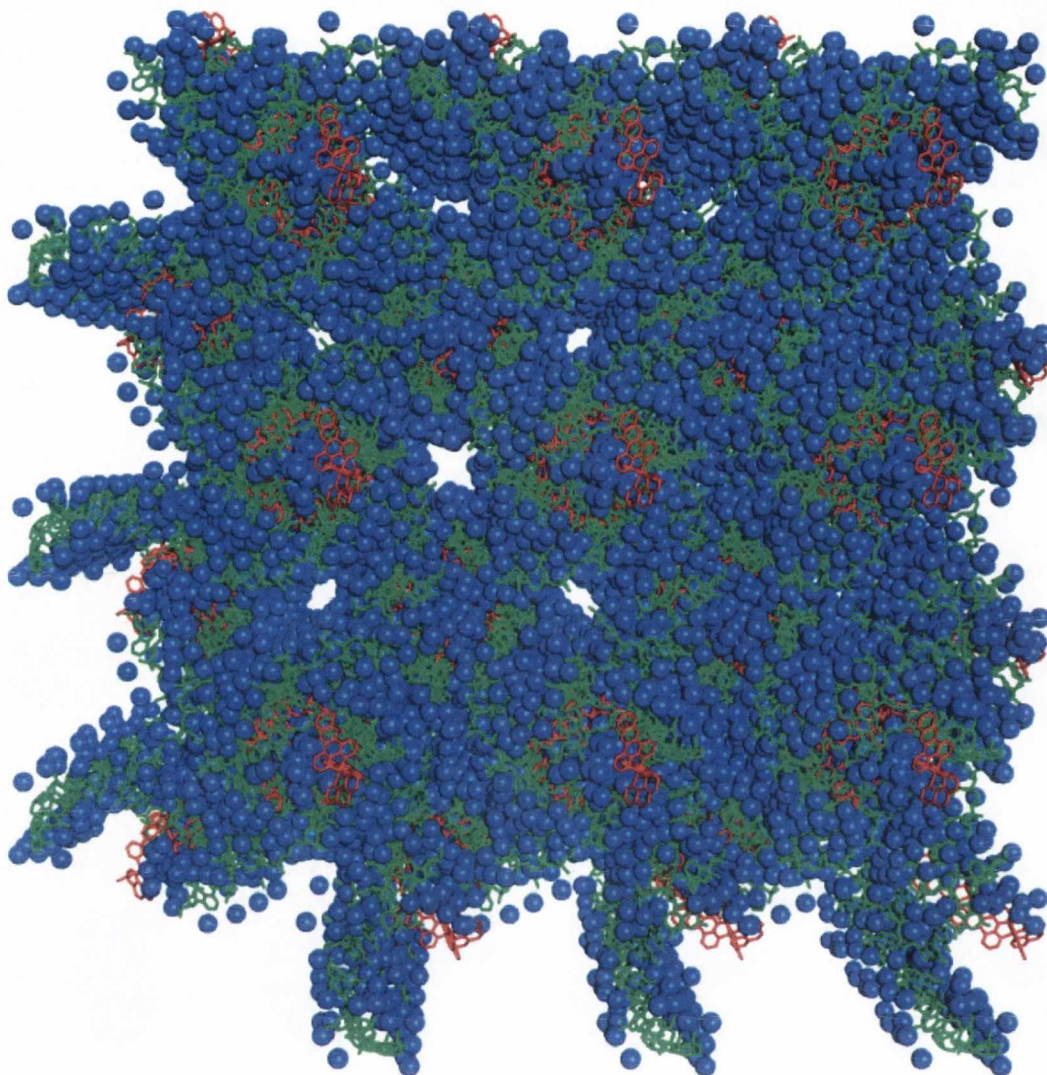


Figure 3.46 – The extended lattice of TCGGCGCCGA with $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ including water molecules. The oligonucleotide is in green, the Λ -Complex coloured red and the water molecules are blue

As seen in Figure 3.47, the major groove was found to be completely hydrated unlike the minor groove, despite it being the smaller of the two. This is as a result of the Λ -complex occupying the minor groove when binding to the oligonucleotide. The two barium ions are located in the major groove, and are completely surrounded by water molecules.

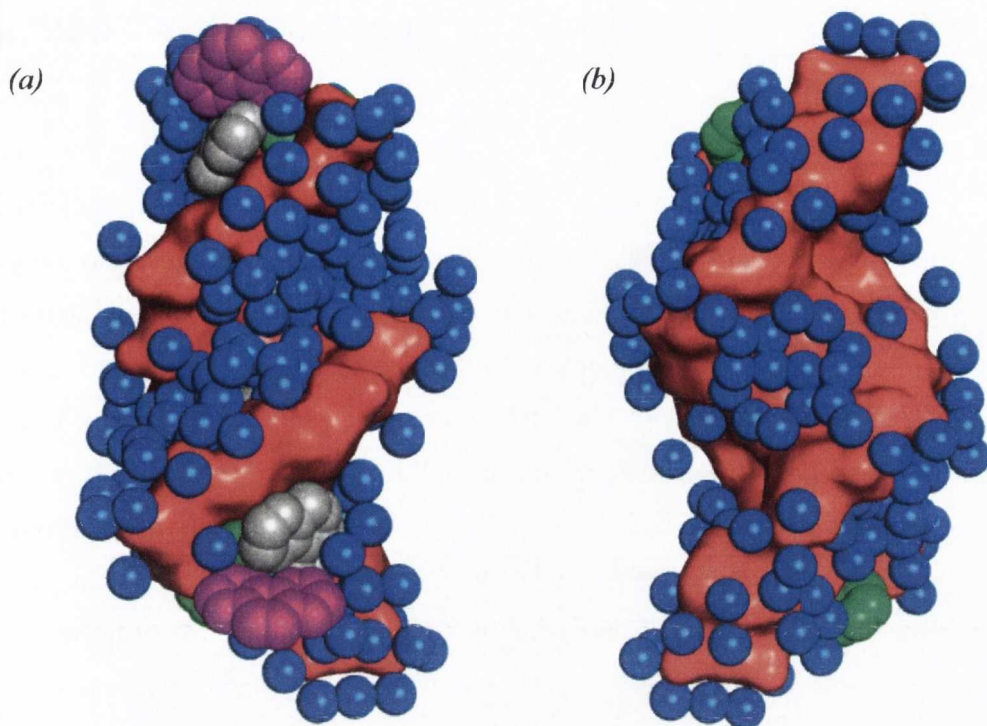


Figure 3.47 – The water molecules resolved in the DNA duplex

(a) A view of the major groove, (b) A view of the minor groove

Interestingly the water molecules do not associate with the ruthenium complex, and in particular do not interact with the dppz. This validates the findings made in previous studies with the $rac\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex and the results in the previous chapter. In water the dppz ligand is free to interact with water, and the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex luminesces. Once bound to DNA however, the dppz is sheltered from the water by the oligonucleotide resulting in a quenching of the luminescence. A further theory offered in the previous chapter is that the reason for the increased quenching of the Δ -complex in comparison to the Λ -complex is due to the ‘right-handed’ Δ -complex being better able to associate with the ‘right-handed’ DNA duplex thus the dppz is better isolated from the water molecules. As can be seen in Figure 3.47, there are a few water molecules in close proximity to the intercalating ligand that may be able to interact with it ever so slightly, thus increasing the Λ -complex’s luminescence. Unfortunately, as we have thus far been unable to crystallize the Δ -complex and so can only guess as to the positioning of water molecules around the Δ -ligands.

3.7 Second Crystal Structure of TCGGCGCCGA with $[\Lambda\text{-Ru(TAP)}_2\text{dppz}]^{2+}$

Crystals formed from macromolecules, particularly DNA, are notoriously delicate and rarely survive the process of obtaining a crystal structure. This is because these crystals are often extremely thin (> 0.05 mm) and cannot withstand the simple movement involved in both the loading and removal from the X-ray machine. The crystals obtained in this work, however, are exceptionally stable as they have a shortest dimension length of at least 0.1 mm. Even under the intense conditions of synchrotron radiation, many of the crystals remained unaffected, if kept cold (180 K). As a result it was believed that these crystals, once formed, would remain stable for the foreseeable future. This was not the case. One of the crystals mounted on the Gemini S-Ultra Diffractometer at the University of Reading showed an increasing build up of ice crystals, which interfere with the diffraction data collection, and was extracted from the cryogenic nitrogen cooling jet to remove the ice, thus allowing it to warm for approximately 30 seconds before being returned to the coolant stream. Data collection from the crystal was resumed and unit cell dimensions were shown to have changed. Following refinement, it was discovered that the structure of this 'altered' crystal differed to that of the original crystal structure obtained.

3.7.1 Data Collection – 'In-House'

A dataset was collected on the Gemini S-Ultra Diffractometer in the Chemistry Department at the University of Reading. The crystal was mounted on a loop and flash cooled to 180K under a nitrogen stream. The oscillation angle was 0.5° and the exposure time was 60 seconds per image. The crystal diffracted to a resolution of 1.92 Å.

The structure crystallised in the tetragonal crystal system with a space group of $P4_32_12$, that same as the original crystal structure. The cell angles also remained the same at $\alpha, \beta, \gamma = 90^\circ$, however, a major difference arises in the cell dimensions. Previously these were $a, b = 42.2$ Å, $c = 39.4$ Å. In this second structure, the dimensions were found to be $a, b = 44.35$ Å, $c = 27.21$ Å, resulting in a slightly longer but much thinner crystal. The asymmetric unit still consists of a single oligonucleotide strand with a two-fold axis of

symmetry, one $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex and one barium ion, however, unlike the original structure a total of only 43 water molecules were refined in the structure, along with a chloride ion and an additional TAP ligand (Figure 3.48). The biggest surprise was in the total volume of the crystals. The original crystals had a total volume of $70256 \text{ \AA}^3$, whereas the second crystal has a total volume of only $53500 \text{ \AA}^3$. This is a decrease in volume by 34%. Considering that the space group and angles of the unit cell remained the same, it was assumed that the crystal had dehydrated. A rare, but not unheard of occurrence in crystallography.

The final refined structure consists of one strand of DNA with a two-fold symmetry axis to generate the complete double stranded DNA structure. This structure has been deposited in the database; PDB ID – 3S80.

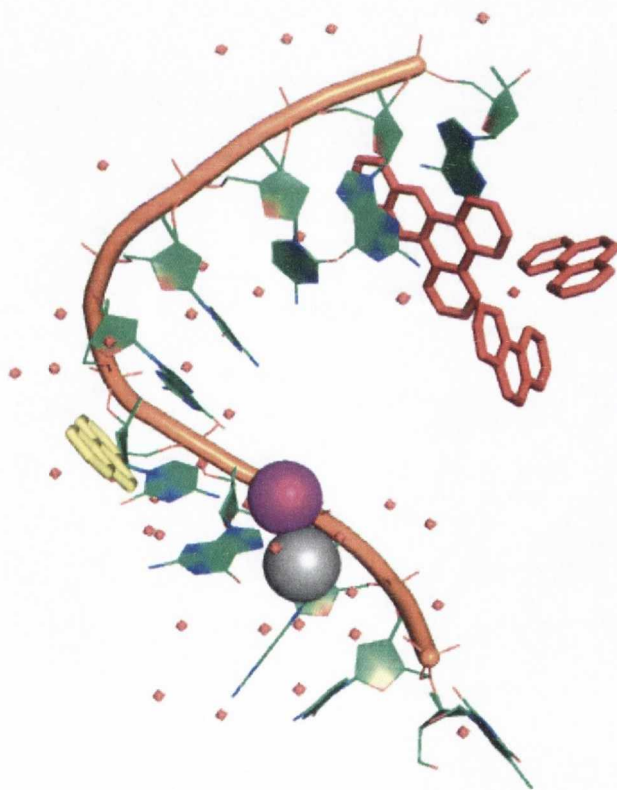


Figure 3.48 - The asymmetric unit cell, $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ is shown in red, Ba^{2+} ion is the grey sphere, Cl^- ion is the magenta sphere, the additional TAP ligand is shown in yellow and forty-three water molecules (red dots)

The Λ -complexes bind to the duplex in the same fashion as previously; intercalation of the dppz ligand at the termini of the duplex and semi-intercalation of the TAP1 ligand at the G3/G4 step inducing a kink, both from the minor groove. Unlike the original structure, however, the intercalation of the dppz ligand results in a flipping out of the terminal thymine (T1) residue instead of the terminal adenine (A10) residue, which is found in the correct conformation for a normal duplex adenine (Figure 3.50). As a result, while three non-covalent cross-linking interactions still occur within this second structure, only one is the same as in the original structure, the semi-intercalation of the TAP1 ligand between G3 and G4. The two barium ions again are located in the major groove, and directly coordinate to the N7 position of G3 and O6 of G4, with five water molecules and a shared chloride ion completing the barium coordination spheres (see Figure 3.49 for a comparison of the original and second duplexes).

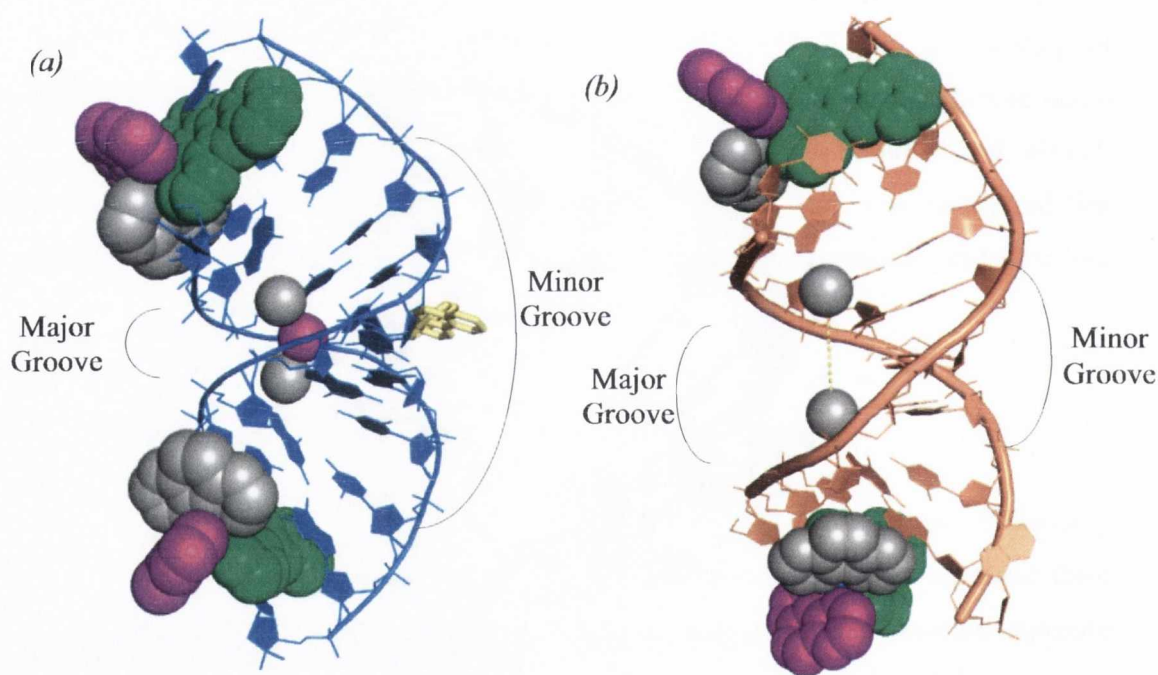
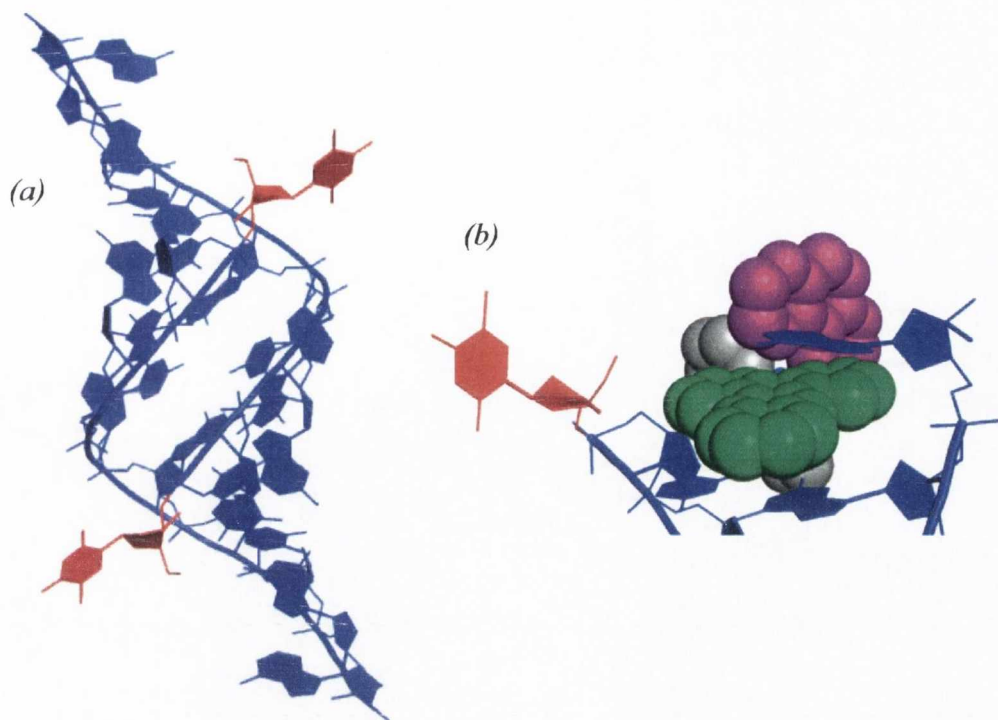


Figure 3.49 – Comparison of the duplexes of (a) the second crystal structure (blue) and (b) the original crystal structure (orange)



*Figure 3.50 – (a) The crystal duplex with the flipped out T1 bases highlighted in red
(b) The intercalation pocket of the dppz ligand in the second structure*

The two other non-covalent cross-linking interactions, previously formed by the association of flipped out adenines with orphaned thymines to link three duplexes (Figures 3.30 and 3.31) do not occur in this structure due the adenine residues resuming a regular duplex conformation (see Figure 3.50b). In this second crystal structure, it is the flipped out thymine residues that causes the non-covalent cross-linking. Previously the duplex into which the TAP1 ligand semi-intercalated (Duplex 2) provided the flipped out adenine residue to bond with the orphaned thymine of Duplex 1. In this second structure, the T1 base that bonds to the orphaned A10 of Duplex 1 is from a different duplex (Duplex 3) than the duplex into which the TAP1 ligand associates (Duplex 2). The flipped out T1 of Duplex 1 bonds to the orphaned A10 at the other terminus of Duplex 3, facilitated by the dramatic kink in the DNA, reinforcing the non-covalent cross-linking interaction between the two DNA helices (see Figure 3.51).

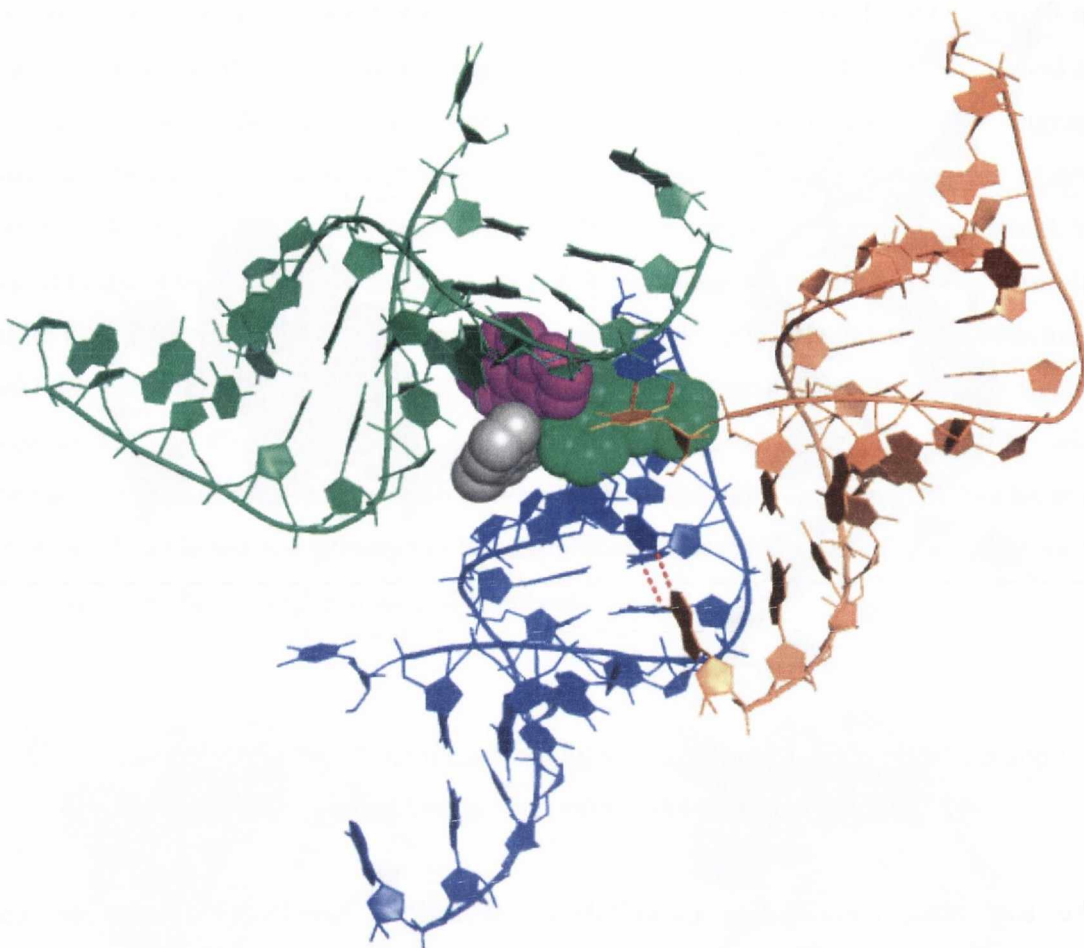


Figure 3.51 – Cross-linking of three duplexes in the second crystal structure
Duplex 1 (blue) – intercalation of the dppz ligand, Duplex 2 (green) – semi-intercalation
of and Duplex 3 (orange) – providing the flipped out T1 and orphaned A10 residues for
reverse Watson-Crick boning to Duplex 1

Inspection of the torsion angles shows that the DNA duplex is of a different A-/B-DNA conformation than that seen in the original crystal structure. The first crystal consists of five A-DNA residues and five B-DNA residues, resulting in a duplex that is structurally intermediate between A- and B-DNA (see Tables 3.8 and 3.9). This second crystal is structurally much closer to the A-DNA conformation by having seven of the ten residues as the A-DNA form and only three as the B-DNA. The values for each of the six rotation angles that determine the sucker puckers were obtained and tabulated in Table 3.12. The δ angles are detailed in Table 3.13 complete with identification of the sucker puckers.

Nucleotide	α	β	γ	δ	ϵ	ζ
T1			-12.129	168.900	-124.028	15.160
C2	-137.706	-110.838	65.879	99.813	-165.921	-97.048
G3	-41.248	163.697	55.567	88.531	-142.602	-83.999
G4	-55.325	-172.944	45.147	155.572	-165.976	-117.258
C5	-83.154	-170.684	42.503	98.479	-139.974	-83.317
G6	-34.250	172.365	26.022	142.901	173.786	-83.651
C7	-82.823	169.725	52.385	74.639	-156.015	-75.030
C8	-67.769	174.909	53.416	82.508	-151.305	-97.857
G9	-51.735	158.091	45.321	103.409	-171.645	-97.633
A10	-54.646	173.298	59.112	87.046		

Table 3.12 – Torsion angles of TCGGCGCCGA in the second structure

Nucleotide	δ	Sugar Pucker	Conformation
T1	168.900	C2'-endo	B-DNA
C2	99.813	C3'-endo	A-DNA
G3	88.531	C3'-endo	A-DNA
G4	155.572	C2'-endo	B-DNA
C5	98.479	C3'-endo	A-DNA
G6	142.901	C2'-endo	B-DNA
C7	74.639	C3'-endo	A-DNA
C8	82.508	C3'-endo	A-DNA
G9	103.409	C3'-endo	A-DNA
A10	87.046	C3'-endo	A-DNA

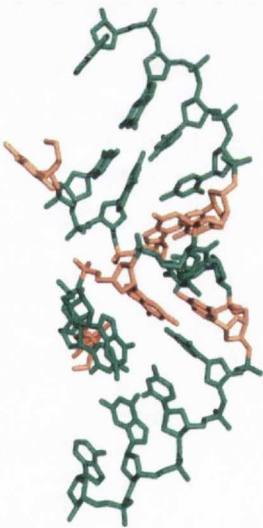


Table 3.13 – δ torsion angles and sugar puckers in the second structure

Duplex DNA, as mentioned in the introduction, may undergo a reversible conformation change to A-DNA when the relative humidity is reduced to 75%. Forty-three water molecules have so far been modelled which is less than the sixty-four for the original structure, supporting the theory of dehydration. However, the data set obtained for the second structure is poorer than that of the first, rendering the number of molecules refined

as potentially inaccurate. From the comparison below in Figure 3.52, it is clear that this second crystal structure is infinitely more of an A-DNA configuration than B-DNA, however, it seems to be even more bent than the A-DNA conformation suggesting that there may be further factors involved in the formation the crystal structure, other than dehydration.

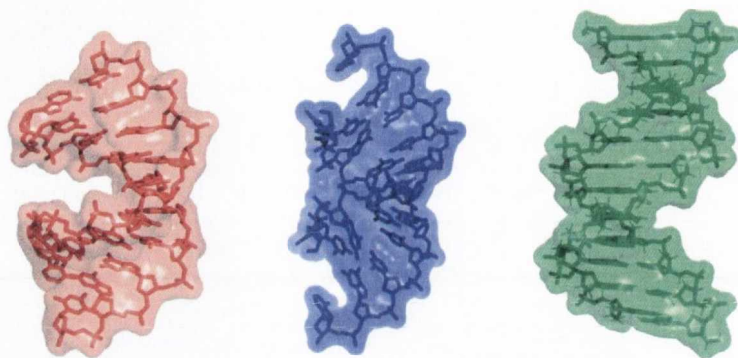


Figure 3.52 – Comparison of A-DNA (red), the second crystal structure (blue) and B-DNA (green)

The main difference between the original and second structures is the appearance of a third molecule semi-intercalating between the C5/G6 step (Figure 3.50). This molecule has been modeled as a lone TAP ligand as it is small, planar and has a C2 axis of symmetry. It is believed that the hydroxyl free radicals generated in crystals by the intense X-rays^{77,78} may interact with the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes resulting in the dissociation of a TAP ligand. However no evidence of a $[\text{Ru}(\text{TAP})\text{dppz}]$ complex has been found in this second structure.

The association of this free TAP ligand to the DNA dramatically alters the G3/G4 and C5/G6 angles (Table 3.14), resulting in a larger kink than was observed in the original structure. This increased kink causes the major groove to further reduce in size, the minor groove to increase and the distance between the two barium ions to decrease from 9.3 Å to only 5.8 Å. To stabilize the increased repulsion between the two bariums ions caused by this shortened distance, a chloride ion associates between the two. The result is the further folding of the duplex upon itself due to the dramatic kinking and is the main cause of the reduced volume recorded for the crystal structure.

	C2-G9	G3-C8	G4-C7	C5-G6	G6-C5	C7-G4	C8-G3	G9-C2
Shear	0.08	-0.19	-0.40	0.23	-0.23	0.40	0.17	-0.08
Stretch	-0.19	-0.02	-0.25	-0.15	-0.15	-0.25	-0.02	-0.19
Stagger	-0.14	0.52	0.02	-0.07	-0.07	0.02	0.52	-0.14
Buckle	-7.00	25.83	-2.12	14.77	-14.77	2.12	-25.83	7.00
Prop-Tw	4.46	-8.65	8.61	-4.30	-4.30	8.61	-8.65	4.46
Opening	-2.95	-0.47	-2.34	3.03	3.03	-2.34	-0.47	-2.95
Shift	0.00	-0.02	-0.46	-0.91	0.00	-0.91	-0.46	-0.02
Slide	0.00	1.50	0.44	-0.02	1.71	-0.02	0.44	1.50
Rise	0.00	2.73	5.01	2.90	4.97	2.90	5.01	2.73
Tilt	0.00	-3.97	-0.55	0.55	0.00	-0.55	0.55	3.97
Roll	0.00	7.29	45.23	2.70	53.51	2.70	45.23	7.29
Twist	0.00	23.18	20.67	34.27	15.98	34.27	20.67	23.18

Table 3.14 - Local base pair and step parameters, calculated with 3DNA v.1.5. The roll angles caused by the semi-intercalation of the TAP1 ligand are highlighted in red. The roll angle caused by the binding of the free TAP ligand is shown in yellow

Table 3.15 summaries the main similarities and differences between the original and second crystal structures.

	Original Crystal Structure	Second Crystal Structure
Space Group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Unit Cell Angles (°)		
α	90	90
β	90	90
γ	90	90
Unit Cell Dimensions (Å)		
a	42.2	44.35
b	42.2	44.35
c	39.4	27.21
Total Volume (Å ³)	70256	53500
Duplex Conformation	A/B Hybrid (50/50)	A/B Hybrid (70/30)
No. of [Λ-Ru(TAP) ₂ dppz] ²⁺	2	2
No. of Barium Ions	2	2
Association of Chloride Ion	No	Yes
Water Molecules Modeled	64	43
Distance between Ba ²⁺ ions (Å)	9.3	5.8
Flipped out Base	Adenine	Thymine
Cross-links	3 interactions	3 interactions
No. of Cross-linked Duplexes	3	3
No. of Kink Sites per Duplex	2	3
Total Angle of Kink	125°	143°
Association of Free TAP Ligand	No	Yes

Table 3.15 – Comparison of the original and second crystal structures

3.8 Crystallisation Attempts with $[\Delta\text{-Ru(TAP)}_2\text{dppz}]^{2+}$

The beautiful crystal structures obtained in this work all contain the Λ -isomer of the $[\text{Ru(TAP)}_2\text{dppz}]^{2+}$ complex. The same crystallisation experiments were also set-up containing the 24 NAMS solutions, the oligonucleotide TGCCGCGGA and $[\Delta\text{-Ru(TAP)}_2\text{dppz}]^{2+}$ (please see the Experimental chapter for full details of crystallisation experiments). Considering the results obtained from the spectroscopic studies (detailed in the previous chapter) and literature reports on the differences between the DNA binding of Λ - and Δ - $[\text{Ru(phen)}_2\text{dppz}]^{2+}$, it was assumed that should crystallisation occur it would be with the Δ -complex. Evidently the converse was true, crystals were obtained with the Λ -isomer and, to date, the Δ -isomer remains in solution. The cause of crystal growth in this study is believed to be the cross-linking interactions that occur between the duplexes. These result in the extended lattices, seen in Figures 3.45 and 3.46, and it is the construction of these lattices that allow the formation of a crystal. The $[\Delta\text{-Ru(TAP)}_2\text{dppz}]^{2+}$ complex must not be able to induce such an arrangement of the oligonucleotides, leading to the speculation that the Δ -isomer does not form cross-linking interactions between duplexes.

As has been shown in this thesis via luminescence and thermal denaturation studies, in conjunction with reports in the literature, ruthenium(II) enantiomers containing ancillary ligands such as phen and TAP, which can span the helical groove of DNA, Δ -isomers bind more tightly by intercalation to right-handed duplexes than do Λ -isomers.^{27,30,79-81} This tighter fit of the Δ -isomer between the base-pairs could reduce the ruthenium-DNA distance, recorded as 6.5 Å for the Λ -isomer. A shorter distance would, in turn, result in less protrusion of the ancillary TAP ligands out of the grooves, reducing the ability of the ligands to interact with a second DNA duplex. A further possibility is due to the sequence selectivity of the two isomers. While both have been shown to have a slight preference for A-T base pairs,⁸² the Λ -complex binds to these sites with a higher preference than the Δ -complex. Indeed the crystal structures obtained in this work show the Λ -complexes binding at the A-T step of the oligonucleotide despite the fact that binding at the terminus of a DNA strand is a less stable interaction than intercalation mid-strand. The Δ -complexes, with a lower affinity for A-T sites, could intercalate mid-strand between a G-

C/G-C or G-C/C-G step to avail of this increased stability. Interaction with the duplex at this point may render semi-intercalation into a second duplex impossible due to the presence of a second Δ -complex occupying the center of the DNA.

Finally, and perhaps the most important prediction of the binding of Δ -[Ru(TAP)₂dppz]²⁺ without access to the crystal structure, is the very nature of the complex itself. Comparisons of the ligand arrangement within the Λ - and Δ -isomers are shown below in Figure 3.53.

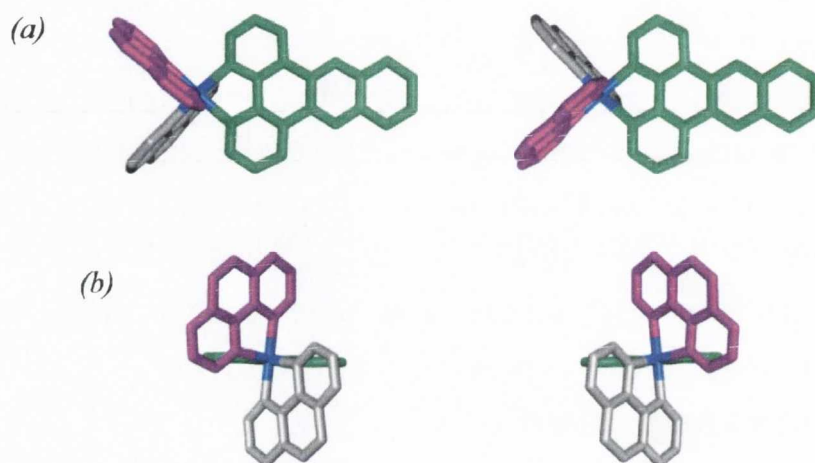


Figure 3.53 – Comparison of the Λ -isomer (left) with the Δ -isomer (right)

(a) View of the dppz ligand, (b) View down the intercalation plane

Part (b) of Figure 3.53 is most telling as it is the view down the actual intercalation axis of the Λ -complex and assumed intercalation axis of the Δ -complex. The angle of the ancillary ligands in the Λ -complex is approximately -25° through the central axis of the ruthenium center while the same angle for the Δ -complex is approximately 25° . The grooves, both major and minor, in DNA run at an angle of approximately 30° , thus a Δ -complex would fit the grooves very well while the Λ -complex would be hindered by the misalignment of its ancillary ligands, as illustrated in Figure 3.57. The ability of the Δ -complex ancillary ligands to fit into the groove would allow for a more complete intercalation of the dppz moiety, resulting in a shortened ruthenium-DNA distance and less of the TAP ligands available for semi-intercalation, as stated previously. This would

effectively prevent the non-covalent cross-linking of duplexes, lattice formation and ultimately crystal growth in the presence of $[\Delta\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$.

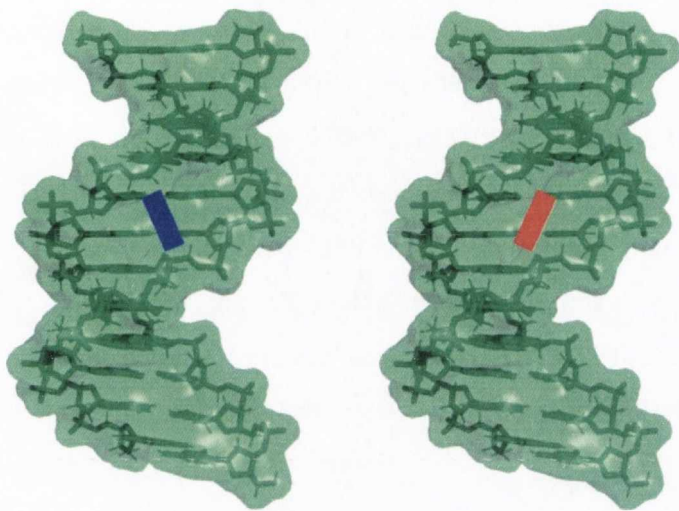


Figure 3.54 – An illustration of the binding of the Λ -isomer (left, blue) and the Δ -isomer (right, red) at the minor groove of a standard B-DNA duplex. The blocks represent the orientation of the ancillary ligands

3.9 Comparison to $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$

Due to this being the first crystal structure of a polypyridyl ruthenium complex bound to DNA submitted to the PDB there was no model with which to directly compare our results. The closest such structure found in the PDB is the binding of the rhodium intercalator $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ to DNA.⁸³ This complex contains an extended aromatic chromophore in the 5,6-chrysenequinone diimine (chrysi) ligand and two aromatic ancillary ligands, bipyridine (bpy) (Figure 3.55). This paper investigates the binding and subsequent photo-cleavage of a mismatched site in the self-complementary DNA dodecamer, CGGAAATTCCCG (the mismatched bases are in italics) The Λ -isomer is mentioned but only in that it failed to cleave the DNA and attempts were not made to crystallise it.

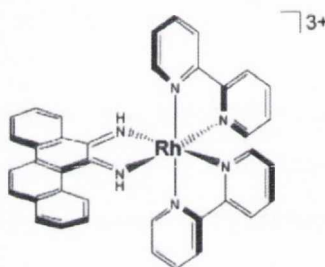


Figure 3.55 – The structural formula of $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+83}$

The $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ complex exhibits two modes of binding, as does our $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$. The main association is via intercalation of the chrysi ligand to two sites of the DNA; the mismatched A-C base step and central A-T/T-A base pair step. The Δ -complex intercalates at the mismatched pairs and binds to this step causing both the adenine and cytosine to be flipped out into the major grooves (the red complex in Figure 3.56). Intercalation at the central matched A-T site is illustrated in blue in Figure 3.56 and is classical intercalation with typical unwinding of the duplex occurring. The matched binding of the complex occurs in the major groove, whereas the mismatched binding occurs with the complex in the minor groove. Interestingly, the binding to this central site has not been detected in solution studies with the rhodium complex and likely is favoured by crystal packing. This is very different to solution studies of the Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complexes conducted with the decamer TGCCGCGCCA (detailed in the previous chapter). The thermal denaturation experiments performed in Reading

showed an increased thermal stability inferred by the binding of both isomers, with the Λ -complex stabilising the oligonucleotide more than the Δ -isomer. This is strong evidence for the occurrence of the cross-linking of duplexes and thus all the binding modes observed in the crystal state are also present in solution.

The distance of the rhodium metal centre to the DNA axis is only 4.7 Å compared to the 6.5 Å observed for the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex. This is typical of the difference in the binding ability of the Λ - and Δ -enantiomers. The Δ -enantiomer with its ancillary ligands aligned to fit into the grooves can bind more tightly to the macromolecule whereas a Λ -enantiomer's ancillary ligands are incorrectly orientated and steric hindrance with the phosphate backbone prevents the complex from binding as tightly. This is a common difference between the binding of Λ - and Δ -isomers to right-handed DNA duplexes, particularly in the narrower minor groove. As is also evident in Figure 3.56, the binding of the rhodium complex does not overly perturb the DNA structure. All the sugars maintain C2'-endo puckering and the duplex, though lengthened by the intercalating complexes, is not kinked at any point. This is a direct contrast to the situation found with the binding of the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex, and is due to absence of semi-intercalation into the DNA.

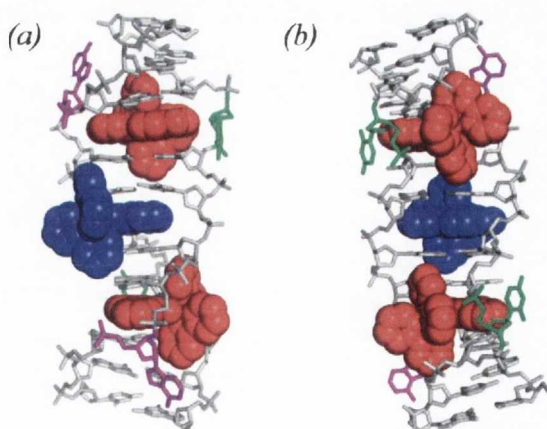


Figure 3.56 – The two intercalative binding sites of $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ to DNA

(a) View down the intercalation axis of the complex (red) bound to the mismatched DNA site (flipped out adenine in pink, flipped out cytosine in green) (b) View down the intercalation axis of the complex (blue) bound to matched DNA. PDB ID: 2011

Non-covalent cross-linking of duplexes has been observed in the rhodium crystal structure, but is dependant on intercalation of the complex to the centre of the duplex, which does not occur in solution, contrary to $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$. Also, the mode of binding through which the non-covalent cross-linking occurs differs for both complexes. In the presence of the Λ -complex semi-intercalation and reverse Watson/Crick bonding results in the cross-linking of duplexes. In contrast, only the central $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ complex induces non-covalent cross-linking via its two ancillary bpy ligands π -stacking with the terminal G-C base pair of two crystallographically related oligonucleotides, as seen in Figure 3.57 below. This association is a much weaker interaction than the semi-intercalation of the TAP ligands, possibly explaining why this is not reported in solution studies of the rhodium complex.

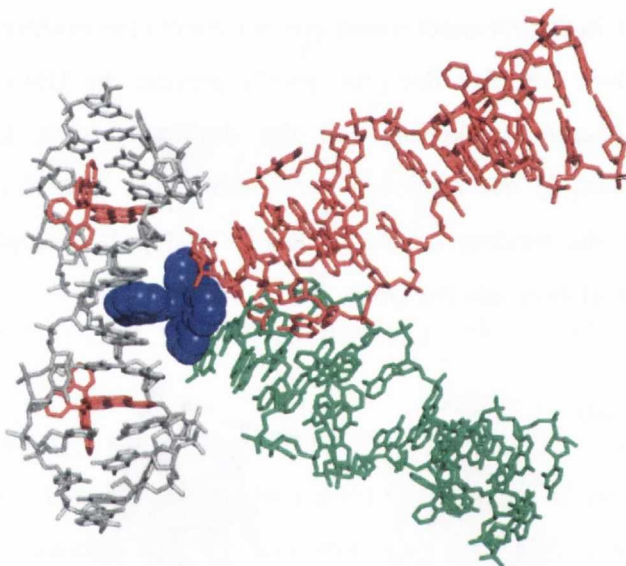


Figure 3.57 – Cross-linking of three duplexes (grey, red, green) by $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$

Table 3.16 summarises the similarities and differences between the crystal structures of the two complexes. It is interesting to note that the fundamental difference is that the rhodium complex is the crystallisation of the Δ -isomer while it is crystals of the Λ -isomer of the ruthenium complex obtained. It is the study of this Δ -isomer that allows us to reach the conclusion that the seemingly failed crystallisation experiments of the ruthenium Λ -

isomer may be due to the fact that the complex binds too deeply into the DNA grooves, preventing the lattice formation observed with the Λ -isomer, crucial for crystal formation.

	$[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$	$[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$
Metal Centre	Rhodium	Ruthenium
Enantiomer	Δ	Λ
Intercalating Ligand	chrysi	dppz
Ancillary Ligands	bpy	TAP
Oligonucleotide Sequence	CGGAAATTCCCG	TGCCGCGGA
Main Binding Mode	Intercalation	Intercalation
Location of Binding	Center of duplex	Terminus of duplex
Groove Binding	Major and Minor	Minor
Cross-linking	yes bpy stacking with DNA terminus	yes TAP semi-intercalates to second DNA duplex
DNA Kinking	no	yes
Metal-DNA Distance	4.7 Å	6.5 Å
Flipped out Bases	cytosine and adenine	adenine
Sequence Selectivity	A-T base pairs	A-T base pairs
Sugar Puckers	12 x C2' <i>endo</i>	5 x C2' <i>endo</i> 5 x C3' <i>endo</i>
Resolution	1.14 Å	1.102 Å
Space Group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2

Table 3.16 – Comparing $[\Delta\text{-Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ to $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$

3.10 Further Crystallisations

Following the success of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with the oligonucleotide TGCCGCGGA, crystallography experiments of both the enantiomers with differing decamer oligonucleotide sequences were conducted in an attempt to obtain crystals with the Δ -isomer and crystals of the Λ -isomer performing classical intercalation at a more central step of the duplex. Each of the crystallography experiments were conducted with the same NAMS solutions used originally (as shown in the Experimental chapter) with the following sequences:

- TCGGCGCCGA (as a control)
- CCGGCGCCGG
- CCGGTACCGG
- TCGGTACCGA
- CAGGTCTACC
- TGGGGT

These sequences were chosen for a variety of reasons. CCGGCGCCGG was used in an attempt to remove the preferential A-T binding site and result in classical intercalation at a more central point. CCGGTACCGG was attempted because of it containing an A-T base pair at the central step of the sequence. TCGGTACCGA was used because of its increased number of A-T sites. For this same reason CAGGTCTACC was attempted but also this sequence contains an A/G step, a purine/purine step. The Λ -complex was shown to have a preference for binding at purine/purine steps, and unlike the previous sequence, the A/G step in this oligonucleotide is not located at the terminus of the duplex. Finally the TGGGGT was chosen as it is a known quadruplex DNA forming sequence. Considering the ability of $[\text{Ru}(\text{bpy})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ to bind to complex secondary structures of DNA,^{84,85} it would be interesting to attempt a crystallographic study of the binding of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with a quadruplex DNA structure.

The results from these experiments have thus far been varied. Crystals were formed with the Λ -complex and TCGGCGCCGA, the control experiment, showing that crystallising conditions were favourable. No crystals have been observed with any of the Δ -complex experiments, regardless of the oligonucleotide or NAMS solution used. However, crystals

were formed with the Λ -complex with the sequences CCGGCGCCGG, CCGGTACCGG and TCGGTACCGA. All of the crystals were formed as rods (Figure 3.58), which is indicative of B-DNA conformation. The crystals formed with the sequence CCGGCGCCGG are unfortunately too small for use on the Gemini-S-Ultra diffractometer, but will be investigated when next we have access to the I02 Beamline at the Diamond facility. Those obtained from the CCGGTACCGG are significantly larger and are currently being investigated at the University of Reading, with the crystals formed with TCGGTACCGA being prepared for neutron crystallographic studies in Grenoble.

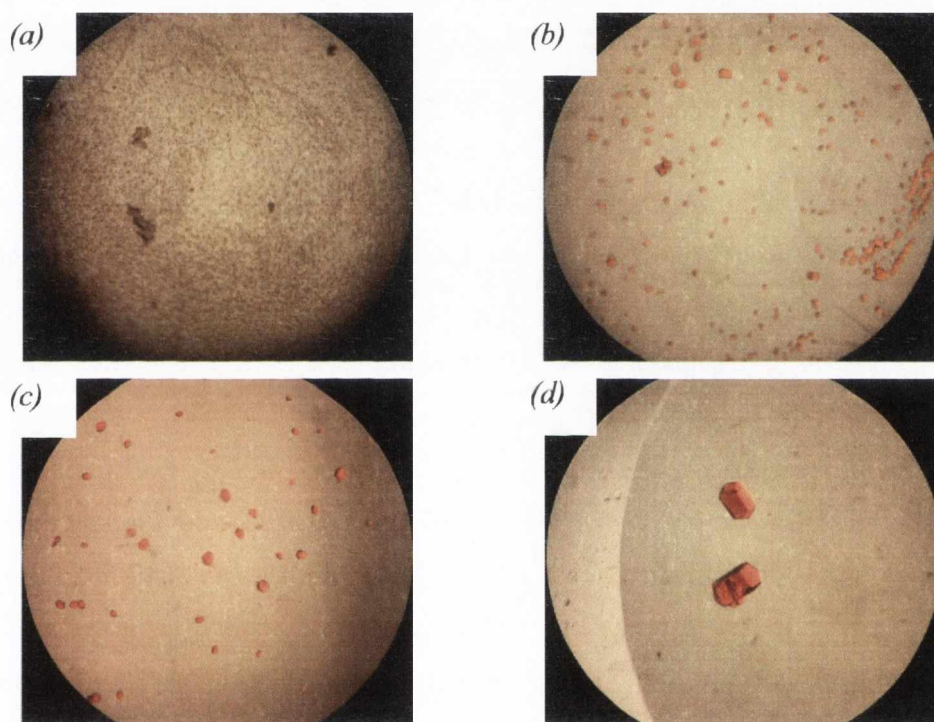


Figure 3.58 – A view down the microscope of the crystals of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ with (a) CCGGCGCCGG, (b) CCGGTACCGG, (c) TCGGCGCCGA, (d) TCGGTACCGA

As with the structures presented in this thesis, all of the crystals obtained with these other oligonucleotide sequences were grown in the presence of barium ions, indicating that a similar structure may be occurring to form a similar extended lattice arrangement.

3.11 Conclusions

The work presented in this chapter is the first example of a crystallographic study of a polypyridyl ruthenium complex binding to DNA. The ensuing crystal structures obtained have demonstrated that while many conclusions drawn from the spectroscopic data of such complexes with DNA are accurate, the binding observed is much more complex than ever presumed.

The results unequivocally show the intercalative binding mode, long suspected as the main method of association to DNA, of a ruthenium complex containing an extended aromatic ligand, and the location of binding being in the minor groove for this Λ -complex. They also confirmed the A-T preference for the binding of the Λ -isomer. The most surprising outcome of these experiments is the observation of the semi-intercalative binding of an ancillary TAP ligand to a second duplex, resulting in the cross-linking of two duplexes. This results in the dramatic kinking of the oligonucleotide, a phenomena rarely seen with the binding of a small molecule, but very common in cellular biology with protein/DNA interactions causing similar kinking which acts as a regulation step of the transcription process within the cell.

The association of the complex to the oligonucleotide also causes the flipping out of the terminal adenine, which is then free to interact with a third duplex and form a second cross-linking of duplexes through reverse Watson-Crick bonding with an orphaned thymine residue. This again has biological implications as the flipping out of a base has been shown to be pivotal in the interaction of some enzymes with DNA, although the mechanism of this interaction is not yet understood.

A further feature of ruthenium-TAP complexes is their ability to cause photo-induced damage to duplex DNA at the guanine residues, and indeed some complexes are known to form photo-adducts with the guanine moieties. As the binding of such complexes to DNA has not previously been seen crystallographically, it was hoped this crystal structure would suggest the route through which photo-adduct formation could occur.

3.11.1 Photo-adduct Formation

The previous chapter wonderfully illustrates the spectroscopic and photo-induced potential of these $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers particularly through fluorescence and time resolved techniques. Recent work has shown that near-UV or visible light irradiation of ruthenium complexes containing TAP ligands in the presence of duplex DNA induces the formation of covalent adducts with guanine.⁸⁶ Of particular interest are the reports of such photo-adducts being formed with $[\text{Ru}(\text{TAP})_3]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{bpy})]^{2+}$,^{86,87} with the photo-adduct of the latter being isolated and identified by mass spectrometry and NMR analysis (Figure 3.59). These adducts are formed by a photochemical reaction causing the formation of a covalent linkage between one of the TAP ligands and the 2-NH₂ group of guanine.

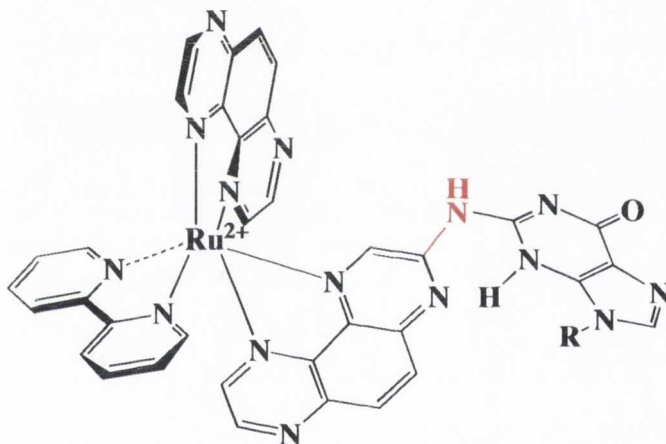


Figure 3.59 – The photo-adduct formed between $[\text{Ru}(\text{TAP})_2\text{bpy}]^{2+}$ and guanine⁸⁶ with the 2-NH₂ group of the guanine shown in red

The time resolved work conducted previously have shown the occurrence of electron transfer from $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to a guanine residue of a duplex^{32,88} though no such photo-adduct has yet been isolated. While the photo-adduct was not seen in the crystal structure obtained with the Λ -complex, inspection of the distance between the TAP ligands and guanine residues allow for speculation of how the reaction may occur.

The two TAP ligands have been labeled TAP1 and TAP2, differentiated by the semi-intercalation of TAP1, and the seemingly inert TAP2. It was initially believed that the

semi-intercalation of the TAP1 ligand between two guanine bases would allow for the relevant CH group of the TAP2 and 2-NH₂ of a guanine to lie in close proximity to facilitate the photochemically induced formation of a covalent bond. Upon inspection of this ligand arrangement however, the orientation of the TAP1 ligand with respect to the 2-NH₂ of the guanine resulted in a distance of 4.5 Å, too great to allow such a bond to form. However, the TAP2 ligand of the complex is positioned in the minor groove and in an orientation required for possible covalent linkage with the 2-NH₂ groups of G3 from one duplex and G4 from the second duplex, see Figure 3.33. The distance between the relevant CH groups and both 2-NH₂ groups is only 3.5 Å (Figure 3.60). This, again, is large in comparison to the C-N bond length of 1.5 Å, however, due to the unhindered environment of TAP2, it is feasible that the ligand would be able to move nearer the 2-NH₂ groups and form the covalent bond, and thus the photo-adduct upon irradiation. This has not yet been attempted with this system, but similar experiments in the solid state have been attempted previously with different systems,⁸⁹ and certainly is an experiment to be considered for these crystals.

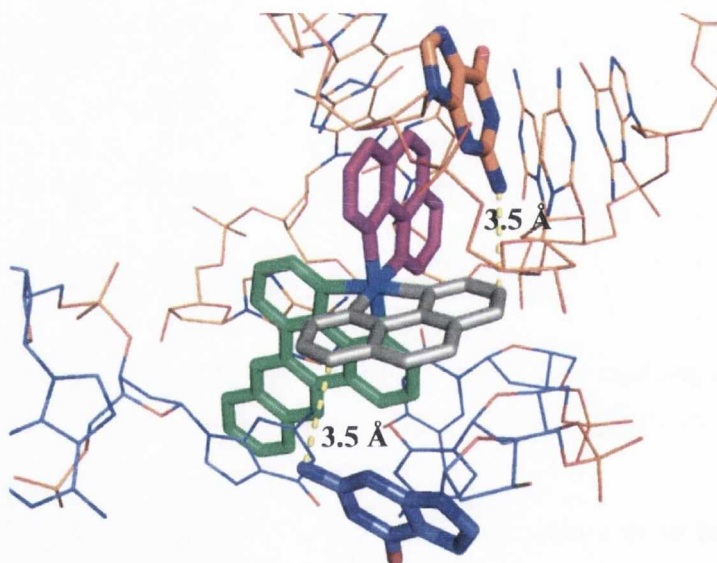


Figure 3.60 – The distances (yellow dashed) between the CH groups of TAP2 (grey) and the 2-NH₂ groups of G3 of Duplex 1 (blue) and G4 of Duplex 2 (orange)

3.11.2 Implications from this work

The crystal structures obtained in this work are accurate to atomic resolution and thus serve as the first unequivocal evidence for the binding of a polypyridyl ruthenium complex to a DNA duplex. Crystal structures are the proverbial 'holy grail' of oligonucleotide chemistry and the formation of these crystals will undoubtedly pave the way for the crystallisation of many such compounds, increasing the understanding of the interaction of small molecules with DNA, the building block of life.

While the long-standing debate of whether such complexes bind from the major or minor groove is not put to rest by these results, the evidence herein reinforces the claim that binding occurs from the minor groove as the Λ -complex studied here is shown to bind at the minor groove. Considering the angles at which the ancillary ligands of a Λ -complex are orientated, binding to the major groove of B-DNA (the larger of the two grooves) was presumed for this molecule as there would be less steric hindrance of the ligands. This has now been shown to be incorrect, with the implication being that such complexes do not bind at the major groove.

Perhaps the most interesting facet of the crystal structure of $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ are biological implications arising from the dramatic kinking of the DNA induced by the semi-intercalation of the TAP1 ligand. DNA kinking has been shown to be pivotal in many biological processes, particularly genetic transcription and repair. Very few small molecules induce kinking to such a degree as to influence such processes, the most notable of which is the anti-tumour agent cis-platin. While the interaction of these two compounds with DNA has already been detailed, the affect of both on the helix are quite similar, leading to the speculation that the $[\Lambda\text{-Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex may have potential as a cytotoxic agent. Experiments with this complex and cells *in vitro* would determine the ability of the complex to penetrate the nucleus and investigate if indeed cytotoxicity is evident. Previous ruthenium complexes have been shown to induce apoptosis *in vitro* though the mechanism of action is not understood. Perhaps the kinking caused by the semi-intercalation of a truncated ancillary ligand may be the answer to this particular conundrum.

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Chapter 4

Investigation of [Ru(TAP)₂(X₂dppz)]²⁺ Analogues

4.1 Introduction

Structural activity relationships (SARS) are crucial in the design of new compounds and complexes in many research-based industries. The pharmaceutical industry is perhaps the leading user of SARS techniques in attempting to create new and more effective drugs. The understanding of a compound's SARS allows for the educated guessing of how the addition or removal of substituents will affect the compound's physical, chemical and electronic structure and predict how the changes would alter the compound's desired ability. In industry, such a technique is used to model possible new target compounds for synthesis, and intricate modelling systems have been devised to predict the likely outcome of utilising the new compound, i.e. will it bind better to a specific binding site. However, in academic research, it is often through the time consuming process of trial and error that the most profound discoveries are made.

To date, many ruthenium polypyridyl complexes have been investigated in terms of their ability to bind to DNA.¹⁻⁵ An extended aromatic ligand is recognised as being crucial to allow full intercalation into DNA as opposed to the weaker semi-intercalation associations such as those formed upon the binding of $[\text{Ru}(\text{phen})_3]^{2+}$.⁶ Recently, the addition of substituents such as methyl groups and fluorine atoms have been shown to effect the binding affinity of intercalating metallo-complexes, particularly those containing a dppz ligand (Figure 4.1).^{5,7-10} Of particular interest to the work thus far presented are the studies regarding the methylation or fluorination of the dppz ligand in the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complex.

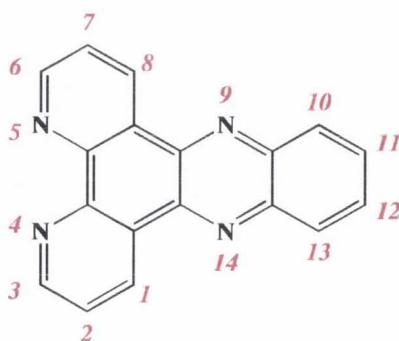


Figure 4.1 – The numbering convention used when substituting around a dppz ligand¹¹

The addition of methyl groups at either the 10,13 or 11,12 positions of the dppz ligand results in disruption to the solvation cage around the ligand. Initially it was believed that only substitution at the 10,13 position would reduce the quenching of the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complex in water due to the steric shielding of the phenazine nitrogens, by the methyl groups, to hydrogen bonding (see Figure 4.2). The presence of methyl groups at these positions did indeed reduce the rate of quenching, however a similar, albeit slightly weaker, effect was also noted when substitution was made at the 11, 12 positions.⁷ The conclusion was that the decreased hydrogen bonding observed for the methylated complexes is due to an entropic effect caused by steric perturbation of the aforementioned solvation cage. Methylation at these positions did not hinder the complex's ability to bind effectively to DNA.

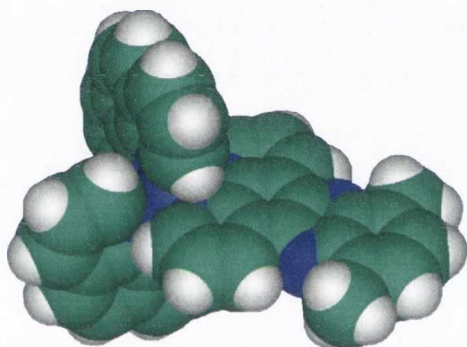


Figure 4.2 – Space filling model of Λ - $[\text{Ru}(\text{phen})_2(10,13\text{-Me}_2\text{dppz})]^{2+}$ illustrating the steric hindrance around the phenazine nitrogen atoms

The reduced quenching effects of the methyl groups were also found to occur upon intercalation to DNA, though on a much smaller scale. The suggestion is that while the methyl groups prevent access from the dppz direction, water molecules can still access the azanitrogens from the groove in which the Ru(II) ion resides and cause a quenching affect, implying that complete intercalation of the complex is prevented by the steric interactions of the ancillary ligands.⁷ The reduction in quenching is larger for the Δ enantiomer than the Λ considering its dppz ligand can intercalate more deeply, thus shortening the distance between the ruthenium centre and the helix and reducing the access of the water molecules.

The fluorination of the dppz ligand of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ has also been the subject of investigation and while in terms of binding, the replacement of two hydrogen atoms with two fluorine atoms has little effect due their being is similar size, the electronic impact is significant. The presence of the more easily reduced F_2dppz ligand lowers the MLCT energy of the complex altering the composition of the excited state through the disappearance of the dppz $^3\pi\pi^*$ state.⁸

To date, investigation of any methylated derivatives of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ have not been reported though such derivatives of similar complexes have been shown to have an increased ability to bind to DNA.⁵ The fluorinated derivative $[\text{Ru}(\text{TAP})_2(11,12\text{-F}_2\text{dppz})]^{2+}$ has been synthesised and its binding affinity for DNA was found to be equal to that of the parent complex.¹² However the enantiomers of this complex have not yet been resolved. Therefore the synthesis of the two complexes $[\text{Ru}(\text{TAP})_2(11,12\text{-Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(11,12\text{-F}_2\text{dppz})]^{2+}$ and investigation of the DNA binding affinity of their enantiomers could yield interesting results.

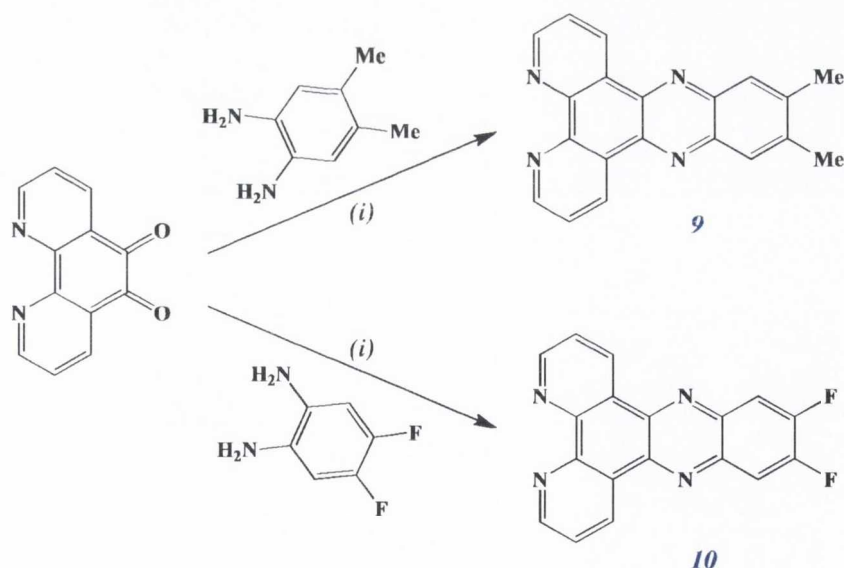
The work presented in this chapter reports the synthesis of these complexes, successful separation of their enantiomers and a thorough investigation of the enantiomers with various DNA sequences, utilizing the techniques of UV/visible absorption, fluorescence, and thermal denaturation.

4.2 Synthesis

$\text{Rac-}[\text{Ru}(\text{TAP})_2\text{dppzMe}_2]^{2+}$ and $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppzF}_2]^{2+}$ were prepared from commercially available starting materials. Full experimental details are provided in Chapter 7.

4.2.1 Synthesis of the dppz derivative ligands Me_2dppz and F_2dppz

The ligands Me_2dppz and F_2dppz were synthesised by adapting the literature method for the synthesis of the dppz ligand.^{2,11,13} Phendione (1 equivalent) and the appropriate diamino compound¹⁴ (2 equivalents) were refluxed in ethanol containing trace *p*-toluene sulphuric acid. The solution was cooled to r.t. and a brown precipitate was obtained. The solid was collected and recrystallized from aqueous ethanol to yield pale yellow needle-like crystals for the Me_2dppz **9** (72% yield) and a cream coloured solid for the F_2dppz ligand **10** (64% yield).

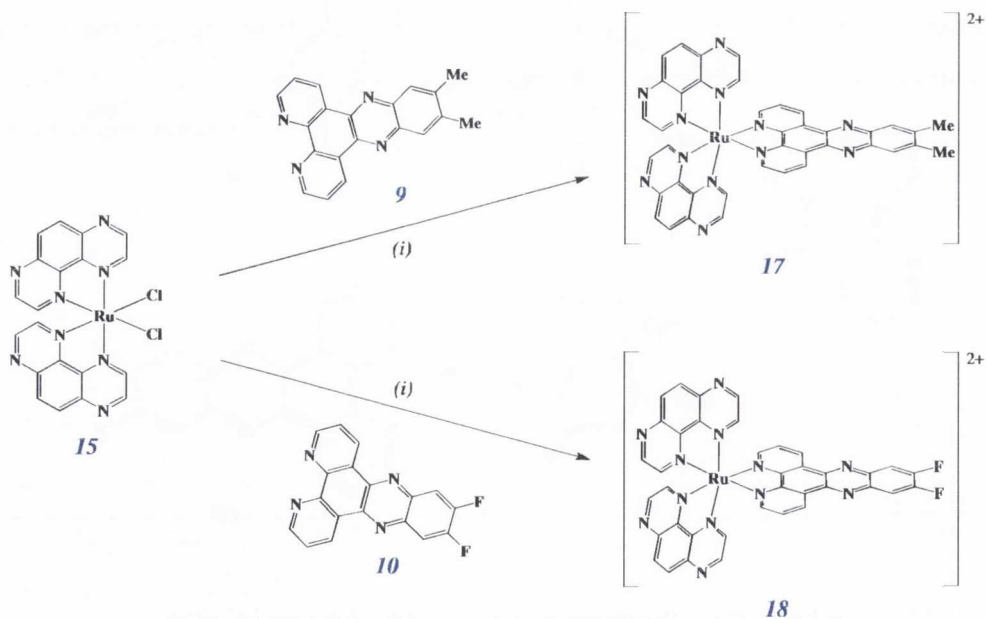


Scheme 4.1 - Synthetic pathway of Me_2dppz and F_2dppz

(i) EtOH, PTSA, 85°C, 3 hours

4.2.2 Microwave Assisted Synthesis of $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$

Following the success of the microwave assisted synthesis of the $\text{rac-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex the same technique was employed for the synthesis of the analogues $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$. The synthesis involved complexation of the appropriate dppzX_2 ligand **9** or **10** with the $\text{Ru}(\text{TAP})_2\text{Cl}_2$ precursor **15** in a degassed solution of ethanol and water (1:1). The $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ complex has not been reported in the literature. The $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex, while not reported in the literature, has been synthesised previously in the Kirsch-DeMesmaeker Group in Brussels.¹² The synthetic route they adopted was to first form the $[\text{Ru}(\text{TAP})_2\text{phendione}]^{2+}$ complex and subsequently react it with 1,2-diamino-4,5-difluorobenzene to yield the $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex. The synthetic method presented in this work, due its utilisation of microwave radiation, both reduces the reaction time from two hours to forty minutes and increases the yield from 52% to 69%. Thus, while the $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex may not be novel, its synthetic route is.



Scheme 4.2 - Synthetic pathway of $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ (i) EtOH/H₂O (1:1), MW, 140°C, 40 mins

Upon forty minutes irradiation in the microwave at 140°C both reaction mixtures changed from a violet coloured solution to a deep red/brown solution, indicating the formation of a tris-polypyridyl Ru(II) complex. Subsequent precipitation of the complexes as their PF₆ salt was achieved by the addition of a concentrated aqueous solution of KPF₆. Isolation of the precipitates yielded a red/brown solid. These were converted to their chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl⁻ form) and the desired products, **17** and **18**, were obtained following purification on an aqueous CM Sephadex C-25 column, using 0.2 M NaCl as the mobile phase. The final products were obtained as red/brown solids for both complexes, with an 82% yield for $[Ru(TAP)_2(Me_2dppz)]^{2+}$ and 69% yield for $[Ru(TAP)_2(F_2dppz)]^{2+}$. Both complexes were characterised by ¹H NMR and MALDI-TOF spectroscopy. Figure 4.3 shows the ¹H NMR spectrum of the racemic $[Ru(TAP)_2(Me_2dppz)]^{2+}$ complex with Table 4.1 identifying the various peaks.

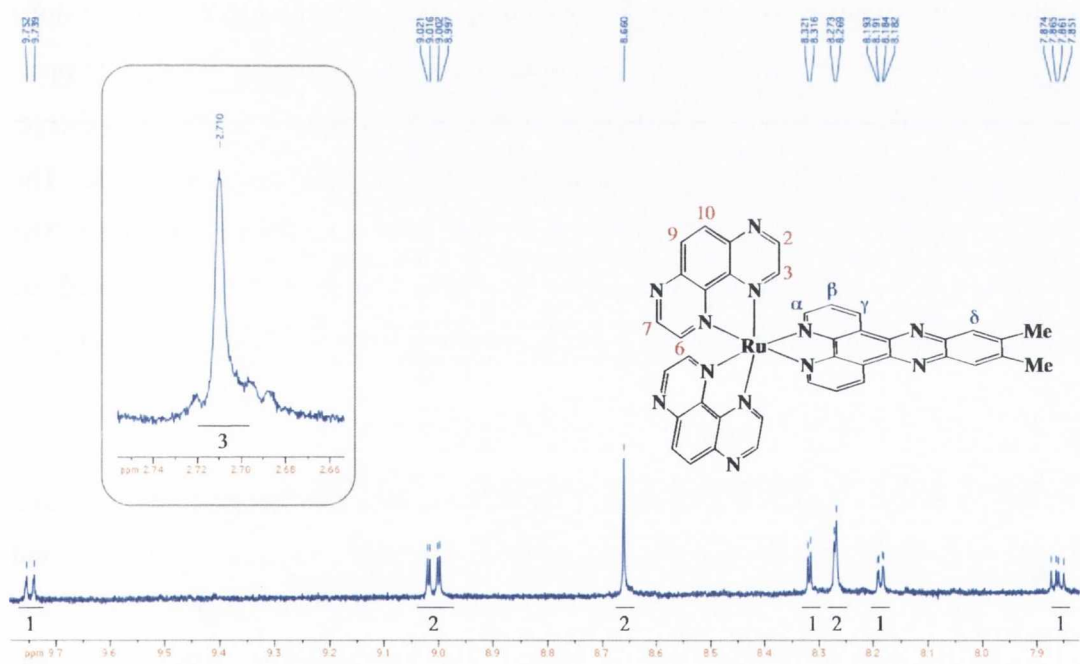


Figure 4.3 – ¹H NMR spectrum of *rac*- $[Ru(TAP)_2(Me_2dppz)]^{2+}$ in CD₃CN

Proton	Identity	$\delta(\text{ppm})$	Mult.	$J_{\text{apparent}} (\text{Hz})$	Int. rel.
TAP	2 and 7	9.09	dd	$J_{3,2} = J_{6,7} = 3$	2
	9 and 10	8.66	s	-	2
	3	8.32	d	$J = 3$	1
	6	8.27	d	$J = 2.4$	2
Me₂dppz	γ	9.74	d	$J_{\gamma,\delta} = 7.8$	1
	δ	8.27	d	$J = 2.4$	2
	α	8.19	dd	$J_{\alpha,\beta} = 5.4$	1
	β	7.86	dd	$J_{\beta,\gamma} = 13.8$	1
	Me	2.71	s	-	3

Table 4.1 – Assignment of peaks in the ^1H NMR spectrum of $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$

Interestingly, the presence of the two methyl groups causes the δ protons of the dppz ligand to shift downfield from 8.41 ppm (reported for $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$)¹² to 8.27 ppm. This shift causes the previously distinct peaks of the dppz δ and TAP 6 protons to merge. The TAP 6 protons are expected to yield a doublet signal and the δ protons a singlet. The peak at 8.27 appears to be a doublet with the second peak concealed by a singlet. The integration of this peak as two protons confirms that this irregular signal is indeed the amalgamation of the two proton environments, resulting from the downfield shifting of the δ protons.

Figure 4.4 shows the ^1H NMR spectrum of the racemic $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex with Table 4.2 identifying the various peaks. Fluorine atoms have a nuclear spin of $1/2$ and as a result, the coupling between hydrogen and fluorine nuclei is quite strong. The effects of a fluorine nucleus can be exhibited on a proton nucleus up to three bonds away, and can be observed as extra peak splitting and increased coupling constants in the ^1H spectrum. Such effects are noted in the spectrum in Figure 4.4. The δ proton signal, which is the closest proton to the fluorine substituents, is now a triplet where before it was a doublet (resulting from coupling to the ϵ proton at position 11). Also, the coupling constant for this signal has increased from 6.5 Hz to 9.6 Hz. The remaining protons are too far removed in the complex to experience such effects.

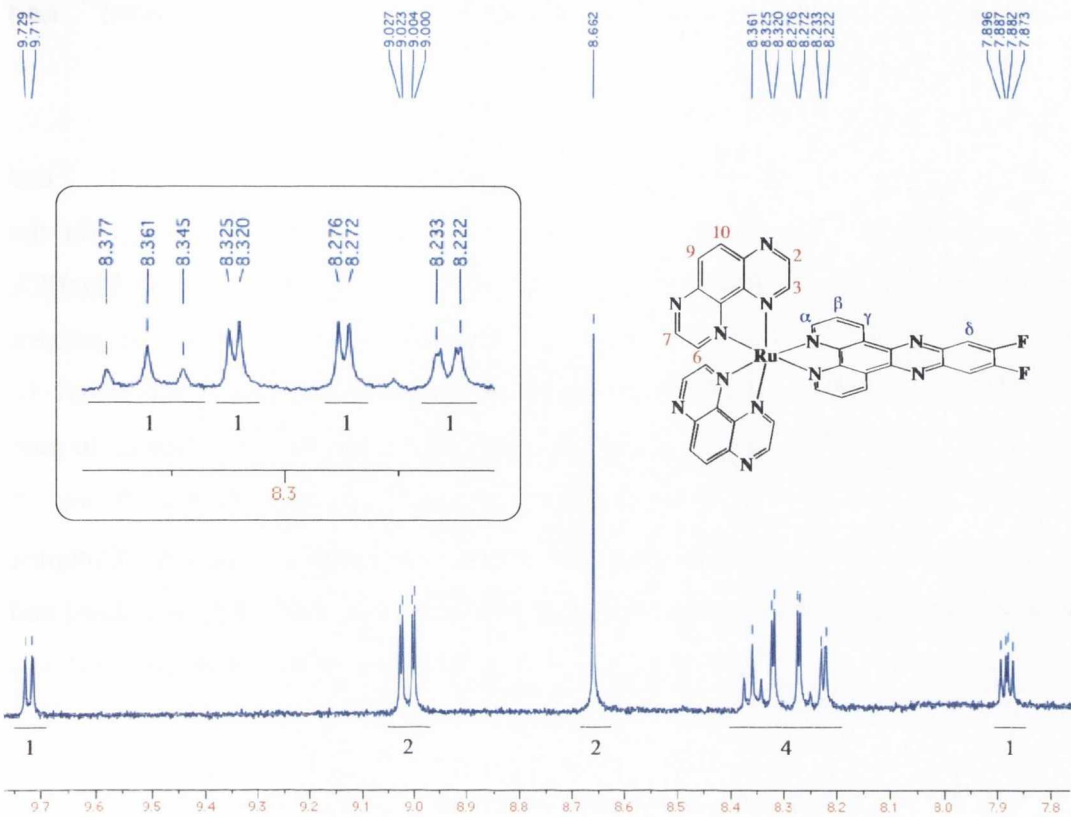


Figure 4.4 – 1H NMR spectrum of $rac-[Ru(TAP)_2(F_2dppz)]^{2+}$ in CD_3CN

Proton	Identity	δ (ppm)	Mult.	J_{apparent} (Hz)	Int. rel.
TAP	2 and 7	9.01	dd	$J_{3,2} = J_{6,7} = 2.4$	2
	9 and 10	8.66	s	-	2
	3	8.32	d	$J = 3.0$	1
	6	8.27	d	$J = 2.4$	1
<i>F</i>₂dppz	γ	9.72	d	$J_{\gamma,\alpha} = 7.2$	1
	δ	8.36	t	$J_{\delta,F} = 9.6$	1
	α	8.23	dd	$J_{\alpha,\beta} = 6.6$	1
	β	7.88	dd	$J_{\beta,\gamma} = 13.8$	1

Table 4.2 – Assignment of peaks in the 1H NMR spectrum of $rac-[Ru(TAP)_2(F_2dppz)]^{2+}$ in CD_3CN

4.2.3 Separation of the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$

The Λ and Δ enantiomers of the complexes $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ were separated following the procedure used previously for the separation of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers. The *rac*- $[\text{Ru}(\text{TAP})_2(\text{X}_2\text{dppz})]\text{Cl}_2$ complex was adsorbed onto the aqueous Sephadex stationary phase in a 1 m perspex column (diameter 2 cm) and the ionic mobile phase of aqueous sodium (–)-dibenzoyl-L-tartrate (0.1 M), neutralised to pH 7, was applied. The complexes were allowed to pass through the column four times to facilitate adequate separation of the Λ and Δ enantiomers before they were collected, with the Λ enantiomer eluting first. Complex purity was confirmed by MALDI TOF mass spectrometry (see Table 4.3 for values) and enantiomeric purity was confirmed by circular dichroism analysis (Figure 4.5 (a) and (b)).

Complex	Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$	Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$
Chemical Formula	$\text{C}_{40}\text{H}_{26}\text{N}_{12}\text{Ru}$	$\text{C}_{38}\text{H}_{20}\text{N}_{12}\text{F}_2\text{Ru}$
Calculated Mass	775.7848	783.7147
Found Mass	775.7857	783.7126

Table 4.3 – MALDI mass spectrometry (TOF MS LD+) analysis of the enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$

Identification of each of the enantiomers from their CD spectra was achieved by comparison with the CD spectra of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers in Chapter 2. Interestingly while the general trend of the spectra are the same for the three complexes, subtle differences are noted in the region between 250 nm and 350 nm. These undoubtedly arise from the substitution at the 11 and 12 positions on the dppz ligand and are compared in Figure 4.6, the spectra of the Λ enantiomers of the three complexes.

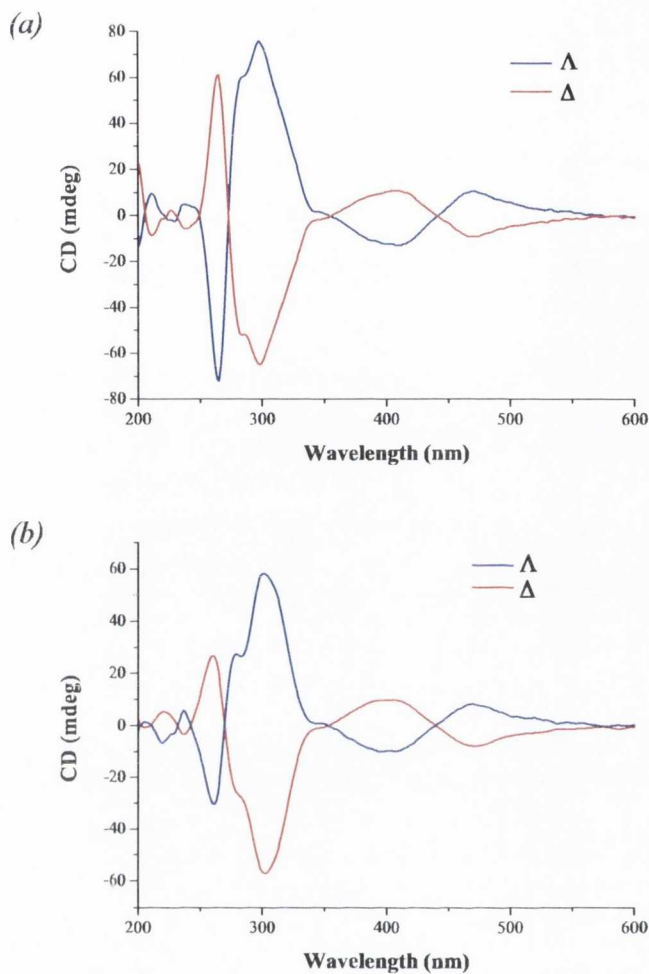


Figure 4.5 – Circular dichroism of the Λ and Δ enantiomers of (a) $[Ru(TAP)_2(Me_2dppz)]^{2+}$ and (b) $[Ru(TAP)_2(F_2dppz)]^{2+}$ in acetonitrile (20 μM)

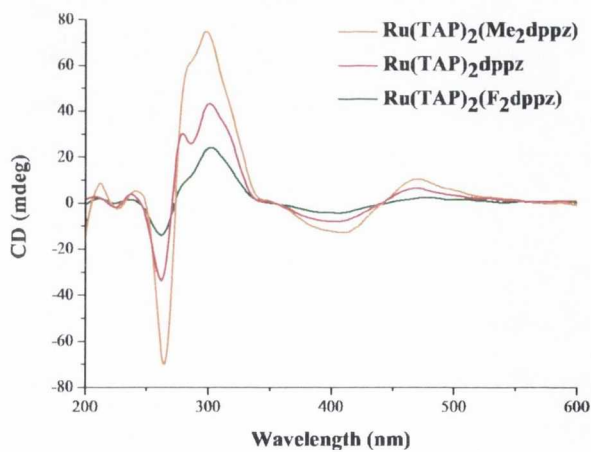


Figure 4.6 – Comparison of the Λ enantiomers of $[Ru(TAP)_2(Me_2dppz)]^{2+}$ (20 μM), $[Ru(TAP)_2dppz]^{2+}$ (10 μM), and $[Ru(TAP)_2(F_2dppz)]^{2+}$ (6 μM) in acetonitrile

4.3 Photophysical Characterisation

Characterisation of the two enantiomers of both the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complexes to confirm that both are identical in every aspect other than chirality was conducted. The UV/vis absorption spectra of the three analogous complexes **8**, **17** and **18** are shown in Figure 4.7 with the corresponding emission spectra are shown in Figure 4.8.

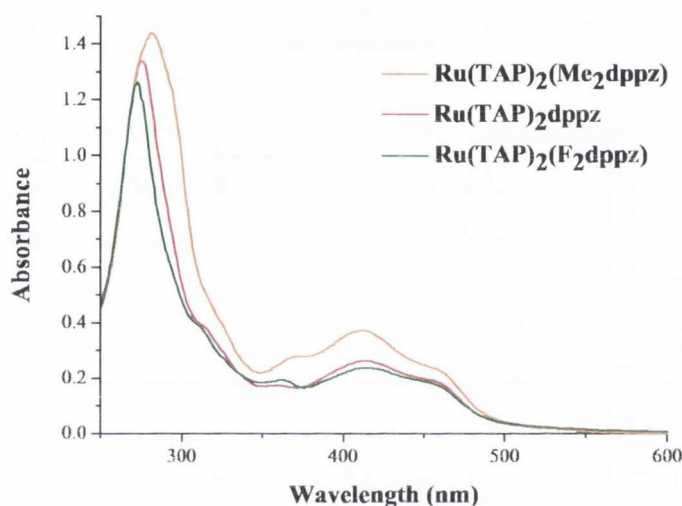


Figure 4.7 - UV/visible absorbance spectra of Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ **8**, Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ **17** and Λ - $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ **18**

As expected, the absorption spectra of the Λ and Δ enantiomers of each complex were identical. Each spectrum contains four different absorption maxima, detailed in Table 4.4, with the spectra of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ comparing favourably to those reported previously.^{12,15}

As mentioned in Chapter 2, the strong band around 275-280 nm in each spectrum is characteristic of the π - π^* intra-ligand transitions of the ancillary TAP ligands, while the very weak absorbance around 365-370 nm is attributed to the π - π^* transitions of the dppz ligand. The final peaks around 410 nm and 456 nm result from the metal-to-ligand charge transfer (MLCT) transitions of the Ru(II) centre, with the former being the Ru(II) $\rightarrow$ dppz MLCT transition and the latter the Ru(II) $\rightarrow$ TAP MLCT transition. It is in this latter area of the spectra that the major differences occur. In the presence of the Me_2dppz ligand the

absorbance at previously at 412 nm shifts to 409 nm and becomes more intense with respect to the band following at approx. 456 nm. Also the band resulting from the IL dppz transitions red-shifts from 362 nm to 371 nm. In the presence of the F₂dppz ligand there is little change in the absorbance band at 412 nm but the IL dppz transition red-shifts to 367 nm and increases in its intensity. All of the bands experiencing alterations correspond to a dppz ligand transition and the differences are expected considering the effects of the substituents have on the electronic structure within the ligand.

Complex	$\lambda_{\text{max}}/\text{nm}$ in CH ₃ CN ($\epsilon/10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$)
$[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$	278, 362, 412 (2.23), 454
$[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$	282, 371, 409 (2.68), 458
$[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$	275, 367, 412 (1.93), 456

Table 4.4 – Principle absorption maxima in acetonitrile for the complexes $\Lambda\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, $\Lambda\text{-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and $\Lambda\text{-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$

The extinction coefficient for the $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex was taken from Dr. Elias’ thesis.¹² The $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ complex, being a novel complex, has no reported extinction coefficient. The typical method through which these values are determined is by calculation of the concentration via dissolution of a known amount of the complex in a known volume of solution. Unfortunately, due to the difficulty in synthesising the complex, only a small amount could be synthesised (< 20 mg). This amount is too small to accurately measure, even with a microbalance. Thus, a more non-conventional method utilising NMR spectroscopy was employed.

A deuterated solution of $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ was made and the concentration determined using the extinction coefficients reported in the literature.¹⁶ A similar solution was made with $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$, obviously of unknown concentration. The UV/visible absorbance spectra of both solutions were obtained before both samples were analysed by NMR. Following this, the two solutions were mixed into one and this mixture was also

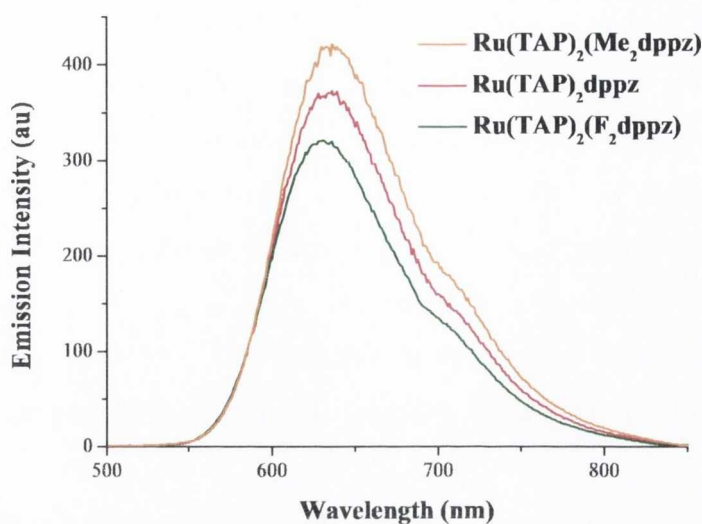
analysed by NMR. The signals specific to the phen and TAP complexes in the mixed solution were identified and the integrations of each performed. A selection of three TAP specific integrated signals was compared to a selection of phen specific signals. Division of the integral values by the molecular weight of the corresponding complex allowed for the calculation of the ratio of the concentration of $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ to $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ in the solution. Knowing this ratio, and the concentration of the $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ complex, determination of the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ concentration and subsequently its extinction coefficient was achieved. The NMR spectra used and the resulting calculations are presented in Appendix C.

The emission spectra of each of the complexes were also obtained in water (Figure 4.8). Excitation into the MLCT bands of each of the complexes at their isosbestic point results in an intense MLCT based emission, reported in Table 4.5.

Complex	λ_{ex} (nm)	λ_{max} (nm)
$[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$	435	635
$[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$	454	638
$[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$	435	636

Table 4.5 – Emission maxima in water for the complexes

Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and Λ - $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$



*Figure 4.8 - Fluorescence spectra of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ **8** (λ_{ex} 435 nm), $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ **17** (λ_{ex} 454 nm) and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ **18** (λ_{ex} 435 nm) in water*

The substitutions on the dppz ligand do not affect the overall ‘reverse’ light switch effect of the complexes. All three analogues are highly luminescent in water, as seen in Figure 4.8 above, and all are expected to quench in the presence of DNA containing G-C base pairs. Indeed Chapter 2 has shown this to be the case for the parent $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex and the $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex is reported to yield the same results as the parent complex in the work submitted in a previous thesis.¹² The enantiomers of the previously unreported $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ complex are shown in the following pages to indeed follow the trend of luminescence quenching the presence of G-C base pairs and enhancement in the presence of A-T nucleotide pairs, but there are subtle differences between the following results and those seen with the un-substituted complex.

4.4 DNA Binding Interactions of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$

Following the success of the binding studies between the enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$, and given that fact that complexes containing methylated dppz ligands have been shown to bind to DNA with a greater affinity than their non-substituted counterparts,⁵ a study into the binding abilities of the Λ and Δ enantiomers of the novel $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complex was undertaken. As previously, the binding of the enantiomers with stDNA, [poly(dG-dC)] and [poly(dA-dT)] were investigated via absorbance and fluorescence titrations, and thermal denaturation where possible.

4.4.1 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with stDNA

4.4.1.1 UV/visible Absorbance Titrations with stDNA – 10 mM sodium phosphate buffer

The interaction of the two enantiomers was first examined with stDNA (salmon testes B-DNA) in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4. The titrations were conducted by adding small aliquots of stDNA (at a stock solution concentration of 1×10^{-2} M) to a buffered solution of an enantiomerically pure complex (approx. 1.7×10^{-5} M) until a plateau in absorbance was reached. For these experiments, DNA additions were continued to a P/D ratio of 30. Each experiment was conducted a minimum of three times, with the results presented here being those deemed most accurate.

Following the investigative methods in Chapter 2, the absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific examination of the π - π^* dppz absorption band at 373 nm and MLCT absorption band at 409 nm both of which are beyond the DNA absorption at 260 nm, allowing for the observation of any spectral shifts induced by DNA binding.

The titration of both the enantiomers with stDNA resulted in significant changes in their absorption spectra. Figure 4.9 shows the recorded absorbance spectra for the Λ and Δ enantiomers, with Figure 4.10 displaying the shift trends observed and accompanying values summarised in Table 4.6.

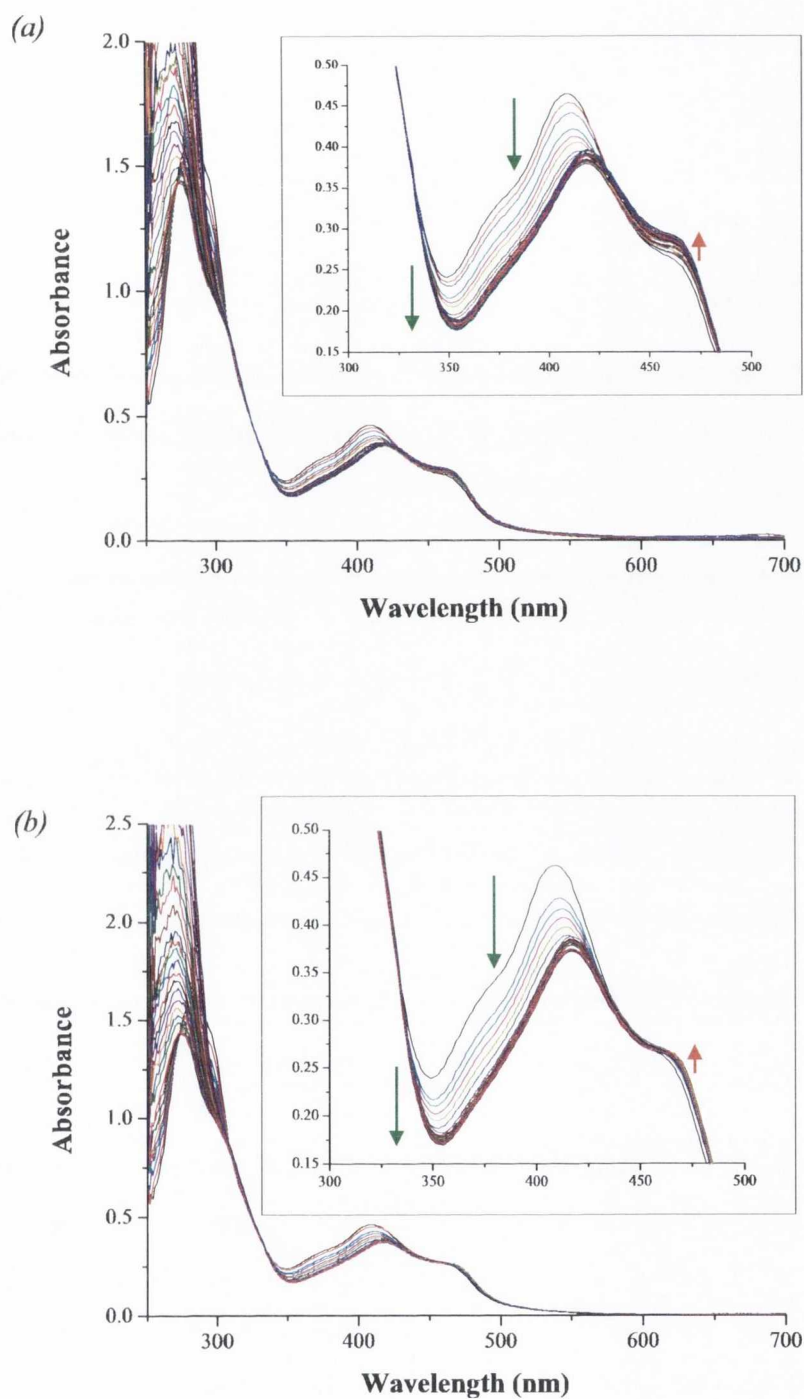


Figure 4.9 - Changes in the UV/vis absorption spectrum of
(a) $\Delta\text{-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ (17.1 μM) and (b) $\Delta\text{-}[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ (17.0 μM)
with increasing additions of stDNA (P/D 30) at 10 mM sodium phosphate buffer

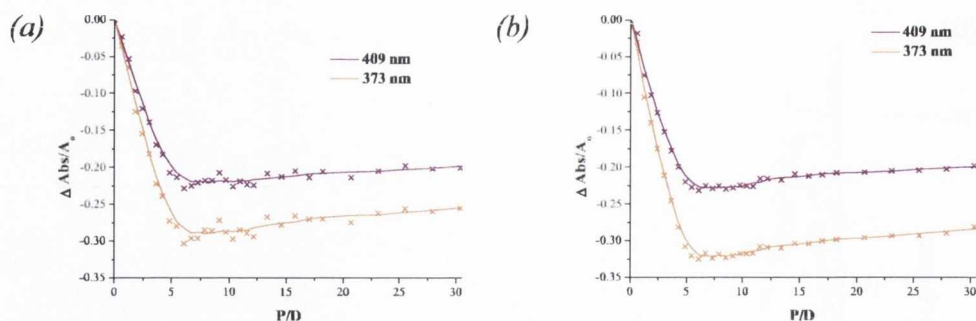


Figure 4.10 – Change in absorbance (ΔAbs) normalised by the absorbance without *stDNA* for (a) Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ and (b) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ in 10 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs_{373}	Max – 30% Rec – 26%	Max – 33% Rec – 28%	4 nm	6 nm
Abs_{409}	Max – 23% Rec – 20%	Max – 24% Rec – 20%	11 nm	9 nm

Table 4.6 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with *stDNA* in 10 mM sodium phosphate buffer

The absorption band of the Λ enantiomer at 275 nm initially decreased until a P/D of 3 was reached where after the absorbance of the DNA (260 nm) became too strong and hid the decay of these transitions. As such, little useful data could be gleaned from this absorbance peak. The band at 373 nm, corresponding to the intra-ligand π - π^* dppz transitions, experienced the most dramatic effects in the form of a hypochromic shift of 30% at P/D 6 before recovering to 26% by a P/D of 30. The absorbance band at 409 nm also underwent a hypochromic shift but only by a maximum of 23% before recovering to a final hypochromism of 20%. The band at 456 nm experienced a hyperchromism to a maximum of 14%.

The absorption band of the Δ enantiomer at 275 nm also initially decreased until a P/D of 5.5 was reached where after the absorbance of the DNA (260 nm) became too strong and hid the further decay of these transitions. As was seen with the Λ enantiomer, the bands at 373 nm and 409 nm underwent hypochromic shifts upon the addition of stDNA. The absorbance band at 373 nm again experienced the most dramatic effects in the form of a hypochromic shift of 33% at P/D 5.5 before recovering to 28% by a P/D of 30. The absorbance band at 409 nm also experienced a hypochromic shift, with the maximum of 24% occurring at a P/D of 5.5, followed by a recovery to 20%. Finally, the band at 456 nm experienced a hyperchromic shift of 4%.

At 456 nm, only a small effect upon the addition of stDNA is detectable for both enantiomers due to this band resulting from the Ru(II) $\rightarrow$ TAP MLCT transition. The hyperchromic shift detected at higher P/D ratios in both compounds is indicative of the reversal of the hypochromicity in absorption which is previously attributed to closely bound metal complexes², as explained in Chapter 2. It is interesting to note that again the hyperchromicity is higher for the Λ enantiomer than the Δ , indicating subtle differences between the binding modes of the two complexes.

As seen in the binding of the parent complex $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$, the bands at 373 nm and 409 nm for the enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ display a pronounced, biphasic absorption behaviour upon the addition of stDNA. Phase 1 occurs at low P/D ratios where significant hypochromic and bathochromic shifts occur as the absorption decreases linearly to a P/D ratio of 5.5. Beyond this P/D, the second phase (Phase 2) is characterised by a slight absorption increase (referred to as recovery) until a plateau is reached. This mirrors the results in Chapter 2 indicating similar binding modes between the methylated and non-substituted enantiomers. The initial non-specific intercalation of the Me_2dppz ligand, characterised by significant hypochromic and bathochromic shifts induced in the spectra,¹⁷ yields the Phase 1 behaviour followed by the cooperative electrostatic binding to the DNA grooves, allowing the dppz moiety to then intercalate only partially between base-pairs, resulting in the small hyperchromic effect of Phase 2.¹⁸

As previously, the extent of the hypochromicity experienced by the enantiomers may be indicative of the complexes' binding affinity for DNA. Although this is only a

generalisation, applying this ‘rule of thumb’ suggests that again the Δ enantiomer of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ has a higher binding ability than the Λ enantiomer. This is indeed expected as the only substitutions made were to the dppz ligand but it is the orientation of the ancillary TAP ligands that affect the depth of intercalative penetration of the dppz moiety. The steric hindrance caused by the left-handed and right-handed alignment of the TAP ligands in Λ and Δ enantiomers respectively is explained in detail in Chapter 2, section 2.4.1.1.

4.4.1.2 Fluorescence Titrations with stDNA – 10 mM sodium phosphate buffer

Fluorescence titrations between the two enantiomeric compounds of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ and stDNA were conducted in parallel to the absorbance titrations described previously. Each spectrum was examined at the excitation wavelength of 454 nm, which corresponds to the isosbestic point observed in the absorbance titration of the Δ enantiomer. The reason for this choice of wavelength is that at the isosbestic point, the fluorescence intensity may be directly related to the relative concentrations of the free and bound enantiomers.

Titration of both the Λ and Δ enantiomers with stDNA (P/D 0 $\rightarrow$ 30) resulted in significant quenching of the emission spectrum compared with the free complexes. As explained in the latter pages of Chapter 2 (section 2.5) it has been shown experimentally that this quenching is due to a proton-coupled electron transfer (PCET) process occurring between the guanine residues in the DNA and the $^3\text{MLCT}$ state of the complexes.¹⁹⁻²¹ The recorded emission spectra for the Λ complex is shown in Figure 4.11(a) with that of the Δ complex in Figure 4.11(b). Contrasts between the two emission spectra are visible from a simple inspection of these two graphs but a comparison of the relative emission intensities I/I_0 (Figure 4.12), where I_0 is the emission measured in the absence of DNA, highlights these differences.

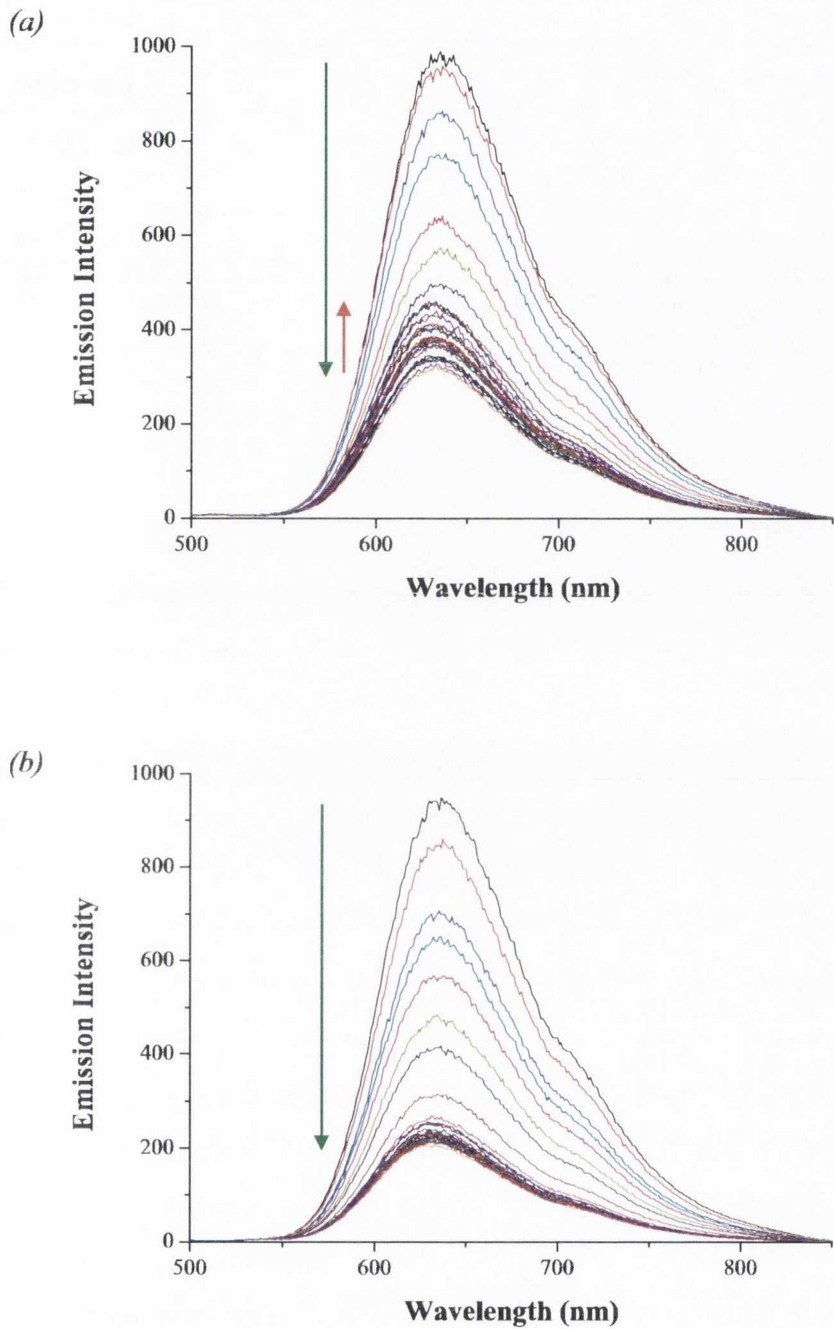


Figure 4.11 - Changes in the emission spectrum of (a) $\Delta-[Ru(TAP)_2(Me_2dppz)]Cl_2$ (17.1 μM) and (b) $\Delta-[Ru(TAP)_2(Me_2dppz)]Cl_2$ (17.0 μM) with increasing additions of stDNA at 10 mM sodium phosphate buffer

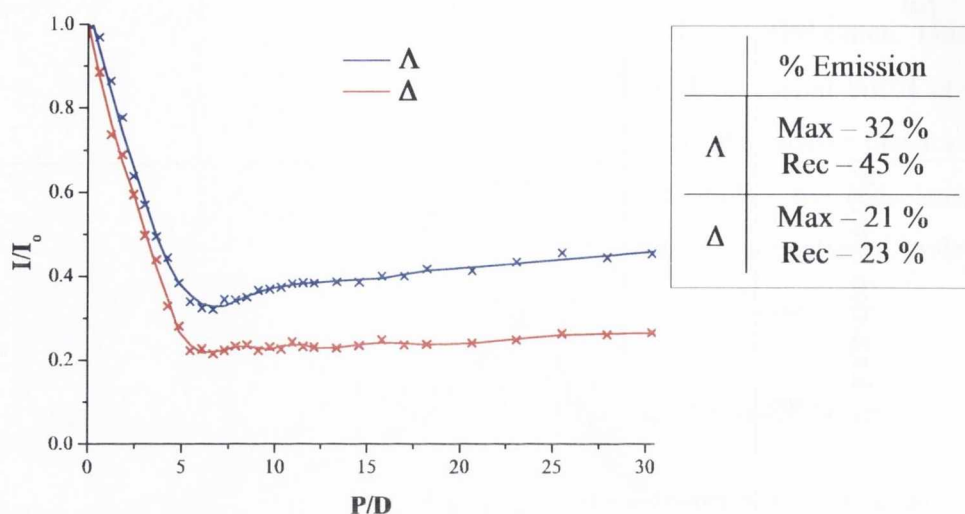


Figure 4.12 – Changes in the relative emission intensities vs increasing $[\text{stDNA}]$ of the Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complexes at 10 mM sodium phosphate buffer

The Λ enantiomer was quenched by 68% with the maximum quenching occurring at a P/D of 6, mirroring the P/D of the maximum hypochromicity recorded in the absorption spectra. The Δ complex was quenched by 79% with the maximum quenching also occurring at a P/D of 5.5. Thus the Δ enantiomer is quenched to a higher degree than the Λ enantiomer, complementing the results from the absorbance titrations that the Δ enantiomer binds more tightly to the DNA. Again, while the Δ complex, once a P/D of 5.5 is reached, remains at an almost constant quenched luminescence, the Λ complex recovers to a luminescence of 0.45 (a decrease from 68% to 55% quenching) at the final P/D of 30. This mirrors the luminescence results from the titrations of the original $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ enantiomers in which the Δ enantiomer experiences a higher degree of quenching and little recovery of luminescence while the Λ enantiomer is less quenched and does indeed recover at higher P/D ratios. Interestingly the degree to which the enantiomers of the methylated complex are quenched is less than that of the non-substituted complex, compared in Table 4.7. This suggests two possibilities. The first is that the changes induced to the electronic structure by the addition of the two methyl groups reduces the proton coupled-electron transfer between the complex and the guanine nucleotides, thus reducing the quenching ability of the DNA. The second is that the enhancement of luminescence caused by the binding to A-T sites may be greater with the methylated enantiomers than with the non-substituted enantiomers. The result of either

theory would be slightly elevated luminescence compared to the non-substituted enantiomers. To determine which of these possibilities is correct, binding studies between the enantiomers and the synthetic homopolymer polynucleotides [poly(dG–dC)] and [poly(dA–dT)] are required.

	Luminescence Quenching	
	Λ	Δ
$[Ru(TAP)_2dppz]Cl_2$	Max – 27% Rec – 33%	Max – 19%
$[Ru(TAP)_2(Me_2dppz)]Cl_2$	Max – 32% Rec – 45%	Max – 21% Rec – 23%

Table 4.7 – Comparison of the luminescence quenching upon the addition of stDNA to the enantiomers of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ in 10 mM sodium phosphate buffer

This second theory also explains the small recovery seen in the Δ enantiomer of the methylated complex, where the relocation affect is known not to occur, but the selective AT binding of any previously unbound ligand would increase the luminescence. The significant recovery of its Λ counterpart is a result of the probable relocation of the Λ enantiomer from the G-C base pairs to which it initially bound to the increasing number of A-T sites that enhance the luminescence of the complex. This relocation ability is facilitated by the fact that the Λ enantiomer does not bind as tightly to DNA as the Δ due to the steric hindrance of its ancillary ligands. Again, the degree of recovery of the methylated Λ (13%) is higher than that of the non-substituted Λ (6%).

The data collected from the absorbance and fluorescence titrations implies that the Δ enantiomer intercalates more deeply than the Λ enantiomer, demonstrated by the ability of the Λ enantiomer to relocate to its preferred A-T sites after the initial non-preferential binding. To further confirm these conclusions, thermal denaturation studies between salmon sperm DNA and the two enantiomers were conducted to determine the relative stability imparted by the binding of each of the enantiomers.

4.4.1.3 Thermal Denaturation Studies with stDNA – 10 mM sodium phosphate buffer

Thermal denaturation studies were performed between the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ and stDNA (150 μM) in 10 mM sodium phosphate buffer, pH 7.4, at a P/D ratio of 20, using degassed solutions. The absorbance at λ_{max} 260 nm was monitored as the temperature was gradually increased from 20°C to 90°C, at a rate of 1°C per minute. The thermal denaturation curves are shown in Figure 4.13.

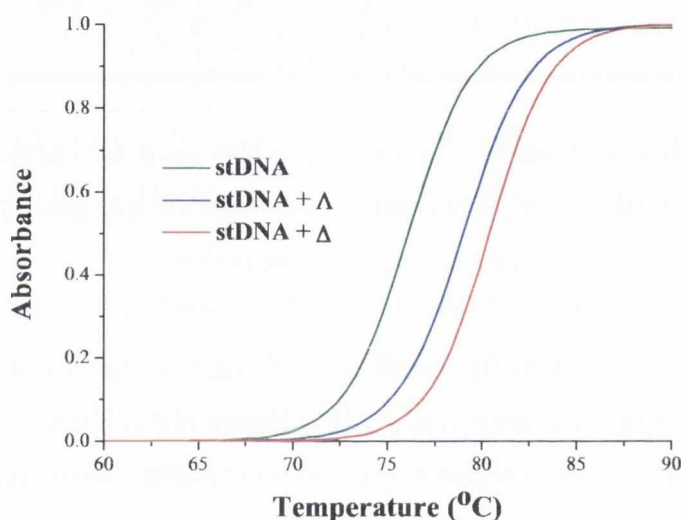


Figure 4.13 – Normalised thermal denaturation curves of dsDNA (150 μM) in the absence of any complex (green), the presence of the Λ enantiomer (blue) and the presence of the Δ enantiomer (red)

In the absence of metal complex, the T_m value for stDNA was recorded as 76°C with significant modulations being measured in the presence of the Λ and Δ enantiomers. Both the enantiomers stabilise the DNA duplex, resulting in increased thermal stability. Binding of the Λ enantiomer increased the T_m temperature by 3°C to 79°C and the Δ enantiomer increased the T_m temperature by 5°C to 81.0°C (Table 4.8).

Complex	T_m	ΔT_m
stDNA	76°C	-
stDNA + Λ	79.0°C	+ 3°C
stDNA + Δ	81.0 °C	+ 5°C

Table 4.8 – The T_m values from the first derivative plots of the melting curves from Figure 4.13

The above figures support the conclusions drawn thus far from the previous photophysical studies, that both complexes bind to and stabilise DNA but the increased ability of the Δ enantiomer to bind more tightly to the duplex and its dppz ligand to penetrate more completely between the base pairs results in the Δ enantiomer having a increased stabilising effect compared to the Λ .

These results compare favourably with those reported for the non-substituted enantiomers in Chapter 2, which report a thermal stabilisation of + 4°C and + 6°C for the Λ and Δ enantiomers respectively. The difference in the values between methylated and non-substituted enantiomers are within the error range of the apparatus (approx. 5%), allowing for the conclusion that the methyl groups do not alter the thermal stability the enantiomeric complexes have on the stDNA.

4.4.1.4 Binding Constant Calculations in Low Salt Conditions – 10 mM sodium phosphate buffer

The binding constants for the interaction of the Λ and Δ enantiomers with salmon sperm DNA at 10 mM sodium phosphate buffer were determined using the McGhee/von Hippel method, as previously, applied to the fluorescence data. The figures produced yielded a binding constant of $1.17 \times 10^8 \text{ M}^{-1}$ for the Λ enantiomer and $7.0 \times 10^8 \text{ M}^{-1}$ for the Δ enantiomer (Table 4.9). The accompanying plots are given in Appendix C.

Complex	Binding Constant $K_b (\text{M}^{-1})$	Binding Site Size n (base pairs)	R^2
Λ	$1.17 \times 10^8 (\pm 0.1)$	2.6 (+ 0.02)	0.99
Δ	$7.0 \times 10^8 (\pm 0.2)$	2.6 (+ 0.02)	0.99

Table 4.9 – stDNA binding constants calculated from the fluorescence data in 10 mM sodium phosphate buffer utilising the McGhee/von Hippel model

From the calculated binding constants, we can confirm that the Δ enantiomer does bind to DNA with a higher affinity than that of the Λ enantiomer. The values calculated above for the two enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ compare favourably to those reported previously for the Λ and Δ enantiomers of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complexes.² As noted with these previous complexes, the calculated values above are extraordinarily high binding constants, which suggest that perhaps these complexes bind to DNA too strongly to allow for the accurate calculation of their binding constants at physiological conditions.

4.4.1.5 ‘Reverse’ Salt Titrations with *stDNA* – 10 mM sodium phosphate buffer

The data obtained thus far allows for the assumption that the Λ enantiomer of the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complex is the more tightly binding of the two enantiomers, a trend echoing the observations from the work presented in Chapter 2. To unequivocally demonstrate that this is indeed the case, and in an effort to determine if the methylation of the dppz ligand has any influence on the ability of the complex to bind to DNA, ‘reverse’ salt titrations were conducted between the enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ and *stDNA* as a means not only to compare the binding of the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with each other but to also compare the relative binding ability of the methylated enantiomers with that of the non-substituted enantiomers.

The ‘reverse’ salt titration consists of increasing the ionic strength of the DNA-ligand solution via the addition of NaCl. Subsequently, the competition for the binding sites and reduction in the electrostatic attraction between the anionic DNA and cationic enantiomers results in the bound ligand being eventually displaced. This is detected in the UV spectra by a decrease in the DNA-ligand band.

The ‘reverse’ salt titration was conducted for each of the enantiomers at 10 mM sodium phosphate buffer conditions. Small aliquots of sodium chloride (4 M) were added to buffered solutions containing the enantiomer in question and duplex DNA at a P/D ratio of 5, so that the complex was fully bound. The absorption spectrum of each addition was recorded. Figure 4.14(a) is the ‘reverse’ titration with the Λ enantiomer and Figure 4.14(b) is the result from the ‘reverse’ titration with the Δ enantiomer. Figure 4.15 compares the percentage of liberated complex in the two enantiomerically pure solutions as the concentration of sodium increases.

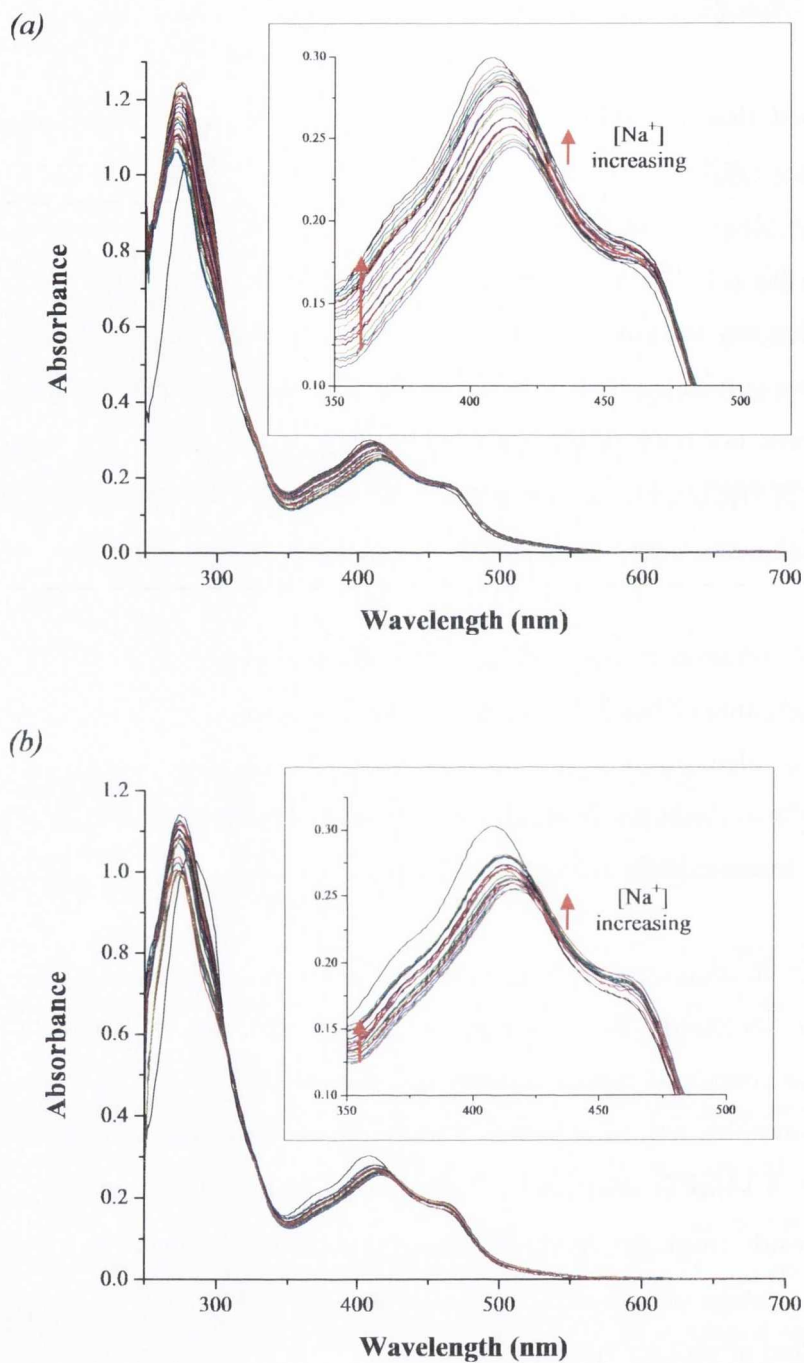


Figure 4.14 – UV/vis absorption spectrum for the ‘reverse’ salt titration of (a) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ (14.3 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ (14.5 μM)
 Note that the Δ enantiomer does not recover to absorbance of the free ligand (black trace) while the Λ enantiomer does

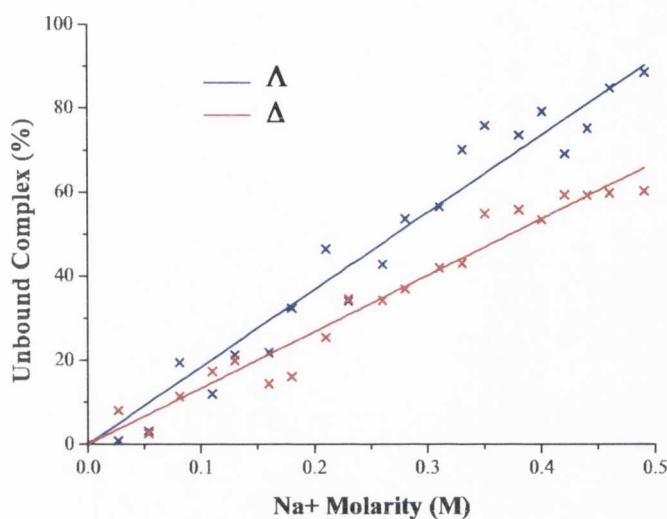


Figure 4.15 - Percentage of the enantiomeric complexes liberated with increasing $[\text{NaCl}]$

The results from the ‘reverse’ salt titration of the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ enantiomers show that Λ complex is almost completely unbound at a $[\text{Na}^+]$ of 0.49 M, whereas at this same end point, the absorbance of the Δ complex has not recovered to that of the free complex, indicating that more salt is required to fully dislodge the Δ enantiomer. This is further proof of the Δ enantiomer’s increased ability to bind more tightly to the DNA duplex than its Λ counterpart via increased penetration between the bases pairs of its intercalating dppz ligand.

An added benefit of conducting these experiments is the ability to now compare the effect of increased salt concentration on the enantiomers of both $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$. Methylation of the dppz ligand did not alter the thermal stability conferred by the enantiomers to stDNA, however the added steric requirement of the methyl groups is anticipated to provide an increased stability to salt displacement. A comparison of the data obtained from the titrations is presented in Figure 4.16, with the accompanying data in Table 4.10. At the final salt concentration of 0.49 M, 95% of the Λ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ had been liberated while only 89% of Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ had unbound. At this same $[\text{NaCl}]$ 63% of the Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ had been liberated while only 57% of Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ had unbound.

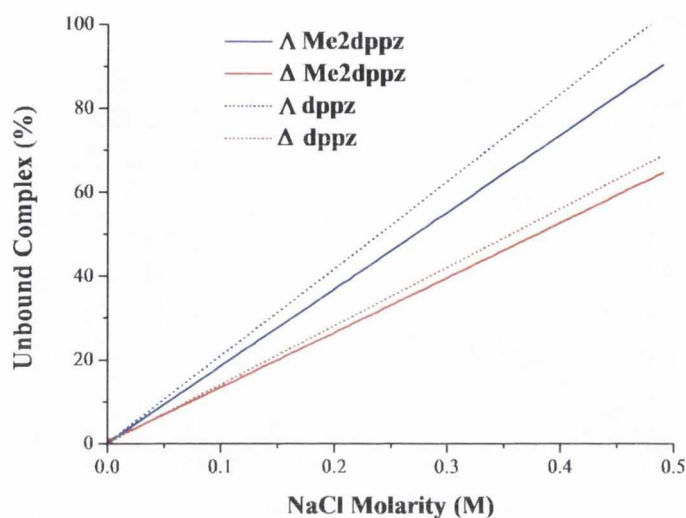


Figure 4.16 – Comparison of the percentage of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ enantiomers liberated with increasing $[NaCl]$

$[NaCl]$ (M)	Δ (% Unbound)		Δ (% Unbound)	
	Me_2dppz	$dppz$	Me_2dppz	$dppz$
0.49	89%	95%	57%	63%

Table 4.10 – Percentage of unbound enantiomeric complexes of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ at the maximum sodium chloride concentration

From this comparative data we can conclude that the methylation of the $dppz$ ligand does indeed increase the stability of the enantiomers to salt displacement compared to the non-substituted complexes. The assumption is that once the $dppz$ ligand has intercalated between the base-pairs, the methyl groups serve as ‘anchors’ due to their size and ability to rotate relative to the plane of the $dppz$ moiety. In the presence of increased salt concentrations, displacement occurs via reduction of the electrostatic attraction between the cationic enantiomers and anionic phosphates of the DNA. Once bound to the DNA, the methylated complexes are further anchored in their binding site by the presence of the methyl groups, thus increasing their resistance to salt displacement.

4.4.1.6 Conclusions from the Binding Studies with *stDNA*

The results obtained from the studies presented thus far initially mirror the results in Chapter 2. The Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ enantiomer is shown to be the better of the two complexes in terms of DNA binding affinity as it experiences increased hypochromicity in the absorption titrations, increased quenching in the fluorescence titrations and confers a higher degree of both thermal and ionic stability compared to that of the Λ enantiomer. This echoes the results from the $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ enantiomeric studies and the literature reports of the binding ability of the two $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ enantiomers.^{2,22-24}

The binding constants calculated for the interaction of the methylated enantiomers with the DNA are in the region of 10^8 , which indicate that both enantiomers have an extremely high affinity for binding to DNA. The Δ enantiomer is indeed shown to have the higher binding affinity of the two, demonstrated by its binding constant being larger than that of the Λ , confirming the indications from the spectroscopic studies. The binding constants between the methylated and non-substituted complexes are similar, with the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ enantiomers having slightly larger values than their $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ counterparts. However, due to the extreme numbers involved in these calculations, any conclusion drawn from comparisons of these values are unsubstantiated at best.

The two binding modes observed during the interaction of the $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ enantiomers with DNA, described in previous studies of similar complexes,^{4,18} are also evident in the binding of both the Λ and Δ enantiomers of the methylated complex. The deduction from this observation is that the methylation of the dppz ligand does not alter the modes through which the complexes bind to the DNA duplexes.

As previously, the Λ enantiomer of the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complex seems to have a preference for A-T binding sites, indicated by its recovery of luminescence.²⁵ Titrations involving solely G-C and A-T duplexes are, however, required to substantiate this observation.

4.4.2 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with $[\text{poly}(\text{dG-dC})]$

The results from the studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ showed that while the complexes have a high binding affinity, their binding preference is for A-T sites over G-C sites. Thus the enantiomers in this work, Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$, were titrated, as before, with the double stranded synthetic polynucleotides $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$ in order to investigate their photophysical properties and determine if any sequence binding specific effects occur

4.4.2.1 UV/visible Absorbance Titrations with $[\text{poly}(\text{dG-dC})]$ – 10 mM sodium phosphate buffer

The interaction of the two enantiomers with the polynucleotide $[\text{poly}(\text{dG-dC})]$ in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4 was first investigated. The titrations were conducted as previously, adding small aliquots of $[\text{poly}(\text{dG-dC})]$ (1.6×10^{-3} M) to a buffered solution of the pure enantiomers (approx. 6.3×10^{-6} M) until a plateau in absorbance was reached. As previously, the absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific observation of the absorbance bands at 373 nm and 409 nm. The titrations resulted in significant changes in the absorption spectra of both enantiomeric complexes and are given in Figures 4.17(a) and 4.17(b) for the Λ and Δ complexes respectively. Figure 4.18 shows the changes in absorbance at the bands 373 nm and 409 nm for the Λ and Δ enantiomers, with Table 4.11 summarising the spectral shifts.

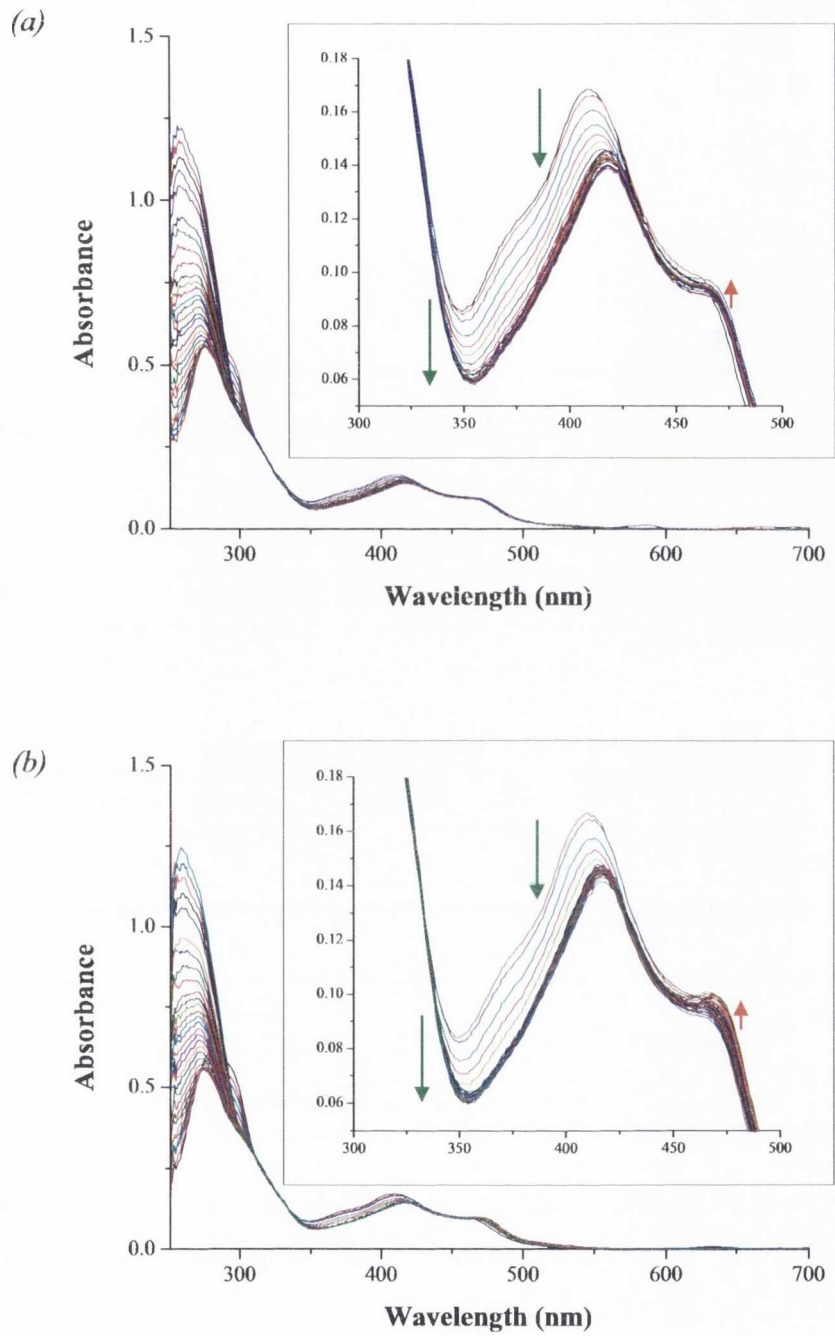


Figure 4.17 - Changes in the UV/vis absorption spectrum of (a) $\Delta-[Ru(TAP)_2(Me_2dppz)]Cl_2$ (6.32 μM) and (b) $\Delta-[Ru(TAP)_2(Me_2dppz)]Cl_2$ (6.35 μM) with increasing additions of $[poly(dG-dC)]$ (P/D 20) at 10 mM sodium phosphate buffer

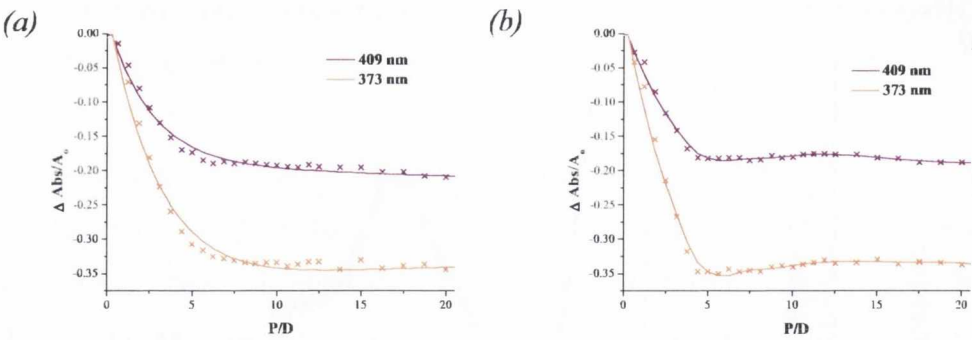


Figure 4.18 – Change in absorbance (ΔAbs) normalised by the absorbance without $[poly(dG-dC)]$ for (a) Λ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ and (b) Δ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ in 10 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₇₃	20%	21%	8 nm	10 nm
Abs ₄₀₉	35%	36%	11 nm	12 nm

Table 4.11 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ with $[poly(dG-dC)]$ in 10 mM sodium phosphate buffer

As with the previously conducted titrations, the band at 456 nm in both enantiomers experiences a small increase in its strength of absorbance, indicative of the reversal of the hypochromic effect of aggregated metal complexes. However unlike the same experiments conducted with the non-substituted enantiomers, the absorption profiles of Λ - and Δ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ exhibit predominately monophasic behaviour at the wavelengths of 373 nm and 409 nm. At low P/D ratios, significant hypochromic and bathochromic shifts occur linearly as the absorption decreases to a P/D of 8 for the Λ enantiomer and 5 for the Δ . At this point an equilibrium between the binding modes of phases 1 and 2 is reached. The hyperchromism of phase two, indicating a second mode of binding does occur with both enantiomers, but on a minute scale compared to that

observed in previous titrations. The implication being that the extent of electrostatic binding of the free complexes to the grooves of the duplex is drastically decreased.

The increased hypochromicity observed with the Δ enantiomer, coupled with the lower P/D ratio of equilibrium compared to that of the Λ suggests that the Δ enantiomer has the higher ability to bind to the [poly(dG–dC)] duplex. Interestingly the P/D ratio of equilibrium is higher for both the enantiomers with [poly(dG–dC)] than stDNA. This again mirrors the data obtained from the binding of the non-substituted enantiomers.

4.4.2.2 Fluorescence Titrations with [poly(dG–dC)] – 10 mM sodium phosphate buffer

To complement the absorbance studies, fluorescence titrations between the two enantiomeric compounds and [poly(dG–dC)] were conducted in parallel. It is expected that the fluorescence data will mirror that of the absorbance by revealing similar G–C specific effects with both enantiomers. The titration was conducted by the addition of small volumes of [poly(dG–dC)] to a solution of each complex in the low salt conditions of 10 mM sodium phosphate buffer at pH 7.4. Each spectrum was examined at an excitation wavelength of 454 nm, which corresponds to the isosbestic point observed for the Δ enantiomer.

The titrations of both the Λ and Δ enantiomers with [poly(dG–dC)] (P/D 0 $\rightarrow$ 20) resulted in significant quenching of the emission spectrum compared with the free complexes. Figure 4.19 displays the fluorescence spectra of the Λ and Δ titration results, with Figure 4.20 comparing the relative emission intensities of both enantiomers at the various P/D ratios.

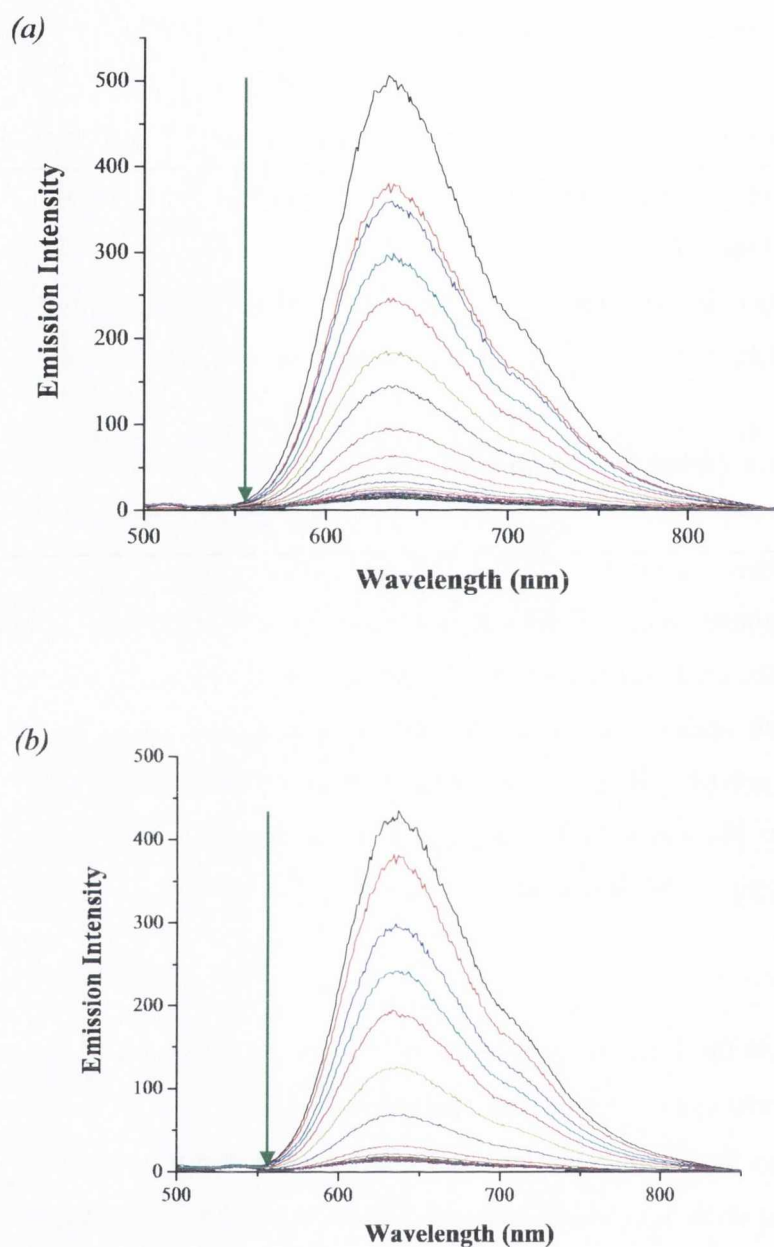


Figure 4.19 - Changes in the emission spectrum of (a) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ ($6.32 \mu\text{M}$) and (b) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ ($6.35 \mu\text{M}$) with increasing additions of $[\text{poly}(\text{dG-dC})]$ at 10 mM sodium phosphate buffer

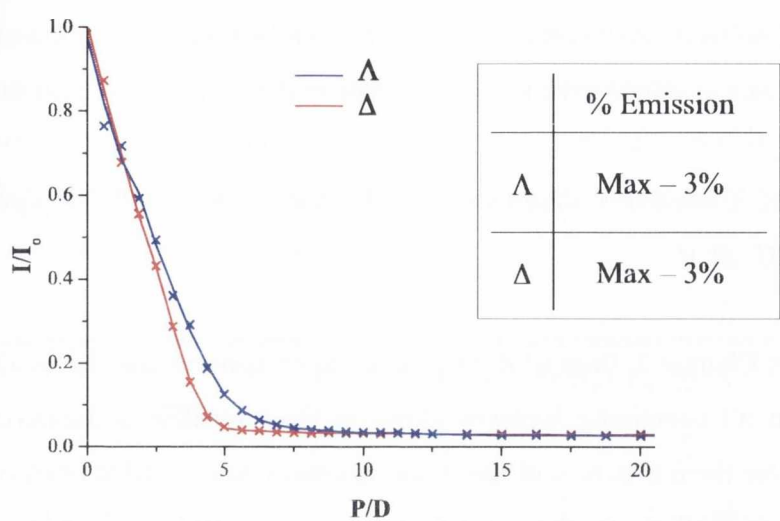


Figure 4.20 – Changes in the relative emission intensities vs increasing ratios of P/D of the Λ - and Δ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ complexes in the presence of poly[dG–dC] in 10 mM sodium phosphate buffer

As is evident from the previous spectra, both enantiomers are highly quenched upon the addition of the polynucleotide [poly(dG–dC)]. The Λ complex was quenched by 97% with the maximum quenching occurring at a P/D of 8. The Δ complex was also quenched by 97% but with the maximum quenching occurring at a P/D of 5.5. In contrast to the results with stDNA, no recovery of luminescence was observed in either enantiomer because there are no A-T sites available for the enantiomers to relocate to and thus enhance luminescence.

The results from the fluorescence titrations do mirror those from the absorbance experiments with the profiles in Figure 4.20 suggesting that the Δ enantiomer is indeed the better binding agent of the two complexes. The P/D ratio of maximum hypochromicity also agrees between the two spectroscopic techniques and again shows the increased affinity the Δ enantiomer has for the duplex binding compared to its Λ counterpart. As observed in the similar experiments with the $[Ru(TAP)_2dppz]Cl_2$ enantiomers, the slopes of the initial phases (P/D ratio 0 $\rightarrow$ 7) of the plots contained in Figure 4.20 further support this argument as it is much steeper for the Δ complex than the Λ , implying that the Δ enantiomer interacts better with the [poly(dG–dC)] at the lower P/D ratios.

The conclusions drawn from the fluorescence data of the titrations with the [poly(dG–dC)] polynucleotide echo those drawn from the absorbance titrations in that the Δ enantiomer again is shown to be the more tightly binding of the two enantiomers.

4.4.2.3 Binding Constant Calculations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with [poly(dG–dC)]

As explained in Chapter 2, thermal denaturation experiments cannot be conducted in the presence of non-AT containing duplexes. However, determination of the intrinsic binding constants, K_b , for the interaction of the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ with [poly(dG–dC)] allows for the evaluation and comparison of their binding affinities.

The binding constants for the interaction of the Λ and Δ enantiomers with [poly(dG–dC)] at 10 mM sodium phosphate buffer were determined using the McGhee/von Hippel method, as previously, applied to the fluorescence data. The figures produced yielded a binding constant of $9.4 \times 10^6 \text{ M}^{-1}$ for the Λ enantiomer and $1.7 \times 10^7 \text{ M}^{-1}$ for the Δ enantiomer (Table 4.12). These binding constants are much lower than those recorded for the stDNA at the same buffer concentrations (Table 4.9), showing that the affinity of both enantiomers for the [poly(dG–dC)] duplex is indeed lower than that for the mixed sequence DNA duplex. As expected, the binding constant for the Δ enantiomer is higher than that of the Λ , confirming the qualitative conclusions drawn from the previous spectra that the Δ enantiomer has a higher binding affinity for [poly(dG–dC)].

Complex	Binding Constant $K_b (\text{M}^{-1})$	Binding Site Size n (base pairs)	R^2
Λ	9.4×10^6 (± 0.4)	2.2 (+ 0.03)	0.99
Δ	1.7×10^7 (± 0.2)	1.8 (+ 0.05)	0.96

Table 4.12 – [poly(dG–dC)] binding constants calculated from the fluorescence data utilising the McGhee/von Hippel model

A comparison of the binding constants of the $[Ru(TAP)_2(Me_2dppz)]Cl_2$ and $[Ru(TAP)_2dppz]Cl_2$ enantiomers in the presence of $[poly(dG-dC)]$ suggests that the methylated enantiomers have a higher binding affinity for duplex DNA than their non-substituted counterparts, as summarised in Table 4.13. The increased binding constant was also in the presence of stDNA, however due to the extreme nature of values involved, no accurate conclusions could be made.

Complex	Binding Constant K_b (M^{-1})	
	Λ	Δ
$[Ru(TAP)_2dppz]^{2+}$	1.2×10^6	3.6×10^6
$[Ru(TAP)_2(Me_2dppz)]^{2+}$	9.4×10^6	1.7×10^7

Table 4.13 – Comparison of the binding constants for the two enantiomers of the $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ complexes with $[poly(dG-dC)]$ in 10 mM sodium phosphate buffer

The spectroscopic data and the binding constant values initially seem to contradict each other when comparing the methylated and non-substituted complexes. The UV/vis and fluorescence data from both sets of enantiomers are quite similar, implying an equally similar binding affinity. However, the binding constants of the methylated enantiomers are calculated as more than three time those of the non-substituted enantiomers in the presence of $[poly(dG-dC)]$, implying an increase in binding affinity. It is at this point that a small, but crucial distinction must be made. The spectroscopic data represents modes and equilibrium points that occur upon the binding of a compound to DNA. Quite often this data mirrors the binding constant data, thus is it interpreted as being indicative of the compound's binding affinity. In cases such as this, however, where two complexes bind to DNA via similar modes and to similar equilibrium points, such an interpretation of spectroscopic data can be misleading.

The binding constant is a useful measure to describe the strength of binding between receptors and their ligands, i.e. DNA and the binding compound. The binding of both the

$[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ complexes to DNA is a dynamic process in so far as the complexes fluctuate between the bound and unbound state. The binding constant is a measure of this fluctuation. In general, the higher the value of the binding constant, the stronger the binding attraction and the smaller the degree of fluctuation. In light of this, the higher binding constants of the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ enantiomers compared to those of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers can be easily explained. As mentioned in the 'reverse' titration (4.4.1.5), once the enantiomer has bound to DNA, the methyl groups serve as 'anchors' due to their size and ability to rotate relative to the plane of the dppz moiety. This reduces the ability of the complex to 'disengage' from the DNA, which serves to further decrease the dynamic nature of the interaction and thus results in larger binding constants.

4.4.3 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with $[\text{poly}(\text{dA-dT})]$

4.4.3.1 UV/visible Absorbance Titrations with $[\text{poly}(\text{dA-dT})]$ – 10 mM sodium phosphate buffer

The interaction of the two enantiomers with the polynucleotide $[\text{poly}(\text{dA-dT})]$ in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4 was conducted following the procedure used in the $[\text{poly}(\text{dG-dC})]$ experiments. The titrations were conducted by, adding small aliquots of $[\text{poly}(\text{dA-dT})]$ (1.7×10^{-3} M) to a buffered solution of the pure enantiomers (approx. 6.6×10^{-6} M) until plateau in absorbance was reached (P/D 20). As previously, the absorbance spectra of both the Λ and Δ enantiomers were recorded, with specific observation of the absorbance bands at 373 nm and 409 nm. The titrations resulted in significant changes in the absorption spectra of both enantiomeric complexes, which are given in Figures 4.21 (a) and (b). Figure 4.22 shows the changes in absorbance at the bands 373 nm and 409 nm for the Λ and Δ enantiomers, with Table 4.14 summarising the spectral shifts.

As with the previously conducted titrations, the band at 456 nm in both enantiomers experiences a hyperchromic effect. Again this is indicative of the reversal of the hypochromic effect of aggregated metal complexes, however, the increase in absorbance in the presence of $[\text{poly}(\text{dA-dT})]$ at this wavelength is significantly larger for both enantiomers (12% - Λ , 11% - Δ) than with stDNA or $[\text{poly}(\text{dG-dC})]$. The implication is that there is a subtle difference in the binding of the complexes to the A-T rich duplex, perhaps with a larger degree of electrostatic binding to the grooves occurring.

At the wavelengths of 373 nm and 409 nm, two phases of absorption are observed upon $[\text{poly}(\text{dA-dT})]$ addition, as also recorded in most of the previous titrations. During phase one, at low P/D ratios, significant hypochromic and bathochromic shifts occur as the absorption decreases linearly to P/D of 4.5. At higher P/D ratios, a recovery in absorbance is noted, phase two. This second phase is a consequence of the second, electrostatic binding mode described previously.

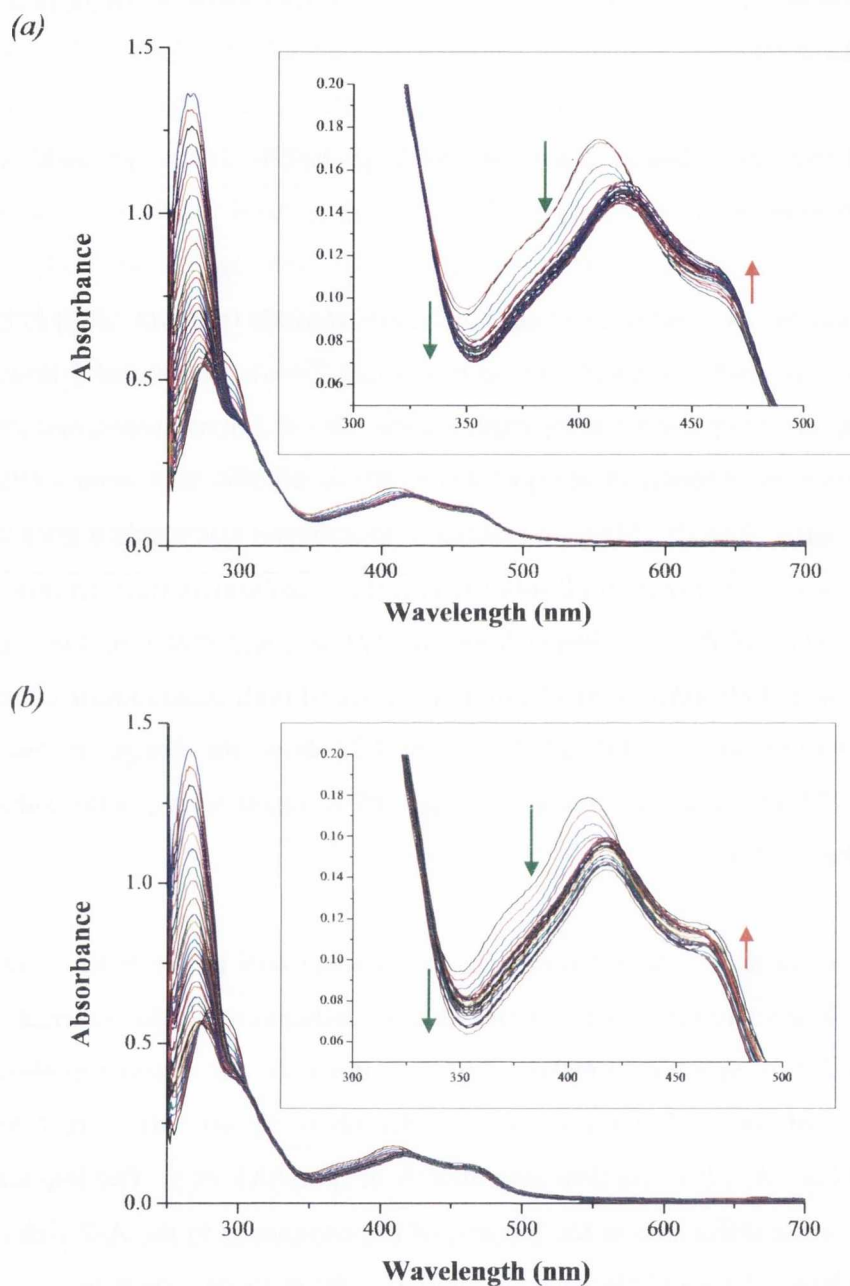


Figure 4.21 - Changes in the UV/vis absorption spectrum of
 (a) Λ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ (6.61 μM) and (b) Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ (6.59 μM)
 with increasing additions of [poly(dA-dT)] (P/D 20) at 10 mM sodium phosphate buffer

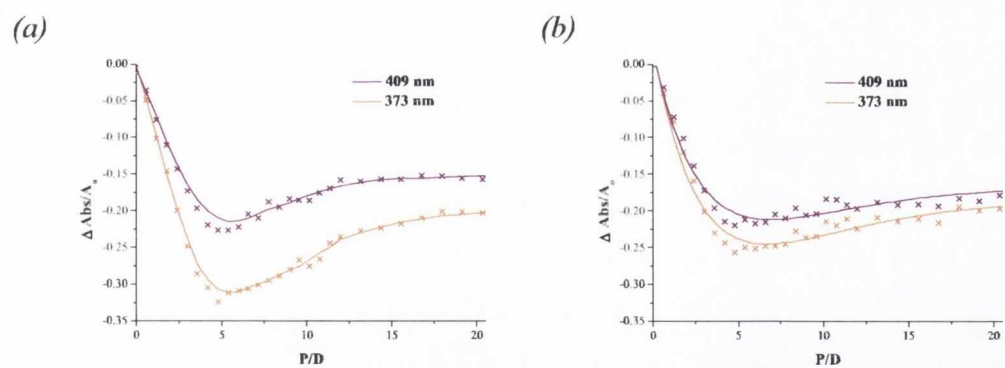


Figure 4.22 – Change in absorbance (ΔAbs) normalised by the absorbance without $[poly(dA-dT)]$ for (a) Λ -[Ru(TAP)₂(Me₂dppz)]Cl₂ and (b) Δ -[Ru(TAP)₂(Me₂dppz)]Cl₂ in 10 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₇₃	Max - 34% Rec - 23%	Max - 26% Rec - 17%	7 nm	7 nm
Abs ₄₀₉	Max - 24% Rec - 16%	Max - 22% Rec - 16%	9 nm	10 nm

Table 4.14 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ -[Ru(TAP)₂(Me₂dppz)]Cl₂ with $[poly(dA-dT)]$ at 10 mM sodium phosphate buffer

Both the Λ and the Δ enantiomers exhibit the same biphasic behaviour in their absorbance and the low P/D ratio of 4.5, common to both enantiomers, at which the maximum hypochromicity occurs suggests that the enantiomers also have a higher binding affinity for A-T sites than G-C, where the P/D binding equilibrium occurred at 8 (Λ) and 5 (Δ).

As observed in the $[poly(dA-dT)]$ titrations with the $[Ru(TAP)_2dppz]Cl_2$ enantiomers, the degree of hypochromicity of the absorption profiles is higher for the Λ enantiomer than the Δ , a reversal of the trend thus far observed in the stDNA and $[poly(dG-dC)]$ experiments. The implication of this is that the Λ enantiomer may in fact have a higher binding affinity for $[poly(dA-dT)]$ than the Δ enantiomer.

The absorbance of the titrations with the $[\text{poly}(\text{dA-dT})]$ suggests that both enantiomers have a preference for A-T sites over G-C sites. This agrees with literature reports of the enantiomers of the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ analogue and the results presented of the $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ enantiomers, all of which exhibit a preference for A-T binding sites.²⁵ Again the biphasic nature of the absorbance profiles indicates that two binding modes are present and the larger degree of hyperchromicity for the Λ enantiomer points to its possible increased ability to intercalate to the duplex compared to its Δ counterpart.

4.4.3.2 Fluorescence Titrations with $[\text{poly}(\text{dA-dT})]$ – 10 mM sodium phosphate buffer

As previously, fluorescence titrations between the two enantiomeric compounds and $[\text{poly}(\text{dA-dT})]$ were conducted in parallel with the absorption studies. The titrations were conducted from a P/D ratio of 0 to 20. The experiments resulted in a significant enhancement in the luminescence of the emission spectrum compared with the free complexes. Figure 4.23 (a) and (b) displays the fluorescence spectra of the Λ and Δ titration results, with Figure 4.24 comparing the relative emission intensities of both enantiomers at the various P/D ratios.

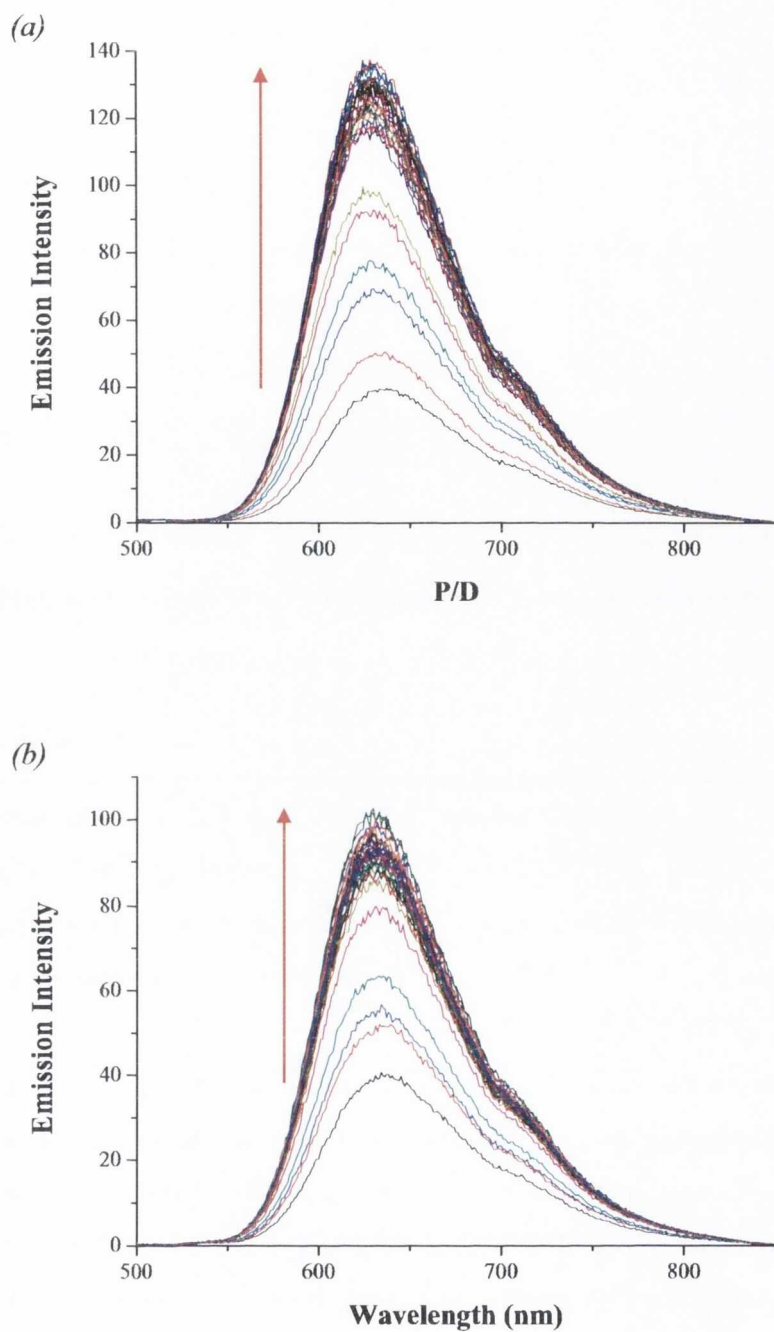


Figure 4.23 - Changes in the emission spectrum of (a) Δ -[Ru(TAP)₂(Me₂dppz)]Cl₂ (6.61 μM) and (b) Δ -[Ru(TAP)₂(Me₂dppz)]Cl₂ (6.59 μM) with increasing additions of poly[dA-dT] at 10 mM sodium phosphate buffer

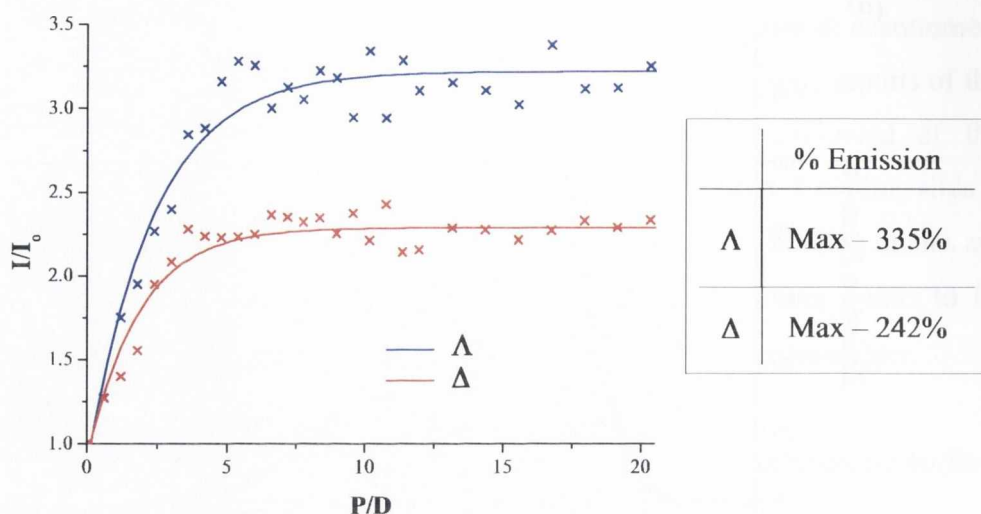


Figure 4.24 – Changes in the relative emission intensities vs increasing ratios of P/D of the Λ- and Δ-[Ru(TAP)₂(Me₂dppz)]Cl₂ complexes in 10 mM sodium phosphate buffer

As is evident from the previous spectra and above plot, the luminescence of both enantiomers is enhanced upon the addition of the polynucleotide [poly(dA–dT)]. The Λ complex was enhanced to 3.35 times its original value with the maximum enhancement occurring at a P/D of 5. The Δ complex was enhanced to 2.42 times its original value with the maximum enhancement occurring at a P/D of 4.5. The phenomenon of luminescence enhancement in the presence of [poly(dA–dT)] is a result of a decrease in the efficiency of the non-radiative deactivation process of the ³MLCT excited state of these complexes induced by the double helix microenvironment and protection from oxygen quenching. This enhancement also occurs in the mixed sequence duplexes, however it is masked by the quenching caused by the PET processes between the guanine nucleosides and Ru(II) polypyridyl centre.

The results from the fluorescence titrations again mirror the absorbance data. The luminescence of the Λ enantiomer is enhanced to a much greater degree than that of the Δ enantiomer, indicating that the [poly(dA–dT)] has a greater effect on the photophysics of the Λ enantiomer. The P/D ratios at which the complexes are fully bound also agree (within error) between the two techniques.

As mentioned in Chapter 2, the reason for the significant enhancement of the Λ enantiomer's luminescence compared to that of the Δ enantiomer is perplexing. However, the results from these latest titrations do validate a theory postulated earlier in this chapter that the methylation of the dppz ligand increases the enantiomers' luminescence enhancement upon binding to A-T rich duplexes. This notion was offered as an explanation for the larger luminescence recovery seen in the binding of the $[Ru(TAP)_2(Me_2dppz)]Cl_2$ enantiomers to stDNA compared to that of the $[Ru(TAP)_2dppz]Cl_2$ enantiomers. Table 4.15 below compares the degree of luminescence enhancement experienced by the four enantiomers, and clearly shows that the methylated complexes experience a larger enhancement effect in the presence of $[poly(dA-dT)]$. This is most likely a result of the steric hindrance caused by the presence of the methyl groups. Once bound to a duplex, and without the presence of guanine residues to quench luminescence, the protection afforded to such complexes from the quenching affects of oxygen becomes apparent. Without the methyl groups, it is possible for small concentrations of oxygen to access the intercalated dppz ligand from the major groove side of the intercalation pocket, thus causing a small degree of quenching. The presence of large methyl groups would hinder the oxygen molecules' access thus reducing this small effect with the result being a small enhancement of luminescence with respect to the non-substituted enantiomers.

Complex	Luminescence Enhancement	
	Λ	Δ
$[Ru(TAP)_2dppz]^{2+}$	325%	218%
$[Ru(TAP)_2(Me_2dppz)]^{2+}$	335%	242%

Table 4.15 - Comparison of the luminescence enhancement of the enantiomers both the $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ complexes with $[poly(dA-dT)]$ in 10 mM sodium phosphate buffer

The conclusions drawn from both the absorbance and fluorescence data of the enantiomers with the polynucleotide $[poly(dA-dT)]$ are that both have an extremely high

binding affinity for the macromolecule, with the Λ enantiomer exhibiting a slightly increased binding ability compared to the Δ .

4.4.3.3 Thermal Denaturation Studies with $[\text{poly}(\text{dA-dT})]$ – 10 mM sodium phosphate buffer

Unlike with the guanine-cytosine base pair, the Watson-Crick bonding between adenine and thymine base pairs involves only two hydrogen bonds. This results in a much lower T_m temperature for the lone $[\text{poly}(\text{dA-dT})]$ duplex, recorded as 54°C , allowing for thermal denaturation studies to investigate the relative stability each enantiomer imparts to the duplex to be performed.

Thermal denaturation studies were conducted between the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{poly}(\text{dA-dT})]$ (150 μM) in 10 mM sodium phosphate buffer, pH 7.4, at a P/D ratio of 20, using degassed solutions. The absorbance at λ_{max} 260 nm was then monitored as the temperature was gradually increased from 20°C to 90°C , at a rate of 1°C per minute. The thermal denaturation curves are given in Figure 4.25 below.

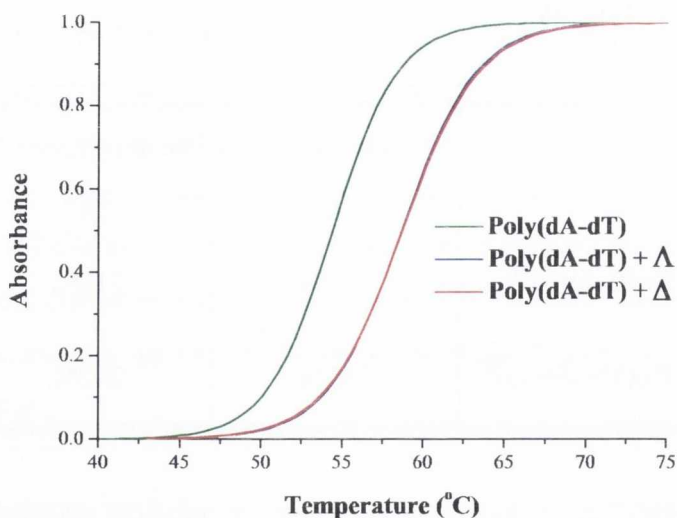


Figure 4.25 – Normalised thermal denaturation curves of $[\text{poly}(\text{dA-dT})]$ (150 mM) in the absence of any complex (green), the presence of the Λ enantiomer (blue) and the presence of the Δ enantiomer (red) in 10 mM sodium phosphate buffer (P/D 20)

In the absence of metal complex, the T_m value for [poly(dA–dT)] was recorded as 54.0°C with significant modulations being measured in the presence of the Λ and Δ enantiomers. Both the enantiomers stabilise the DNA duplex, resulting in increased thermal stability. Binding of both the enantiomers increased the T_m temperature by 5°C to 59.0°C (Table 4.16). The fact that both enantiomers stabilise the [poly(dA–dT)] duplex to the same degree supports the conclusions drawn from the spectroscopic data that the two have a similar affinity for A-T binding sites. The increase in stability (5°C) is indicative of the complexes’ increased preference for and ability to bind to A-T sites.

Complex	T_m	ΔT_m
[poly(dA–dT)]	54.0°C	-
[poly(dA–dT)] + Λ	59.0°C	+ 5°C
[poly(dA–dT)] + Δ	59.0°C	+ 5°C

Table 4.16 – The T_m values from the first derivative plots of the melting curves from Figure 4.25

Interestingly, the fact that melting profiles of both the enantiomers are the same indicates that both enantiomers have similar degrees of intercalation into the duplexes. As noted in the melting experiments conducted in the presence of stDNA, the thermal stability conferred upon the binding of methylated and non-substituted complexes is the same. This trend continues for the [poly(dA–dT)] experiments. The methylated enantiomers stabilise the duplex by 5°C while the non-substituted enantiomers stabilise it by 6°C. This small difference is within the margin of error for the apparatus and thus the four enantiomers are deemed to be equal.

A noticeable difference between the [poly(dA–dT)] experiments of the methylated enantiomers compared to the non-substituted enantiomers is that in the case of the former both the Λ and Δ denaturation curves are identical. If the difference between the two non-substituted enantiomers is attributed to the ability of the Λ enantiomer to form cross-

linking interactions between the duplexes, then the absence of any such differences in Figure 4.23 implies that the methylated Λ complex does not form such interactions in solution. Naturally without a crystallographic study to confirm, this is merely conjecture. As such, a possible explanation for the similarity in the melting curves is that the A-T duplex, being inherently more flexible than the G-C duplex may allow the Λ enantiomer to fit more completely into the grooves, facilitating a deeper penetration of the dppz ligand between the base-pairs resulting in its ability to bind as effectively as the Δ enantiomer.

4.4.3.4 Binding Constant Calculations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ with $[\text{poly}(\text{dA-dT})]$

Unfortunately due to the very abrupt changes in the I/I_0 plot at low P/D ratios for the two enantiomers with $[\text{poly}(\text{dA-dT})]$, determining the binding constants via fitting according to the McGhee/von Hippel method was not possible. From this, however, we can conclude that the binding constants for these interactions are larger than those recorded previously with both the stDNA and $[\text{poly}(\text{dG-dC})]$, indicating the binding preference of $[\text{poly}(\text{dA-dT})] > \text{stDNA} > [\text{poly}(\text{dG-dC})]$, echoing that found with the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ enantiomers.

4.4.4 Conclusions from the binding studies of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$

The results presented in the chapter confirm the trends reported for the enantiomers of intercalating ruthenium polypyridyl complexes in the literature and the observations presented in Chapter 2 of this thesis. The Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ both display a very high affinity for binding to duplex DNA, with the Δ enantiomer being the slightly better binding agent of the two. This is a result of its chirality matching that of the duplex DNA, allowing for a better fit into the binding site, and thus a more complete intercalation of its dppz ligand.

Two methods of DNA binding reported in the literature for similar complexes were observed to occur in the presence of both enantiomers.^{1,4,18} The most prevalent binding mode is the non-specific intercalation of the dppz ligand between DNA base pairs, dominating at lower P/D ratios. At higher P/D ratios, a second binding mode occurs. This method of binding is believed to be electrostatically driven whereby the complexes bind to the grooves of the DNA, perhaps allowing the dppz ligands to partially intercalate between base pairs.¹⁸ Evidence for this electrostatic binding mode is found in the hyperchromic effect of the absorbance spectrum that is characteristic of the second phase in the absorbance profile.

Interpretation of the spectroscopic data indicates that the Δ enantiomer has a higher binding affinity for DNA than the Λ . The ‘reverse’ salt titration was conducted to provide evidence for this difference in binding ability. The addition of sodium chloride results in the eventual displacement of the bound ligand and through monitoring the absorbance spectrum of the solution, determination of the concentration of liberated complex is possible. The results from these experiments with the two enantiomers of the methylated complex revealed that after the addition of 0.49 M sodium chloride, 32% more of the Λ enantiomer had been released from the DNA than the Δ , thus providing evidence that the Δ enantiomer does indeed have a higher binding affinity for DNA.

Investigation of the enantiomers’ binding potential with the homopolymers [poly(dG–dC)] and [poly(dA–dT)] revealed slight variations in the binding affinities. While both enantiomers do bind to both polynucleotides, a trend in the K_b was noted in

the binding ability for the complexes as being $[\text{poly}(\text{dA-dT})] \geq \text{stDNA} \geq [\text{poly}(\text{dG-dC})]$. The Δ enantiomer was found to bind more effectively to the $[\text{poly}(\text{dG-dC})]$ duplex than the Λ enantiomer while in the presence of A-T duplexes, both enantiomers seemed to be of equal binding affinity, as demonstrated by their identical melting curves. The explanation for the A-T preference of both the enantiomers, and also the increased binding affinity of the Λ in the $[\text{poly}(\text{dA-dT})]$ environment may lie in the inherent flexibility of A-T duplexes which can seemingly better accommodate complexes of opposite chirality, as explained in Chapter 2 (2.4.6).

4.4.5 Comparison of the binding studies of the enantiomers of $[Ru(TAP)_2dppz]Cl_2$ and $Ru(TAP)_2(Me_2dppz)Cl_2$

It is clear from a comparison of the results presented in this chapter with those presented in Chapter 2 that methylation of the dppz ligand of $[Ru(TAP)_2dppz]Cl_2$ does indeed have an effect on the DNA binding ability of its enantiomers. The most significant effect of methylation is the increased binding affinity both enantiomers have for the DNA duplex. The spectroscopic data shows the binding modes and their equilibrium points (indicated by the P/D ratio of maximum hypochromicity) to be equal for both sets of enantiomers, non-specific intercalation dominating initially with electrostatic interactions prevailing at higher DNA concentrations. The calculated binding constants, however, are quite different, as table 4.17 summarises.

	Binding Constants K_b (M^{-1})			
	$\Lambda-[dppz]^{2+}$	$\Delta-[dppz]^{2+}$	$\Lambda-[Me_2dppz]^{2+}$	$\Delta-[Me_2dppz]^{2+}$
stDNA	1.4×10^7 (5)	1.2×10^8 (5)	1.12×10^8 (5.5)	7.0×10^8 (5.5)
[poly(dG-dC)]	1.2×10^6 (12)	3.6×10^6 (8)	9.4×10^6 (8)	1.7×10^7 (5)
[poly(dA-dt)]	5	5	4.5	4.5

Table 4.17 – Comparison of the calculated binding constants of the enantiomers of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ with various DNA sequences in 10 mM sodium phosphate buffer. P/D ratios of binding mode equilibrium are given in brackets

These binding constants indicate that methylation of the dppz ligand increases the affinity of the enantiomers for duplex DNA. This is ascribed to the ability of the methyl moieties to act as ‘anchors’, holding the complex in the intercalative pocket and preventing its dynamic disassociation from the double helix.

The main trends in the fluorescence data remain the same. Upon binding to stDNA all four complexes are quenched, with both Δ enantiomers experiencing a higher degree of

quenching than the Λ enantiomers. In the presence of $[poly(dG-dC)]$, all four enantiomers are quenched to similar levels, within experimental error, while in a $[poly(dA-dT)]$ environment, the luminescence of all four enantiomers is enhanced, with the two Λ enantiomers experiencing a higher degree of enhancement than their Δ counterparts. Interestingly however, the non-substituted complexes are quenched to a higher degree than the methylated enantiomers in the presence of stDNA, while their luminescence is not enhanced to the same level as that of the methylated enantiomers, as summarised in Table 4.18.

	Fluorescence Quenching/Enhancement (%)			
	$\Lambda-[dppz]^{2+}$	$\Delta-[dppz]^{2+}$	$\Lambda-[Me_2dppz]^{2+}$	$\Delta-[Me_2dppz]^{2+}$
stDNA	0.27	0.19	0.32	0.22
$[poly(dG-dC)]$	0.05	0.04	0.03	0.03
$[poly(dA-dT)]$	325	218	335	242

Table 4.18 – Comparison of fluorescence data of the enantiomers of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(Me_2dppz)]^{2+}$ with various DNA sequences in 10 mM sodium phosphate buffer

While the differences in luminescence between the methylated and non-substituted enantiomers are small, only those of the $[poly(dG-dC)]$ can be fully explained by experimental error. Having determined the binding affinity for the methylated enantiomers is higher, it would be logical to assume that the quenching upon binding to stDNA would be larger than that of the non-substituted complexes. However, the presence of the methyl groups causes a block at the major groove side of the intercalation pocket (considering the enantiomers bind via the minor groove). This helps to prevent any solvated oxygen from interacting with the intercalating dppz ligand and thus reduce the subsequent quenching that would otherwise occur. The result of this is that the methylated enantiomers do not experience as much quenching as their $[Ru(TAP)_2dppz]^{2+}$ cousins. This explanation also applies to the increased enhancement noted with the methylated complexes.

Finally, the addition of methyl groups was found to have no effect on the thermal stability imparted by the enantiomers to any of the duplex DNA. All the determined melting points agreed within experimental error, though they did further support the conclusions that the Δ enantiomer is better able to intercalate than the Λ . As expected, however, was the discovery that methylation increased the enantiomeric stability to salt displacement. As shown in Figure 4.16 and Table 4.8, at the maximum recorded sodium concentration of 0.49 M, less of the methylated enantiomers had been liberated from the duplex than their non-substituted counterparts. The assumption is that once the dppz ligand has intercalated between the base-pairs, the methyl groups serve as ‘anchors’ due to their size and ability to rotate relative to the plane of the dppz moiety.

The ultimate conclusions drawn from the results presented in this work is that the methylation of the intercalating moiety of a ruthenium polypyridyl complex increases the DNA binding affinity of the complex and, once bound, reduces the disturbance to the photophysics from the dissolved oxygen in the solvent. It also infers a degree of environmental stability by withstanding increased sodium chloride concentrations, indicating that should such complexes be utilised in medicine, perhaps a methylated analogue would be preferable.

4.5 DNA Binding Interactions with $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$

As mentioned earlier, the *rac*- $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ complex was synthesised in the Kirsch-De Mesmaeker Group in Brussels and reported in the thesis of Dr. Benjamin Elias.¹² In this work, binding studies of *rac*- $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ with mixed sequence B-DNA are presented along with the mention that the same studies were conducted using the *rac*- $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ complex and yielded the same results. The conclusion was that the substitution of hydrogen atoms for fluorine at the 11 and 12 position of the dppz ligand did not alter the binding affinity the complex has for double stranded DNA. However, the accompanying data for the *rac*- $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ experiments was not presented, thus preventing our interpretation of the data. To validate Dr. Elias' statement, spectroscopic titrations of the Λ and Δ enantiomers of $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ were conducted with stDNA following the procedure employed throughout this project.

4.5.1 Spectroscopic Titrations of Λ - and Δ - $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ with stDNA

4.5.1.1 UV/visible Absorbance Titrations with stDNA – 10 mM sodium phosphate buffer

The interaction of the two enantiomers was studied with stDNA in low salt concentrations of 10 mM sodium phosphate buffer, pH 7.4. The titrations were conducted by adding small aliquots of stDNA (7.2×10^{-3} M) to a buffered solution of an enantiomerically pure complex (approx. 2.7×10^{-5} M) until a plateau in absorbance was reached. For these experiments, DNA additions were continued to a P/D ratio of 20.

The titration of both the enantiomers with stDNA resulted in significant changes in their absorption spectra. Figure 4.26 shows the recorded absorbance spectra for the Λ and Δ enantiomers, with Figure 4.27 displaying the shift trends observed and accompanying values summarised in Table 4.19.

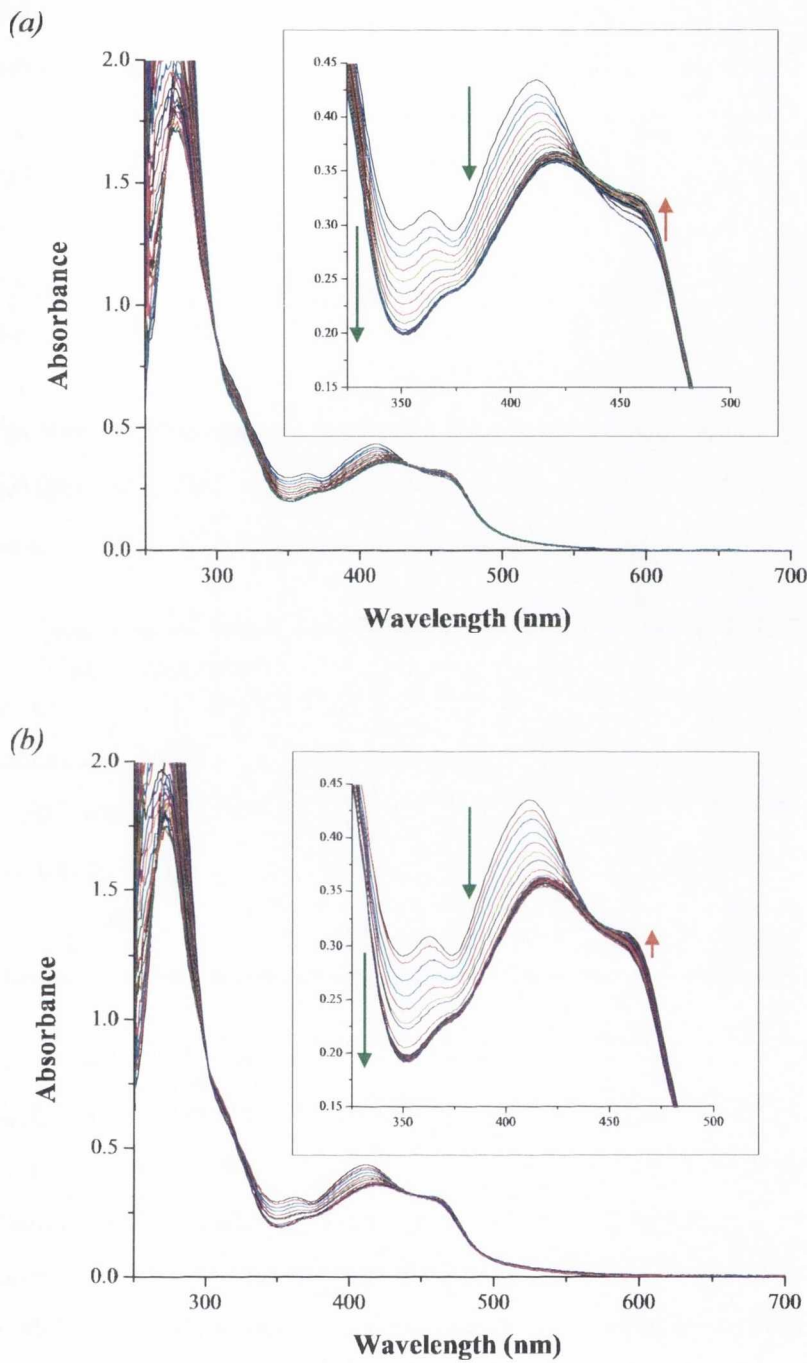


Figure 4.26 - Changes in the UV/vis absorption spectrum of (a) Δ -[Ru(TAP)₂(F₂dppz)]Cl₂ (26.6 μ M) and (b) Δ -[Ru(TAP)₂(F₂dppz)]Cl₂ (27.1 μ M) with increasing additions of stDNA (P/D 20) at 10 mM sodium phosphate buffer

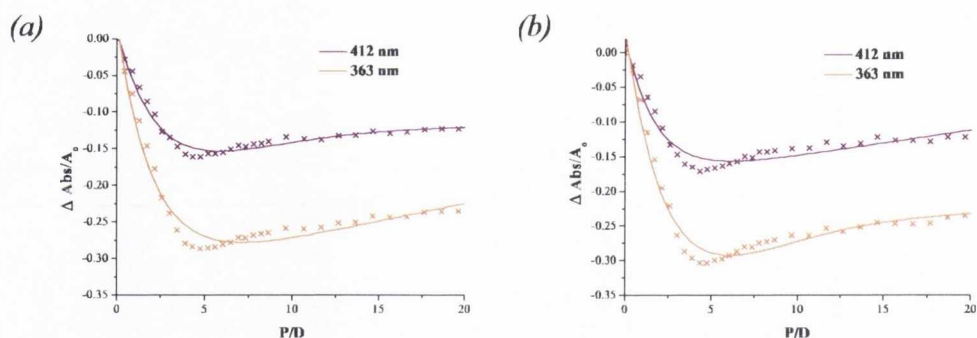


Figure 4.27 – Change in absorbance (ΔAbs) normalised by the absorbance without *stDNA* for (a) Λ - $[Ru(TAP)_2(F_2dppz)]Cl_2$ and (b) Δ - $[Ru(TAP)_2(Me_2dppz)]Cl_2$ in 10 mM sodium phosphate buffer

Enantiomer	Hypochromicity		Bathochromicity	
	Λ	Δ	Λ	Δ
Abs ₃₆₃	Max – 28% Rec – 23%	Max – 31% Rec – 23%	4 nm	6 nm
Abs ₄₁₂	Max – 16% Rec – 12%	Max – 17% Rec – 12%	11 nm	9 nm

Table 4.19 – Summary of UV/vis shifts in the absorbance bands for the titrations of Λ - and Δ - $[Ru(TAP)_2(F_2dppz)]Cl_2$ with *stDNA* in 10 mM sodium phosphate buffer

The same trends are noted in these titrations as in the previous studies. The absorption band of both enantiomers at 275 nm initially decreased until a P/D of 3 was reached where after the absorbance of the DNA (260 nm) became too strong and hid the decay of these transitions. The band at 363 nm, corresponding to the intra-ligand π - π^* dppz transitions, experienced the most dramatic effects in the form of a hypochromic shifts in both spectra (28% - Λ , 31% - Δ at a P/D of 5) before a small recovery is observed. A hyperchromic shift is also noted initially in the band at 412 nm for both enantiomers, followed by a small increase in absorbance. The less pronounced band at 456 nm was found to undergo a hyperchromic shift upon the addition of DNA. This effect was larger with the Λ enantiomer (13%) than with the Δ enantiomer (5%), and while the hyperchromicity is indicative of the separation of closely bound metal complexes², as

explained in Chapter 2, this difference again suggests subtle differences between the binding modes of the two complexes.

Following the trends observed in the titrations of the $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ enantiomers, the absorbance bands at 363 and 412 nm display a pronounced, biphasic behaviour upon the addition of stDNA. Phase 1 occurs at low P/D ratios where significant hypochromic and bathochromic shifts occur as the absorption decreases linearly to a P/D ratio of 5. Beyond this P/D, the second phase (Phase 2) is characterised by a slight absorption increase (referred to as recovery) until a plateau is reached. These results mirror those of the unsubstituted enantiomers in Chapter 2, including the P/D ratio at which maximum hypochromicity occurs.

As previously, the extent of the hypochromicity experienced by the enantiomers may be indicative of the complexes' binding affinity for DNA. Although this is only a generalisation, applying this 'rule of thumb' suggests that again the Δ enantiomer of $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ has a higher binding ability than the Λ enantiomer, a trend common to the enantiomers of both $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$.

4.5.1.2 Fluorescence Titrations with stDNA – 10 mM sodium phosphate buffer

As conducted with the parent complex, fluorescence titrations between the two enantiomeric compounds of $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ and stDNA were conducted in parallel to the absorbance titrations described previously. Each spectrum was examined at the excitation wavelength of 435 nm, which corresponds to the isosbestic point observed in the absorbance titration of the Δ enantiomer.

Titration of both the Λ and Δ enantiomers with stDNA (P/D 0 $\rightarrow$ 20) resulted in significant quenching of the emission spectrum compared with the free complexes. As explained in the latter pages of Chapter 2 (section 2.5) it has been shown experimentally that this quenching is due to a proton-coupled electron transfer (PET) process occurring between the guanine residues in the DNA and the $^3\text{MLCT}$ state of the complexes.¹⁹⁻²¹ The recorded emission spectra for the Λ complex is shown in Figure 4.28(a) with that of the Δ complex in Figure 4.28(b). Contrasts between the two emission spectra are visible from a simple inspection of these two graphs but a comparison of the relative emission

intensities I/I_0 (Figure 4.29), where I_0 is the emission measured in the absence of DNA, highlights these differences.

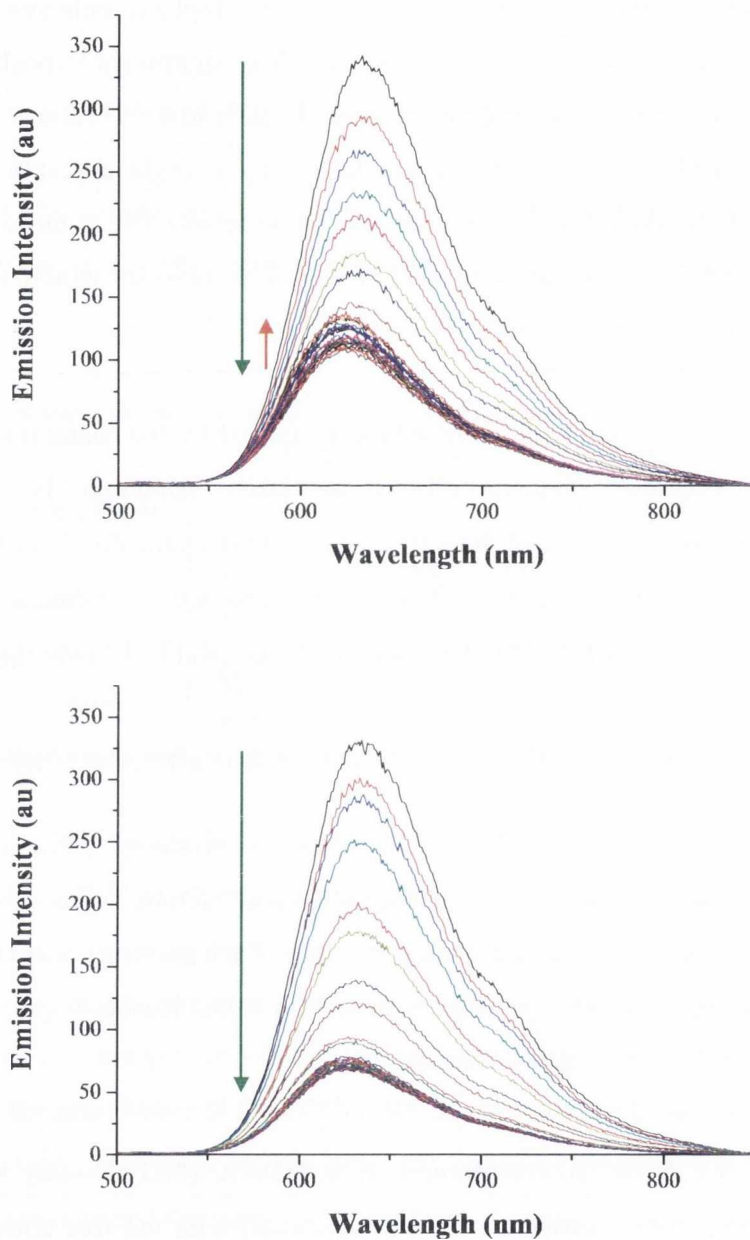


Figure 4.28 - Changes in the emission spectrum of (a) $\Delta\text{-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ ($26.6 \mu\text{M}$) and (b) $\Delta\text{-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ ($27.1 \mu\text{M}$) with increasing additions of *dsDNA* at 10 mM sodium phosphate buffer

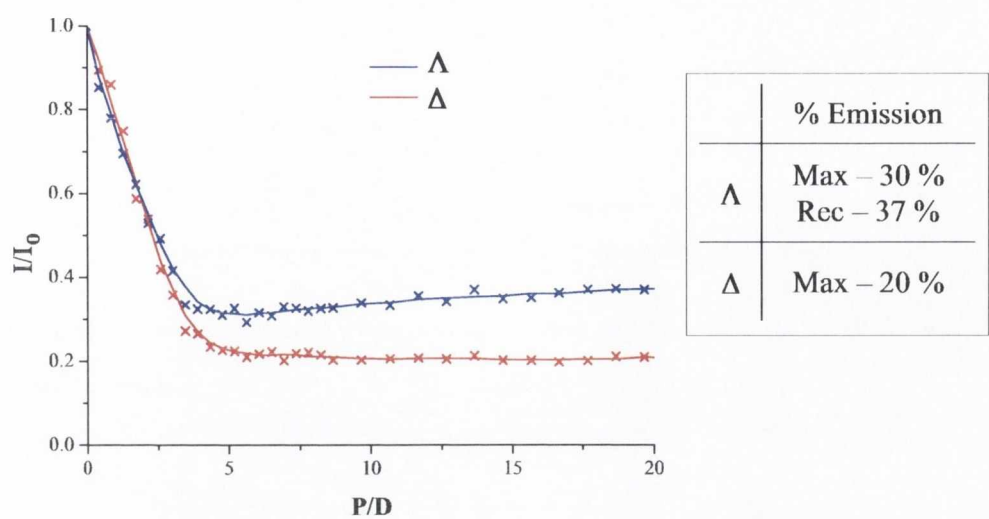


Figure 4.29 – Changes in the relative emission intensities vs increasing [stDNA] of the Λ- and Δ-[Ru(TAP)₂(F₂dppz)]Cl₂ complexes at 10 mM sodium phosphate buffer

The Λ enantiomer was quenched by 70% with the maximum quenching occurring at a P/D of 5, mirroring the P/D of the maximum hypochromicity recorded in the absorption spectra. The Δ complex was quenched by 80% with the maximum quenching also occurring at a P/D of 5. Thus the Δ enantiomer is quenched to a higher degree than the Λ enantiomer, complementing the results from the absorbance titrations that the Δ enantiomer binds more tightly to the DNA. Again, while the Δ complex, once a P/D of 5 is reached, remains at an almost constant quenched luminescence, the Λ complex recovers to a luminescence of 0.37 (a decrease from 70% to 63% quenching) at the final P/D of 20. This mirrors the luminescence results from the titrations of the original [Ru(TAP)₂dppz]Cl₂ enantiomers in which the Δ enantiomer experiences a higher degree of quenching and little recovery of luminescence while the Λ enantiomer is less quenched and does indeed recover at higher P/D ratios. The degree to which the enantiomers of the fluorinated complex are quenched is approximately equal to that of the original dppz complex and are compared in Table 4.20.

	Luminescence Quenching	
	Λ	Δ
$[Ru(TAP)_2dppz]Cl_2$	Max – 27% Rec – 33%	Max – 19%
$[Ru(TAP)_2(F_2dppz)]Cl_2$	Max – 30% Rec – 37%	Max – 20%

Table 4.20 – Comparison of the luminescence quenching upon the addition of stDNA to the enantiomers of $[Ru(TAP)_2dppz]^{2+}$ and $[Ru(TAP)_2(F_2dppz)]^{2+}$ in 10 mM sodium phosphate buffer

The data collected from the absorbance and fluorescence titrations implies that the Δ enantiomer binds more completely than the Λ enantiomer, demonstrated by the ability of the Λ enantiomer to relocate to its preferred A-T sites after the initial non-preferential binding. The similarity in the quenching experienced by both the dppz and F_2dppz sets of enantiomers indicates that the presence of the fluorine atoms does not hinder the solvated oxygen atoms from interacting with the phenazine nitrogens of the dppz ligand, thus the luminescence quenching is approximately the same level.

4.5.1.3 Binding Constant Calculations in Low Salt Conditions – 10 mM sodium phosphate buffer

The binding constants for the interaction of the Λ and Δ enantiomers with salmon sperm DNA at 10 mM sodium phosphate buffer were determined using the McGhee/von Hippel method, as previously, applied to the fluorescence data. The figures produced yielded a binding constant of $1.2 \times 10^7 \text{ M}^{-1}$ for the Λ enantiomer and $1.1 \times 10^8 \text{ M}^{-1}$ for the Δ enantiomer (Table 4.21). The accompanying plots are given in Appendix C.

Complex	Binding Constant $K_b \text{ (M}^{-1}\text{)}$	Binding Site Size n (base pairs)	R^2
Λ	$1.2 \times 10^7 \text{ (+ 0.2)}$	2.2 (+ 0.1)	0.99
Δ	$1.1 \times 10^8 \text{ (+ 0.1)}$	2.1 (+ 0.1)	0.97

Table 4.21 – *st*DNA binding constants calculated from the fluorescence data in 10 mM sodium phosphate buffer utilising the McGhee/von Hippel model

From the calculated binding constants, we can confirm that the Δ enantiomer does bind to DNA with a higher affinity than that of the Λ enantiomer. The values calculated above for the two enantiomers of $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ compare favourably to those reported previously for the Λ and Δ enantiomers of the $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ and $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complexes.² As noted with these previous complexes, the calculated values above are extraordinarily high binding constants, which suggests that perhaps these complexes bind to DNA too strongly to allow for the accurate calculation of their binding constants at physiological conditions. However, a comparison of these values to those calculated for $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ show the fluorinated enantiomers to have a very similar binding affinity to the non-substituted enantiomers.

The previously studied complexes of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ have shown that the conclusions drawn from the initial UV/visible and fluorescence spectroscopic data have been validated by further investigative methods, such as ‘reverse’ salt titration, thermal denaturation studies and synthetic homopolymer polynucleotides titrations. Due to time constraints, the same experiments were not conducted with the enantiomers of the $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$ complex, however interpretation of the UV/visible and fluorescence spectroscopic data and the calculated binding constants support the findings in Dr. Elias’ thesis that the fluorination of the dppz ligand at the 11 and 12 position has little effect on the DNA binding capability of the racemic complex, or indeed its two enantiomers.

4.6 Conclusions

The initial aspect of the work presented in this chapter is synthetic in nature. Firstly the synthesis and subsequent characterisation of the novel complex $\text{rac-Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})^{2+}$ is described. Secondly the development of a novel synthetic route involving microwave radiation to yield the $\text{rac-}[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ complex in a shorter reaction period with a higher yield than previously reported is significant. Finally, the separation of the two enantiomers of both the methylated and fluorinated complexes is the first reported enantiomeric separation of these complexes.

Binding studies conducted between the fluorinated enantiomers and stDNA yielded data very similar to that obtained for the same study with $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, leading to the conclusion that fluorination at the 11 and 12 position of the dppz ligand does not affect the DNA binding affinity of the enantiomers or any ensuing photophysics.

In depth binding studies of the methylated enantiomers with stDNA and the synthetic homopolymer polynucleotides $[\text{poly}(\text{dG-dC})]$ and $[\text{poly}(\text{dA-dT})]$ showed that while the major binding trends observed in Chapter 2 are maintained, notably the higher binding affinity of the Δ enantiomer compared to the Λ and the presence of two binding modes (non-specific intercalation and electrostatic groove binding), the presence of methyl groups at the 11 and 12 position of the dppz ligand increased the DNA binding affinity of both enantiomers. This effect has previously been reported with similar complexes.⁵ Interestingly, the presence of the methyl groups does not increase the thermal stability conferred by the enantiomers, but does increase stability to salt concentration effects. This results from the methyl groups acting as an anchor once the dppz ligand has intercalated, reducing the readiness with which the complex can disengage from the intercalation site.

A small decrease in luminescence quenching was noted upon DNA binding of the methylated enantiomers compared to that of their non-substituted counterparts. This is attributed to the bulkiness of the methyl groups reducing the extent to which the solvated oxygen molecules can access the phenazine nitrogen atoms of the dppz ligands whilst in the intercalation pocket. Solvated oxygen molecules can interact with these nitrogen

atoms from either the minor or major groove in the absence of methylation, causing a small increase in luminescence quenching. The methyl groups at the 11 and 12 positions reduce the access of the oxygen from the major groove, thus reducing the degree of quenching experienced by the complex, resulting in the marginal luminescence increase observed in the data.

We can conclude from this work that while the presence of fluorine groups at the 11 and 12 positions of the dppz ligand of $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ has little effect on the enantiomers' DNA binding ability or associated photophysics, methyl groups at these same positions result in an increased binding affinity of both enantiomers and enhanced luminescence.

Overall, the work presented in this chapter increases our understanding of the nature by which ruthenium polypyridyl complexes associate to DNA, and provides insights into the structural activity relationships of various substituents and ligands when considering complexes with high DNA affinities and photo-oxidative potentials.

4.7 References

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Chapter 5

*Increasing Molecular Complexity:
Bi-nuclear Ruthenium Complexes*

5.1 Introduction

Following the success in obtaining the two enantiomers for the various mono-nuclear ruthenium complexes, and the increasing literature reports pertaining to increased DNA binding potential¹⁻⁶ and enantiomeric separation⁷⁻⁹ of bi-nuclear complexes, it was decided to increase the molecular complexity from the compounds discussed in the previous chapters to a bi-nuclear ruthenium bis(TAP) derivative. Building on the desire to further study ruthenium bis(TAP) complexes it was decided to attempt the separation of the enantiomers of the bi-nuclear complex $[(\text{TAP})_2\text{Ru}-\text{TPAC}-\text{Ru}(\text{TAP})_2]^{4+}$ where TPAC = tetrapyrido[3,2-*a*:2',3'-*c*:3'',2''-*h*;2''',3'''-*j*]acridine (Figure 5.1).

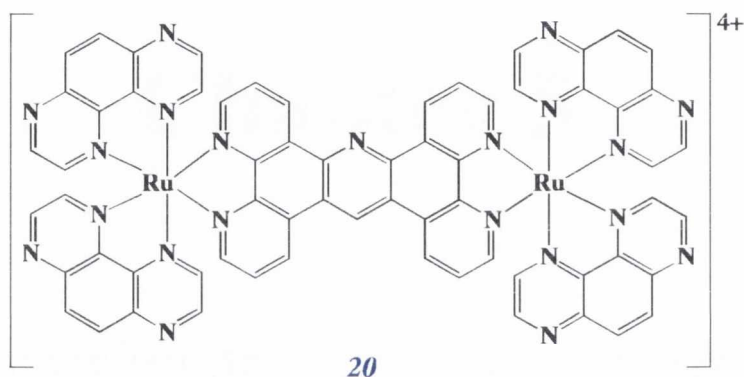
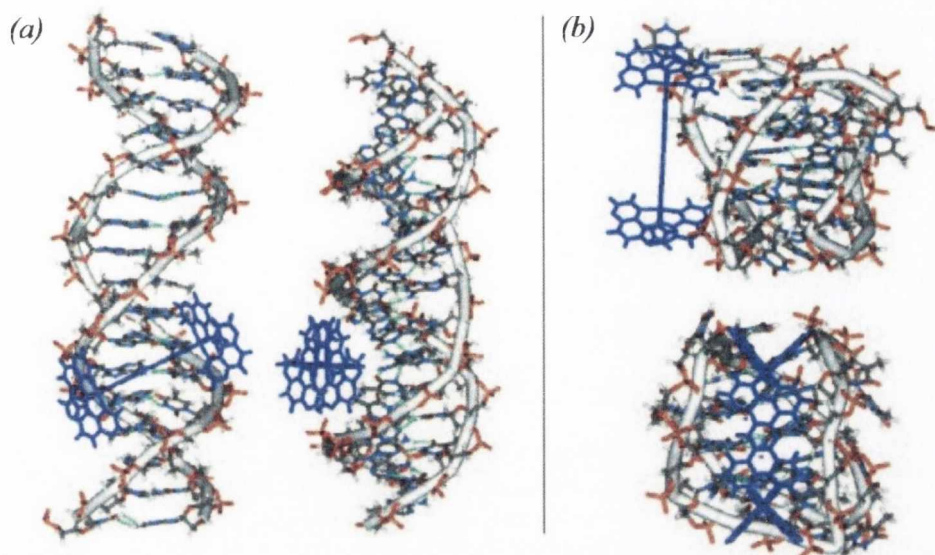


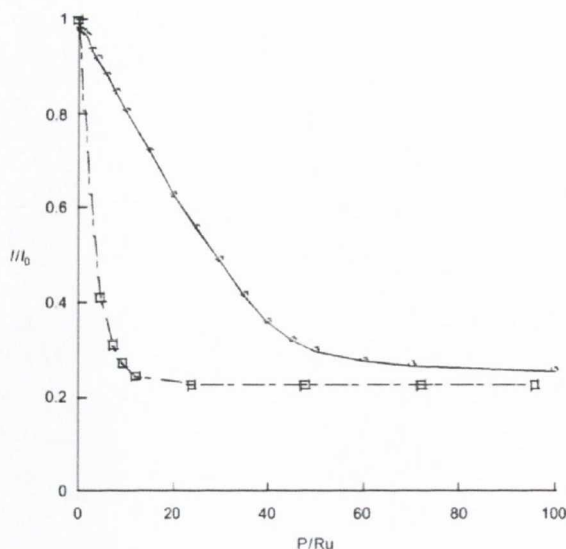
Figure 5.1 – Structural formula of $[(\text{TAP})_2\text{Ru}-\text{TPAC}-\text{Ru}(\text{TAP})_2]^{4+}$

This complex had been synthesised as an isomeric mix in the Kirsch-DeMesmaecker Group with the intention of increasing the photoreactive potential of DNA binding ruthenium complexes and as a possible inter-duplex cross-linking complex.¹⁰⁻¹² Subsequent studies with the isomeric complex and DNA showed that the complex did not form cross-linking interaction, nor did it intercalate into the duplex. Instead it was found to bind to the major groove of duplex DNA.¹² This same study also investigated the ability of the bi-nuclear complex to bind to quadruplex DNA and showed similar binding trends (Figure 5.2). This is highly topical as there is currently much research into possible sensitizers in photodynamic therapy, and a ruthenium complex selective for quadruplex DNA that can caused short-range photo-oxidative DNA damage would be greatly received by the medical community.



*Figure 5.2 – Molecular models of **20** binding to (a) the major grooves of B-DNA and (b) between the intramolecular grooves of human telomeric (quadruplex) DNA
Image taken from a recent *European Journal of Chemistry* publication¹²*

The complex was shown to have a higher affinity for the quadruplex DNA ($7.6 \times 10^6 \text{ M}^{-1}$, 10 mM sodium cacodylate buffer) compared to that for the B-DNA ($2.9 \times 10^6 \text{ M}^{-1}$, 10 mM sodium cacodylate buffer).¹² As the complex contains TAP ligands as the ancillary moieties, the luminescence is observed in an aqueous solution and quenched upon binding to DNA. The quenching profiles differ greatly depending on the nature of the DNA. In the presence of quadruplex DNA, the complex is quenched to its maximum at a much lower P/D ratio than with duplex DNA (calf thymus), suggesting the complex's increased affinity for the quadruplex structure and its potential photooxidative ability, Figure 5.3.



*Figure 5.3 – Changes in the relative emission intensities vs increasing ratios of P/D of **20** in the presence of CT-DNA (circles) and human telomeric DNA (squares)*
Image taken from a recent European Journal of Chemistry publication¹²

Indeed photoreaction studies were also undertaken and the photodamaging effect of the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex was found to be impressive, particularly in the presence of the human telomeric DNA sequence.¹² It was for this reason that obtaining the three optical isomers – $\Lambda\Lambda$, $\Lambda\Delta$ and $\Delta\Delta$ – would be very interesting. From the studies presented in Chapters 2 and 4, the Δ enantiomer of the mono-nuclear dppz complex was found to have a higher binding affinity for duplex DNA than its Λ counterpart, and due to its ability to intercalate more deeply between the base pairs, is assumed to have the greater photooxidative potential of the two enantiomers, as suggested by the nanosecond results. Investigation of the three optical isomers of the bi-nuclear complex could reveal similar trends, yielding one enantiomer as having the highest quadruplex affinity, which would greatly increase its attractiveness as a phototherapeutic complex.

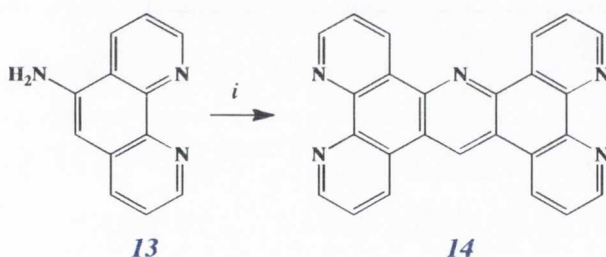
The work presented in these remaining pages discusses the attempts made at obtaining the enantiomers of the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex. This could not have been undertaken without the synthetic collaboration with the Kirsch-DeMesmaeker Group in Brussels. Under the direction of Prof. Andrée Kirsch-DeMesmaeker and with guidance from Dr. Kathleen Servaty the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex was synthesised in the Brussels laboratory. Later, a novel synthesis utilising microwave radiation was developed here in our laboratory at Trinity College Dublin.

5.2 Synthesis

The $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]\text{Cl}_4$ complex was prepared from the reaction of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ with the TPAC ligand utilizing both the reported literature method¹⁰, conducted during collaboration with Prof. Andr  e Kirsch-DeMesmaecker and her research group at the Universit   Libre de Bruxelles, Belgium, and a novel procedure involving microwave technology. Full experimental details are provided in Chapter 7.

5.2.1 Synthesis of the TPAC ligand

In the work, the TPAC ligand was not synthesized by the reported literature method,¹³ but according to a new synthetic route developed within the Kirsch-DeMesmaecker Group that has yet to be reported. This new synthesis reduces the main reaction time from 8 days to one hour. The 5-NH₂-phen **13** was synthesised as described in the literature.¹⁴



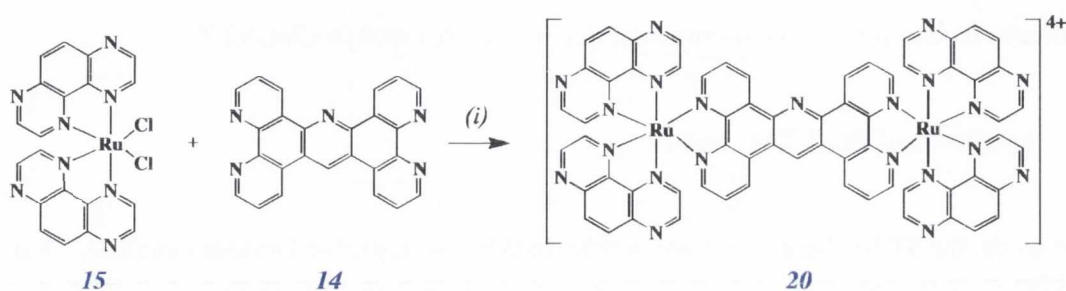
Scheme 5.1 – Synthesis of the TPAC ligand

(i) PFA, HCl, MW 100°C, 1 hour

5-NH₂-phen **13** (2 equivalents) and paraformaldehyde (PFA) (1 equivalent) were added to concentrated hydrochloric acid (6 M, 5 ml) and subjected to microwave radiation for an hour at a temperature of 100°C. A change in the colour of the solution from red to bright orange indicated the success of the reaction. Neutralisation of the solution via the drop-wise addition of sodium hydroxide (2 N) to a pH of 7, indicated by a pH probe and a colour change in the solution of orange to yellow. The desired ligand was collected by filtration and dried by dessication. TPAC **14** was obtained as a brown solid at a 78% yield. Characterisations by ¹H NMR and HRMS agree with literature values.¹³

5.2.2 Synthesis of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]\text{Cl}_4$

Synthesis of the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex involved the complexation of a TPAC ligand **14** with the $\text{Ru}(\text{TAP})_2\text{Cl}_2$ precursor **15** in a degassed solution of ethylene glycol.



Scheme 5.2 - Synthetic pathway of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$

(i) Ethylene glycol, MW, 140°C, 40 mins

Two synthetic methods were employed in obtaining the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]\text{Cl}_4$ complex. The first, utilising the more conventional method of heating, reflux, is reported in the literature and was the method followed while working in the laboratory in Brussels.¹⁰ This synthesis involves a reaction time of 30 hours and a yield of 49%. The second synthesis was developed in the Trinity laboratory following the successful use of microwave radiation in the synthesis of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ and its substituted analogues.

$\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (2.5 equivalents) and TPAC **14** (1 equivalent) were added to ethylene glycol (2 ml) and subjected to microwave radiation for forty minutes at a temperature of 140°C. A colour change of the reaction solution from purple to dark brown indicated the success of the reaction. Addition of an aqueous solution of KPF_6 was added to the cooled reaction solution and the subsequent red/brown precipitate was collected and washed via centrifugation. The product was converted to its chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl^- form) and the desired product **20** was obtained following purification on an aqueous CM Sephadex C-25 column with 0.2 M NaCl as the mobile phase. A red/brown solid was recovered in 28% yield. While the reaction time was drastically shortened, the yield was reduced by almost 50%. Investigation of the side products provided an answer for this low yield, and is explained in section 5.4. The

complex was characterised by ^1H NMR and MALDI TOF spectroscopy, both of which agree with the literature values.¹⁰ Figure 5.4 shows the ^1H NMR spectrum of the $[(\text{TAP})_2\text{Ru}\text{--TPAC}\text{--Ru}(\text{TAP})_2]^{4+}$ complex with Table 5.1 identifying the various peaks.

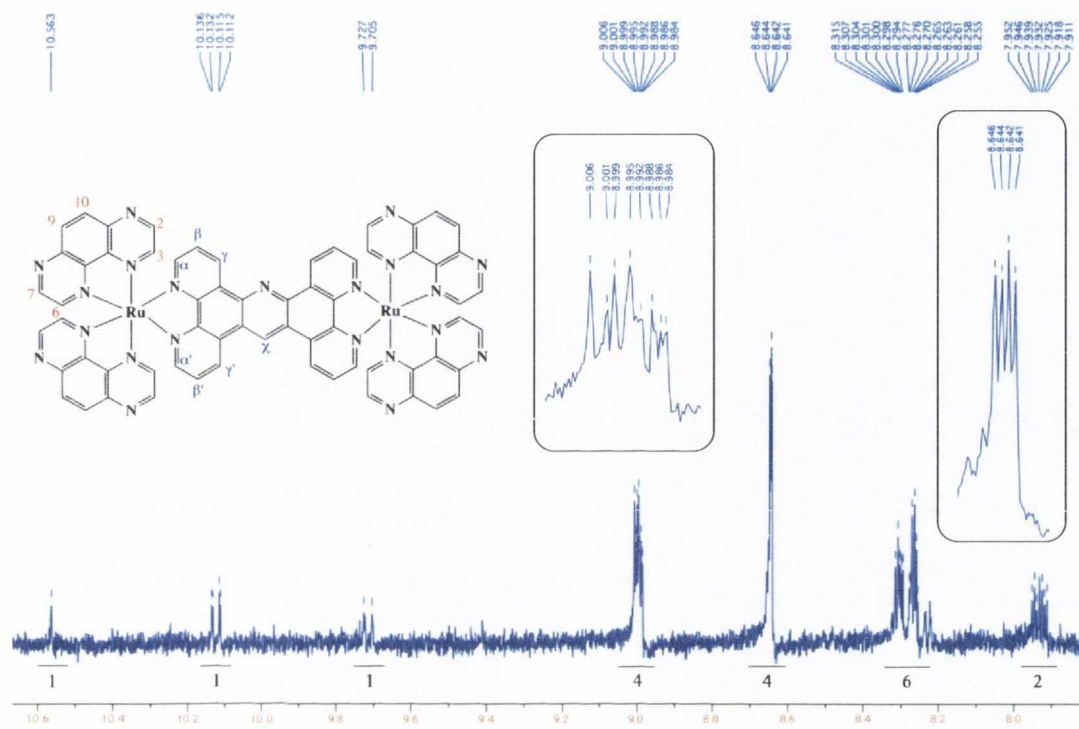


Figure 5.4 – ^1H NMR spectrum of $[(\text{TAP})_2\text{Ru}\text{--TPAC}\text{--Ru}(\text{TAP})_2]^{4+}$ in CD_3CN

Proton	Identity	$\delta(\text{ppm})$	Mult.	J_{apparent} (Hz)	Int. rel.
TAP	2 and 7	9.09	m	-	4
	9 and 10	8.64	m	-	4
	3 and 6	8.28	m	-	6*
TPAC	χ	10.56	s	-	1
	γ	10.11	dd	$J = 9.6$	1
	γ'	9.70	d	$J = 8.8$	1
	α and α'	8.28	m	-	6*
	β and β'	7.93	m	-	2

Table 5.1 - Assignment of peaks in the ^1H NMR spectrum of $[(\text{TAP})_2\text{Ru}\text{--TPAC}\text{--Ru}(\text{TAP})_2]^{4+}$ in CD_3CN

* same signal

The complex was further characterised by UV/vis absorption and fluorescence spectroscopy, shown below in Figure 5.5.

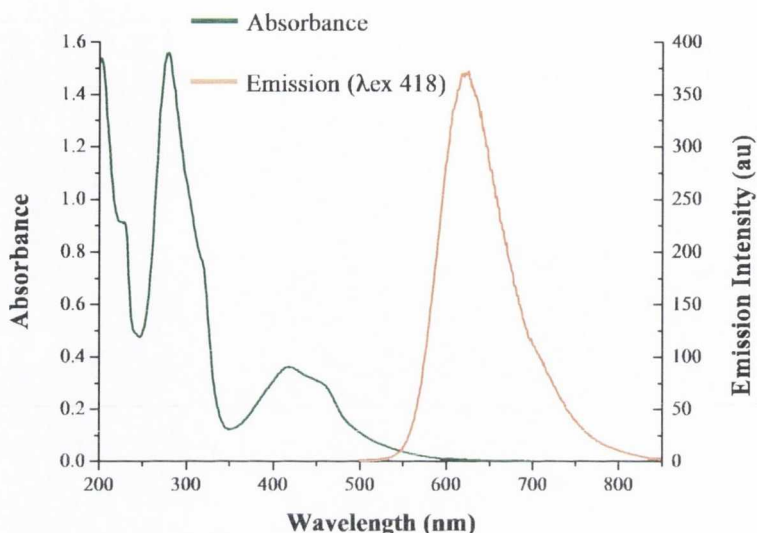


Figure 5.5 – UV/visible absorbance (green) and emission (orange) spectra of $[(TAP)_2Ru-TPAC-Ru(TAP)_2]^{4+}$ in H_2O

Five different absorption maxima are found in the UV/vis absorption spectrum. The strong bands at 230 nm and 279 nm are characteristic of the $\pi-\pi^*$ intra-ligand transitions of the ancillary TAP ligands while the very weak band at 317 nm is attributed to the $\pi-\pi^*$ transitions of the TPAC ligand. The final bands at 418 nm and 460 nm result from the MLCT $Ru(II) \rightarrow TPAC$ and $Ru(II) \rightarrow TAP$ transitions respectively.

The lifetime complex was recorded previously as 739 ns in an oxygenated environment and 919 ns in a de-oxygenated environment of argon.¹⁵ Similarly the associated quantum yields were observed as 2.5×10^{-2} and 3.1×10^{-2} in oxygenated and argon environments respectively.¹⁵

As no isosbestic points for the interaction of this complex with DNA have been reported, the excitation wavelength was chosen as the MLCT maxima of 418 nm. This yielded the spectrum shown in Figure 5.5 with a maximum occurring at 426 nm.

5.3 Attempts at Separation of the Enantiomers

There are a number of reports detailing the separation of bi-nuclear polypyridyl ruthenium and osmium diastereoisomers, ie. *meso* complex ($\Lambda\Lambda$) from the racemic complexes ($\Lambda\Lambda$ and $\Delta\Delta$), utilising the Sephadex column methods employed for the separation enantiomers of the mono-nuclear ruthenium complexes (Figure 5.6).^{16,17} The difference between separating the diastereoisomers and separating enantiomers is the nature of the anionic salt in the mobile phase. For diastereoisomeric separation, an aqueous solution of sodium toluene-*p*-sulfonate (0.25 M) had been previously employed and successfully resolved the *meso* complex from the racemic with a number of related bi-nuclear ruthenium complexes.^{16,17}

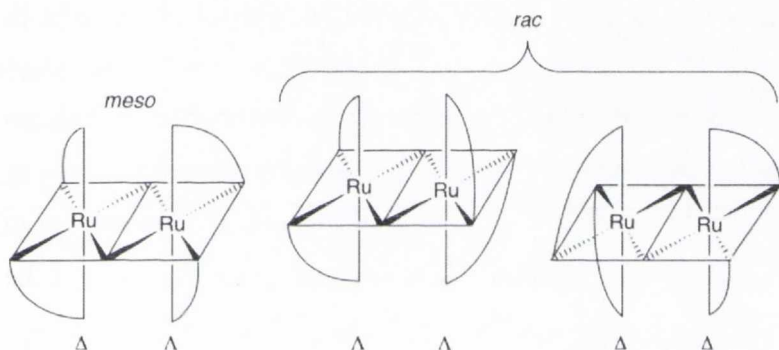


Figure 5.6 – Schematic view of the isomeric possibilities of the general dinuclear species.

Image taken from a publication in Dalton Transactions¹⁷

The sodium toluene-*p*-sulfonate is expected to have a differential association with the alternative relative orientations of the heterocyclic ancillary ligands in the two forms (*meso* or *rac*) resulting in a separation similar to that reported with the enantiomeric separation of the mono-nuclear complexes. Sodium toluene-*p*-sulfonate has only been shown to separate the diastereoisomers. Use of another anion, such as sodium (–)-dibenzoyl-L-tartrate, would facilitate the enantiomeric separation on a separate column.

5.3.1 Attempted Separation of the diastereoisomers - meso from $\Lambda\Lambda/\Delta\Delta$

Separation of the two diastereoisomers of the $[(TAP)_2Ru-TPAC-Ru(TAP)_2]Cl_4$ complex was attempted. The aqueous solution of the chloride salt was adsorbed onto the aqueous CM Sephadex stationary phase in a 1 m perspex column (diameter 2 cm) and the cationic mobile phase of the sodium toluene-*p*-sulfonate (0.25 M) was applied. This resulted in a slight shrinking of the Sephadex due to the toluene-*p*-sulfonate cations reducing the intramolecular repulsion of the sulfonate groups of the sephadex. Upon saturation of the column with the mobile phase, the $[(TAP)_2Ru-TPAC-Ru(TAP)_2]Cl_4$ complex was passed through the column a number of times to facilitate separation.

It was expected that the bi-nuclear complex would not separate as quickly as its mono-nuclear cousin, however, after an excess of 25 passes through the column (equivalent to a week), no separation had occurred. The bright orange band of the ruthenium complex, which was expected to separate into two smaller orange bands simply moved as one band, but became exceptionally broad. It was eluted from the column in a number of fractions, whose circular dichroism was recorded. There was no signal from any of the fractions. CH COSY analysis was used to confirm whether the first and last fractions contained different stereoisomers as every other technique available would have shown both diastereoisomers as the same complex. Unfortunately the resulting spectra did not yield any significant differences.

Precautions were taken in ensuring overloading of the column (whose limits are approx. 65 mg of compound) did not occur, thus the separation process was attempted again at a lower loading concentration, unfortunately with the same results. Also varying the concentration of the eluent was also attempted (0.1 M, 0.25M and 0.5 M) with no successful outcomes, leading to the conclusion that the application of sodium toluene-*p*-sulfonate does not separate the diastereoisomers of $[(TAP)_2Ru-TPAC-Ru(TAP)_2]Cl_4$.

5.3.2 Attempted Separation of the $\Lambda\Lambda$, meso and $\Delta\Delta$ optical isomers

Following the failure of the sodium toluene-*p*-sulfonate, it was decided to attempt a separation of the three optical isomers utilising the sodium (-)-dibenzoyl-L-tartrate (0.1 M) which was used previously to separate the Λ and Δ enantiomers of the $[\text{Ru}(\text{TAP})_2(\text{X}_2\text{dppz})]^{2+}$ complexes. The expectation was that three bands would form on the column, with the leading band being the $\Lambda\Lambda$ optical isomers followed by the $\Lambda\Delta$ and finally the $\Delta\Delta$ optical isomers. As before, after an excess of 25 passes through the column, no separation had occurred. The orange band of the complex became extensively broadened without any single concentrated areas that might indicate the presence of a separated enantiomer. Again the band was eluted from the column in a number of fractions, whose circular dichroism was recorded. The CD spectra of the three optical isomers should be different, but the spectra of all the collected fractions were identical, each showing no signal, indicating that again an attempt at the separation of the optical isomers was unsuccessful. Again varying the eluent concentration was attempted (0.25 M and 0.5 M) without success.

The reports in the literature describing the successful separation of diastereoisomers and enantiomers of bi-nuclear complexes are of compounds containing relatively small bridging ligands – 2,2'-bipyrimidine (bipym),¹⁸ 2,3-bis(2-pyridyl)pyrazine (bpypz)¹⁶ and 1,4,5,8,9,12-hexaazatriphenylene (HAT).¹⁹ Compared to the TPAC bridging ligand of this study, these smaller ligands are, at most, only two thirds the size (Figure 5.7).

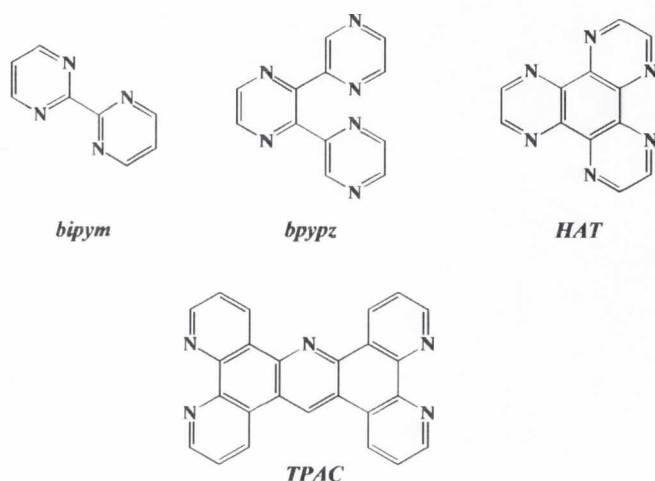
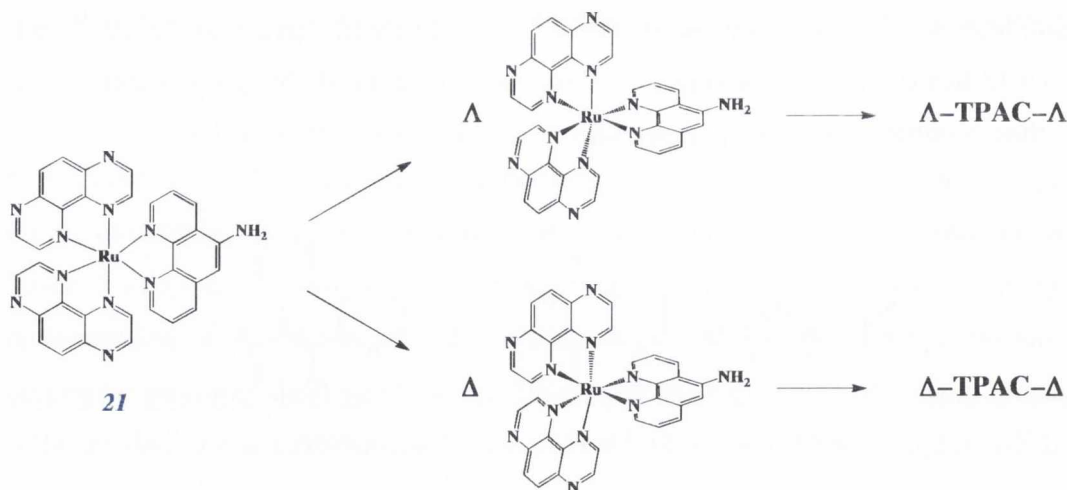


Figure 5.7 – Structural formulae of various bridging ligands

The result of a larger bridging ligand is that the intramolecular distance between the two ruthenium centres, and thus the two sets of ancillary ligands, is larger. This increased distance may prevent the association of the toluene-*p*-sulfonate cations required to facilitate the diastereomeric separation. The explanation for why the tartaric acid did not separate the enantiomers remains elusive.

5.3.3 Stereo-specific synthesis

After the frustration of the unsuccessful attempts of separating the three enantiomers of the bi-nuclear TPAC complex, a possible synthetic route was devised. Unlike the synthesis of the bi-nuclear derivative, the mono-nuclear $[\text{Ru}(\text{TAP})_2\text{TPAC}]^{2+}$ complex cannot be synthesised through the simple complexation of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ with the TPAC ligand. Its synthesis is achieved through initial formation of $[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ **21** precursor followed by reaction with paraformaldehyde and a second 5-NH₂-phen ligand. If the precursor could be separated into its Λ and Δ enantiomers, perhaps the reaction of a $\Lambda\text{-}[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ with a second a $\Lambda\text{-}[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ would yield the $\Lambda\Lambda\text{-}[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ and similarly with the Δ , as depicted in Scheme 5.3.



*Scheme 5.3 – Proposed stereospecific synthesis of $\Lambda\Lambda$ - and $\Delta\Delta$ -
 $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$*

The $[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ complex was synthesised following literature methods (yield 52%) and purified on a CM Sephadex column with a 0.2 M NaCl eluent.¹⁰ The successful synthesis of the complex was confirmed by MALDI spectroscopy and an aqueous solution of the complex was applied to the Sephadex column. Considering the relative simplicity of the complex, and the desire for its separate enantiomers, eluent used was an aqueous solution of sodium (–)-dibenzoyl-L-tartrate (0.25 M). The complex was allowed to pass through the column ten times before it was determined that the enantiomers of this complex did not separate. As with the bi-nuclear complex, the orange band moved through the column and became broader but did not separate into two distinct bands, which would indicate the presence of the two enantiomers. As previously, the band was eluted from the column in a number of fractions, whose circular dichroism was recorded and shown to have no significant signal. The separation of the $[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ precursor did not occur on the column, preventing the possible stereo-specific synthesis of the $\Lambda\Lambda$ and $\Delta\Delta$ optical isomers of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$.

5.3.4 Binding Affinity-based DNA-Assisted Stereoisomer Separation (BADASS)

Following the unsuccessful attempts at the separation of the diastereoisomers and enantiomers of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ using a CM Sephadex column, a second, less conventional method was attempted. Affinity chromatography – involving biologically specific interactions as a means of separation – has long been used in the purification and isolation of biomolecules.^{20,21} Common applications of the technique include the purification of antibodies,²² glycoproteins,²³ enzymes²⁴ and hormones.²⁵ Often immobilised nucleic acids are employed in the role of the affinity ligand with the isolation of the target molecule being achieved via their specific or non-specific interactions. More recently this technique has been applied to the chromatographic resolution of enantiomeric mixtures of metal complexes.^{26,27} The enantiomers of complexes such as $[\text{Ru}(\text{phen})_3]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{ppz})]^{2+}$ were separated by means of elution through a DNA-hydroxylapatite column²⁸ and, later, the resolution of dinuclear complexes such as $[(\text{Me}_2\text{bpy})_2\text{Ru-bpm-Ru}(\text{Me}_2\text{bpy})_2]^{4+}$ was achieved.²⁹ As this method has proven successful with a number of polypyridyl ruthenium complexes, its use in the separation of the optical isomers of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ was attempted.

5.3.4.1 Chromatographic Set-up and Results

The ability of immobilised streptavidin (a 60,000 dalton protein purified from bacteria) to strongly bind biotinylated substances has been exploited in numerous affinity chromatography applications.²⁹⁻³² Accordingly, the utilization of this interaction in the immobilisation of selected non-duplex oligonucleotides to a stationary phase for use in the DNA-affinity chromatography of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ was chosen. HiTrap Streptavidin HP columns (1 ml), purchased from Amersham Biosciences, contain a medium consisting of streptavidin immobilised on Sepharose. Equilibration of the column with the buffer solution (10 mM sodium phosphate/0.075 M sodium chloride/pH 7.5) and subsequent loading of the biotinylated oligonucleotide (biotin-ATATATATATAT 5' → 3') was conducted. Once the DNA was immobilised and washed, the column was tested with an aqueous solution of $[\text{Ru}(\text{phen})_3]^{2+}$ to ensure its resolution capability. Using a buffer of 5 mM sodium phosphate/0.0375 M sodium chloride/pH 7.5, two bands were observed to pass down the column. The first was a diffuse, fast moving band, identified as the Δ - $[\text{Ru}(\text{phen})_3]^{2+}$ enantiomer. The second,

more coherent band eluted approximately five hours after the first, utilising a more concentrated buffer solution (20 mM sodium phosphate/0.15 M sodium chloride/pH 7.5). This was identified as the Λ -[Ru(phen)₃]²⁺ enantiomer by circular dichroism (Figure 5.8) and MALDI TOF spectroscopy.

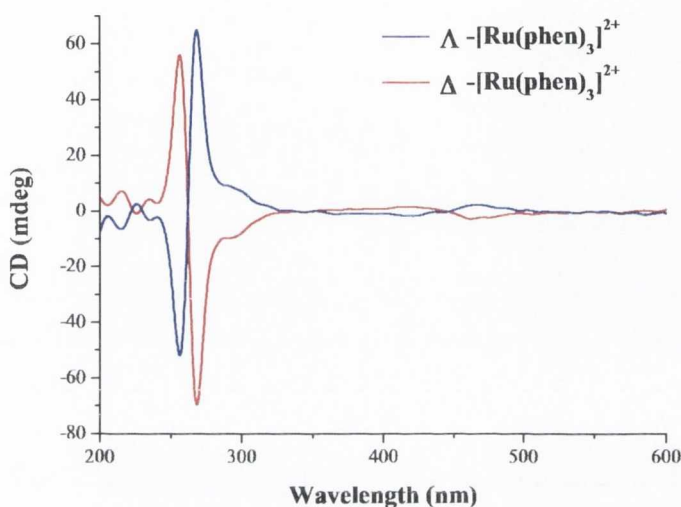


Figure 5.8 – CD spectrum of the Λ and Δ enantiomers of [Ru(phen)₃]²⁺ in CH₃CN

Confident of the resolution capability of the biotin column approximately 50 nmol of the [(TAP)₂Ru-TPAC-Ru(TAP)₂]⁴⁺ complex was loaded. Initial elution was attempted with the same buffer solution of 10 mM sodium phosphate/0.075 M sodium chloride/pH 7.5 after purification through a 0.45 μ m filter. When no movement of the complex band (deep brown) was observed after a period of 48 hours, the buffer was increased to twice its concentration (20 mM sodium phosphate/0.15 M sodium chloride/pH 7.5). This did not result in any movement of the complex, prompting a further doubling of the buffer concentration. When this proved unsuccessful, a buffer containing 10 mM sodium phosphate/0.075 M sodium chloride/pH 7.5 and 2% methanol was attempted. The methanol concentration was subsequently increased to 5%, 10% and finally 20% in an attempt to elute the complex, but no movement occurred beyond the initial adsorption of the bi-nuclear complex to the column. Each buffer was allowed to pass through the column for 48 hours at a rate of 1 ml min⁻¹. The details are recorded in Table 5.2.

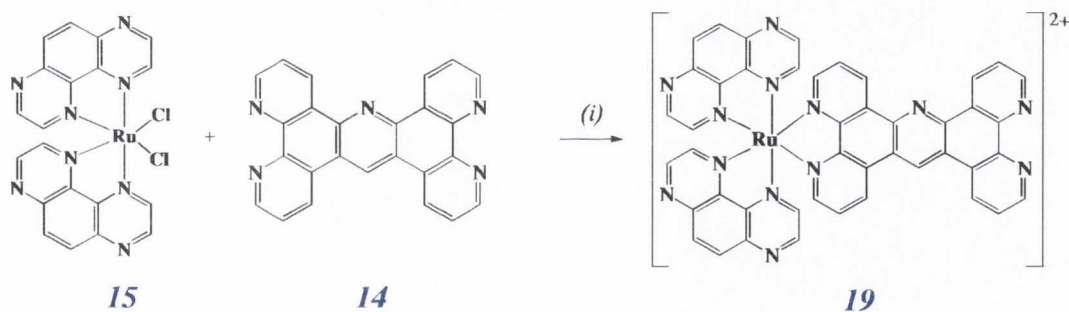
Buffer Concentration	pH	% Methanol	
10 mM sodium phosphate 0.075 M sodium chloride	7.5	0	X
20 mM sodium phosphate 0.15 M sodium chloride	7.5	0	X
40 mM sodium phosphate 0.3 M sodium chloride	7.5	0	X
10 mM sodium phosphate 0.075 M sodium chloride	7.5	2	X
10 mM sodium phosphate 0.075 M sodium chloride	7.5	5	X
10 mM sodium phosphate 0.075 M sodium chloride	7.5	10	X
10 mM sodium phosphate 0.075 M sodium chloride	7.5	20	X

*Table 5.2 – Buffer concentrations used in the attempted
BADASS chromatography of $[(TAP)_2Ru-TPAC-Ru(TAP)_2]^{4+}$*

While unable to satisfactorily explain the lack of success in the resolution of the $[(TAP)_2Ru-TPAC-Ru(TAP)_2]^{4+}$ complex using the BADASS technique, suggestions have been made that perhaps the sheer size of the complex prevents its passage through the immobilised DNA or that the increased number of nitrogen atoms compared to previously separated complexes may prevent movement due to the increased number of hydrogen bonds possible between the complex and the oligonucleotide. However these are only speculation. The conclusion drawn from the many attempts made at the resolution of the *meso*, $\Lambda\Lambda$ and $\Delta\Delta$ enantiomers of the $[(TAP)_2Ru-TPAC-Ru(TAP)_2]^{4+}$ complex is that such a process is not possible under the conditions thus far explored.

5.4 Synthesis of $[(\text{TAP})_2\text{Ru}-\text{TPAC}]^{2+}$

An interesting development to emerge from the synthesis and subsequent purification of the bi-nuclear $[(\text{TAP})_2\text{Ru}-\text{TPAC}-\text{Ru}(\text{TAP})_2]^{4+}$ complex was the significantly reduced yield when microwave radiation was employed in the reaction - 49%¹⁰ vs 28%. Purification on a CM Sephadex column (0.2 M NaCl eluent) first yielded a purple band, corresponding to the unreacted $\text{Ru}(\text{TAP})_2\text{Cl}_2$ complex, followed by two distinct orange bands. The latter of these bands was identified as the bi-nuclear complex and utilised in the aforementioned resolution attempts. The former band, found to have an identical absorption spectrum as the $[(\text{TAP})_2\text{Ru}-\text{TPAC}-\text{Ru}(\text{TAP})_2]^{4+}$ complex, was shown to be the mono-nuclear derivative $[(\text{TAP})_2\text{Ru}-\text{TPAC}]^{2+}$. This was unexpected as the reported synthesis of this mono-nuclear complex states that the conventional method of complexation of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ with the TPAC ligand does not yield the desired compound.¹⁰ The reported synthesis of $[(\text{TAP})_2\text{Ru}-\text{TPAC}]^{2+}$ first involves the synthesis of the $[\text{Ru}(\text{TAP})_2(5\text{-NH}_2\text{-phen})]^{2+}$ **21** with subsequent reaction with a further equivalent of 5-NH₂-phen.¹⁰ As a result, during the conventional method reported for the synthesis of the bi-nuclear complex, none of the mono-nuclear derivative was formed. However, discovery of this complex during the microwave synthesis of the bi-nuclear complex explains the comparatively low yield of 28%.



Scheme 5.4 - Synthetic pathway of $[(\text{TAP})_2\text{Ru}-\text{TPAC}]^{2+}$

(i) Ethylene glycol, MW, 140°C, 40 mins

Following this discovery, the mono-nuclear $[(\text{TAP})_2\text{Ru}-\text{TPAC}]^{2+}$ complex was thus synthesised following the procedure outlined for the bi-nuclear complex, reacting only one equivalent of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ with 1.5 equivalents of TPAC. The result was the

collection of $[(\text{TAP})_2\text{Ru-TPAC}]^{2+}$ **19** in 52% yield. The complex was characterised by ^1H NMR and MALDI TOF spectroscopy, both of which agree with the literature values.¹⁰ The ^1H NMR spectrum for the $[(\text{TAP})_2\text{Ru-TPAC}]^{2+}$ complex is given in Figure 5.9 below, with the assignment of the chemical shifts detailed in Table 5.3.

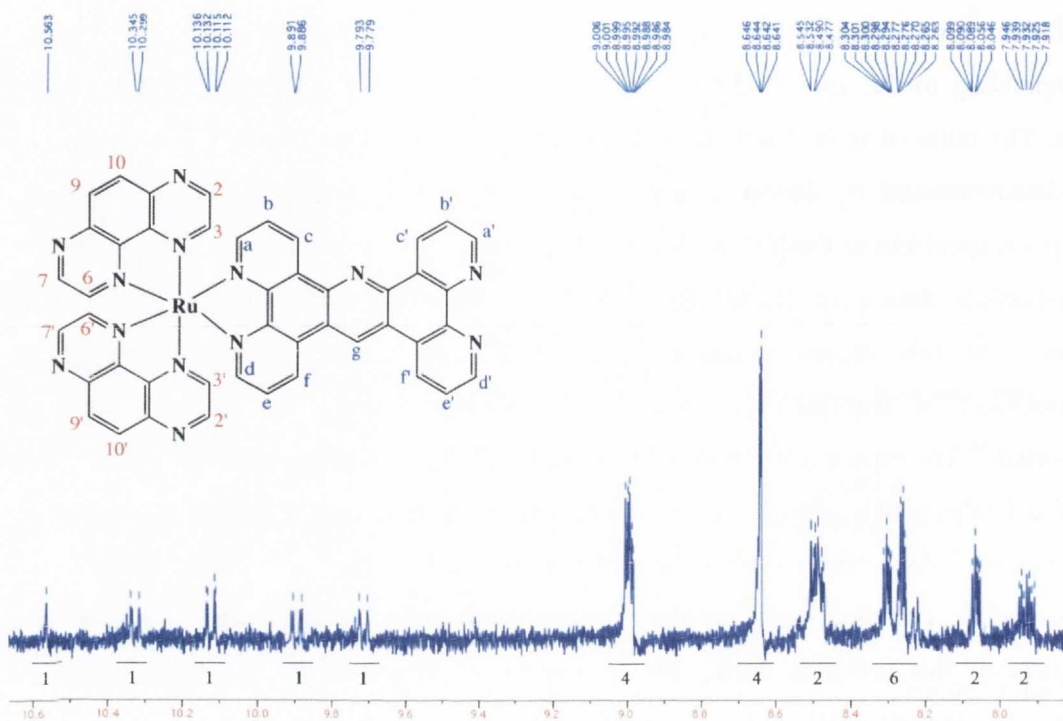


Figure 5.9 – ^1H NMR spectrum of $[(\text{TAP})_2\text{Ru-TPAC}]^{2+}$ in CD_3CN

Proton	Identity	$\delta(\text{ppm})$	Mult.	$J_{\text{apparent}}(\text{Hz})$	Int. rel.
TAP	2 and 7	9.00	m	-	4
	9 and 10	8.64	m	-	4
	3 and 6	8.28	m	-	6*
TPAC	g	10.56	s	-	1
	c, f, c' or f'	10.32	d	$J = 7.7$	1
	c, f, c' or f'	10.12	d	$J = 8.5$	1
	c, f, c' or f'	9.89	d	$J = 7.4$	1
	c, f, c' or f'	9.79	d	$J = 8.1$	1
	a, d or a', d'	8.52	m	-	2
	a, d or a', d'	8.28	m	-	6*
	b, e or b', e'	8.09	m	-	2
	b, e or b', e'	7.93	m	-	2

Table 5.3 - Assignment of peaks in the ^1H NMR spectrum of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ in CD_3CN
* same signal

5.5 Conclusions

As previously explained, the desire for increased specificity coupled with increased photo-oxidative potential of ruthenium complexes in photodynamic therapies is of great relevance to many industries. To this end, bi-nuclear metallo-complexes are the subject of intense research, often with the aim of targeting quadruplex DNA. The bi-nuclear $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex has previously demonstrated significant potential as a photo-oxidative agent with regard to DNA and is shown to have a preference for quadruplex DNA over duplex DNA. Considering the results from the mono-nuclear complexes in the previous chapters, it was anticipated that the enantiomers of the bi-nuclear complex would demonstrate differing degrees of affinity for DNA, perhaps with one having an affinity higher than the multi-isomeric complex.¹² To this end, many attempts were made to separate the two diastereoisomers of $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$, followed by attempts at separating the three enantiomers, and finally an attempted stereo-specific synthesis of two of the enantiomers, each without success.

However, a novel method for the synthesis of the $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ complex, reducing the reaction time from 30 hours to 40 minutes was developed and reported in this work. Furthermore, a novel reaction for the mono-nuclear derivative $[(\text{TAP})_2\text{Ru-TPAC}]^{2+}$ was devised, reducing the reaction time for this complex from eight days to 40 minutes.

To complete this study, a method of obtaining the three enantiomers of the bi-nuclear complex $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$ is required, perhaps by the use of a different anionic salt to facilitate separation on the CM Sephadex column, synthesis of an enantiomeric precursor, or by some other method not investigated in this work. It is my belief that startling differences will be found between the binding potential of each of the enantiomers, making the most effective binder of the three enantiomers a very attractive agent in the field of medicine and particularly photodynamic therapy.

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Chapter 6

Conclusions and Future Work

6.1 Conclusions

The overall aim of this thesis was to synthesise and resolve the enantiomers of various ruthenium polypyridyl complexes, incorporating the TAP (1,4,5,8-tetraazaphenanthrene) ligand, and investigate the binding potentials of each separate enantiomer with various DNA sequences. The work herein described encompasses two exciting areas of chemistry that are constantly evolving. The first of these involved the use of various spectroscopic methods to study the binding affinity and modes of enantiomeric ruthenium polypyridyl complexes with duplex DNA (Chapters 2 and 4). These studies culminated in the use of ultrafast transient absorption and time resolved infrared techniques to elucidate the differences the enantiomers have upon sub-second energy transfer processes of DNA. The second area of chemistry utilised during this project is crystallography (Chapter 3). Often referred to as an 'art form', crystallography is renowned for its frustrations in attempting to obtain crystals, but superb images and insights once the allusive crystals have been grown.

6.1.1 Binding Studies of $[\text{Ru}(\text{TAP})_2(\text{X}_2\text{dppz})]^{2+}$

Chapters 2 and 4 detail the synthesis of three ruthenium polypyridyl analogues: $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$, $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ (a novel compound) and $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$, and resolution into their component enantiomers: Λ and Δ . A thorough investigation of these enantiomers with various sequences of DNA was conducted and revealed that all six enantiomers displayed the 'light-switch off' phenomenon in the presence of mixed sequence DNA and $[\text{poly}(\text{dG}-\text{dC})]$ but the 'light-switch on' effect in the presence of $[\text{poly}(\text{dA}-\text{dT})]$.

The binding studies determined that for each of the three complexes, the binding preference for both enantiomers was $[\text{poly}(\text{dA}-\text{dT})] > \text{stDNA} > [\text{poly}(\text{dG}-\text{dC})]$. All three Δ enantiomers displayed an increased binding affinity for G-C containing DNA sequences compared to that of the Λ , with both enantiomers having an equal binding affinity for $[\text{poly}(\text{dA}-\text{dT})]$. The binding modes for all six enantiomers also remained constant, with non-specific intercalation dominating at lower P/D ratios and cooperative electrostatic binding to the DNA grooves at higher P/D ratios. While the fluorination of

the dppz complex at positions 11 and 12 had very little effect upon the DNA binding affinity of either enantiomer, methylation at this position significantly increased the binding affinity of both enantiomers. This is due to the methyl groups acting as ‘anchors’ once the complex has bound to DNA, increasing the strength of the binding interaction.

Following the work conducted previously with the *rac*-[Ru(TAP)₂dppz]²⁺ complex, ultrafast investigations of the two enantiomeric complexes Λ -[Ru(TAP)₂dppz]²⁺ and Δ -[Ru(TAP)₂dppz]²⁺ in the presence of [poly(dG–dC)] were performed in an effort to determine if the steric differences between the enantiomers would have an effect on the rate at which the proton-coupled electron transfer (PCET) and back electron transfer (BET) processes occur. This project was conducted in collaboration with Prof. George in the University of Nottingham and the Rutherford Appleton Laboratory, UK. While accurate information regarding the PCET process was allusive due to it occurring on a sub-nanosecond timescale, much data regarding the slower BET process was collected. Determination of the lifetimes of the intermediate excited species and rate at which the BET reaction occurs in D₂O showed that for the Δ enantiomer, the process occurred at a rate twice that of the Λ enantiomer. This difference is attributed to varying distances between the ruthenium centre and excited state guanine residue, with the Δ enantiomer assumed to have a significantly shorter distance than the Λ enantiomer, due to the configuration of its ancillary ligands allowing for a deeper penetration of the dppz intercalating moiety. This enantiomeric effect did not occur in the presence of water, suggesting that the nature of the exchangeable proton/deuterium ion is important in discerning such behaviour.

6.1.2 Crystallography

Chapter 3 discusses the crystallographic work conducted in collaboration with the University of Reading and the Diamond Light Source facility. The results presented in this chapter are the first example of a crystallographic study of a ruthenium polypyridyl complex binding to an oligonucleotide. There has been much debate as to whether such complexes bind to B-DNA in either the major or the minor grooves, with evidence presented in support of both arguments. However, without a crystallographic study, such a debate is not easily reconciled. As such this work shows unequivocally that the Λ -

$[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ complex binds to DNA in the minor groove in addition to confirming the main mode through which the complex binds as intercalation of the elongated aromatic dppz ligand. Perhaps the most interesting revelation to emerge from this study, however, is the observation of the semi-intercalative mode of binding. This binding mode has long been suspected as the method through which complexes with small aromatic ligands, such as $[\text{Ru}(\text{phen})_3]^{2+}$ associate to DNA but has not before been observed crystallographically.

There are a number of biologically significant aspects to arise from this study. Firstly the association of the complex to the oligonucleotide causes a flipping out of the terminal adenine. This nucleobase is then free to interact with a second duplex and forms a cross-linking interaction through reverse Watson-Crick bonding with its orphaned thymine residue. This is of biological interest because the flipping out of nucleobases has been previously shown to be pivotal in the interaction of some enzymes with DNA, although the mechanism of this interaction is not yet understood. Secondly, the occurrence of the semi-intercalative binding of an ancillary TAP ligand to a third duplex, results in a second cross-linking interaction of duplexes. This causes a dramatic kinking of the oligonucleotide, a phenomena rarely seen with small molecule binding, but very common in cellular biology. Some protein/DNA interactions cause similar kinking which acts as a regulation step of the transcription process within the cell. Such kinking has been noted with the potent anti-tumour agent *cis*-platin and identified as the source of its cytotoxicity. Finally, ruthenium-TAP complexes are known for their ability to cause photo-induced damage to duplex DNA at the guanine residues, occasionally forming photo-adducts. The method through which these photo-adducts are formed is not yet known, however, the ability to now determine the inter-atomic distances has allowed for more accurate proposals of the reaction mechanism.

6.1.3 Bi-nuclear Ruthenium Complexes

In an attempt to create a binding agent with a preference for quadruplex DNA, the molecular structure was increased in complexity to a bi-nuclear ruthenium complex incorporating the TAP ligands and a TPAC bridging ligand. This complex, $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]^{4+}$, was initially synthesised in collaboration with Prof. Andrée Kirsch-DeMesmaeker at the Université Libre de Bruxelles, Belgium. Following the success with

the enantiomeric separations of the mono-nuclear ruthenium-TAP complexes, attempts were made at separating the three bi-nuclear enantiomers $\Lambda\Lambda$, $\Delta\Delta$ and $\Lambda\Delta$ using initially the CM Sephadex C-25 column with various mobile phases and secondly, binding affinity-based DNA-assisted stereoisomer separation. Surprisingly neither of these techniques resulted in the separation of the enantiomers.

However, during the course of this project, a novel method for the synthesis of the bi-nuclear complex utilising microwave heating was devised which, in turn, lead to the development of a novel synthetic pathway of the mono-nuclear $[\text{Ru}(\text{TAP})_2\text{TPAC}]^{2+}$ compound via simple complexation.

6.2 Future Work

The work presented in this thesis represents a further step in our understanding of ruthenium complex chemistry, and how it interacts with the building block of life, DNA.

In terms of the solution studies into the binding of the enantiomeric complexes Λ - and Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ to DNA, this thesis reports a rather thorough investigation. The proposed enantiomeric effect, while shown to occur on the back electron transfer process in D_2O , has not been corroborated for the proton-coupled forward electron transfer process. To this end, picosecond transient absorbance and time resolved infrared spectroscopic investigations could be performed to demonstrate this theorised effect. Similarly, nanosecond and picosecond experiments could be performed utilising the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]^{2+}$ enantiomers to investigate the effect methyl substituents may have the rate of reaction for the processes.

Despite frustrations with regard to the separation of the bi-nuclear enantiomers, continued attempts should be made to achieve this goal. There are very few enantiomeric studies of binding agents preferential for quadruplex DNA, and judging from the studies of the mono-nuclear enantiomeric binding complexes, significant differences between the binding of the bi-nuclear enantiomers is anticipated. Such a binding agent could prove to be a powerful tool in developing new compounds to aid in the struggle against cancer and many other diseases.

The crystallographic work conducted during the course of this project is, perhaps, the crowning achievement of this thesis. To date, there are very few examples of ruthenium compounds in the crystallographic database (the PDB) and no such examples of a ruthenium polypyridyl complex binding to an oligonucleotide, despite their being the result of intense research over the past 25 years. The discovery and publication of the crystallographic method will undoubtedly lead to an explosion of crystal structures of similar ruthenium complexes with a variety of oligonucleotide sequences. Unfortunately only the Λ enantiomer was found to crystallise in this study, however crystallisation of the Δ -[Ru(TAP)₂dppz]²⁺ enantiomer, and indeed the more widely researched [Ru(phen)₂dppz]²⁺ and its enantiomers are sure to follow, improving our understanding of ruthenium complexes and their role in cytotoxicity and potential in future medical therapies.

Chapter 7

Experimental

This chapter describes the procedures used during the course of this work, detailing the exact conditions for each experiment conducted.

7.1 Experimental Materials

7.1.1 Reagents and Solvents

All solvents were reagent grade except where otherwise stated. All chemicals were purchased from Sigma Aldrich, Sigma Genosys and Fluka. Millipore water was used in all aqueous preparations. Samples of [poly(dA-dT)] and [poly(dG-dC)] were purchased from Amersham Biosciences.

7.1.2 Buffer Solutions

Sodium phosphate buffer (7.4 pH) was utilised in each of the experiments conducted with DNA. The buffer was made by first creating Solution A, an aqueous solution of $\text{NaH}_2\text{PO}_4 \cdot 5\text{H}_2\text{O}$ (0.156 g in 5 ml H_2O), and Solution B, an aqueous solution of Na_2HPO_4 (0.142 g in 5 ml H_2O). A combination of Solution A (1.95 ml) and Solution B (3.05 ml) with water (5 ml) yielded a 100 mM sodium phosphate buffer solution (10 ml, 7.4 pH).

7.1.3 Metal Complex Solutions

Metal complex stock solutions, prepared by dissolving and vortexing the chloride salts in water and the potassium hexafluorophosphate salts in acetonitrile, were kept in the dark to avoid degradation. Experimental samples were freshly prepared before each experiment from stock solutions of accurately determined concentration using accurate calibrated micropipettes (Eppendorph P10, P20, P200 and P1000). Typically, concentrations ($< 4 \times 10^{-5}$ M) were sufficiently low to avoid re-adsorption corrections. In experiments involving spectroscopy, the nucleic acids concentrate was added stepwise in small volumes, allowing time for equilibrium, and corrections were made for the dilution of the sample.

7.1.4 DNA solutions

DNA solutions were made utilising either 10 mM sodium phosphate or 100 mM sodium phosphate buffer solutions. Salmon testes DNA was obtained from Sigma Aldrich. Approximately 0.02 g of DNA was allowed to dissolve in buffered solution (1 ml) overnight. The solution was vortexed to ensure a homogeneous solution and centrifuged at 10,000 rpm for 20 minutes to remove any insoluble impurities. The supernatant was collected and stored at 4°C.

All the experiments involving the interaction of complexes with DNA were prepared directly in the cuvette. The DNA concentration per nucleotide was determined spectrophotometrically using the molar absorption coefficient $6600 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm.¹

7.2 Experimental Apparatus and Techniques

7.2.1 Isomeric Resolution Chromatography

CM Sephadex C-25 (Sigma Aldrich) was used to resolve the various isomers detailed in this thesis. The compound, which was loaded onto the top of the column as an aqueous solution, was eluted 0.1 M (-)-O, O'-dibenzoyl-L-tartaric acid monohydrate aqueous solution. The Λ enantiomer eluted before the Δ enantiomer.

7.2.2 Nuclear Magnetic Resonance Spectroscopy

Samples were recorded using a Bruker Advance III spectrometer, operating at 600 and 400 MHz in appropriate solvents. Shifts are referenced relative to the internal solvent signals. NMR data was processed using iNMR Version 2.6.3.

7.2.3 UV-vis Absorption Spectroscopy

Absorption spectra and optical densities were recorded on a Varian Cary 200 spectrophotometer or a Shimadzu UV-2401 PC UV-vis spectrophotometer in an appropriate range. Solutions were measured in 4 cm³ (10 mm x 10 mm) quartz cuvettes. Wavelength range was 200-700 nm with a scan rate of 600 nm min⁻¹. Concentration of the ruthenium complexes was in the order of 10⁻⁵ M so as to achieve a maximum absorption in the range of 0-2 in the spectrum. Extinction coefficient are accurate to 5%.

The extinction coefficients ϵ were calculated using the Beer-Lambert Law (Equation 7.1). The value of ϵ is usually reported at a wavelength of a maximum absorption peak and is denoted as $\epsilon_{\max}$. In order to determine $\epsilon_{\max}$ for the ruthenium complexes, solutions of known concentrations were prepared and their absorption spectra were obtained from which $\epsilon_{\max}$ was calculated.

$$A = -\log\left(\frac{I}{I_0}\right) = \epsilon cl \quad (7.1)$$

7.2.4 Photoluminescence

Emission measurements were performed using a Varian Cary Eclipse spectrometer. Solutions were measured in 4 cm³ (10 mm x 10 mm) quartz cuvettes. The solvents used were HPLC grade and the concentrations of the compounds under investigation were determined via UV-visible absorption measurements. Lifetime measurements were performed on a Horiba Jobin Yvon Fluorolog TCSPC. The excitation source was a 458 nm NanoLED

7.2.5 Linear and Circular Dichroism Spectroscopy

Linear and Circular dichroism spectra were recorded at a concentration corresponding to an optical density of approximately 1.0, in buffered solution, on a JASCO J-815 Circular Dichroism Spectropolarimeter equipped with a Linear Dichroism Accessory (LD) or a JASCO J-810-150S CD spectropolarimeter (CD). Spectra were typically accumulated over six cycles.

7.2.6 Mass Spectrometry

MALDI-TOF measurements were recorded on a Waters MALDI Q-TOF Premier system and ESI-MS measurements were performed using an LCT Orthogonal Acceleration TOF Electrospray mass spectrometer. Negative and positive ionisation modes were used where appropriate.

7.2.7 Nanosecond Time-Correlated Single Photo Counting

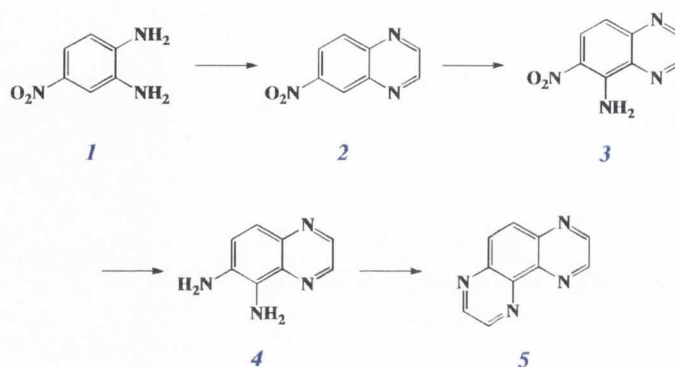
Fluorescence lifetime were recorded on an Edinburgh Instruments FL920 TCSPC spectrometer, with excitation from a 450 nm pulsed diode laser (Hamamatsu Photonics) onto a Peltier-cooled red-sensitive R2658P PMT (Hamamatsu Photonics).

7.2.8 Thermal Denaturation

The thermal denaturation experiments were performed on a thermoelectrically coupled Perkin Elmer LAMBDA 25 UV/Vis Spectrophotometer.

7.3 Preparation of Ligands

7.3.1 1,4,5,8-tetraphenanthrene (TAP)



1) 4-nitro-1,2-benzenediamine

Purchased from Sigma Aldrich, CAS number: 99-56-9

2) 6-nitroquinoxaline

4-nitrobenzene-1,2-diamine **1** (4 g, 27.95 mmol, 1 eq.) was suspended in ethanol (100 ml) before an aqueous solution of oxalaldehyde (40%, 6 ml) was added and the mixture heated at reflux for 2 hrs. The solvent was removed under reduced pressure before being dissolved in water (200 ml) and the aqueous layer extracted with dichloromethane (DCM) (4 x 50 ml). The solvent was again removed under reduced pressure after being dried over MgSO_4 . The resulting orange solid was recrystallised twice from isopropanol (2 x 150 ml) to yield golden crystalline needles (4.07 g, 79%).

δ_{H} (600 MHz, DMSO-d_6): 8.36 (d, 1H, $J=9.04$ H-3), 8.57 (dd, 1H, $J=2.52$, $J=9.04$, H-2), 8.91 (d, 1H, $J=2.52$, H-1), 9.17 (s, 2H, H-4 and H-5).

HRMS (m/z -ES): Found: 176.0474 $\text{C}_8\text{H}_6\text{N}_3\text{O}_2$ (Calc. Mass: 176.0460).

3) 6-nitroquinoxaline-5-amine

Sodium metal (2.3 g, 100 mmol, 3.3 eq.) was added to distilled methanol (125 ml). A solution of hydroxylamine (3.13 g, 45 mmol, 1.5 eq.) in distilled methanol (50 ml) was added after cooling to 0°C on ice. The resulting sodium chloride precipitate was allowed to settle before the supernatant solution was added to a well stirred suspension of 6-nitroquinoxaline **2** (5.25 g, 30 mmol, 1 eq.) in boiling distilled methanol (250 ml). The

resulting brown reaction mixture was heated under reflux for 90 mins before being cooled to 0°C on ice. The resulting yellow precipitate was collected by suction filtration before being recrystallised from a mixture of acetic acid and water (3:1, 150 ml) to yield bright yellow crystalline needles (2.49 g, 41%).

δ_{H} (600 MHz, DMSO- d_6): 7.19 (d, 1H, $J=9.52$, H-3), 8.30 (d, 1H, $J=9.52$, H-2), 8.53 (s, 2H, NH_2) 8.94 (d, 1H, $J=2.04$, H-4/5), 9.10 (d, 1H, $J=2.00$, H-4/5).

HRMS (m/z -ES): Found: 191.0568 $\text{C}_8\text{H}_7\text{N}_4\text{O}_2$ (Calc. Mass: 191.0569).

4) quinoxaline-5,6-diamine

6-nitroquinoxalin-5-amine **3** (1.5 g, 7.86 mmol, 1 eq.) and 10% Pd/C (0.2 g) were added to ethanol (80 ml) and the mixture heated at reflux for 1 hr. Hydrazine monohydrate chloride (98%, 7.89 g, 157.6 mmol, 20 eq) was added and the mixture was again heated at reflux for 1 hr. The resulting red/black mixture was filtered hot through a pad of celite and the solid washed with DCM (80 ml). The solvent was removed under reduced pressure yielding a blood red solid which was dried under high vacuum (1.24 g, 98%). m.p.147-148°C.

δ_{H} (600 MHz, DMSO- d_6): 5.16 (s, 2H, NH_2), 5.28 (s, 2H, NH_2), 7.20 (d, 1H, $J=8.52$, H-2/3), 7.26 (d, 1H, $J=9.04$, H-2/3), 8.51 (d, 1H, $J=2.00$, H-4/5), 8.58 (d, 1H, $J=2.00$, H-4/5).

HRMS (m/z -ES): Found: 161.0816 $\text{C}_8\text{H}_9\text{N}_4$ (Calc. Mass: 161.0827).

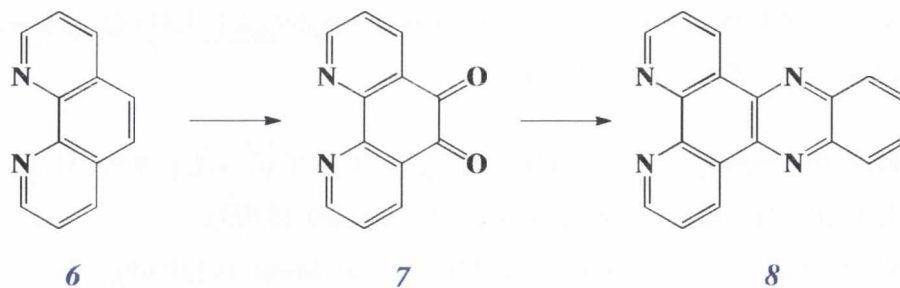
5) 1,4,5,8-tetraazaphenanthrene (TAP)

Quinoxaline-5,6-diamine **4** (1 g, 6.24 mmol, 1 eq.) was suspended in ethanol (100 ml) before an aqueous solution of oxalaldehyde (40%, 5 ml) was added and the mixture heated at reflux for 2 hrs. The solvent was removed under reduced pressure before being dissolved in water (100 ml) and the aqueous layer extracted with DCM (5 x 40 ml). The solvent was again removed under reduced pressure after being dried over MgSO_4 . The resulting orange solid was recrystallised from isopropanol (50 ml) to yield yellow crystalline needles (0.83 g, 69%).

δ_{H} (600 MHz, DMSO- d_6): 8.38 (s, 2H, H-3 and H-3'), 9.21 (d, 2H, $J=2.04$, H-2 and H-2'), 9.24 (d, 2H, $J=2.04$, H-1 and H-1').

HRMS (m/z -ES): Found: 183.0685 $\text{C}_{10}\text{H}_7\text{N}_4$ (Calc. Mass: 183.0671).

7.3.2 Dipyrido[3,2-a:2',3'-c]phenazine (dppz)



6) 1,10-phenanthroline

Purchased from Sigma Aldrich, CAS number: 66-71-7

7) 1,10-phenanthroline-5,6-dione (phendione)

This compound was synthesised following a method described in the literature for the oxydation of 1,10-phenanthroline **6**². 1,10-phenanthroline **6** (5.00 g, 27.7 mmol) was dissolved gradually with constant stirring in concentrated sulfuric acid (30 ml). Potassium bromide (2.5 g, 24.3 mmol) was added followed by nitric acid (70%, 15 ml). The mixture was refluxed for 40 minutes before the condenser was removed and the bromine vapours allowed to escape during 15 minutes of gentle boiling. The mixture was allowed to cool to r.t. before being added to ice (400 g) and slowly neutralized to pH 7 with the addition of 10 M sodium hydroxide (150 ml). After 30 minutes the solution was filtered and any solid was extracted with boiling water. The insoluble material was removed from the cooled extract by filtration and the aqueous fractions were extracted with DCM. The organic phase was washed with water and dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure. The residue was recrystallized from toluene (400 ml) to yield yellow/orange needle-like crystals (2.64 g, 45%).

δ_{H} (600 MHz, CDCl_3): 9.13 (dd, 1H, $J=4.7$), 8.52 (dd, 1H, $J=1.8$), 7.63 (dd, 1H, $J=7.9$).

HRMS (m/z -ES): Found: 210.1878 $\text{C}_{10}\text{H}_7\text{N}_4$ (Calc. Mass: 210.1882).

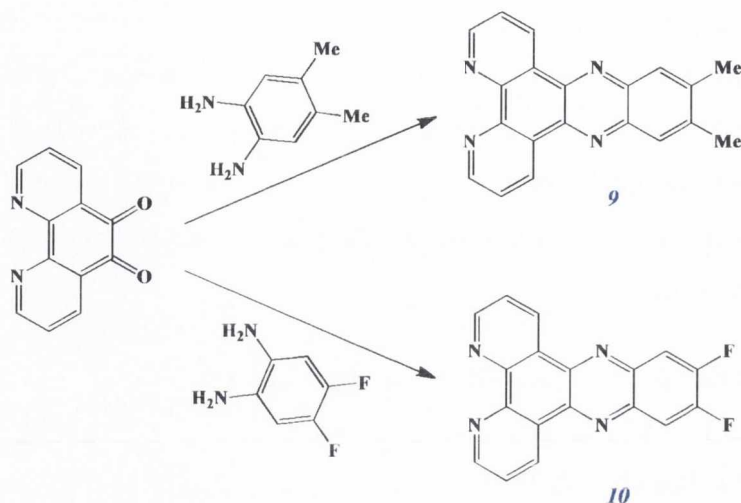
8) dipyrido[3,2-*a*:2',3'-*c*]phenazine (dppz)

This compound was obtained via the condensation reaction between phendione **7** and 1,2-phenylenediamine as described in the literature³. Phendione **7** (0.515g, 2.45 mmol) was dissolved in ethanol (15 ml) and added to a solution containing 1,2-phenylenediamine (0.53 g, 4.91 mmol) in ethanol (10 ml) and trace *p*-toluene sulphuric acid. The mixture was refluxed for 3 hours before allowing some ethanol (approx. half) to boil off. The remaining solution was cooled to r.t. and a brown precipitate was obtained. The solid was collected and recrystallized from aqueous ethanol to yield brown/orange needle-like crystals (0.512 g, 74%).

δ_{H} (600 MHz, CDCl_3): 9.46 (dd, 1H, $J=8.3$), 9.19 (dd, 1H, $J=1.6$), 8.34 (dd, 1H, $J=6.6$), 8.04 (dd, 1H, $J=5.1$), 7.92 (dd, 1H, $J=3.3$).

HRMS (m/z -ES): Found: 282.2993 $\text{C}_{18}\text{H}_{10}\text{N}_4$ (Calc. Mass: 282.2988).

7.3.3 Dimethyl-dipyrido[3,2-a:2',3'-c]phenazine (Me₂dppz) and Difluoro-dipyrido[3,2-a:2',3'-c]phenazine (F₂dppz)



9&10) dimethyl-dipyrido[3,2-a:2',3'-c]phenazine (Me₂dppz) and difluoro-dipyrido[3,2-a:2',3'-c]phenazine (F₂dppz)

These compounds were obtained via the condensation reaction between phendione **7** and appropriate diamino compound. Phendione **7** (0.515g, 2.45 mmol) was dissolved in ethanol (15 ml) and added to a solution containing the appropriate diamino compound (4.91 mmol) in ethanol (10 ml) and trace *p*-toluene sulphuric acid. The mixture was refluxed for 3 hours before allowing some ethanol (approx. half) to boil off. The remaining solution was cooled to r.t. and a brown precipitate was obtained. The solid was collected and recrystallized from aqueous ethanol to yield pale yellow needle-like crystals for the Me₂dppz **9** (0.547 g, 72% yield) and a cream coloured solid for the F₂dppz ligand **10** (0.49 g, 64% yield).

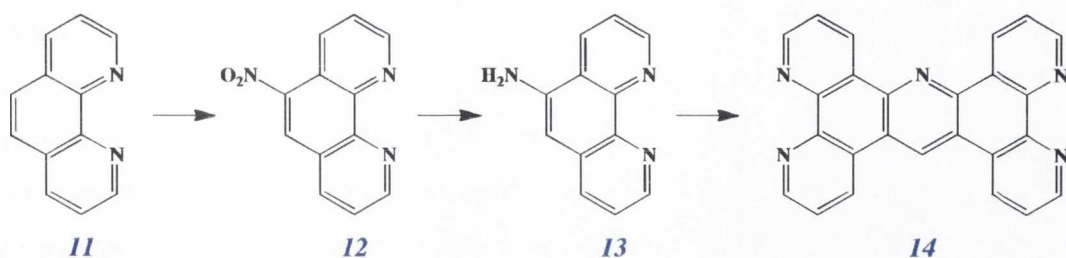
Me₂dppz **9** - δ_{H} (600 MHz, CD₃CN): 8.38 (s, 2H, H-3 and H-3'), 9.21 (d, 2H, J=2.04, H-2 and H-2'), 9.24 (d, 2H, J=2.04, H-1 and H-1').

HRMS (*m/z* -ES): Found: 310.3512 C₂₀H₁₄N₄ (Calc. Mass: 310.3520).

F₂dppz **10** - δ_{H} (600 MHz, CD₃CN): 8.38 (s, 2H, H-3 and H-3'), 9.21 (d, 2H, J=2.04, H-2 and H-2'), 9.24 (d, 2H, J=2.04, H-1 and H-1').

HRMS (*m/z* -ES): Found: 318.2804 C₁₈H₈N₄F₂ (Calc. Mass: 318.2797).

7.3.4 Tetrapyrido[3,2-a:2',3'-c:3'',2''-h:2''',3'''-j]acridine (TPAC)



This compound was synthesised following a method unreported as yet in the literature but optimised in the Kirsch-De Mesmaeker laboratories, Université Libre de Bruxelles, Belgium.

12) 5-nitro-1,10-phenanthroline (5-NO₂-phen)

1,10-phenanthroline **6** (5.011g, 27.8 mmol) was dissolved in fuming sulphuric acid (25 ml). Concentrated nitric acid (14 ml) was added and the mixture refluxed at 170°C for 30 minutes. The reaction mixture was cooled to room temperature and neutralised with NaOH (2M). The resulting precipitate was collected and washed with iced water via filtration. The product was collected as a pale yellow solid (3.238g, 14.7 mmol, 53%).

δ_H (600 MHz, CDCl₃): 9.36 (dd, 1H, J=4.4), 9.29 (dd, 1H, J=4.3), 9.05 (dd, 1H, J=1.9), 8.67 (s, 1H), 8.45 (dd, 1H, J=1.7), 7.92 (dd, 1H, J=8.8), 7.89 (dd, 1H, J=8.1). **HRMS (*m/z* -ES):** Found: 225.2039 C₁₂H₇N₃O₂ (Calc. Mass: 225.2029).

13) 5-amino-1,10-phenanthroline (5-NH₂-phen)

Hydrazine hydrate (5ml) was diluted in ethanol (100 ml) and added drop-wise to a solution of 5-NO₂-phen **12** and Pd/C (600 mg) in ethanol (100 ml). The reaction mixture was stirred at 70°C for 15 minutes before being cooled to room temperature and filtered. Water (20 ml) was added to the collected filtrate and the resultant precipitate was isolated. The product was collected as a yellow solid (2.387 g, 12.2 mmol, 91%).

δ_H (600 MHz, CDCl₃): 9.20 (dd, 1H, J=4.4), 8.93 (dd, 1H, J=3.7), 8.28 (dd, 1H, J=1.9), 8.02 (dd, 1H, J=1.4), 7.65 (dd, 1H, J=8.3), 7.48 (dd, 1H, J=8.1), 6.95 (s, 1H), 4.35 (s, 2H). **HRMS (*m/z* -ES):** Found: 195.2187 C₁₂H₉N₃ (Calc. Mass: 195.2200).

14) tetrapyrido[3,2- α :2',3'-c:3'',2''-h:2''',3'''-j]acridine (TPAC)

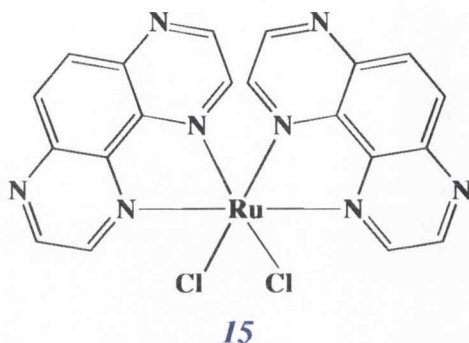
5-NH₂-phen **13** (82 mg, 0.42 mmol) and paraformaldehyde (6.9 mg, 0.23 mmol) were placed in a microwave tube (8 ml) with a magnetic stirrer and 6 N hydrochloric acid (5 ml). The tube was sealed and placed in the microwave for 1 hour at 100 °C, 150 W max. The solution was transferred to a conical flask and neutralised (pH 7) adding sodium hydroxide (2 N) drop-wise. The resulting precipitate was collected via centrifugation and washed with water and ethanol and dried with ether. The compound was purified by recrystallisation from a minimum of hot ethanol to yield a pale yellow solid (62.8 mg, 0.16 mmol, 78%).

δ_H (600 MHz, CDCl₃): 8.46 (dd, 2H, J=1.2), 8.40 (dd, 1H, J=3.7), 8.28 (dd, 2H, J=1.8), 7.83 (s, 1H), 7.73 (dd, 2H, J=8.3), 7.67 (d, 2H, J=7.8), 7.06 (dd, 2H, J=4.1), 6.82 (dd, 2H, J=4.1).

HRMS (m/z -ES): Found: 383.4049 C₂₅H₁₃N₅ (Calc. Mass: 383.4042).

7.4 Preparation of Ruthenium Polypyridyl Complexes

7.4.1 Ruthenium bis-(1,4,5,8-tetraazaphenanthrene) dichloride ($\text{Ru}(\text{TAP})_2\text{Cl}_2$)

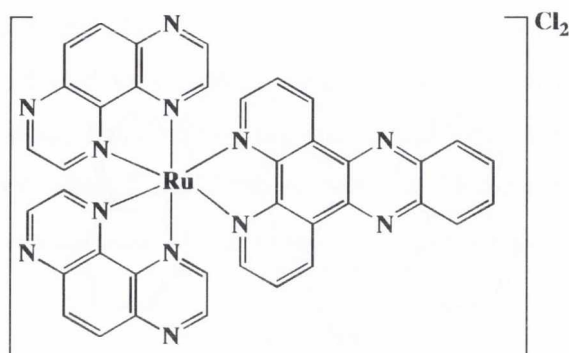


The synthesis of this metal complex was taken from methods reported in the literature⁴. Ruthenium (III) chloride hydrate (578 mg, 2.79 mmol), TAP (1000 mg, 5.48 mmol) and lithium chloride (270 mg, 6.37 mmol) were dissolved in anhydrous DMF (40 ml) and allowed to reflux. The progress of the reaction was followed via absorbance spectroscopy and the reaction halted after the appearance of the spectrum corresponding to the trichelated complex $[\text{Ru}(\text{TAP})_3]^{2+}$, which occurs after approximately 40 minutes. The reaction mixture was cooled to rt, transferred to a beaker and placed in a dessicator containing acetone (250 ml). The solution was left in the dark for 3 days to precipitate out. The solid was obtained via centrifugation, washed in a minimum of water with acetone and purified by recrystallization from ethylene glycol/acetone (5:20, 5 ml) to yield deep purple crystals (688.34 mg, 1.3 mmol, 46%).

δ_{H} (600 MHz, $\text{DMSO}-d_6$): 10.21 (d, 1H, $J=2.7$), 9.51 (d, 1H, $J=2.7$), 8.65 (m, 2H), 8.52 (dd, 1H, $J=9.3$), 8.35 (d, 1H, $J=2.8$).

MALDI (TOF MS LD+): Found: 536.3381 (M^{2+}). $\text{C}_{20}\text{H}_{12}\text{N}_8\text{Cl}_2\text{Ru}$ (Calc. Mass: 536.3389).

7.4.2 Ruthenium bis-(tetraazaphenanthrene) dipyridophenazine dichloride ([Ru(TAP)₂dppz]Cl₂)

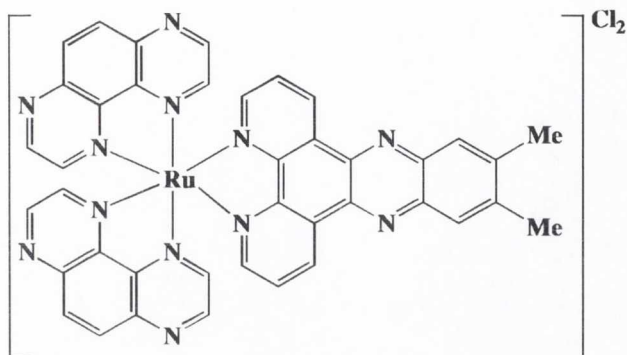
**16**

Ru(TAP)₂Cl₂ **15** (81 mg, 0.15 mmol) and dppz **8** (28 mg, 0.15 mmol) were placed in a microwave tube (10 ml) with a magnetic stirrer and a solution of ethanol and water (7 ml, 1:1). The tube was sealed evacuated with argon for 15 minutes before being placed in the microwave. Upon forty minutes irradiation in the microwave at 140°C the reaction mixture changed from a violet coloured solution to a deep red/brown solution. The reaction mixture was filtered and subsequent precipitation of the complex from the filtrate as its PF₆ salt was achieved by the addition of a concentrated aqueous solution of KPF₆. Isolation of the precipitate yielded a red/brown solid. This was converted to its chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl⁻ form) and the desired product, **16**, was obtained following purification on an aqueous Sephadex C-25 column with 0.2 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (90 mg, 0.11 mmol, 74% yield).

δ_{H} (600 MHz, CD₃CN): 9.7 (d, 1H, J=8), 8.95 (2d, 2H), 8.61 (m, 2H), 8.44 (d, 1H, J=3.0), 8.41 (d, 1H, J=6.5), 8.35 (d, 1H, J=3.0), 8.14 (d, 1H, J=5.5), 8.07 (dd, 1H, J=3.3), 7.83 (dd, 1H).

MALDI (TOF MS LD+): Found: 747.7307 (M²⁺) C₃₈H₂₂N₁₂Ru (Calc. Mass: 747.7317).

7.4.3 Ruthenium bis-(tetraazaphenanthrene) dimethyl-dipyridophenazine dichloride ($[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$)



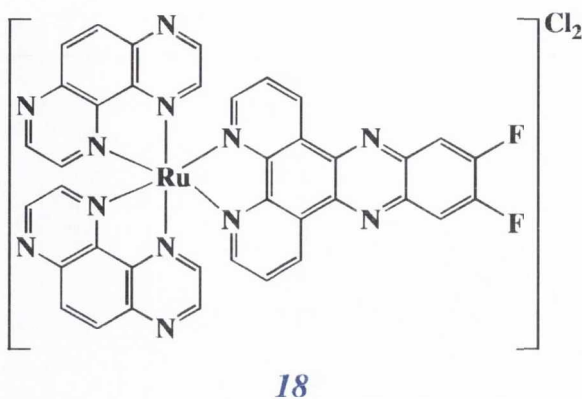
17

This complex was synthesised following the same precedure used in the synthesis of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ **16** with the only difference being the starting materials. $\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (81 mg, 0.15 mmol) and Me_2dppz **9** (31 mg, 0.1 mmol) were reacted in the microwave as before. The product, **17**, was obtained following purification on an aqueous Sephadex C-25 column with 0.2 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (69 mg, 0.82 mmol, 82% yield).

δ_{H} (600 MHz, CD_3CN): 9.74 (d, 1H, $J=7.8$), 9.09 (2d, 2H, $J=3$), 8.66 (s, 2H), 8.32 (d, 1H, $J=3$), 8.27 (d, 2H, $J=2.4$), 8.19 (dd, 1H, $J=5.4$), 7.86 (dd, 1H, $J=13.8$), 2.71 (s, 3H).

MALDI (TOF MS LD+): Found: 775.7857 (M^{2+}) $\text{C}_{40}\text{H}_{26}\text{N}_{12}\text{Ru}$ (Calc. Mass: 775.7848).

7.4.4 Ruthenium bis-(tetraazaphenanthrene) difluoro-dipyridophenazine dichloride ($[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]\text{Cl}_2$)

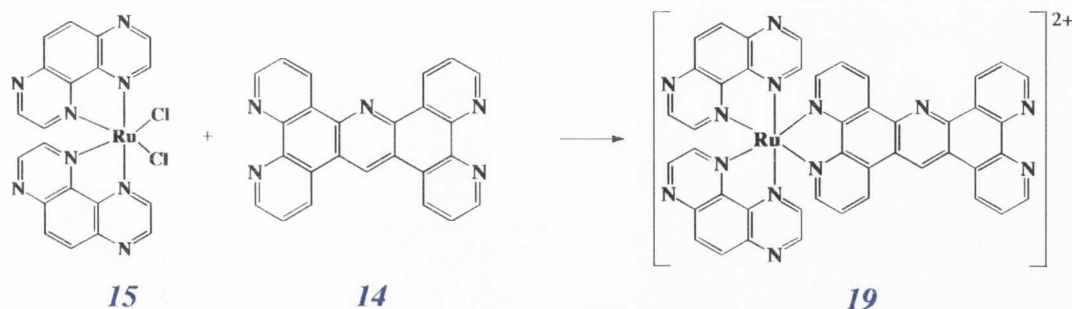


This complex was synthesised following the same procedure used in the synthesis of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ **16** with the only difference being the starting materials. $\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (81 mg, 0.15 mmol) and F_2dppz **10** (47.8 mg, 0.15 mmol) were reacted in the microwave as before. The product, **18**, was obtained following purification on an aqueous Sephadex C-25 column with 0.2 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (88.4 mg, 0.1 mmol, 69% yield).

δ_{H} (600 MHz, CD_3CN): 9.70 (d, 1H, $J=1.4$), 8.99 (2d, 2H, $J=2.8$), 8.63(m, 2H), 8.32 (t, 1H, $J=9.4$), 8.27 (2d, 2H), 8.19 (dd, 1H, $J=5.4$), 7.86 (dd, 1H, $J=8.4$).

MALDI (TOF MS LD+): Found: 783.7147 (M^{2+}) $\text{C}_{38}\text{H}_{20}\text{N}_{12}\text{F}_2\text{Ru}$ (Calc. Mass: 783.7126).

7.4.5 Ruthenium bis-(tetraazaphenanthrene) tetrapyridoacridine dichloride ($[(\text{TAP})_2\text{Ru-TPAC}]\text{Cl}_2$)

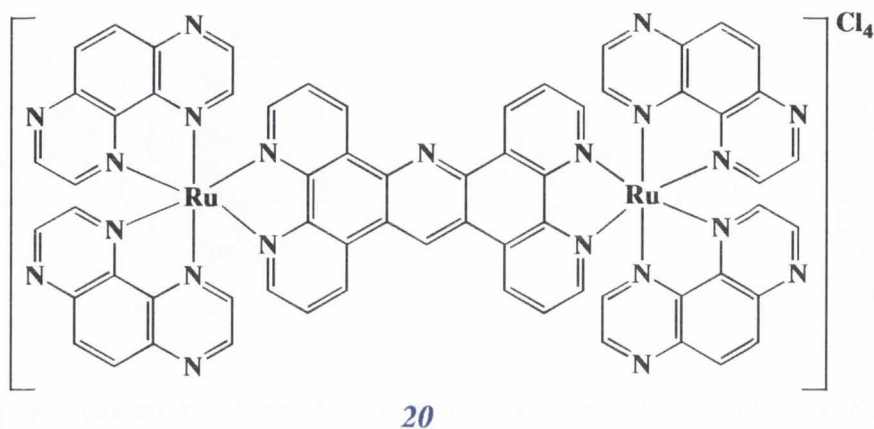


$\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (14 mg, 0.03 mmol) and TPAC **14** (10 mg, 0.026 mmol) were placed in a microwave tube (5 ml) with a magnetic stirrer and ethylene glycol (2 ml). The tube was sealed evacuated with argon for 15 minutes before being placed in the microwave. Upon forty minutes irradiation in the microwave at 140°C the reaction mixture changed from a violet coloured solution to a deep red/brown solution. Water (5 ml) was added to the reaction mixture and the solution was filtered to remove any unreacted insoluble material. The complex was precipitated from the filtrate by the addition of a concentrated aqueous solution of KPF_6 . Isolation of the precipitate yielded a red/brown solid. This was converted to its chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl-form) and the desired product, **19**, was obtained following purification on an aqueous Sephadex C-25 column with 0.4 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (5 mg, 0.014 mmol, 52% yield).

δ_{H} (400 MHz, CD_3CN): 10.56 (s, 1H), 10.32 (d, 1H, $J=7.7$), 10.12 (d, 1H, $J=8.5$), 9.89 (d, 1H, $J=7.4$), 9.79 (d, 1H, 8.1), 9.00 (m, 4H), 8.64 (m, 4H), 8.52 (m, 2H), 8.28 (m, 6H), 8.09 (m, 2H), 7.93 (m, 2H).

MALDI (TOF MS LD+): Found: 848.8378 (M^{2+}) $\text{C}_{45}\text{H}_{25}\text{N}_{13}\text{Ru}$ (Calc. Mass: 848.8371).

7.4.6 Ruthenium tetra-(tetraazaphenanthrene) tetrapyridoacridine tetrachloride $[(\text{TAP})_2\text{Ru-TPAC-Ru}(\text{TAP})_2]\text{Cl}_4$

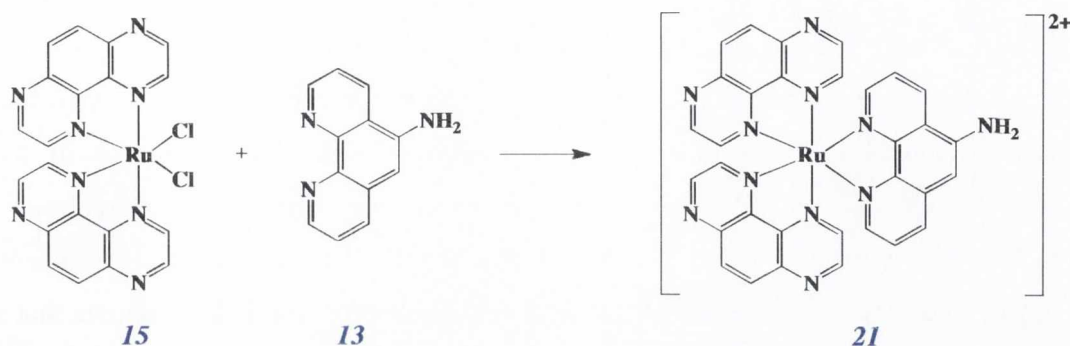


This complex was synthesised following the same procedure used in the synthesis of $[(\text{TAP})_2\text{Ru-TPAC}]\text{Cl}_2$ **19** with the only difference being the starting materials. $\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (30 mg, 0.06 mmol) and TPAC **14** (10 mg, 0.026 mmol) were reacted in the microwave as before. The desired product, **20**, was obtained following purification on an aqueous Sephadex C-25 column with 0.4 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (10.6 mg, 0.007 mmol, 28% yield).

δ_{H} (400 MHz, CD_3CN): 10.56 (s, 1H), 10.11 (dd, 1H, $J=9.6$), 9.70 (d, 1H, $J=8.8$), 9.09 (m, 4H), 8.64 (m, 4H), 8.28 (m, 6H), 7.93 (m, 2H).

MALDI (TOF MS LD+): Found: 1314.2623 (M^{4+}) $\text{C}_{65}\text{H}_{37}\text{N}_{21}\text{Ru}_2$ (Calc. Mass: 1314.2700).

7.4.7 Ruthenium tetra-(tetraazaphenanthrene) 5-amino-phenanthroline $[(\text{TAP})_2\text{Ru}-(5\text{-NH}_2\text{-phen})]\text{Cl}_2$



$\text{Ru}(\text{TAP})_2\text{Cl}_2$ **15** (140 mg, 0.3 mmol) was dissolved in a methanol/water solution (1:1, 20 ml). 5- NH_2 -phenanthroline **13** (58 mg, 0.3 mmol) was also dissolved in a methanol/water solution (1:1, 20 ml) and added drop-wise to the reaction mixture. The solution was stirred at room temperature for 4 hours. The methanol was removed under reduced pressure and the complex was precipitated from the remaining water by the addition of a concentrated aqueous solution of KPF_6 . Isolation of the precipitate yielded an orange solid. This was converted to its chloride salt by stirring with Amberlite IRA-400 ion exchange resin (Cl-form) and the desired product, **21**, was obtained following purification on an aqueous Sephadex C-25 column with 0.4 M NaCl as the mobile phase. The final product was obtained as a red/brown solid (5 mg, 0.014 mmol, 52% yield).

δ_{H} (600 MHz, CD_3CN): 9.20 (dd, 2H, $J=4.3$), 8.98 (dd, 4H, $J=2.97$), 8.63 (m, 4H), 8.39 (dd, 2H, $J=8.6$), 8.28 (dd, 1H, $J=1.9$), 7.98 (dd, 1H, $J=1.4$), 7.65 (dd, 1H, $J=8.4$), 7.50 (dd, 1H, $J=8.0$), 6.95 (s, 1H), 4.27 (s, 2H).

MALDI (TOF MS LD+): Found: 195.2214 (M^{2+}) $\text{C}_{45}\text{H}_{25}\text{N}_{13}\text{Ru}$ (Calc. Mass: 195.2200).

7.5 Separation of the Enantiomers

The separation of the Λ - and Δ - enantiomers of the mono-ruthenium polypyridyl complexes was achieved using a Perspex column (1 m x 2 cm) containing CM Sephadex C-25 as the stationary phase connected to a peristaltic pump to facilitate recycling through the column. An anionic eluent is used to expedite the separation of the enantiomers. Unless they occupy a formal coordination site on the metal centre, anions have classically been considered as isolated from the complex in a polar environment, being part of a second-sphere solvent 'cage'. However, recent studies have shown that a variety of anions can strongly associate with a cationic ruthenium complex containing amide-functionalised polypyridyl ligands.⁵⁻⁷ Such specific interactions rely heavily upon hydrogen bonding, in addition to electrostatic factors that facilitate the association.⁸⁻¹⁰

7.5.1 General Procedure

The 1 m Perspex column (GE Healthcare) was washed thoroughly with water before use. The bottom of the column was sealed with an AC 26 adaptor (GE Healthcare) and filled with a small amount of water (10 ml). The capillary tubing from the adaptor was attached to the Miniplus3 peristaltic pump (Gilson) and the water pumped at a flow rate of 1 ml/min. The CM Sephadex C-25, previously suspended in water was poured into the column and allowed to settle approximately 10 cm from the top.

Once the Sephadex had settled, the aqueous solution of the racemic complex was pipetted above the stationary phase and allowed to adsorb by the continual application of water. Upon the total absorption of the complex (i.e. the water above the Sephadex is now colourless) a further 5cm of Sephadex was applied gently to avoid disturbing the adsorbed complex. Once this Sephadex had settled the mobile phase (0.1 M sodium dibenzoyl-L-tartaric acid pH 7.0) was applied to the column at a flow rate of 1 ml/min.

Application of the anionic mobile phase resulted in a slight shrinking of the Sephadex in the column due to the anions reducing the intra-molecular repulsion between the sulfonate groups (see Figure 2.5). As such, a continual 'top up' of stationary phase to the top of the column was required until the complex (now moving with the mobile phase)

reached the bottom adaptor. At this point the pump was halted to allow the recently added stationary phase to settle. Following this, a second AC 26 adaptor was fixed to the top of the column, ensuring the base of the adapter was flush with the top of the stationary phase. The capillary tube from this second adaptor was fitted to the output of the peristaltic pump so as to allow the recycling of the mobile phase and complex through the column. The pump was then restarted at the slower rate of 0.1 ml/min. This slower pace was to reduce the amount of mixing of enantiomers that occurs while off-column in the capillary tubes.

After a number of passes through the column (species dependant) the various fractions were collected and the complexes precipitated from the mobile phase as PF_6^- salts.

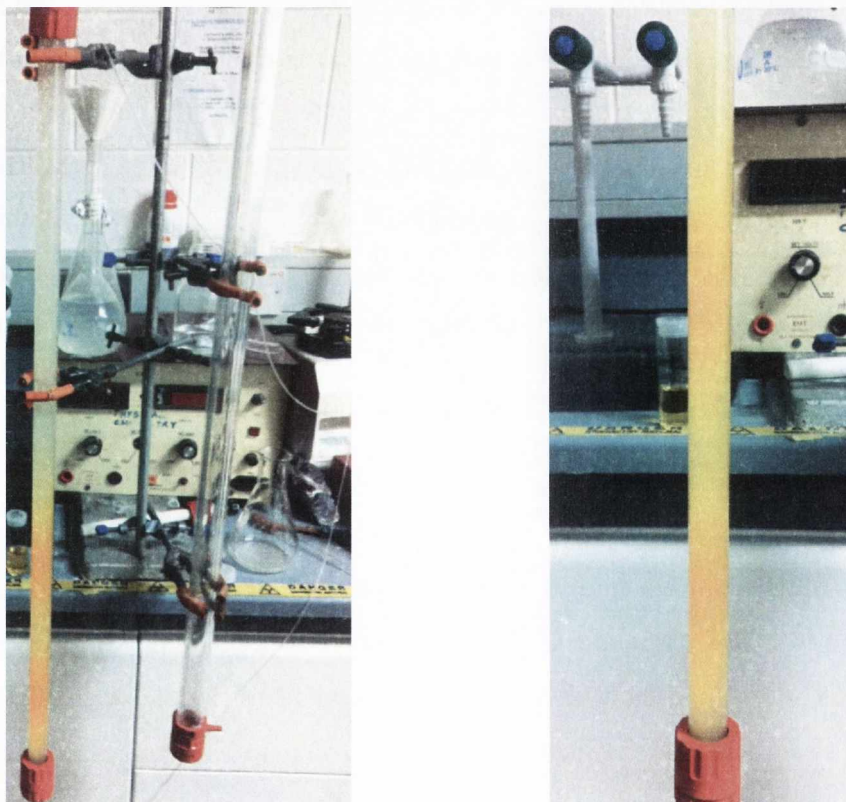


Figure 7.1 – Images of the CM Sephadex C-25 column separating the enantiomers of $[\text{Ru}(\text{TAP})_2(\text{F}_2\text{dppz})]^{2+}$ (the front orange band is the Δ enantiomer with the Λ enantiomer trailing)

7.6 Nanosecond Transient Absorption and Time-Resolved Infrared Spectroscopy

7.6.1 Sample Considerations

A commercial cell from Harrick Scientific Corporation was used. The instruments used have flow cell capabilities, however, with our systems the volume of sample needed would make our experiments extremely expensive, due to the necessity to maintain concentration in the mM range to afford a good signal. Therefore, static samples of low-volume (60 μ l) together with a commercial Teflon spacer (100 μ m, from Harrick Scientific, U.S.) between two 25 mm CaF_2 plates (Krystron Limited, U.K.) were used. In an attempt to minimise sample degradation, the cell was randomly oscillated in both the x and y directions (perpendicular to the direction of the laser beam) using an in-house x-y stage and electric motors at a rate of approximately 100 mm/s. The number of delays is user defined as are the delay times themselves. Acquisition time for each delay is also variable and in our experiments, data acquisitions were over 30 seconds. Within a cycle the delay times are randomised by the software, with a typical spectrum consisting of four accumulations, i.e. four full cycles of all delay times. Negative time delays are necessary so as to provide a baseline.

7.6.2 Sample preparation

The samples were each prepared in a 10 mM sodium phosphate buffer of either D_2O or H_2O , depending on the experiment. The concentration of the complex in each sample of 60 μ l was 25×10^{-5} M, and the P/D ratio of [poly(dG-dC)] to complex was 20:1 to ensure the highest degree of complex binding. The sample (60 μ l) was prepared in an ependorph vial and mixed thoroughly using a vortex. Once loaded between the CaF_2 plates, being careful to ensure no air bubbles were formed in the sample, the Harrick Cell was securely sealed. The UV/vis absorption spectrum was taken before and after each experiment to examine for any degradation.

7.7 Crystallisation Techniques

The crystallisation experiments were performed in the laboratory of the HeliX Group at the University of Reading. Following their advice with regard to the concentrations of complex and oligonucleotide (TCGGCGCCGA), initial experiments of a P/D of 1:1 were screened utilizing the solutions from the Nucleic Acid Mini Screen kit (Hampton Research) and an oligonucleotide concentration of 1 mM.

The crystallisation technique used was vapour diffusion via the sitting drop method (explained in section 3.2.5.1). The complex solutions and oligonucleotide solution were made to the desired concentrations by dissolution in nanopure water. A large quantity of 35% methyl-2,2-pentane diol (MPD) solution (100 ml) was also obtained using nanopure water.

The 24-well plate (Crychem) was removed from its sterile packaging and each of the 24 reservoirs was filled with 35% MPD solution (1 ml). Following this, one of the 24 solutions from the NAMS kit (6 μ l) was added to each of the 24 wells via micropipette, followed by the oligonucleotide (1 μ l, 1mM) and finally followed by the desired complex (1 μ l, 1mM). Once each of the wells had been loaded, the entire tray was sealed with transparent crystallographic sealant tape, thereby isolating the environment within each of the wells from the atmosphere and each other. The lid was then fitted and the tray was placed in a 4°C environment to slow the vapour diffusion process. A slower process generally yields more crystals of bigger size and higher quality.

Due to the precise nature of crystallography, and the extreme environmental sensitivity displayed by crystals, every precaution must be taken to minimise the possibility of contamination. As such, the Crychem 24-well plate is retrieved new from its sterile package after all the preparations have been made. Disposable pipette tips are used for the addition of the various solutions to the wells and only four of the wells are open to the environment at any one time. The remaining 20 are covered with the plate lid to reduce contamination.

Initially only four trays were set up with a view to crystallisation, detailed below in Table 7.1

	Complex	[Complex]	Oligonucleotide Sequence	[Oligonucleotide]	NAMS Solutions
Tray 1	Δ -Ru(TAP) ₂ dppzCl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 2	Λ -Ru(TAP) ₂ dppzCl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 3	Δ -Ru(TAP) ₂ dppzCl ₂	2 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 4	Λ -Ru(TAP) ₂ dppzCl ₂	2 mM	TCGGCGCCGA	1 mM	1 to 24

Table 7.1 – Initial crystallisation experiments with the enantiomers of [Ru(TAP)₂dppz]Cl₂

The trays were kept at 4°C and checked periodically. After a week crystals were discovered in the wells B5, B6, D2 and D3 of Tray 2. These wells corresponded to the NAMS solutions containing barium ions thus leading to the conclusion that barium ions are required for the crystallisation process. The details of which are discussed in Chapter 3.

- B5 – pH 6.0, 80 mM sodium chloride, 20 mM barium chloride
- B6 – pH 6.0, 80 mM potassium chloride, 20 mM barium chloride
- D2 – pH 7.0, 80 mM sodium chloride, 20 mM barium chloride
- D3 – pH 7.0, 80 mM potassium chloride, 20 mM barium chloride

Following the success with the Λ enantiomer, a series of trays were setup with a view to the crystallisation of various ruthenium polypyridyl complexes with a selection of oligonucleotides, utilising only the NAMS solutions containing barium ions. The details of these trays are presented in Table 7.2.

	Complex	[Complex]	Oligonucleotide Sequence	[Oligonucleotide]	NAMS Soltuions
Tray 5	Δ -Ru(TAP) ₂ dppzCl ₂	1 mM	TCGGCGCCGA CCGGCGCCGG CCGGTACCGG TCGGTACCGA CAGGTCTACC TGGGGT	1 mM	11 12 20 21
Tray 6	Δ -Ru(TAP) ₂ dppzCl ₂	0.5 mM	TCGGCGCCGA CCGGCGCCGG CCGGTACCGG TCGGTACCGA CAGGTCTACC TGGGGT	1 mM	11 12 20 21
Tray 7	Δ -Ru(TAP) ₂ dppzCl ₂	1 mM	CCGGCGCCGG	1 mM	1 to 24
Tray 8	Δ -Ru(TAP) ₂ dppzCl ₂	1 mM	CCGGTACCGG	1 mM	1 to 24
Tray 9	Δ -Ru(phen) ₃ Cl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 10	Δ -Ru(phen) ₃ Cl ₂	1 mM	CCGGCGCCGG	1 mM	1 to 24
Tray 11	Δ -Ru(phen) ₃ Cl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 12	Δ -Ru(phen) ₃ Cl ₂	1 mM	CCGGCGCCGG	1 mM	1 to 24
Tray 13	<i>rac</i> -Ru(phen) ₂ dppzCl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 14	<i>rac</i> -Ru(phen) ₂ dppzCl ₂	1 mM	CCGGCGCCGG	1 mM	1 to 24
Tray 15	<i>rac</i> -Ru(phen) ₂ dppzCl ₂	1 mM	TCGGTACCGA	1 mM	1 to 24
Tray 16	<i>rac</i> -Ru(phen) ₂ dppzCl ₂	1 mM	TCGGCGCCGA CCGGCGCCGG CCGGTACCGG TCGGTACCGA CAGGTCTACC TGGGGT	1 mM	11 12 20 21
Tray 17	<i>rac</i> -Ru(TAP)(dppz) ₂ Cl ₂	1 mM	TCGGCGCCGA	1 mM	1 to 24
Tray 18	<i>rac</i> -Ru(TAP)(dppz) ₂ Cl ₂	1 mM	CCGGCGCCGG	1 mM	1 to 24
Tray 19	<i>rac</i> -Ru(TAP)(dppz) ₂ Cl ₂	1 mM	TCGGTACCGA	1 mM	1 to 24

Table 7.2 - Crystallisation experiments with a variety of ruthenium complexes and oligonucleotide sequences

The trays were kept at 4°C and checked periodically. After a week crystals were discovered in Trays 1, 15 and 16. These crystals were of varying size and quality, as seen in Figure 3.58. No crystallisation results have thus far been obtained with these crystals due poor diffraction. The indication being that an optimisation of the complexes with the differing oligonucleotide sequences is required.

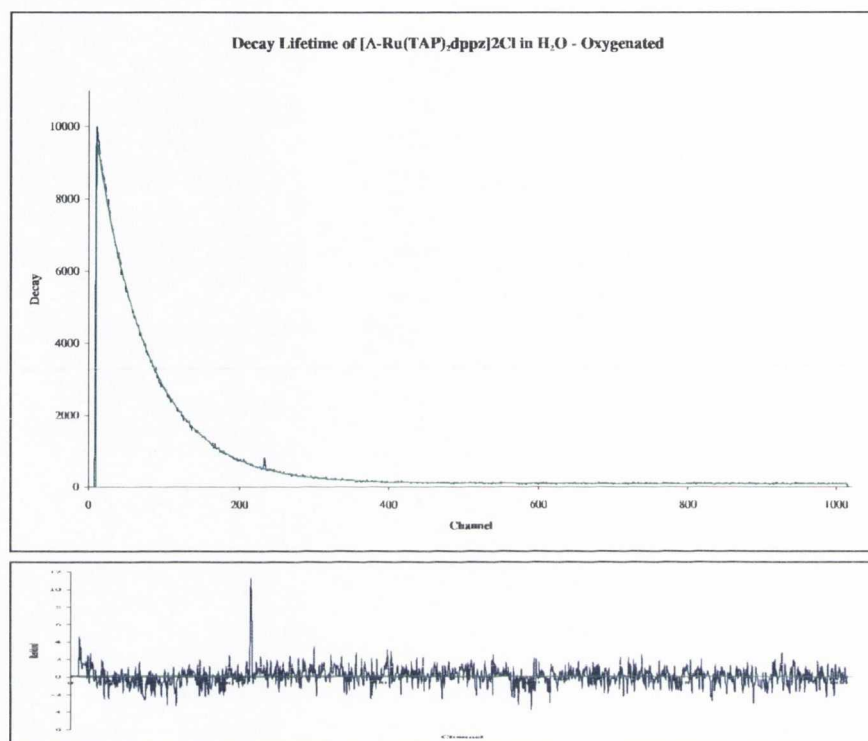
7.8 References

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Appendices

Appendix A – From Chapter 2

The following plots are the lifetimes obtained of the Λ - and Δ -[Ru(TAP)₂dppz]²⁺ enantiomers in water, both oxygenated and deoxygenated.



Calculated using 1 exponential

The initial paramters are:

Shift Value = Fixed @ 0 ch; 0 sec
T1 Estimate = 75.38141 ch; 7.342866E-07 sec

Prompt and decay LO = 11 ch; 1.071505E-07 sec

Prompt and decay HI = 1013 ch; 9.867584E-06 sec

Background on prompt = 0

Time calibration = 9.740951E-09 sec/ch

The fitted parameters are: SHIFT = 0 ch

T1 = 70.69042 ch; 7.93292E-07 sec S.Dev = 5.601795E-08 sec

A = 90.67992 S.Dev = 0.370613

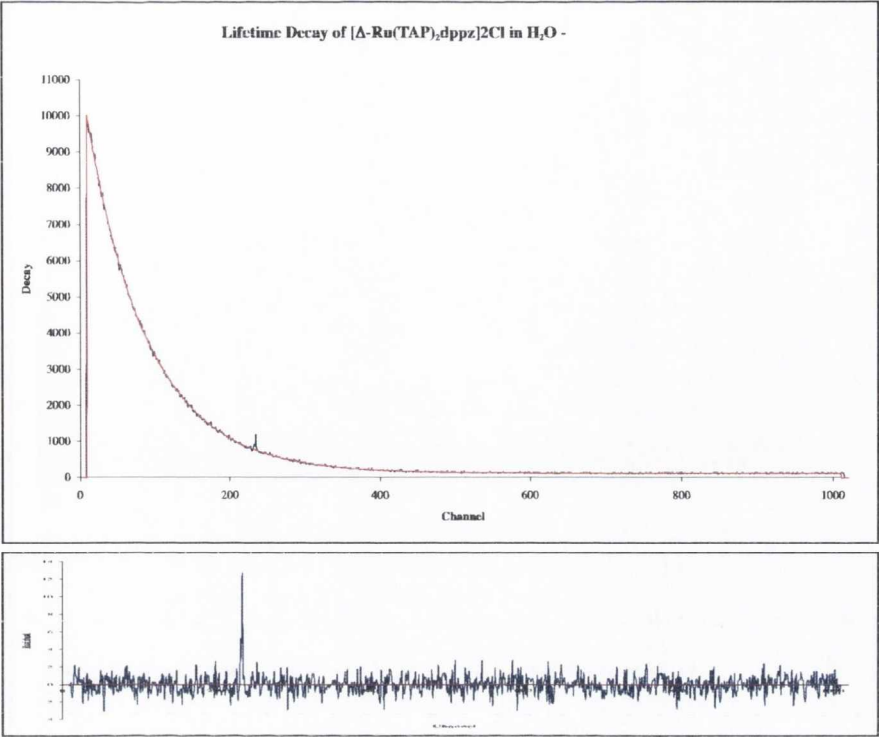
B1 = 9453.396 [100.00 Rel.Ampl] S.Dev = 11.92085

CHISQ = 1.318487 [1000 degrees of freedom]

Chi-squared Probability = 4.1393E-09 percent

Durbin-Watson Parameter = 1.689138

Figure A-1 – Lifetime of Λ -[Ru(TAP)₂dppz]Cl₂ in water (Oxygenated)



Calculated using 1 exponential

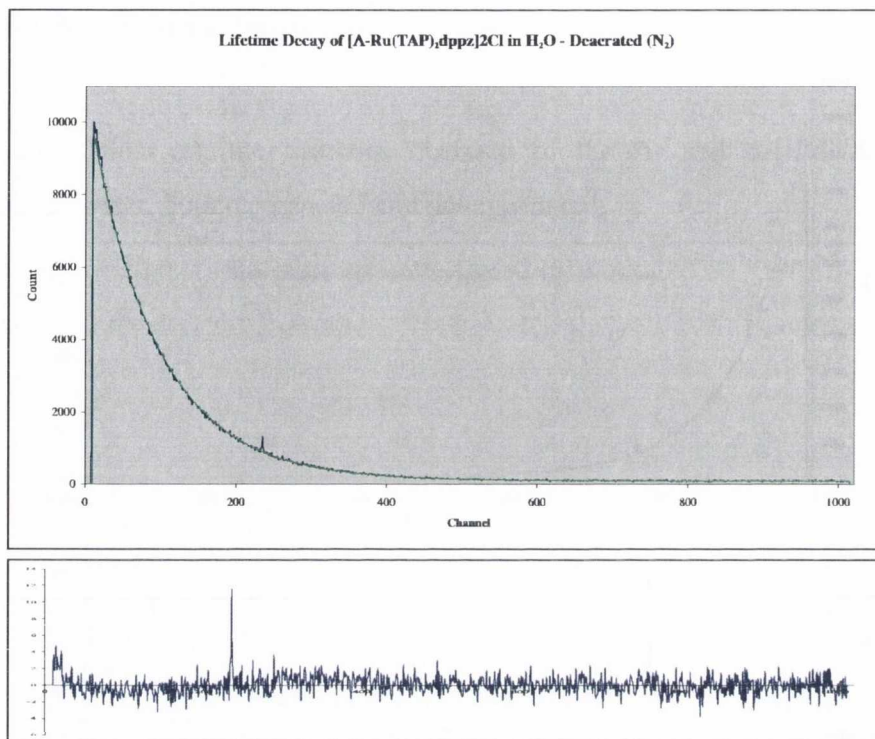
The initial paramters are:
Shift Value = Fixed @ 0 ch; 0 sec
T1 Estimate = 82.23273 ch; 8.010251E-07 sec
Prompt and decay LO = 10 ch; 9.740951E-08 sec
Prompt and decay HI = 1010 ch; 9.838361E-06 sec
Background on prompt = 0
Time calibration = 9.740951E-09 sec/ch

The fitted parameters are: SHIFT = 0 ch

T1 = 81.43044 ch; 8.012099E-07 sec S.Dev = 4.844778E-08 sec
A = 123.4714 S.Dev = 0.4456319
B1 = 9895.486 [100.00 Rel.Ampl] S.Dev = 11.48951
CHISQ = 1.187354 [998 degrees of freedom]

Chi-squared Probability = 3.6882E-03 percent
Durbin-Watson Parameter = 1.812192

Figure A-2 – Lifetime of Δ-[Ru(TAP)2dppz]Cl2 in water (Oxygenated)



Calculated using 1 exponential

Prompt data : (none)

Decay data : Decay

The initial paramters are:

Shift Value = Fixed @ 0 ch; 0 sec

T1 Estimate = 90.17448 ch; 9.183852E-07 sec

Prompt and decay LO = 11 ch; 1.071505E-07 sec

Prompt and decay HI = 1012 ch; 9.857843E-06 sec

Background on prompt = 0 (manual)

Time calibration = 9.740951E-09 sec/ch

The fitted parameters are:

SHIFT = 0 ch

T1 = 90.65387 ch; 9.20349E-07 sec S.Dev = 6.413241E-08 sec

A = 94.40298 S.Dev = 0.4087977

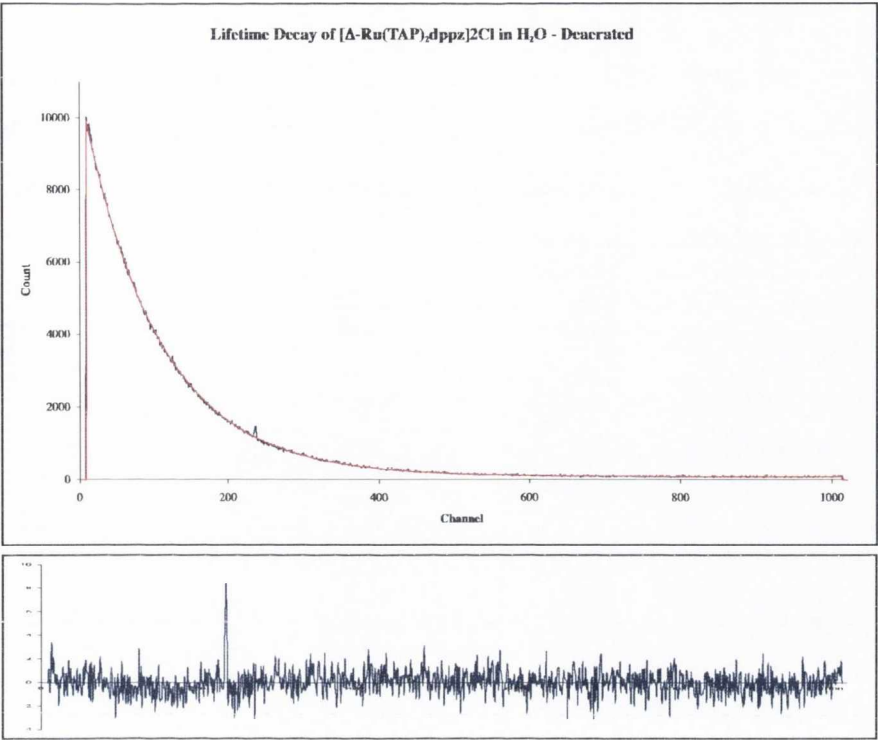
B1 = 9545.59 [100.00 Rel.Ampl] S.Dev = 10.655

CHISQ = 1.361171 [999 degrees of freedom]

Chi-squared Probability = 1.7190E-11 percent

Durbin-Watson Parameter = 1.66623

Figure A-3 – Lifetime of $\Lambda\text{-}[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ in water (De-oxygenated)



Calculated using 1 exponential

Prompt data : (none)
Decay data : Decay

The initial paramters are:
Shift Value = Fixed @ 0 ch; 0 sec
T1 Estimate = 93.87321 ch; 9.144144E-07 sec

Prompt and decay LO = 10 ch; 9.740951E-08 sec
Prompt and decay HI = 1012 ch; 9.857843E-06 sec

Background on prompt = 0 (manual)
Time calibration = 9.740951E-09 sec/ch

The fitted parameters are:
SHIFT = 0 ch

T1 = 102.4387 ch; 9.144505E-07 sec S.Dev = 5.830086E-08 sec
A = 91.517 S.Dev = 0.4263726
B1 = 9793.935 [100.00 Rel.Ampl] S.Dev = 10.18554
CHISQ = 1.183236 [1000 degrees of freedom]

Chi-squared Probability = 5.1067E-03 percent
Durbin-Watson Parameter = 1.825495

Figure A-4 – Lifetime of Δ-[Ru(TAP)2dppz]Cl2 in water (De-oxygenated)

The following plots are the Scatchard Plots resulting from the fluorescence data recorded from the various titrations of the Λ - and Δ -[Ru(TAP)₂dppz]²⁺ enantiomers. These were formulated following the McGhee von Hippel technique and allowed for the determination of the binding constants reported earlier in this thesis.

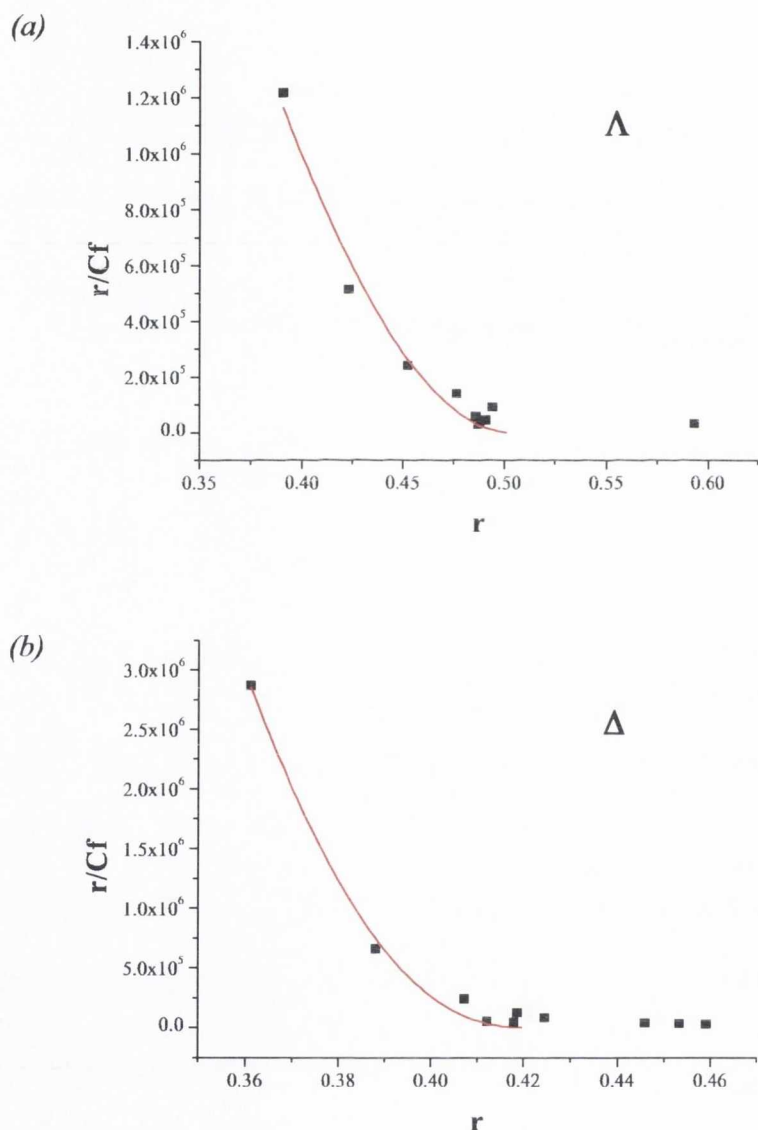


Figure A-5 – Scatchard plots of (a) Λ - and (b) Δ -[Ru(TAP)₂dppz]²⁺ binding to stDNA in 10 mM sodium phosphate buffer. Calculated from the fluorescence data and fitted using the McGhee von Hippel technique on OriginPro 8.0

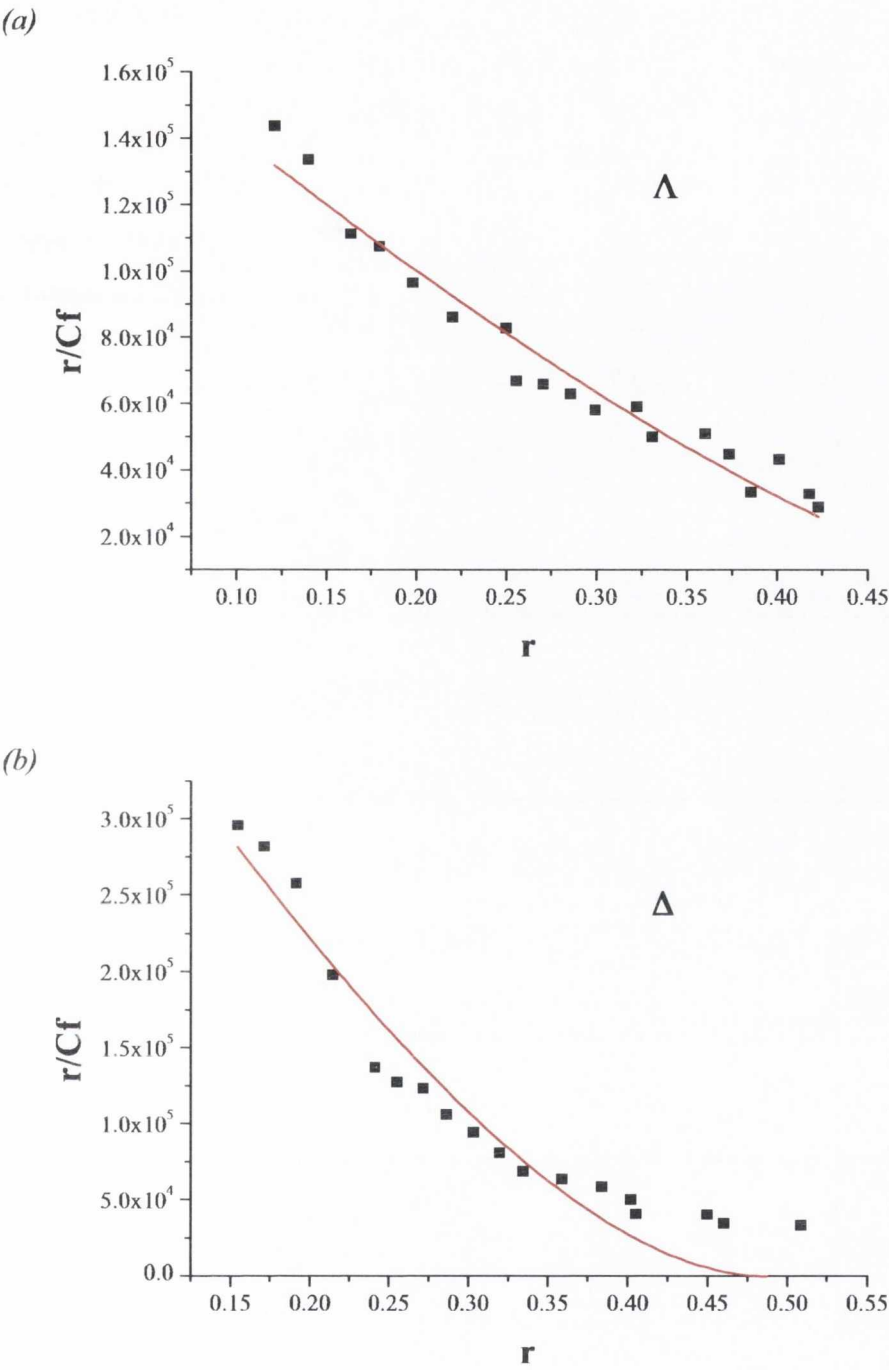


Figure A-6 – Scatchard plots of (a) Λ - and (b) Δ -[Ru(TAP)₂dppz]²⁺ binding to stDNA in 100 mM sodium phosphate buffer. Calculated from the fluorescence data and fitted using the McGhee von Hippel technique on OriginPro 8.0

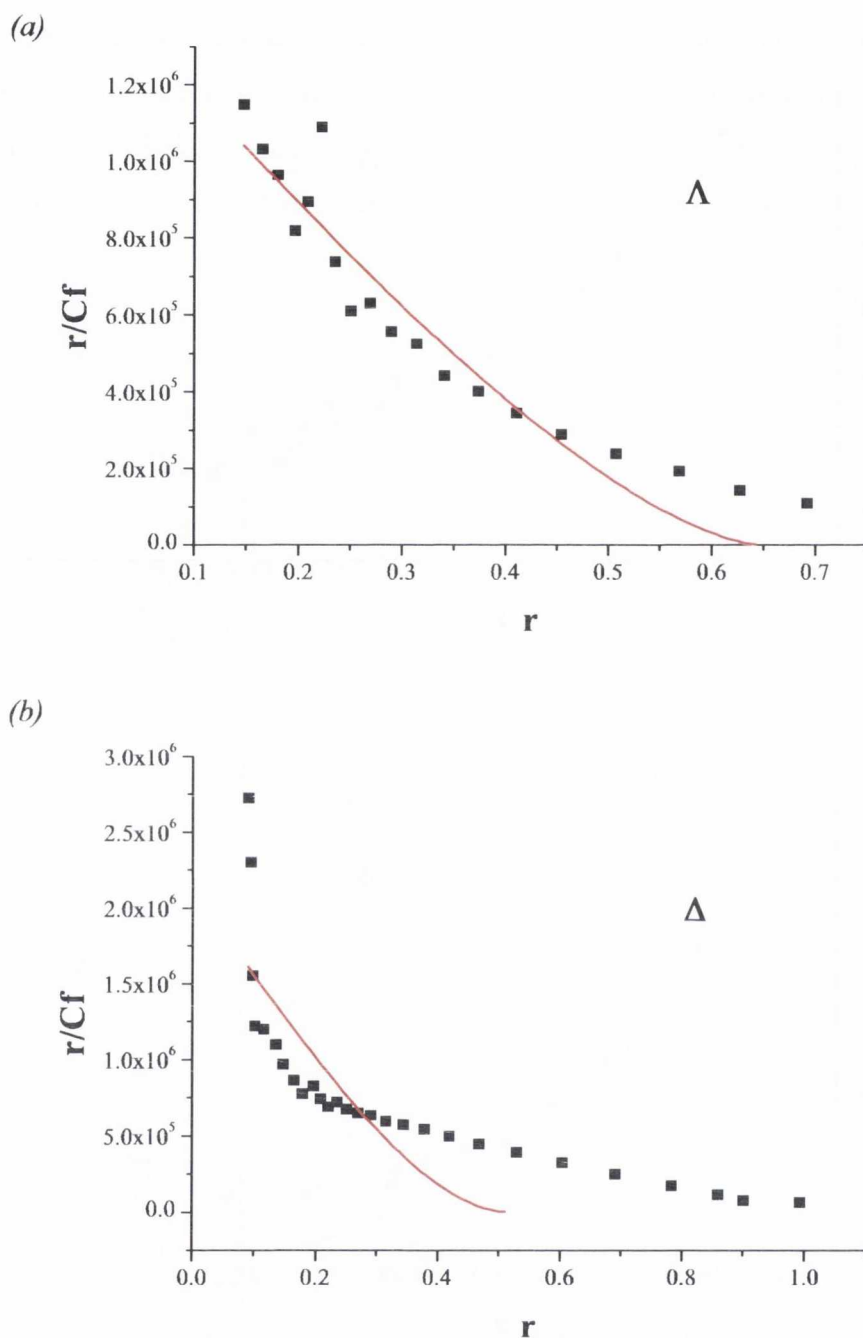


Figure A-7 – Scatchard plots of (a) Λ - and (b) Δ - $[\text{Ru}(\text{TAP})_2\text{dppz}]^{2+}$ binding to $[\text{poly}(\text{dG-dC})]$ in 10 mM sodium phosphate buffer. Calculated from the fluorescence data and fitted using the McGhee von Hippel technique on OriginPro 8.0

Appendix B – From Chapter 3

Figure B-1 contains the data of the Nucleic Acid Mini Screen (NAMS) from Hampton Research that is formulated to assist in the determination of preliminary crystallization conditions of nucleic acid fragments. Each Nucleic Acid Mini Screen kit contains 24 unique reagents, 1.0 ml each plus a 250 ml volume of dehydrant (35% v/v MPD). All solutions are formulated using ultra-pure Type 1 water and are sterile filtered.

Nucleic Acid Mini Screen™

HR2-118 Reagent Formulation

Tube Number	Precipitant	Tube Number	Buffer	Tube Number	Polyamine	Tube Number	Monovalent Ion	Tube Number	Divalent Ion
1.	10% v/v MPD	1.	40 mM Na Cacodylate pH 5.5	1.	20 mM Cobalt Hexamine	1.	None	1.	20 mM Magnesium Chloride
2.	10% v/v MPD	2.	40 mM Na Cacodylate pH 5.5	2.	20 mM Cobalt Hexamine	2.	80 mM Sodium Chloride	2.	20 mM Magnesium Chloride
3.	10% v/v MPD	3.	40 mM Na Cacodylate pH 5.5	3.	20 mM Cobalt Hexamine	3.	12 mM Sodium Chloride / 80 mM Potassium Chloride	3.	None
4.	10% v/v MPD	4.	40 mM Na Cacodylate pH 5.5	4.	20 mM Cobalt Hexamine	4.	40 mM Lithium Chloride	4.	20 mM Magnesium Chloride
5.	10% v/v MPD	5.	40 mM Na Cacodylate pH 6.0	5.	12 mM Spermine tetra-HCl	5.	80 mM Potassium Chloride	5.	20 mM Magnesium Chloride
6.	10% v/v MPD	6.	40 mM Na Cacodylate pH 6.0	6.	12 mM Spermine tetra-HCl	6.	80 mM Potassium Chloride	6.	None
7.	10% v/v MPD	7.	40 mM Na Cacodylate pH 6.0	7.	12 mM Spermine tetra-HCl	7.	80 mM Sodium Chloride	7.	20 mM Magnesium Chloride
8.	10% v/v MPD	8.	40 mM Na Cacodylate pH 6.0	8.	12 mM Spermine tetra-HCl	8.	80 mM Sodium Chloride	8.	None
9.	10% v/v MPD	9.	40 mM Na Cacodylate pH 6.0	9.	12 mM Spermine tetra-HCl	9.	80 mM Sodium Chloride / 12 mM Potassium Chloride	9.	20 mM Magnesium Chloride
10.	10% v/v MPD	10.	40 mM Na Cacodylate pH 6.0	10.	12 mM Spermine tetra-HCl	10.	12 mM Sodium Chloride / 80 mM Potassium Chloride	10.	None
11.	10% v/v MPD	11.	40 mM Na Cacodylate pH 6.0	11.	12 mM Spermine tetra-HCl	11.	80 mM Sodium Chloride	11.	20 mM Barium Chloride
12.	10% v/v MPD	12.	40 mM Na Cacodylate pH 6.0	12.	12 mM Spermine tetra-HCl	12.	80 mM Potassium Chloride	12.	20 mM Barium Chloride
13.	10% v/v MPD	13.	40 mM Na Cacodylate pH 6.0	13.	12 mM Spermine tetra-HCl	13.	None	13.	80 mM Strontium Chloride
14.	10% v/v MPD	14.	40 mM Na Cacodylate pH 7.0	14.	12 mM Spermine tetra-HCl	14.	80 mM Potassium Chloride	14.	20 mM Magnesium Chloride
15.	10% v/v MPD	15.	40 mM Na Cacodylate pH 7.0	15.	12 mM Spermine tetra-HCl	15.	80 mM Potassium Chloride	15.	None
16.	10% v/v MPD	16.	40 mM Na Cacodylate pH 7.0	16.	12 mM Spermine tetra-HCl	16.	80 mM Sodium Chloride	16.	20 mM Magnesium Chloride
17.	10% v/v MPD	17.	40 mM Na Cacodylate pH 7.0	17.	12 mM Spermine tetra-HCl	17.	80 mM Sodium Chloride	17.	None
18.	10% v/v MPD	18.	40 mM Na Cacodylate pH 7.0	18.	12 mM Spermine tetra-HCl	18.	80 mM Sodium Chloride / 12 mM Potassium Chloride	18.	20 mM Magnesium Chloride
19.	10% v/v MPD	19.	40 mM Na Cacodylate pH 7.0	19.	12 mM Spermine tetra-HCl	19.	12 mM Sodium Chloride / 80 mM Potassium Chloride	19.	None
20.	10% v/v MPD	20.	40 mM Na Cacodylate pH 7.0	20.	12 mM Spermine tetra-HCl	20.	80 mM Sodium Chloride	20.	20 mM Barium Chloride
21.	10% v/v MPD	21.	40 mM Na Cacodylate pH 7.0	21.	12 mM Spermine tetra-HCl	21.	80 mM Potassium Chloride	21.	20 mM Barium Chloride
22.	10% v/v MPD	22.	40 mM Na Cacodylate pH 7.0	22.	12 mM Spermine tetra-HCl	22.	40 mM Lithium Chloride	22.	80 mM Strontium Chloride / 20 mM Magnesium Chloride
23.	10% v/v MPD	23.	40 mM Na Cacodylate pH 7.0	23.	12 mM Spermine tetra-HCl	23.	40 mM Lithium Chloride	23.	80 mM Strontium Chloride
24.	10% v/v MPD	24.	40 mM Na Cacodylate pH 7.0	24.	12 mM Spermine tetra-HCl	24.	None	24.	80 mM Strontium Chloride / 20 mM Magnesium Chloride

Nucleic Acid Mini Screen contains twenty-four unique reagents. To determine the formulation of each reagent, simply read across the page.

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Solutions for Crystal Growth

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Figure B-1 – Nucleic acid mini screen formulations from Hampton Research

Appendix C – From Chapter 4

The following is the NMR spectra and subsequent calculations used to determine the extinction coefficient for the $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complex. The $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ complex, $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ complex and $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2/[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ mixed sample were dissolved in D_2O .

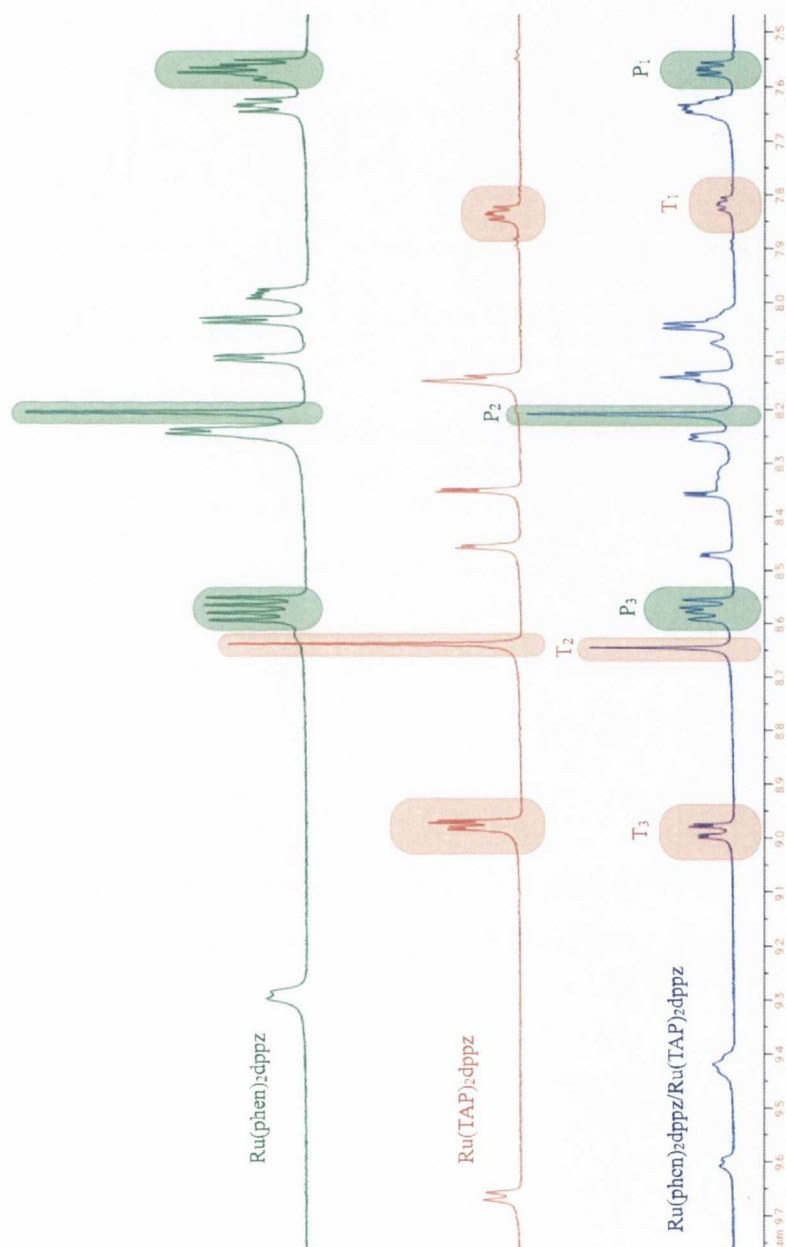


Figure C-1 – ^1H NMR spectra of $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$, $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ and $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2/[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ samples

	Integral Value	<u>Integral Value</u> Appropriate MW	Ratio TAP : Phen	Average Ratio
TAP 1 Signal	1.0000	1.1811x10 ⁻³	1 : 0.8819	1 : 0.8695
Phen 1 Signal	0.8486	1.0416x10 ⁻³		
TAP 2 Signal	0.9536	1.1263x10 ⁻³	1 : 0.8516	
Phen 2 Signal	0.8194	1.0058x10 ⁻³		
TAP 3 Signal	2.4762	2.9246x10 ⁻³	1 : 0.8749	
Phen 3 Signal	2.0846	2.5588x10 ⁻³		

Concentration of $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ in UV/vis sample = 2.175×10^{-5} M

$\therefore$ Concentration of $[\text{Ru}(\text{TAP})_2\text{dppz}]\text{Cl}_2$ in UV/vis sample = 2.501×10^{-5} M

Using the Beer-Lambert Law

$$A = \epsilon cl$$

$$A_{409} = 0.670 = \epsilon \cdot 2.501 \times 10^{-5} \cdot l$$

$$\epsilon = \frac{0.670}{2.501 \times 10^{-5}}$$

$$\epsilon = 26,800 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$$

Figure C-2 – Calculations determining the extinction coefficient of $[\text{Ru}(\text{TAP})_2(\text{Me}_2\text{dppz})]\text{Cl}_2$ from the NMR spectra

The following spectra are the Scatchard Plots resulting from the fluorescence data recorded from the various titrations of the Λ - and Δ -[Ru(TAP)₂(Me₂dppz)]²⁺ enantiomers. These were formulated following the McGhee von Hippel technique and allowed for the determination of the binding constants reported earlier in this thesis.

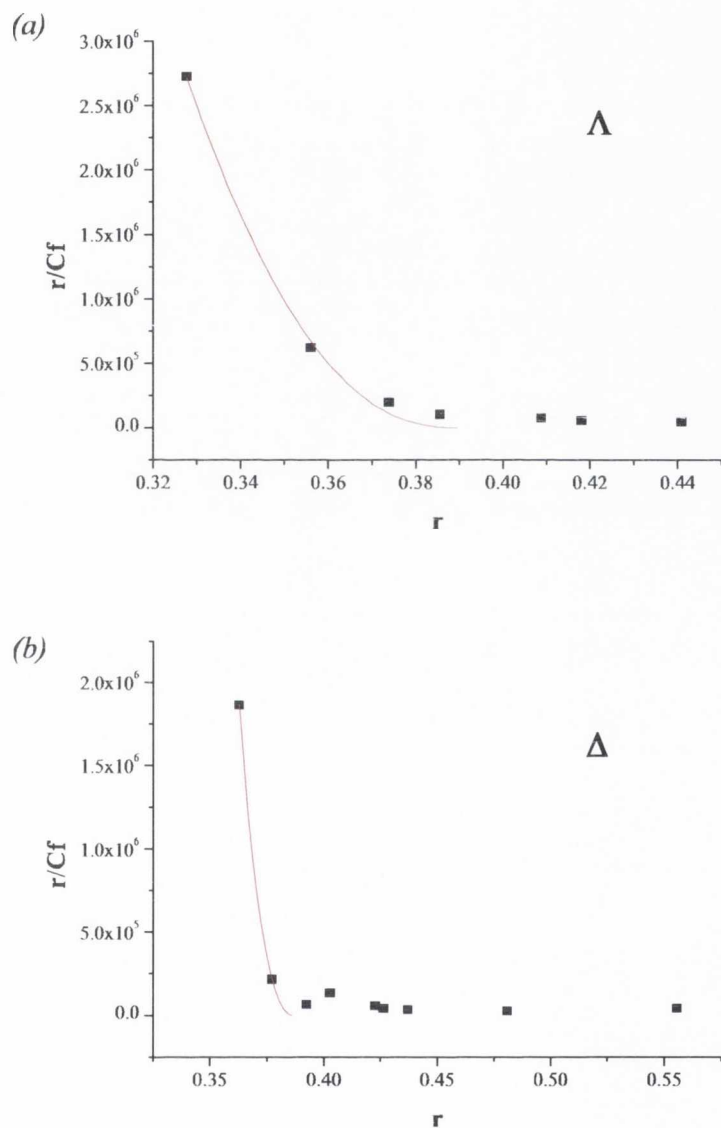


Figure C-3 – Scatchard plots of (a) Λ - and (b) Δ -[Ru(TAP)₂(Me₂dppz)]²⁺ binding to stDNA in 10 mM sodium phosphate buffer. Calculated from the fluorescence data and fitted using the McGhee von Hippel technique on OriginPro 8.0

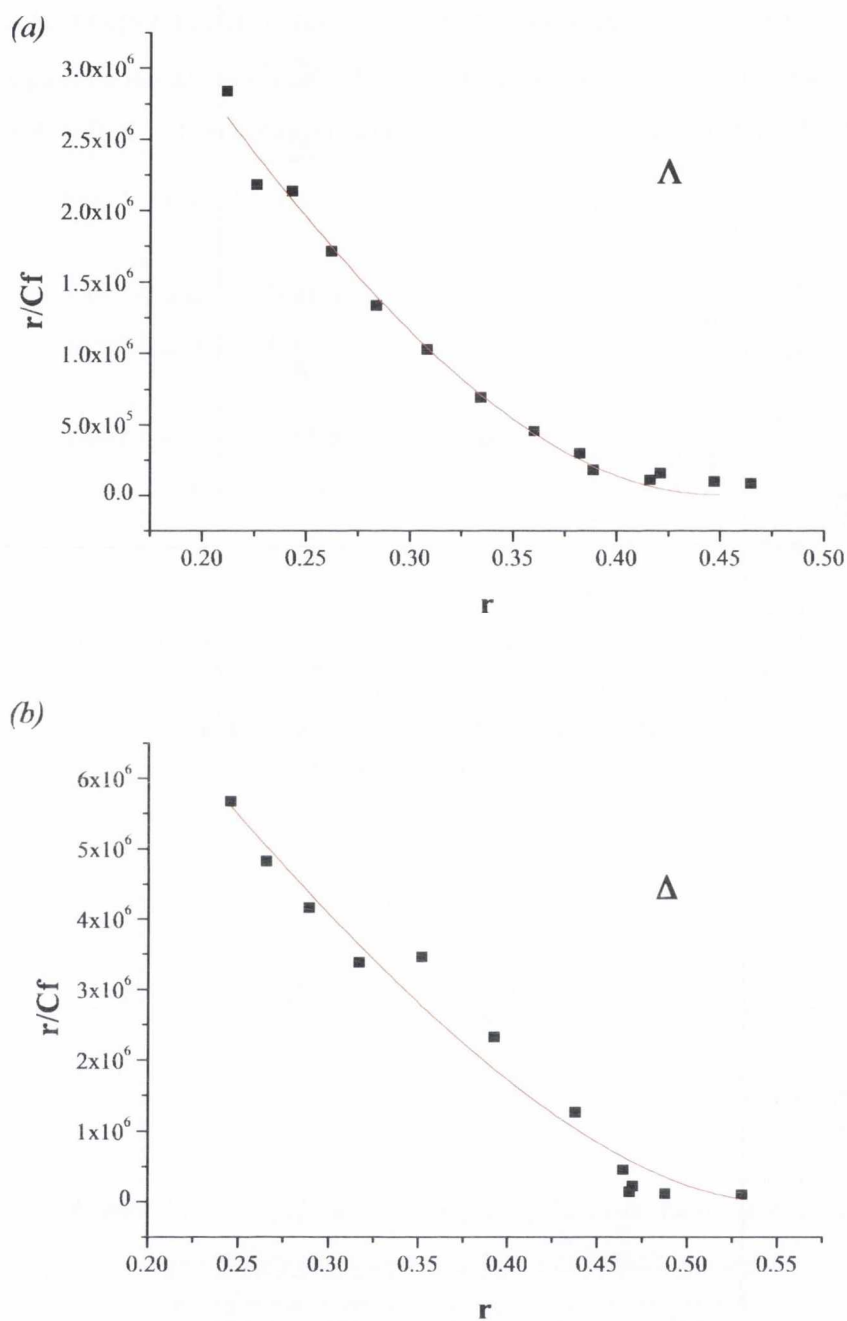


Figure C-4 – Scatchard plots of (a) Δ - and (b) Δ -[Ru(TAP)₂(Me₂dppz)]²⁺ binding to (poly[dG-dC]) in 10 mM sodium phosphate buffer. Calculated from the fluorescence data and fitted using the McGhee von Hippel technique on OriginPro 8.0

The following spectra are the Scatchard Plots resulting from the fluorescence data recorded from the various titrations of the Λ - and Δ -[Ru(TAP)₂(F₂dppz)]²⁺ enantiomers. These were formulated following the McGhee von Hippel technique and allowed for the determination of the binding constants reported earlier in this thesis.

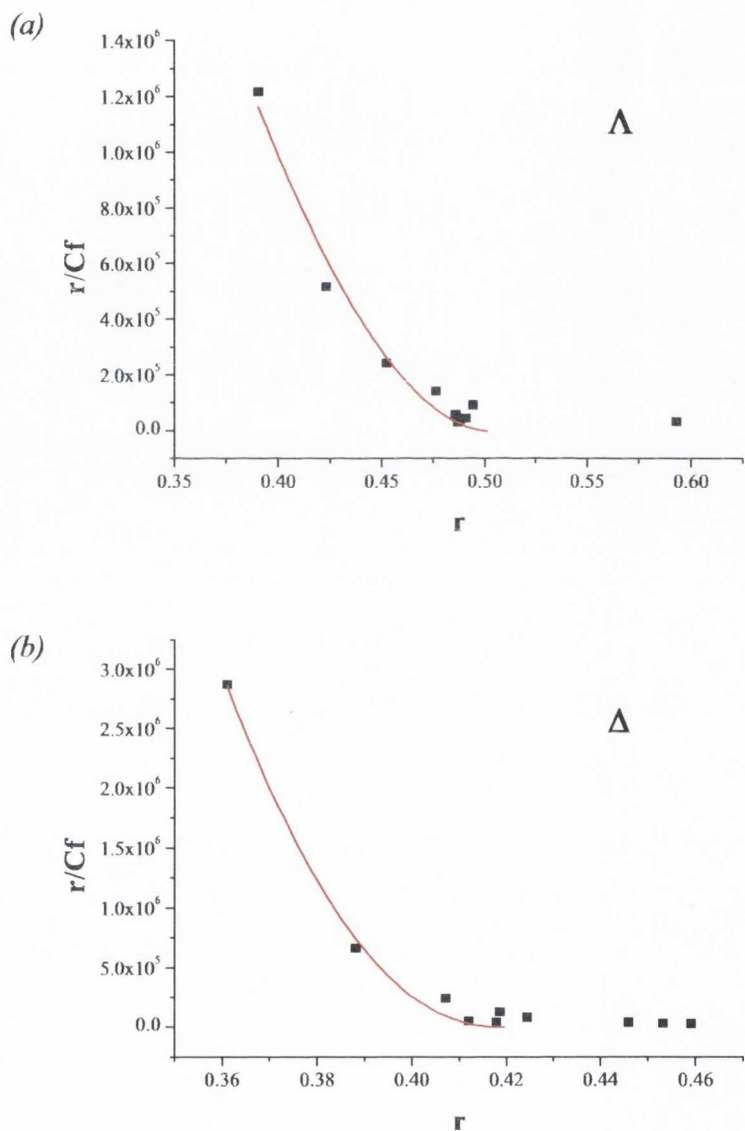


Figure C-5 – Scatchard plots of (a) Λ - and (b) Δ -[Ru(TAP)₂(F₂dppz)]²⁺ binding to stDNA in 10 mM sodium phosphate buffer. Calculated from the fluorecence data and fitted using the McGhee von Hippel technique on OriginPro 8.0