



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

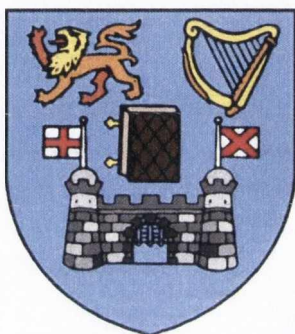
By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Diquaternerised and Ru(II) Based Polypyridyl Conjugates as Biological Probes and Phototherapeutic Agents

Robert B. P. Elmes

March 2011



**University of Dublin
Trinity College**

**Based on research carried out under the direction of
Prof. Thorfinnur Gunnlaugsson**

*A thesis submitted to the School of Chemistry,
University of Dublin, Trinity College for the degree of
Doctor of Philosophy*



Thesis 9615

Declaration

This Thesis is submitted for the degree of Doctor of Philosophy to the University of Dublin, Trinity College and has not been submitted before for any degree or examination to this or any other university. Other than where acknowledged, all work described herein is original and carried out by the author alone. Permission is granted so that the Library may lend or copy this Thesis upon request. This permission covers only single copies made for study purposes, subject to normal conditions of acknowledgment.

Rob

Acknowledgements

First of all, I would like to thank my supervisor, Professor Thorfinnur Gunnlaugsson for his endless enthusiasm, encouragement, advice, and support over the past three and a half years. Without you none of this would have been possible so Thorri, my friend, thank you so much!! I would also like to thank Professor Clive Williams for bringing the biological side of this project to life. With two people like Clive and Thorri behind it, this project was a pleasure to be a part of and I hope what has been started will continue for many years to come. I would also like to acknowledge the input of Dr. Susan Quinn and Professor John Kelly for wise words, helpful discussions and of course the use of various instruments.

A huge 'Thank you' must go to Dr. John O'Brien, Dr. Manuel Ruether, Dr. Dilip Rai, Dr Bernard St. Denis and Dr. Martin Feeney for all of their help. Special thanks must go to John for his help with NMR. With demanding aromatic structures no challenge was too much hassle, even at 5 o'clock on a Friday. Thanks John!

Thanks to all of the members of the Williams research group; Suzanne, Trevor, Kim and of course Luisa who has made my introduction to biochemistry so much fun and who has made my ability to 'relax' that bit easier! My thanks, of course, have to go to all members of the TG group, past and present, for all their encouragement and support over the last three and a half years, and especially during the writing of this Thesis. A big 'thanks' to Cidalia, Aline, Niamh, Danni, Gary, Doireann, Rebecca, Jen and Christophe. Thanks to Joe, Swagata and especially Dawn and Dave for their 'attention to detail' proof-reading, and to Steve and Oxana for endless advice. Thank you so much to Emma for reading my Introduction even when she had a much more important lady to deal with! I want to thank 'the best postdoc ever', Celia, for her help and encouragement throughout my PhD. Even though she needs to work on her 'Chinese' she is always a great source of amusement. Thanks to Stefano for his dancing and falling into large piles of leaves! Thanks to Jon for lots of proof reading of Chapters and for always being up for a couple of stress relieving Friday evening pints. Obviously special thanks have to go to Laura and Elaine who have been not only unbelievably helpful over the last few months and weeks but who have been great friends over the last two and a half years! Thanks Guys!! Of course thanks go to my fellow Dublin Chemistry classmates Brian and Sam for all the good times during our time as PhD students. Especially thanks to Brian for being there to share in my writing up woes and for putting up with my incessant ridiculous questions! My heading colours and margin layouts wouldn't have been the same without you!

I need to thank all of my friends who have made sure that chemistry hasn't entirely taken over my life over the past three and half years. Thanks to the Bear, Liam, Robbie, Ste and Kendrick without whom my life would have been extraordinarily boring. I can't thank my 'favourite person' Kate enough for looking after me so well for the last few years and making sure my stress levels didn't 'creep' up too much! Invariably they did but you were always on hand to relax me!

I want to thank D-More for all his financial assistance from day one! After seven and a half years, your long term investment is finally coming to fruition! Thanks to my brothers Steven, Simon and Pete, my new sister Lucy and my 'uncle' John who have all helped me in one way or another during my time as a student. My thanks also go to my 'supergran' who has looked after me since I was in nappies and continues to do so to this day!

Finally, I want to thank my Mum who has been there for me for as long as I can remember. Be it financial, emotional or moral support you have always been there for me! Mum I can't thank you enough!

Summary

This Thesis entitled ‘Diquaternerised and Ru(II) Based Polypyridyl Conjugates as Biological Probes and Phototherapeutic Agents’ is divided into 8 Chapters. Chapter 1, the introduction, gives a brief introduction to the cell cycle and its relevance to cancer treatment and is followed by a brief description of the structure of DNA and the different binding modes by which molecules may interact with it. A description of the photophysical properties of Ru(II) polypyridyl complexes and their DNA binding ability is also given and is supported by relevant examples from the literature. A brief introduction to photodynamic therapy (PDT) follows with particular emphasis on the applicability of certain Ru(II) conjugates. The introduction then deals with the excited state reactivity of the 1,8-naphthalimide chromophore and leads to a short review of gold nanoparticle (AuNP) conjugates and their applicability to biological use. This Chapter concludes with a description of ongoing research within the Gunnlaugsson research group and also describes the aims of the research conducted in each of the subsequent Chapters.

In Chapter 2, a series of Ru(II) complexes based on a new ligand, pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine, or pdppz, are described. The synthesis of each derivative is discussed, in addition to their photophysical and DNA binding properties. This is followed by an evaluation of their excited state reactivity with DNA and their cellular uptake and photocytotoxicity.

Chapter 3 deals with the ability of an organic quaternerised derivative of pdppz to bind to DNA. Due to its extended aromatic surface, in concert with its cationic character, this species is shown to possess rich photophysical properties that are monitored to evaluate its DNA binding interactions, using various spectroscopic techniques. The synthesis, photophysical evaluation, DNA binding and photocleavage ability, as well as its cellular uptake and cytotoxicity is discussed in this Chapter.

In Chapter 4, Ru(II)(TAP)₂-1,8-naphthalimide conjugates with a rigid arrangement of the subunits is discussed. The effect the inclusion of the π deficient TAP ancilliary ligands has on the photophysical and DNA binding properties of these conjugates is explored, where both the solution photophysical properties and the DNA binding responses are evaluated. Furthermore, their ability to photocleave DNA and be internalised in cancer cells is also discussed with a view to their potential as phototherapeutic agents.

Chapter 5 details the ability of Ru(II)(bpy)₃-1,8-naphthalimide conjugates to function as fluorescent anion sensors. The spectroscopic and colorimetric effects on the Ru(II) excited state upon anion binding as well as preliminary NMR binding data is detailed.

In Chapter 6, the synthesis and photophysical properties of two bis-Ru(II) (bpy)₃-bis-1,8-naphthalimide containing Tröger's base derivatives are described. The effect the substitution pattern around the Ru(II) centre has on the synthesis and photophysical properties of these moieties is explored, in addition to a preliminary description of their DNA binding ability and applicability to biological imaging.

Chapter 7 details the synthesis and photophysical evaluation of a series of alkyl thiol functionalised Ru(II) complexes and their ability to target DNA. This is followed by a description of their use in the synthesis of Ru(II) stabilised water-soluble, luminescent AuNP conjugates. The synthesis, physical and photophysical evaluation of each conjugate is discussed and is followed by preliminary DNA binding and cellular uptake studies.

Finally, in Chapter 8, general experimental procedures are outlined and the synthesis and characterisation of each of the compounds discussed is given. Subsequently, literature references are provided and are followed by the Appendix which provides spectroscopic and titration data to support work described in the main text.

Abbreviations

A	adenine
AcO ⁻	acetate (CH ₃ CO ₂ ⁻) anion
AuNP	Gold nanoparticle
br s	broad singlet
bpy	bipyridine
C	cytosine
CD	circular dichroism
conc.	concentrated
d	doublet
dd	doublet of doublets
dt	doublet of triplets
δ	chemical shift
DCM	dichloromethane
DLS	dynamic light scattering
DMAP	4-(N, N-dimethylamino)pyridine
DMF	<i>N</i> , <i>N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ε	molar extinction coefficient
eq.	equivalents
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ESI	electrospray ionisation
Et ₃ N	triethylamine
Et ₂ O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
G	guanine
HRMS	high resolution mass spectrometry
hrs	hours
Hz	hertz
IR	infrared
ISC	inter system crossing
<i>J</i>	coupling constant (expressed in Hz)

λ	wavelength
LD	linear dichroism
m	multiplet
MALDI-TOF	matrix assisted laser desorption ionisation, time of flight
MeCN	acetonitrile
MeOH	methanol
mins	minutes
MLCT	metal to ligand charge transfer
m.p.	melting point
m/z	mass charge ratio
nm	nanometre
NMR	nuclear magnetic resonance
PS	photosensitiser
PBS	phosphate buffered saline
Pd/C	palladium on carbon catalyst
PET	photoinduced electron transfer
phen	1,10-phenanthroline
ppm	parts per million
q	quartet
ROS	reactive oxygen species
s	singlet
SPR	surface plasmon resonance
stDNA	salmon testes DNA
t	triplet
T	thymine
TAP	1,4,5,8 tetraazaphenanthrene
TBA	tetra <i>n</i> -butylammonium
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
T _m	DNA thermal melting
TOAB	tetraoctylammonium bromide
UV/Vis	ultraviolet/visible

Contents

Chapter 1: Introduction	1
1.1 Cancer and DNA as a Target	1
1.2 DNA Structure	10
1.3 DNA Binding Interactions	12
1.3.1 Major Groove Recognition	13
1.3.2 Minor Groove Recognition	13
1.3.3 Electrostatic Binding	14
1.3.4 Covalent Bonding	15
1.3.5 Intercalation	16
1.4 Ruthenium(II) Polypyridyl Complexes	18
1.4.1 Photophysical Properties	18
1.4.2 Structurally Simple Ru(II) Complexes	20
1.4.3 Strategies to Increase DNA Binding Ability	20
1.5 Photodynamic Therapy and DNA - A Therapeutic Target	26
1.6 Ru(II) Complexes as DNA Photocleavage Agents	28
1.6.1 Singlet Oxygen Sensitisation	29
1.6.2 π - Deficient Ru(II) Polyazaaromatic Complexes	30
1.6.3 Ru(II) Polyazaaromatic Complexes with Appended Functional Species	32
1.7 1,8-Naphthalimides as DNA Photocleavage Agents	34
1.8 Gold Nanoparticles and Medical Application	39
1.8.1 Gold Nanoparticles Conjugates as PDT Agents	40
1.8.2 Transition Metal – Gold Nanoparticle Conjugates	42
1.9 Recent Advances within the Gunnlaussón Group	44
1.10 Conclusion	48
1.11 Work Described within this Thesis	50

Chapter 2: A Family of Ru(II) Complexes Based on a Novel Extended Aromatic Polypyridyl Ligand	47
2.1 Introduction:	46
2.2 Synthesis.....	48
2.2.1 Synthesis of TAP, 25 and pdppz, 52	48
2.2.2 Microwave Assisted Synthesis of Complexes 53-55	50
2.3 Photophysical Characterisation	54
2.3.1 Photophysical Characterisation of Ligand 52	54
2.3.2 Photophysical Characterisation of Complexes 53, 54 and 55	55
2.4 DNA Binding Interactions.....	58
2.4.1 Spectroscopic Titrations of 53-55 with DNA.....	59
2.4.2 Spectroscopic Titrations of 53-55 with DNA at High Ionic Strength	64
2.4.3 Spectroscopic Titrations of 53-55 with Synthetic Homopolymer Polynucleotides	70
2.4.4 Ethidium Bromide Displacement Assays of 53 – 55	78
2.4.5 Thermal Denaturation Studies of 53 – 55	79
2.4.6 Circular Dichroism Studies of 53 – 55 in the Presence of DNA.....	81
2.4.7 Linear Dichroism Studies of 53 – 55 in the Presence of DNA	83
2.4.8 Summary of DNA Binding Studies	85
2.5 Singlet Oxygen Sensitisation Ability of 53 -55	87
2.6 DNA Photocleavage efficiency of 53 – 55	88
2.7 Cellular Uptake and Localisation Studies of 54 and 55	91
2.7.1 Confocal Microscopy Studies of 54 and 55	92
2.7.2 Transmission Electron Microscopy Studies of 55	95
2.8 Cellular Phototoxicity Studies of 54 and 55	96
2.9 Conclusions and Future Perspectives	98
Chapter 3: An Organic pdppz Derivative as a DNA Probe and Photocleavage Agent ..	101
3.1 Introduction.....	101
3.2 Synthesis and Characterisation of 67	103

3.3 Photophysical Characterisation of 67	104
3.4 DNA Binding Interactions	105
3.4.1 UV/Vis Absorption Titrations of 67 with DNA	105
3.4.2 Fluorescence Emission Titrations of 67 with DNA	107
3.4.3 UV/Vis Absorption Titrations of 67 with Synthetic Homopolymer Polynucleotides...	110
3.4.4 Fluorescence Emission Titrations of 67 with Synthetic Homopolymer Polynucleotides	112
3.4.5 Ethidium Bromide Displacement Assays of 67	114
3.4.6 Thermal Denaturation Studies with 67	115
3.4.7 Linear and Circular Dichroism Studies with 67	116
3.4.8 Summary of DNA Binding Studies of 67	117
3.5 DNA Photocleavage Efficiency Studies of 67	118
3.6 Confocal Microscopy Cellular Localisation Study of 67	120
3.7 Preliminary Cellular Toxicity Studies with 67	122
3.8 Conclusions and Future Perspectives	123
Chapter 4: Π-Deficient Rigid Ru(II)-mono-1,8-Naphthalimide Conjugates	124
4.1 Introduction	124
4.2 Synthesis	126
4.2.1 Synthesis of nitro-1,8-naphthalimide substituted bipyridine 72	126
4.2.2 Complexation of 72 with Ru(TAP) ₂ Cl ₂	128
4.3 Photophysical Characterisation	131
4.4 DNA Binding Interactions of 70 and 71	133
4.4.1 Spectroscopic Titrations of 70 and 71 with DNA	134
4.4.2 Spectroscopic Titrations of 70 and 71 with DNA at High Ionic Strength	138
4.4.3 Spectroscopic Titrations of 70 and 71 with Synthetic Homopolymer Polynucleotides	143
4.4.4 Ethidium Bromide Displacement Assays of 70 and 71	149
4.4.5 Thermal Denaturation Studies of 70 and 71	150

4.4.6 Circular Dichroism Studies of 70 and 71 in the Presence of DNA.....	151
4.4.7 Linear Dichroism Studies of 70 and 71 in the Presence of DNA	152
4.4.8 Summary of DNA Binding Studies of 70 and 71	153
4.5 DNA Photocleavage efficiency of 70 and 71	155
4.6 Preliminary Cellular Uptake and Localisation Studies of 70 and 71	157
4.7 Conclusions and Future Work.....	159
Chapter 5: A Ru(II)-Mono-1,8-Naphthalimide Conjugate for Colorimetric and Luminescence Anion Sensing	161
5.1 Introduction:	161
5.2 Photophysical Evaluation of 48	163
5.3 Spectroscopic Titrations of 48 with Anions	164
5.3.1 Preliminary UV/Vis Absorption and Emission Titration of 48 with Anions	164
5.3.2 Absorption and Fluorescence Titrations of 48 with AcO ⁻	166
5.3.3 Absorption and Fluorescence Titrations of 48 with H ₂ PO ₄ ⁻	168
5.3.4 Absorption and Fluorescence Titrations of 48 with Cl ⁻	169
5.3.5 Absorption and Fluorescence Titrations of 48 with F ⁻	170
5.3.6 Discussion of Spectroscopic Titrations of 48 with Anions	173
5.4 ¹ H NMR Studies of 48 with Anions	176
5.5 Conclusions and Future Perspectives	177
Chapter 6: Synthesis, Photophysical and DNA Binding Studies of Ru(II) 4-Amino-1,8-Naphthalimide Derived Tröger's Bases.....	179
6.1 Introduction.....	179
6.2 Synthesis of 89 and 90	182
6.2.1 Synthesis of bipyridine functionalised 4-amino-1,8-naphthalimide precursors	183
6.2.2 Synthesis of bipyridine functionalised 4-amino-1,8-naphthalimide containing Tröger's bases.....	184
6.2.3 Complexation of 100 and 101 with Ru(bpy) ₂ Cl ₂	186
6.3 Photophysical Characterisation of 89 and 90	189
6.3.1 Photophysical properties of ligands 100 and 101	189

6.3.2 Photophysical Characterisation of Complexes 89 and 90	190
6.4 DNA Binding Interactions of 89 and 90	192
6.4.1 Spectroscopic Titrations of 89 and 90 with DNA.....	192
6.4.2 Thermal Denaturation Studies of 89 and 90	194
6.5 Preliminary Cellular Uptake and Localisation Studies of 89 and 90	197
6.6 Conclusions and Future Perspectives	198
Chapter 7: Luminescent Ru(II) Polypyridyl Functionalised Gold Nanoparticles	201
7.1 Introduction.....	201
7.2 Synthesis of Complexes 105 - 107	203
7.2.1 Synthesis of alkyl thiol functionalised phenanthroline 108	204
7.2.2 Synthesis of alkyl thiol functionalised Ru(II) complexes 105 – 107	205
7.3 Photophysical Characterisation of 105 - 107	208
7.4 DNA Binding Interactions of 105 , 106 and 107	210
7.4.1 Spectroscopic Titrations of 105 - 107 with DNA.....	210
7.4.2 Ethidium Bromide Displacement Assays	215
7.4.3 Thermal Denaturation Studies of 105 – 107	216
7.4.4 Circular Dichroism Studies of 105 – 107 in the Presence of DNA.....	217
7.4.5 Summary of DNA Binding Studies of 105 – 107	218
7.5 Synthesis of Ru(II) Functionalised AuNP-105 , AuNP-106 and AuNP-107	219
7.6 Characterisation of Ru(II) Functionalised AuNP-105 , AuNP-106 and AuNP-107	221
7.6.1 Dynamic Light Scattering Studies of AuNP-105 , AuNP-106 and AuNP-107	221
7.6.2 Transmission Electron Microscopy Studies of AuNP-105 , AuNP-106 and AuNP-107	222
7.7 Photophysical Studies of Ru(II) Functionalised AuNP-105 , AuNP-106 and AuNP-107	223
7.8 DNA Binding Interactions of AuNP-105 , AuNP-106 and AuNP-107	227
7.8.1 Spectroscopic Titrations of AuNP-105 , AuNP-106 and AuNP-107 with DNA.....	227
7.8.2 Ethidium Bromide Displacement Assays	231
7.8.3 Thermal Denaturation Studies of AuNP-105 , AuNP-106 and AuNP-107	232

7.8.4 Circular Dichroism Studies of AuNP-105 , AuNP-106 and AuNP-107 in the Presence of DNA	233
7.8.5 DNA Induced Aggregation of AuNP-105 , AuNP-106 and AuNP-107 Observed by Confocal Microscopy	234
7.8.6 Summary of DNA Binding Studies of AuNP-105 , AuNP-106 and AuNP-107	236
7.9 DNA Photocleavage Efficiency of AuNP-105 , AuNP-106 and AuNP-107	237
7.10 Cellular Uptake and Localisation Studies of AuNP-105 , AuNP-106 and AuNP-107 ..	240
7.10.1 Confocal Microscopy Cellular Localisation Study of AuNP-105 , AuNP-106 and AuNP-107	240
7.10.2 TEM Cellular Localisation Study of AuNP-105 , AuNP-106 and AuNP-107	242
7.11 Overall Conclusions and Future Perspectives	243
Chapter 8: Experimental Section	251
8.1 General Experimental Techniques	246
8.1.1 Calculation of binding constants from UV/Vis Titration Data	248
8.1.2 Calculation of binding constants from Fluorescence Titration Data.....	248
8.1.3 Calculation of binding constant from ethidium bromide displacement assays	248
8.2 General Biological Procedures.....	248
8.4 Materials.....	248
8.5 General Synthetic Procedures	248
Procedure 1: Complexation of pdppz ligand, 52 with Ru(L-L) ₂ Cl ₂	248
Procedure 2: Chalcone formation.....	248
Procedure 3: 4-(nitrophenyl)-2,2'-bipyridine formation.....	248
Procedure 4: 4-(nitrophenyl)-2,2'-bipyridine reduction.....	248
Procedure 5: 4-[N-phenyl-4-nitro-1,8-naphthalimide]-2,2'-bipyridine formation	248
Procedure 6: 4-[N-phenyl-4-nitro-1,8-naphthalimide]-2,2'-bipyridine reduction	248
Procedure 7: Formation of Bis-1,8-naphthalimide containing Tröger's base derivatives	248
Procedure 8: Complexation of Bis-1,8-naphthalimide containing Tröger's base derivatives with Ru(bpy) ₂ Cl ₂	248

Procedure 9: Complexation of alkyl thiol functionalised 1,10-phenanthroline ligand with $\text{Ru}(\text{L-L})_2\text{Cl}_2$	248
Procedure 10: Synthesis of $\text{Ru}(\text{II})$ stabilised AuNPs	248
8.6 Synthesis and characterisation of compounds described in Chapter 2	248
8.7 Synthesis and characterisation of compounds described in Chapter 3	248
8.8 Synthesis and characterisation of compounds described in Chapter 4	248
8.9 Synthesis and characterisation of compounds described in Chapter 6	248
8.10 Synthesis and characterisation of compounds described in Chapter 7	248
Chapter 9: References	272
Appendices	285
Appendix 2	285
Appendix 3	301
Appendix 4	303
Appendix 5	314
Appendix 6	317
Appendix 7	323
Publications	341

Chapter 1:

Introduction

Since the discovery of the deoxyribonucleic acid (DNA) double helix, there has been considerable interest in the development of molecules that selectively bind DNA and that exhibit photophysical properties sensitive to the binding event. Such species show significant potential as nucleic acid probes, diagnostic agents and novel therapeutic agents. Moreover, through synthetic design, the development of molecules that have the ability to recognise specific sequences on DNA and also that can be activated photochemically has become an intensively studied area of research.

This thesis focuses on the interaction and reaction of spectroscopically active molecules with DNA and evaluates their potential for biological application in areas such as imaging and photoactivated cancer therapies. A number of approaches have been employed to enhance both DNA binding affinity and phototoxic effect mainly involving the use of Ru(II) polypyridyl based species. This Chapter will look to introduce the various topics discussed in this thesis and starts with a brief discussion of the relationship between cancer and the biochemical processes involved in the cell cycle. This will be followed by a short description of DNA structure and the factors governing its recognition, followed by a discussion of some relevant examples of small DNA binding molecules from the literature. The remainder of this Chapter will review some recent development in the areas of Ru(II) polypyridyl complexes, 1,8-naphthalimides and gold nanoparticles (AuNPs) and will finish with a review of selected work undertaken within the Gunnlaugsson group.

1.1 Cancer and DNA as a Target

Cancer is a leading cause of death worldwide accounting for 7.9 million deaths (around 13% of all deaths) in 2007 and, furthermore, deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths expected in 2030.¹ The name cancer is a broad term used to describe more than 200 diseases that can affect any part of the body. It is a disease of the cell that occurs due to failures in the mechanisms that regulate cell growth and division. The mechanisms by which cancer arises are generally considered to be as a result of changes to the genome that may lead to abnormal gene regulation, failure of expression of genes or alteration of a gene, all leading to abnormal cell function. The transformations that occur are then passed on from the individual cells to its progeny resulting in the accumulation of cells with abnormal function.²

Cell proliferation itself is a complex cellular process that is regulated by a balance between growth stimulating and growth suppressing factors.³ Each dividing cell goes through a complex sequence of events that results in the formation of two daughter cells. This series of events is collectively termed the cell cycle and a diagrammatic representation is shown in Figure 1.1.

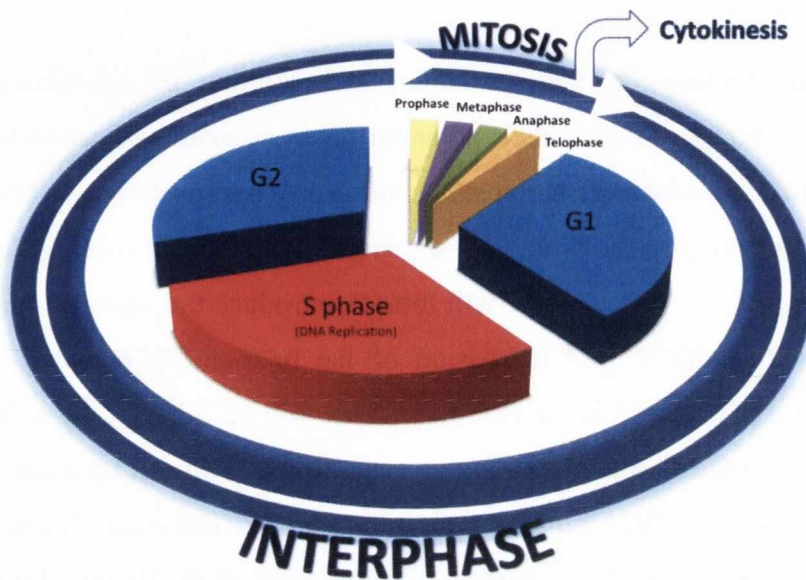


Figure 1.1 Schematic representation of the cell cycle

The major phases of the cell cycle are G_0 , G_1 , S , M and G_2 .⁴ Resting cells are in the G_0 phase and healthy cells spend most of their time in this phase. In a dividing cell, the mitotic (M) phase alternates with interphase; a growth period. The first part of interphase, called G_1 , is induced by external growth factors and is followed by S phase, when the chromosomes replicate; the last phase is G_2 and, like G_1 , is a gap phase in which the cell grows by producing proteins and cytoplasmic organelles in preparation for the final mitotic (M) phase; cell division.⁴ Briefly, M phase is divided into 4 main phases; (i) prophase (incorporating prometaphase), (ii) metaphase, (iii) anaphase and (iv) telophase. During these stages of mitosis the chromosomes condense, align and separate before the cell elongates leading, finally, to division of the cell cytoplasm and separation (cytokinesis), (Figure 1.2).⁴ Activated checkpoints within the cell cycle give cells time to repair any damage to the genome that may have occurred. However, if damage is not corrected cells will undergo apoptosis, which is a form of programmed cell death. This process prevents any mutations being carried through to progeny cells.³ Often cancer cells find a way to circumvent the apoptotic mechanism resulting in cell accumulation and tumor progression where these defects in regulatory circuits lead to the breakdown of normal cellular proliferation and homeostasis.⁵

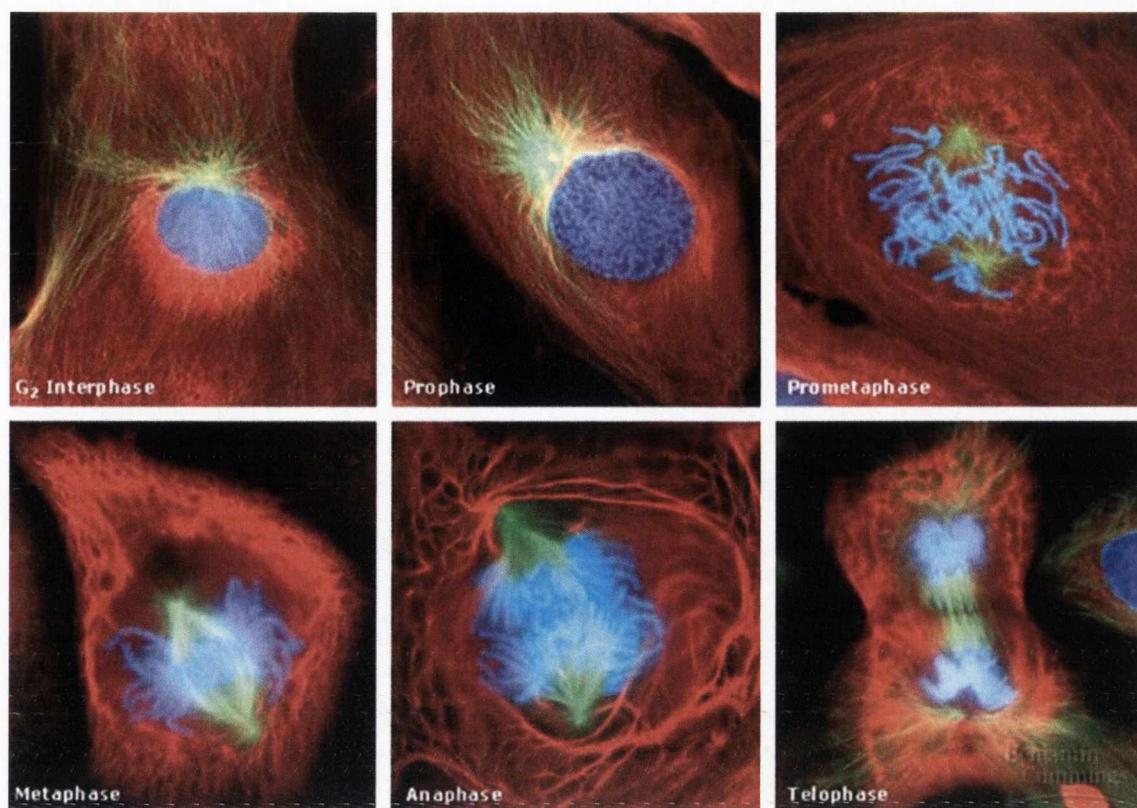


Figure 1.2 Confocal microscopy images showing G_2 interphase and the different stages of mitosis; Prophase, Prometaphase, Metaphase, Anaphase and Telophase ⁴

Much effort within medicinal chemistry has been devoted towards the development of anti-cancer drugs for the treatment of tumors. Chemotherapy is the use of cytotoxic compounds to kill or inhibit rapidly proliferating cancer cells in preference to the body's own healthy cells.⁶ Such cytotoxic agents can take effect by inhibiting any of the processes that take place during the cell cycle.

On a molecular level, cancers are expressed through protein action but actually originate at the deoxyribonucleic acid (DNA) level, in the code or in its processing. The information amplification from DNA to proteins, makes DNA a particularly attractive medicinal target, since in principle, a single drug molecule per cell could change the expression of a gene on DNA where multiple drug molecules per cell are required to act on the multiple copies of proteins.⁷ Thus DNA is extremely important in the design and development of novel anti-cancer therapeutics and consequently it continues to be one of the main molecular targets in medicinal chemistry. The regulation of gene expression is central to all processes that occur in the body and targeting of DNA with small or medium molecules selective for certain gene sequences will enable the specific regulation of gene expression, important in many fields of medical research for new cancer therapies and novel drug design.⁸

1.2 DNA Structure

The discovery of the right handed double helix, by Watson and Crick in April 1953,⁹ immediately provided fundamental new insights into the nature of genetic events. It presented more profound levels of understanding of the processes of gene regulation, transcription and translation, mutation/carcinogenesis, and drug action at the atomic and molecular levels. These advances in nucleic acid structural studies have given rise to knowledge that is now being exploited in various fields of medicine for both therapeutic and diagnostic ends.

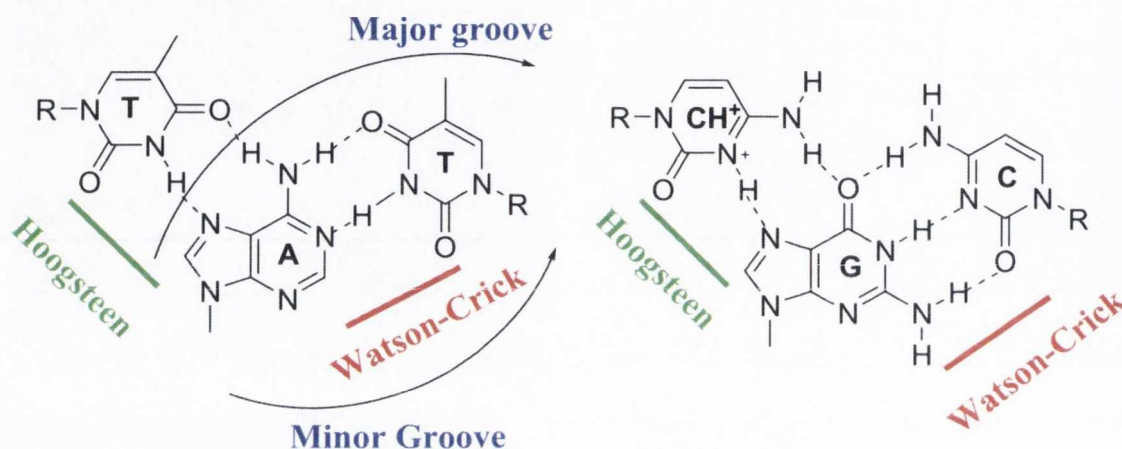


Figure 1.3 Structures of the DNA bases showing the Watson-Crick and Hoogsteen base pairs and position of the grooves

DNA is a biological polymer consisting of two strands arranged in a right handed anti-parallel double helix. The strands consist of a number of heterocyclic nitrogenous bases linked by a negatively charged sugar - phosphate backbone. Unlike most proteins, which can be made up from a pool of 20 different amino acids, DNA primarily contains 4 bases; the purines - *adenine* (A) and *guanine* (G), and the pyrimidines - *thymine* (T) and *cytosine* (C), (Figure 1.3).

Each base contains numerous hydrogen bond donor and acceptor sites which allow for specific hydrogen bonding interactions to occur between bases on opposite nucleotide chains, known as Watson-Crick base pairing. According to the Watson-Crick base pairing rules *adenine* always pairs with *thymine* and *guanine* always pairs with *cytosine*. Appropriate geometrical correspondence of hydrogen bond donors and acceptors ensures only the "right" pairs form stably and it is on this premise that DNA-binding enzymes can recognise specific base pairing patterns that identify particular regulatory regions of genes. Hoogsteen base pairing can also occur with the functional groups that protrude into the major groove giving rise to the formation of secondary structures of DNA such as triplex DNA and G quadruplex DNA (Figure 1.3). Base stacking effects are also especially important in the secondary

structure of DNA. Base stacking interactions are due to dispersion attraction, short-range exchange repulsion, and electrostatic interactions. Where base pairing provides specificity in DNA structure it is these stacking interactions and hydrophobic forces that provide the primary means by which the helix is stabilised.¹⁰⁻¹² B-DNA, the classic helical structure described by Watson and Crick, describes the right handed anti-parallel double helix of DNA structure. However, there also exists a number of other forms that depend very much on environmental conditions such as humidity, temperature, pH, and salt concentration. The structure initially derived for B-DNA was from X-ray diffraction patterns of DNA fibres at 92% relative humidity.⁹ When the relative humidity is reduced to 75 %, B-DNA may undergo a reversible conformational change to A-DNA. This structure is again a right handed helix but is somewhat wider than that of B-DNA. A third conformation is the left handed double helix Z-DNA, which forms for complementary polynucleotides with alternating *guanine* and *cytosine* residues at high salt concentration. The backbone of Z-DNA is irregular compared to A- or B-DNA since the residues have very distinct conformations, resulting in a 'zig-zag' arrangement of phosphate groups. The structural features of all three conformations are summarised in Table 1.1,¹² and depicted in Figure 1.4.

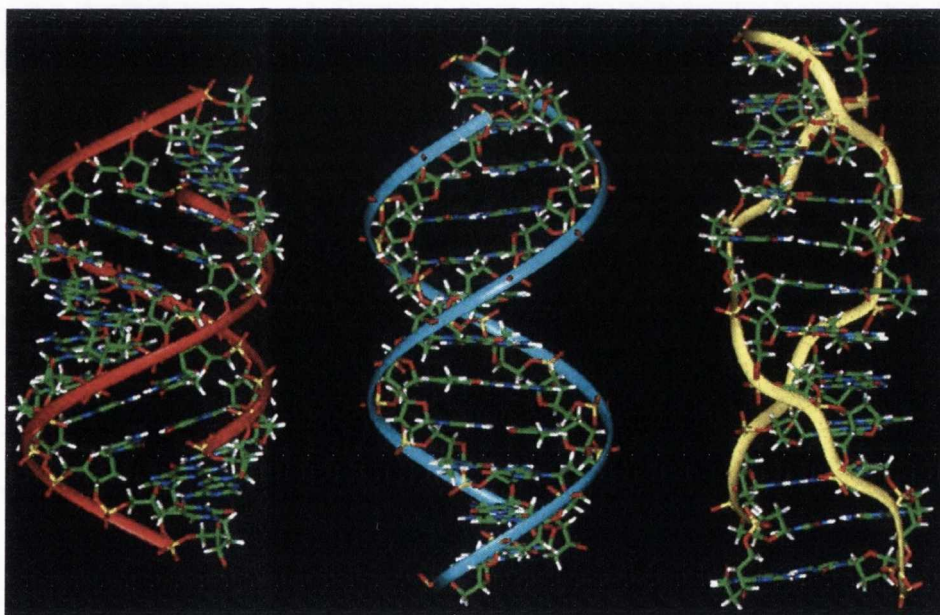


Figure 1.4 Representation of possible structural forms of DNA. Left to right; A-DNA, B-DNA and Z-DNA¹²

Table 1.1 Average helix parameters for A-DNA, B-DNA and Z-DNA¹²

	A	B	Z
Helix	Right handed	Right handed	Left handed
Diameter	~26 Å	~20 Å	~18 Å
Base pairs per helical turn	11	10	12
Helical twist per base pair	32.7°	36°	-9°, -51°
Helix rise per base pair	2.56 Å	3.4 Å	3.7 Å
Base tilt to the helix axis	20°	-6°	-7°
Major groove	Narrow and deep	Wide and deep	Flat
Minor groove	Wide and shallow	Narrow and deep	Narrow and deep
Glycosidic bond	Anti	Anti	Anti for pyrimidines, syn for purines
Sugar pucker	C3'-endo	C2'-endo	C2'-endo for pyrimidines, C3'-endo for purines

The classical B-DNA right handed helix, which is the most prevalent in nature, gives rise to a number of structural features which are amenable to binding. The hydrophobicity, the presence of heteroatoms and the level of unsaturation of the four nucleobases, together with the specificity of hydrogen bonding and π -stacking interactions between them, make DNA chemically accessible and ideal for DNA specific drug-target interactions.⁷ A large number of clinically important drugs and antibiotics are believed to exert their primary biological action through DNA interaction and subsequent inhibition of template function.¹¹

1.3 DNA Binding Interactions

Molecular recognition of B-DNA can take place in 5 distinct ways: (i) major groove recognition; (ii) minor groove recognition; (iii) electrostatic binding; (iv) covalent bonding to the bases and (v) intercalation. The binding of small molecules can involve one or a combination of these interactions. The main body of this thesis will detail the binding of chemical agents to DNA and as such each of these interactions will be discussed and examples of each of the binding modes given in the following sections.

1.3.1 Major Groove Recognition

Proteins frequently achieve DNA sequence recognition by binding in or around the major groove and forming specific hydrogen-bond contacts to the edges of the base pairs. Major groove recognition usually involves cylindrical binding units based on α helices, with the units being just the right size and shape to fit snugly into the major groove but being too large for the minor groove.¹¹ Synthetic oligonucleotides and peptide nucleic acids (PNAs) may be designed to bind in the major groove, which in doing so give rise to triple helix (triplex) formation. Hoogsteen base pairing is important in forming such structures. Stretches of triple helix can be formed by oligonucleotides of an appropriate sequence binding to long duplex DNA molecules as depicted in Figure 1.5.¹³

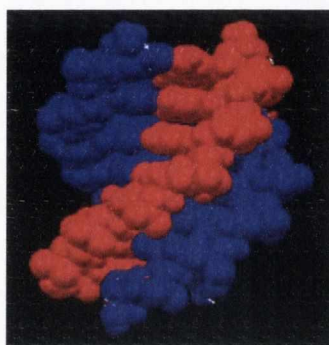


Figure 1.5 NMR structure of Hoogsteen, pyrimidine-purine-pyrimidine DNA Triplex. The Watson–Crick strands are shaded blue and the additional strand in the major groove red¹³

Such a sequence with a covalently attached strand cleavage agent has been shown to bind and cleave a unique site on a yeast chromosome, demonstrating the exceptionally high specificity for a target duplex sequence that a triplex formation can achieve.¹⁴

1.3.2 Minor Groove Recognition

The minor groove is a very important aspect of DNA structure as this is where the edges of the bases are exposed to the environment, thus providing functional groups accessible to hydrogen bond interactions. The common structural characteristics shared by most minor groove binding molecules are: (i) positive charge(s), (ii) linked aromatic and/or heteroaromatic rings and (iii) an approximately crescent shape. Typically *adenine-thymine* regions of B-DNA have a narrower minor groove than *guanine-cytosine* regions, and this allows a snug fit of the drug against the walls of the groove allowing close van der Waal's contacts. Binding in the minor groove may also involve the concave curvature of the inner surface of the bound molecule complementing that of the convex surface of the minor groove itself. Van der Waal's and hydrophobic interactions with the groove walls are the dominant factors in overall stability and strength of binding when the ligand is bound in the groove.

However, directed hydrogen bonding, though a smaller contributor to overall stability, is responsible for directing binding to particular sites and sequences.⁸ Studies on this class of compounds have led to the development of synthetic polyamides such as TRIBIZ, **1**.¹⁵ This groove binding molecule containing three linked benzimidazole heterocycles is able to bind tightly to the DNA helix with little changes in DNA structure (Figure 1.6).

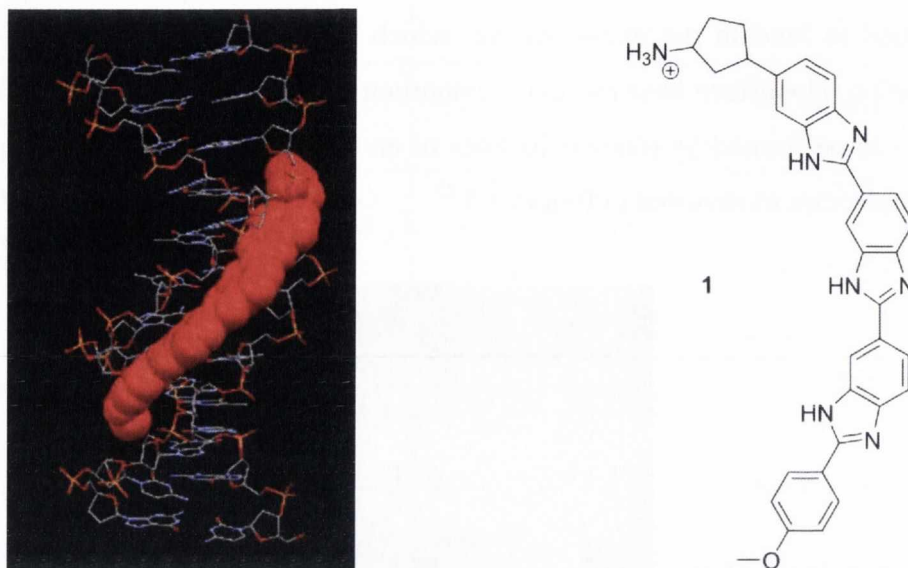
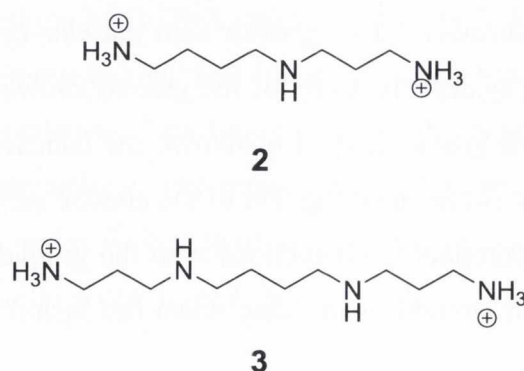


Figure 1.6 Crystal structure of the complex between TRIBIZ, **1** and duplex DNA. TRIBIZ is shown in red¹⁵

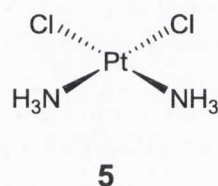
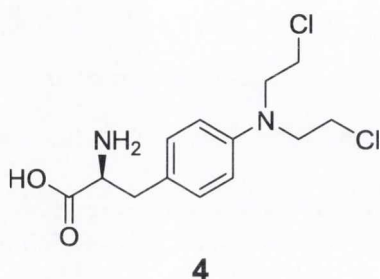
1.3.3 Electrostatic Binding

DNA is a polyanion due to the broad electronegative cloud that surrounds the phosphate backbone. There are several types of molecule that can interact with this outside edge, mainly *via* non-specific electrostatic interactions. In aqueous solution metal ions such as Na^+ and Mg^{2+} are bound, however, polyamines such as spermine, **2** and spermidine, **3** are classic examples of outside edge-binding drugs. Polyamines of this type are ubiquitous in mammalian cells and are thought to play multiple roles in cellular function. Due to the non-specific nature of binding the exact mechanisms of action are unknown. However, it is likely that polyamines neutralise the negative charges on the DNA backbone and hence promote DNA packaging, a process vital to cell proliferation.¹⁶



1.3.4 Covalent Bonding

A further major mode of DNA recognition, and one that has been extensively exploited in cancer chemotherapy, is covalent attachment of a small molecule to the DNA bases. This most commonly occurs at the N7 atoms of *guanine* and *adenine*, located in the DNA major groove. Alkylating agents of this type were originally derived from World War I poison gases giving analogues such as L-phenylalanine mustard, **4** which still finds use against various cancers.¹⁷



Among the most successful of the clinical alkylating agents used to treat cancers is cisplatin, **5** which binds to DNA with the displacement of two chlorides and the formation of two metal-coordination bonds to N7 of two adjacent purine DNA bases on the same strand (Figure 1.7).^{18,19} There is a strong preference for *guanine* over *adenine* with over 70% of the lesions being found at *guanine-guanine* sites and around 20% at *guanine-adenine*.⁷ Binding of **5** to DNA causes significant distortion of helical structure and results in inhibition of DNA replication and transcription leading to cellular apoptosis and eventually suppression of tumor growth.²⁰

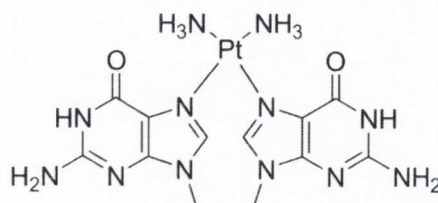
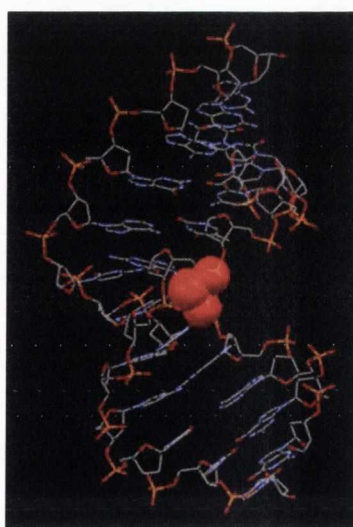


Figure 1.7 Crystal structure of the 1,2-intrastrand adduct formed between cisplatin, **5** and DNA. Also shown is a schematic representation of the adduct formed by the cross-linking of **5** with the N7 atoms of two guanine bases^{18, 19}

1.3.5 Intercalation

Many chemical species that target DNA are characterised by possession of an extended planar heteroaromatic ring system, a chromophore, which is approximately the same size as a DNA base pair. It is this planar aromatic moiety that targets DNA by becoming inserted between adjacent base pairs in an intercalative manner as depicted in Figure 1.8. Early studies have provided evidence that the DNA helix unwinds slightly to create a space into which the flat polycyclic aromatic species can be inserted.^{21,22} The interaction can be characterised by a lengthening of the DNA helix and a local unwinding around the intercalated species with stabilisation achieved through polarisation forces and van der Waal's dispersion interactions between the planar species and the DNA base pairs surrounding it. Structurally simple intercalators such as proflavine, **6** and ethidium, **7** are known to be non-specific binders although a small preference is observed for pyrimidine-3',5'-purine sequence steps especially C:G steps.²³ This is thought to be as a result of a low activation energy (< 20 kcal/mol) for "opening up" the dinucleotide phosphate base pair from the DNA structure to one into which the intercalator can fit.²⁴

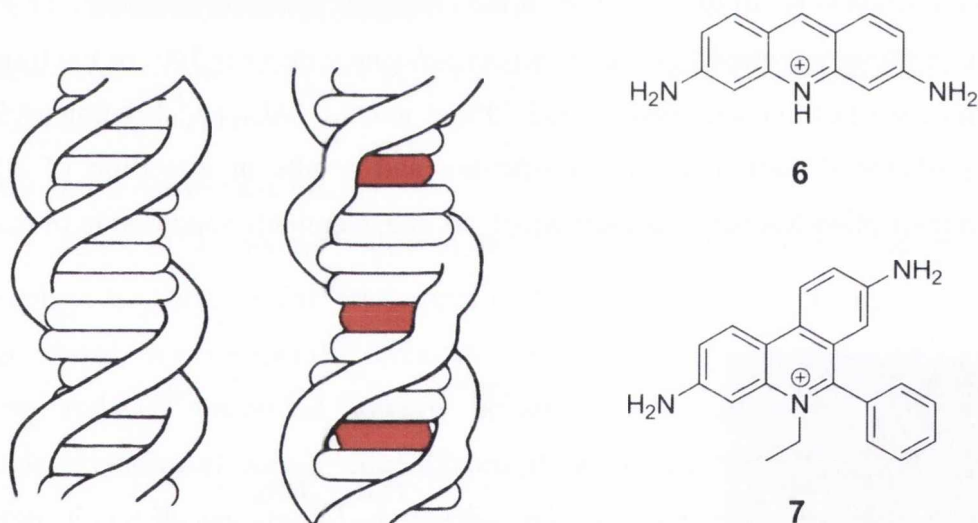
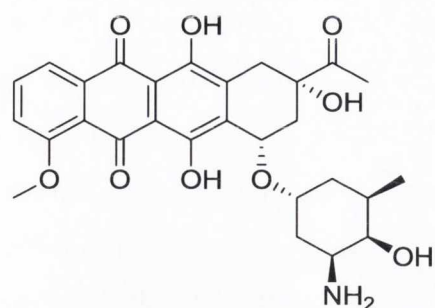
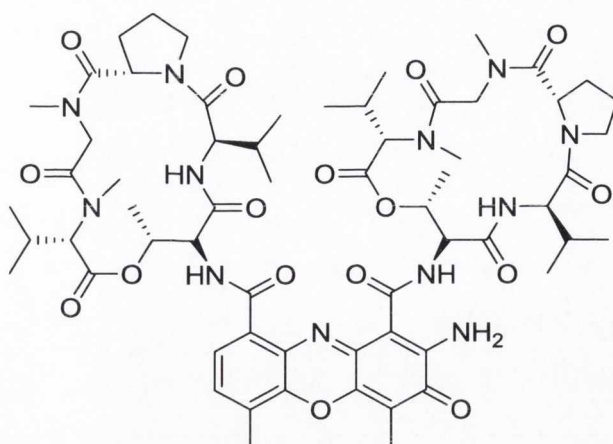


Figure 1.8 A schematic representation of a planar drug molecule binding intercalatively into a DNA double helix causing base pair extension and unwinding. Drug molecules are shown in red

More complex intercalating species have appended groups including side chains, sugar rings or peptide units such as the anti-tumor drugs daunomycin, **8** and actinomycin D, **9**. These side groups reside in the grooves where they are further stabilised by van der Waal's interactions and can hydrogen bond to adjacent bases to impart a degree of sequence specificity.²⁵

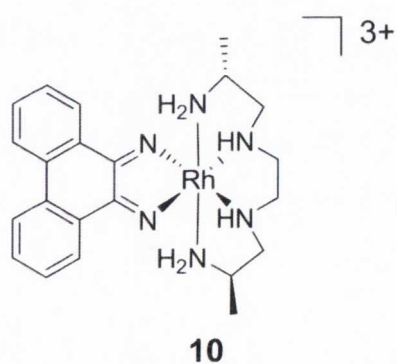
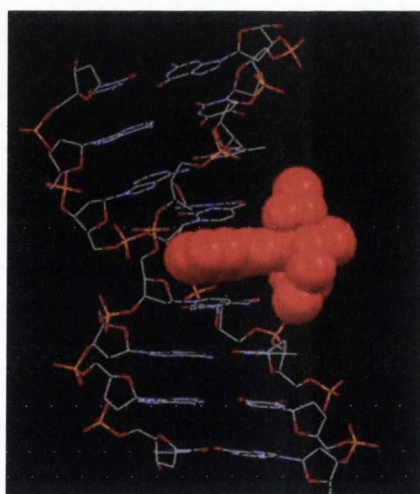


8



9

Another method utilised for increasing the binding affinity of intercalating species for DNA is the imparting of a positive charge into the design by incorporating a transition metal. A number of metal containing intercalating molecules have been synthesised as probes of DNA structure, some of which will be discussed in the following section with this being a major theme throughout this Thesis. The rhodium complex $[\text{Rh}(\text{phi})(\text{Me}_2\text{trien})]^{3+}$, **10** was developed by Barton *et al.* using an octahedral metal centre.²⁶ The molecular design used was based on a large planar ligand structure that can insert into DNA and to which the metal can be attached. While the planar unit inserts, the metal and additional co-ligands are left to reside in one of the DNA grooves, as shown in Figure 1.9.



10

Figure 1.9 Crystal structure of $[\text{Rh}(\text{phi})(\text{Me}_2\text{trien})]^{3+}$ intercalated between the central base pairs of a DNA duplex structure²⁶

Metal ions and transition metal complexes of this type, because of their cationic character, three-dimensional structural profiles, and propensity for performing hydrolysis, redox, or photoreactions, have a natural aptitude for interacting with DNA.²⁷ The well-developed synthetic chemistry of ruthenium is of particular interest as it provides for many approaches to innovative new metallopharmaceuticals.²⁸ In the past, ruthenium complexes have attracted much attention due to their many advantages over platinum based complexes currently in use as a result of reduced toxicity, a novel mechanism of action and a different spectrum of activity.²⁹ Within this class of complexes lies a subgroup of compounds containing polypyridyl ligands. These complexes show significant DNA binding and cleavage properties, as well as rich spectroscopic properties that have been intensely investigated.³⁰ The following section will detail a brief overview of Ru(II) polypyridyl complexes and a number of relevant examples from the literature will be described.

1.4 Ruthenium(II) Polypyridyl Complexes

Ruthenium(II) polypyridyl metal complexes have many and varied applications³¹ and have received significant attention over the past 25 years due to their excellent photophysical properties and strong binding affinity with DNA.^{32,33} Due to their positive charge, complexes of this type bind tightly to DNA *via* electrostatic binding to the phosphate backbone. In addition, by varying the ligands around the metal centre it has been possible to modify the interaction,³⁴ where they may also bind by either groove binding or intercalation.³⁵

1.4.1 Photophysical Properties

The photophysical properties and selective photochemistry of Ru(II) polypyridyl complexes in the presence of DNA yields systems that enable simple methods to study the binding process. Characteristic photostability, water solubility, effective DNA binding affinity as well as useful photophysics and photochemistry all combine to make Ru(II) polypyridyl complexes excellent candidates for potential cancer therapeutics as well as spectroscopic DNA probes and photoreagents.

In order to understand how Ru(II) polypyridyl complexes interact and photoreact with DNA an explanation of their photophysics is necessary. The most studied complex in this series is Ru(bpy)₃²⁺, **11** and as such the photophysical mechanisms of this complex will be described. An energy level diagram depicting the mechanism is shown in Figure 1.10.³²

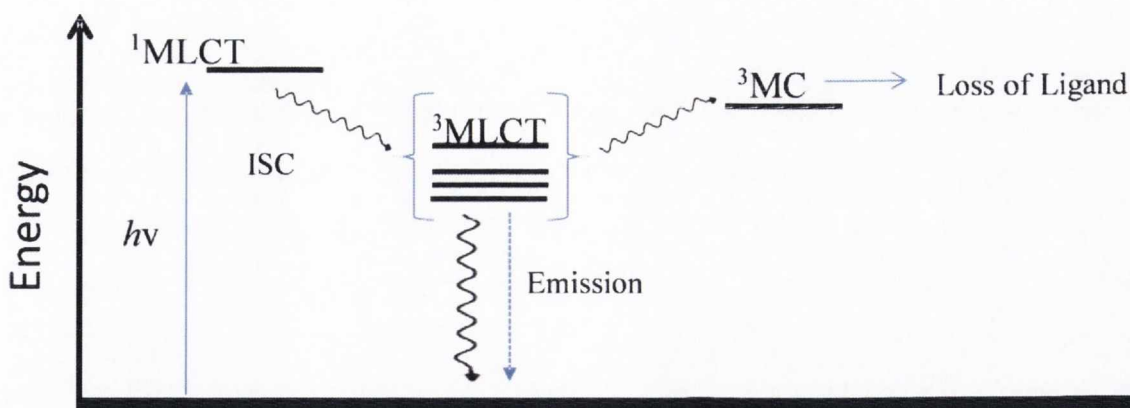


Figure 1.10 Energy level diagram for the lowest excited states of $\text{Ru}(\text{bpy})_3^{2+}$

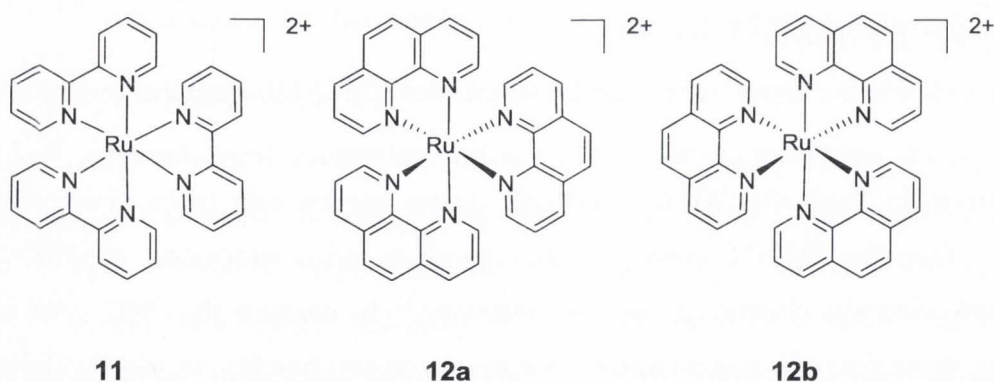
Upon irradiation in the visible absorption band of $\text{Ru}(\text{bpy})_3^{2+}$, **11** a singlet metal to ligand charge transfer (MLCT) excited state is populated. This state deactivates rapidly by intersystem crossing (ISC) to the lowest lying triplet MLCT state ($^3\text{MLCT}$). The $^3\text{MLCT}$ state corresponds to three energy states that are very close in energy to each other and a fourth state that is slightly higher in energy. Deactivation of the $^3\text{MLCT}$ state can occur *via* three major pathways.

1. Radiative deactivation which is responsible for the observed emission in the wavelength region of 600 nm.
2. Radiationless activation which generally controls the lifetime of the $^3\text{MLCT}$ state in many $\text{Ru}(\text{II})$ polypyridyl complexes.
3. Conversion to the upper triplet metal-centred state (^3MC) by thermal activation.

Interestingly, both the $^3\text{MLCT}$ and ^3MC states exhibit two types of excited state reactivities, where the $^3\text{MLCT}$ state generally involves redox processes, exhibiting both oxidation and reduction depending on the conditions.³² In contrast the ^3MC state usually results in the promotion of a non-bonding electron into an anti-bonding σ orbital. This causes distortion along the Ru-N bonds and can result in the loss of one ligand.³² Due to the favourable properties of $\text{Ru}(\text{II})$ polypyridyl complexes mentioned above, an enormous amount of research has been undertaken in the field. The following section will deal with some relevant examples from the literature.

1.4.2 Structurally Simple Ru(II) Complexes

Early examples of Ru(II) polypyridyl complexes as DNA binders involved complexes that had been previously well characterised in other contexts. These simple systems were based on bipyridine (bpy), **11** and phenanthroline (phen), **12** complexes of ruthenium. It was observed that while **11** bound to DNA relatively weakly, (mainly through electrostatic interactions), the interaction of $[\text{Ru}(\text{phen})_3]^{2+}$ was much stronger.²⁷ This was rationalised by the cationic nature of these complexes where both **11** and **12** were expected to be attracted to the negatively charged backbone of DNA, while **12**, with slightly extended heteroaromatic ligands might insert between the base-pairs of B-form double-stranded DNA (*ie.* bind *via* intercalation). Being a second row transition metal, ruthenium typically adopts an octahedral conformation. Chirality around this octahedral metal centre arises from the disposition of the chelating ligands clockwise or anti-clockwise in a left (Λ), **12a**, or right (Δ), **12b** handed helix about the C_3 axis. Studies by Barton *et.al.* revealed stereospecific binding of the Δ - isomer over the Λ - isomer of $[\text{Ru}(\text{phen})_3]^{2+}$.³⁶ It was originally proposed that one of the phen ligands intercalated, with the two non-intercalated ligands of the Δ -isomer fitting tightly along the groove. However, subsequent studies by Satyanarayana *et al.* identified viscosity measurements suggesting that neither the Δ - nor the Λ -enantiomer binds to DNA by classical intercalation but by partial non-classical intercalation, resulting in a kink in the helix.³⁷

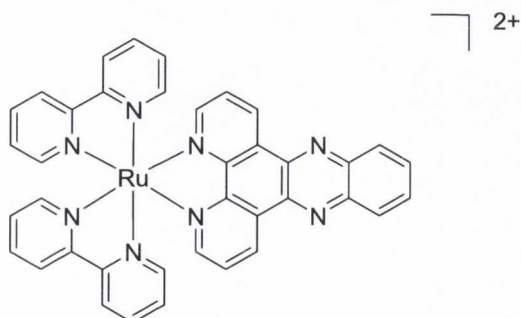


1.4.3 Strategies to Increase DNA Binding Ability

The main drawbacks associated with the two systems discussed in the previous section, with regards to their DNA binding properties, were low affinity and relatively small changes in their photophysical properties. The obvious way of overcoming these shortcomings was by changing the ligands surrounding the metal centre so as to provide a means to vary the nature and strength of binding to both nucleic acids and other biomolecules. One of those strategies utilised was extension of the aromatic ligands to provide an extended aromatic platform for intercalation.

1.4.3.1 Intercalation as a Platform for Binding to DNA

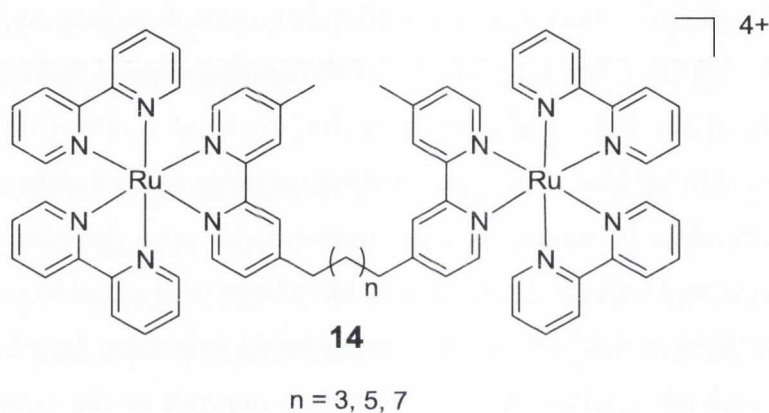
While all cationic complexes are expected to be attracted to the negatively charged DNA backbone, complexes possessing extended heteroaromatic ligands might be expected to insert between the base-pairs of B-form double-stranded DNA. Many complexes with extended aromatic heterocyclic ligands have been designed to date with a view towards improving their intercalation ability, the most impressive of these being those incorporating the dipyrido[3,2-a:2',3'-c]phenazine (dppz) ligand. The first complex studied containing this ligand was $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, **13**, originally synthesised by Sauvage and co-workers.³⁸ Barton *et al.* described the interaction of the complex with DNA,³⁵ and showed that in aqueous solution no photoluminescence was observed at ambient temperature, but in the presence of double helical DNA intense photoluminescence was observed; the difference between the 'on' and 'off' state of this complex has been described as the 'light switch' effect.³⁵ This reflected the shielding of the intercalating dppz ligand, being inserted from the major groove side of the DNA helix, from the bulk solvent.³⁹ The MLCT transition was shown to be directed to the phenazine ring, and the resulting excited state subject to efficient non-radiative deactivation by hydrogen bond formation between the phenazine nitrogens of the dppz and solvent molecules.⁴⁰ Many studies have been carried out to determine the exact mechanism of the 'light switch' effect which is proposed to involve bright and dark states in equilibrium with each other, whose relative population depends on the local environment.^{41,42} Intercalation has also been supported as a mode of binding by using linear dichroism and fluorescence studies.⁴³⁻⁴⁵ This effect has been advantageous for the development of DNA probes for two main reasons. Firstly, the binding affinity for DNA of such complexes is high, in the region of $10^6 - 10^7 \text{ M}^{-1}$,³⁵ and secondly the enhanced photophysical properties of these complexes are useful for monitoring the binding process. The extended polyazaaromatic system of dppz and appended planar aromatic moieties have yielded systems with high binding affinity to DNA which are particularly useful as DNA probes.



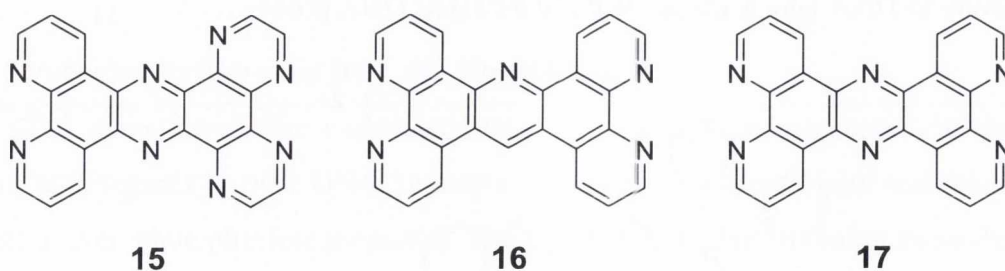
13

1.4.3.2 Bimetallic Ru(II) Complexes

Research has also shown that the introduction of a second metal centre to the binding species gives rise to increased DNA binding ability. For instance, Kelly *et al.* described a series of dimeric systems, where two $[\text{Ru}(\text{bpy})_3]^{2+}$ or $[\text{Ru}(\text{phen})_2\text{bpy}]^{2+}$ units are covalently linked by polyalkyl chains of various lengths.⁴⁶⁻⁴⁸ As described in section 1.4.2, $[\text{Ru}(\text{bpy})_3]^{2+}$ interacts with DNA relatively weakly (mainly through electrostatic interactions). However, the dimeric species, **14**, was shown to exhibit a 100-fold increase in binding affinity for DNA. The effect of ionic strength variations on the binding equilibrium saw a strengthening of electrostatic interactions due to the high positive charge of **14**. The important ionic character of this construct was also found to improve its binding resistance to increasing NaCl concentrations up to, and even above, physiological ionic strength conditions.⁴⁸



A more rigid arrangement of the two metal complex cores in bimetallic species has also been explored using complexes based on planar aromatic bridging ligands such as 1,10-phenanthroline[5,6-b]1,4,5,8,9,12-hexaazatriphenylene (PHEHAT), **15**, (tetrapyrido[3,2- α :2',3'-c:3'',2''-h:2''',3'''-j]acridine (TPAC), **16** and tetrapyrido[3,2-a:2',3'-c:3'',2''-h:2'',3''-j]phenazine (TPPHZ), **17**.⁴⁹⁻⁵¹



A particular example is **18**, a dinuclear mono-intercalating Ru(II) complex developed by Thomas *et al.*, that displays high affinity binding to both duplex and quadruplex DNA.⁵² Photophysical and biophysical studies indicate that, even at high ionic strengths, this complex binds to duplex DNA, through intercalation, with high affinities ($1.1 \times 10^7 \text{ M}^{-1}$ in 25 mM

NaCl buffer).⁵² Furthermore, this study also indicated that **18** binds to quadruplex DNA with affinities that are comparable to, or higher, than those obtained for duplex DNA binding. Isothermal Titration Calorimetry (ITC) data for **18** also clearly showed that binding to quadruplex sequences was about 5 kJ mol^{-1} more favourable than binding to duplex DNA. In addition, binding to quadruplex DNA was accompanied by a distinctive “quadruplex light switch” effect, in which the emission was blue-shifted and considerably more enhanced relative to duplex binding.⁵² In a subsequent publication, the same research group reported the application of **18** as an *in cellulo* nuclear DNA stain, well tolerated by live cells, for use with both luminescence and transition electron microscopy (Figure 1.11), where **18** was shown to be taken up by live cells *via* a structure-specific and temperature dependent non-endocytotic mechanism.⁵³ Interestingly, **18** exhibited potential as a new imaging agent due to the existence of multiple emission maxima upon binding to DNA, suggesting that the complex may find future application as a DNA structural probe, specifically for quadruplex DNA.⁵³

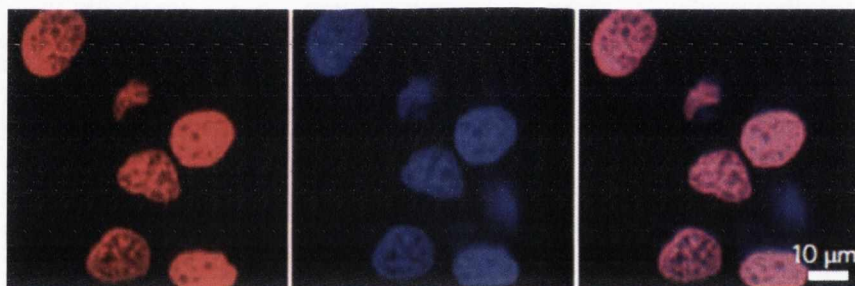
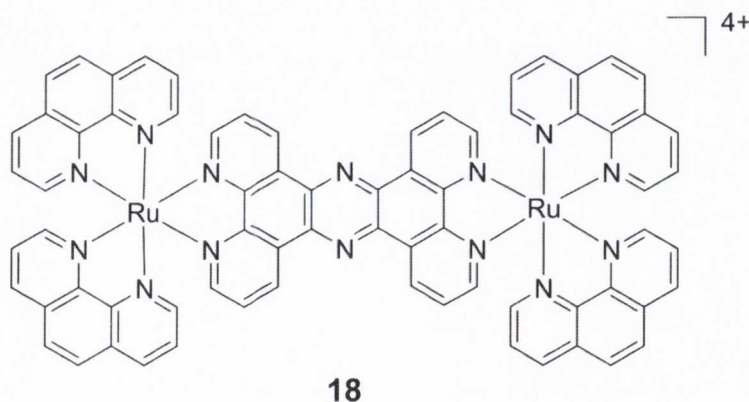


Figure 1.11 Co-staining of **18** (red, left) with DNA-specific dye DAPI (4',6-diamidino-2-phenylindole; blue, centre) and the overlay image (right)



Lincoln and Nordén reported a more flexible dimeric system, based on covalently linked dppz units, **19**. This bis-intercalator is different from others studied, because the intercalating units are linked at the point through which they would usually be expected to insert into the DNA duplex. The homochiral enantiomers (Δ - Δ , Λ - Λ) and the meso compound (Δ - Λ), were synthesised and studied in the presence of DNA.⁵⁴⁻⁵⁶ Flow linear dichroism (LD)

results supported a bis-intercalative binding mode for all three stereoisomers. The observation of a ‘light switch’ effect upon binding to DNA for these stereoisomers further supported the intercalation hypothesis, as shielding of the ring nitrogens of the dppz from the aqueous environment was required for emission detection. Taking into account the binding site of four base pairs determined from binding titrations, it was concluded that the two $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ moieties were intercalated two base pairs apart from each other and were bound in the same groove (Fig. 1.12).^{54,57} As shown by LD and molecular modeling, the Δ - and Λ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ subunits presented a distinct orientation of their phen ancillary ligands with respect to the DNA grooves. The positioning of the phen ligands of the Δ - enantiomer allowed for a slightly ‘deeper’ insertion of the dppz ligand into the base pair stack, which accounted for the significantly higher luminescence quantum yield of Δ - Δ -**19** compared to its enantiomer Λ - Λ -**19** upon binding to DNA.⁴³ Strikingly, both Δ - Δ -**19** and Λ - Λ -**19** were shown to exhibit extremely high binding constants ($1.7 \times 10^{10} \text{ M}^{-1}$ at 50 mM NaCl) corresponding to a four orders of magnitude increase with respect to their monomeric enantiomers ($3.2 \times 10^6 \text{ M}^{-1}$ at 50 mM NaCl).⁵⁶

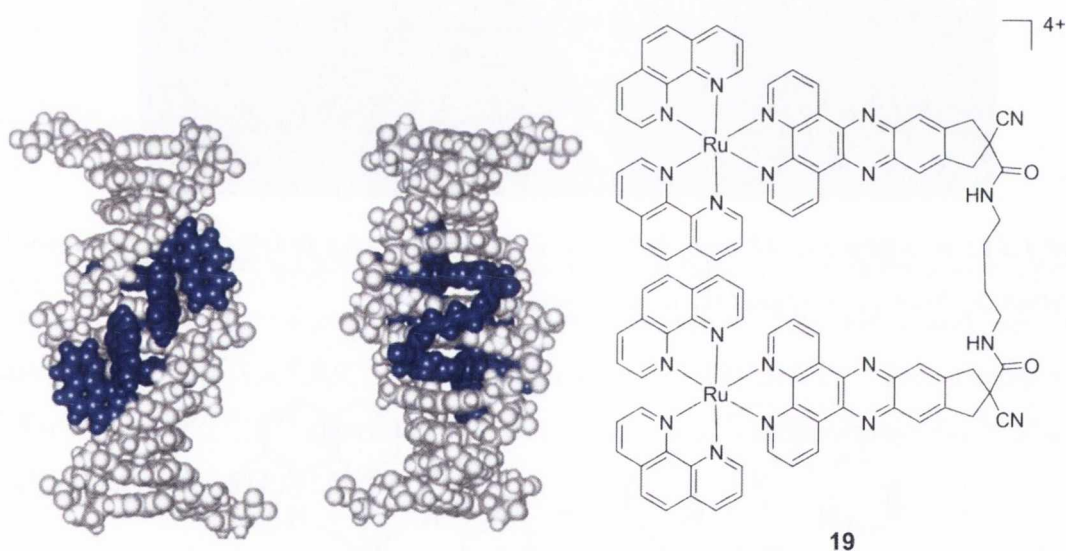


Figure 1.12 Molecular model of the binding geometry of Δ - Δ -**19** showing the two intercalated Δ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ units in the minor groove (left) and the linker in the major groove (right)

Whereas intercalation of the monomeric $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ can be assumed to proceed by simple insertion of the dppz ligand, a threading mechanism of $\text{Ru}(\text{phen})_2$ moieties, either by direct crossing through the core of DNA, or through passage of the tether across the DNA helix, is required for **19** to reach, or to leave, its final bis-intercalative binding mode. Such a threading mechanism implies considerable DNA conformational changes, which would possibly involve base pairs opening and, therefore, association and dissociation rates

would be slowed down considerably. The study of the binding kinetics revealed remarkably slow dissociation kinetics for the stereoisomers of **19** with rate constants two to three orders of magnitude smaller than that observed for $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$. It was therefore concluded that the threading mechanism involves the passage of the bulky $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ subunits across the DNA helix, and the final bis-intercalation geometry was proposed to be reached from a threaded mono-intercalated intermediate by a slow reorganisation process involving several DNA bound intermediates.⁵⁶ Bis-intercalating ruthenium complexes of this type represent a novel type of DNA-threading compound that binds DNA with extremely high affinity. Furthermore, such findings subsequently provided new possibilities for the design of excellent fluorescent markers for DNA in the study of chromosome spindle arrangements as demonstrated in Figure 1.13.⁵⁸

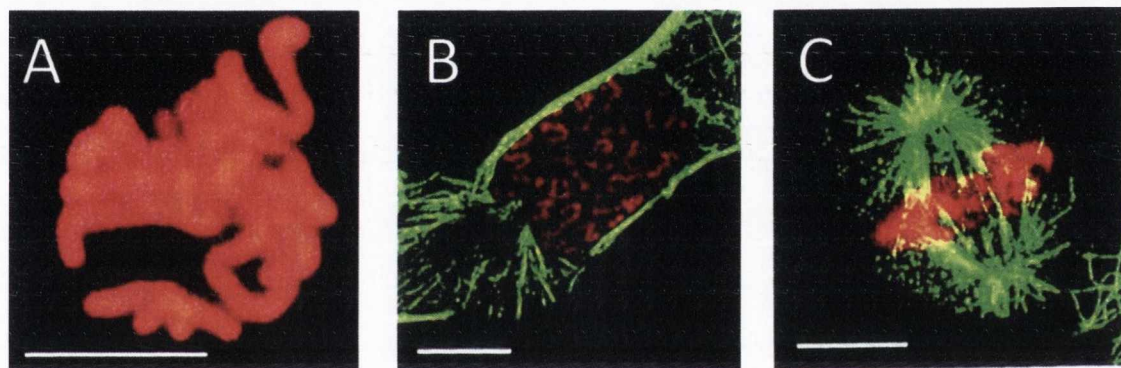


Figure 1.13 A: Fixed cells in which the chromosomes have been stained with **19**. B and C show additional microtubule staining. Lines correspond to 20 μM ⁵⁸

The strategy of incorporating two metal centres into a single complex has proven to be extremely successful; having afforded systems with increased DNA binding affinity and enhanced photophysical responses compared to their monomeric derivatives. As a result Ru(II) complexes are the focus of numerous research works with various applications mainly due to their attractive photophysical and photochemical properties.³¹ In biological research areas, in particular, they are developed mainly as efficient photosensors of biological molecules. Perhaps more interestingly, Ru(II) complexes also display attractive photochemistry and as such their excited state reactivity with DNA has been explored extensively with a view to developing artificial metallonucleases for medicinal application.^{32,59,60} Moreover, species that can photoreact with DNA offer an alternative approach to the development of photoactive chemotherapy agents, the potential of which will be discussed in the following section.

1.5 Photodynamic Therapy and DNA - A Therapeutic Target

Since the pioneering use of cis-platin in the 1960's as a chemotherapeutic agent, DNA targeting molecules have been used as both anti-cancer therapeutics as well as probes for DNA. However, many modern cancer chemotherapy agents suffer from several drawbacks such as a lack of DNA sequence specificity and cell specificity causing numerous harmful side effects. Photodynamic therapy (PDT) is a modern treatment that uses two independently non-toxic components, light and a chromophoric molecule or photosensitiser (PS) to locally photosensitise tissues and to selectively kill targeted cells.⁶¹ Following administration of the PS, the cancerous lesion of interest is irradiated with visible or near-infrared (NIR) light, promoting a set of photochemical reactions, giving rise to fluorescence for the purpose of imaging in addition to inducing apoptosis and/or necrosis and, hopefully, resulting in ablation of the cancerous lesion (Figure 1.14).⁶²

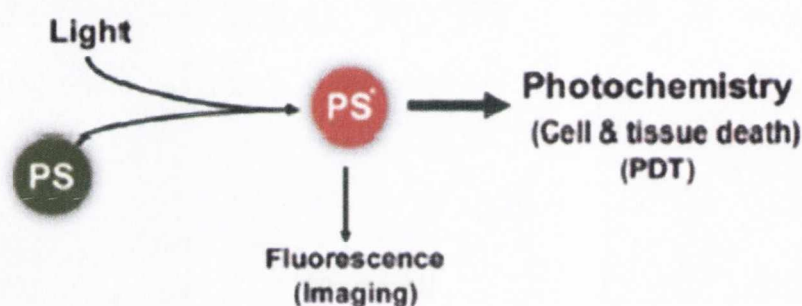


Figure 1.14. Schematic representation of PDT where PS is a photoactivatable multifunctional agent, which upon light activation can serve as both an imaging agent and a therapeutic agent⁶²

Using conventional light sources, a single quantum of light is typically absorbed, causing the absorbing molecule to be electronically excited. The electronic energy levels between which transitions occur by absorption of ultraviolet-visible light ($\lambda = 200\text{--}700\text{ nm}$) may be represented by the simplified energy level diagram (Figure 1.15). The electronic states are represented by the singlet states, S_n , and the triplet states, T_n . The singlet excited states are rather short-lived, while the triplet state lifetimes are longer lived, in the microsecond to millisecond range. With the absorption of a single photon of light, the molecule is promoted to an excited singlet state, for example, S_1 . From this excited state, it may initiate photochemistry (depending on its chemical structure and lifetime) or undergo intersystem crossing to a triplet state, T_1 . From S_1 , the excited molecule may also relax back to S_0 by non-radiative decay and generate heat or may re-emit radiation as fluorescence which forms the basis of fluorescence imaging. In general, T_1 is longer lived than the first excited singlet state, so that the biologically relevant photochemistry is often mediated by this state. T_1 can initiate photochemical reactions directly, giving rise to reactive free radicals, or transfer its energy to

the ground state oxygen molecules ($^3\text{O}_2$) to give rise to $^1\text{O}_2$ molecules. The electronic excitation to produce $^1\text{O}_2$ requires at least 20 kcal mol^{-1} , which places limits on the longest absorption wavelength of the PS.⁶² If the energetics are appropriate, photo-oxidative reactions may occur by $^1\text{O}_2$ mediation which is a generally accepted mode of action for most PSs currently under investigation, although other competing mechanisms exist.⁶³

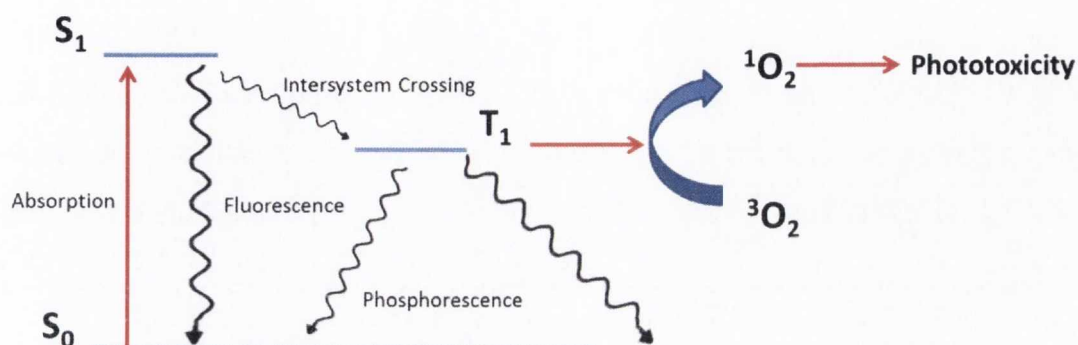
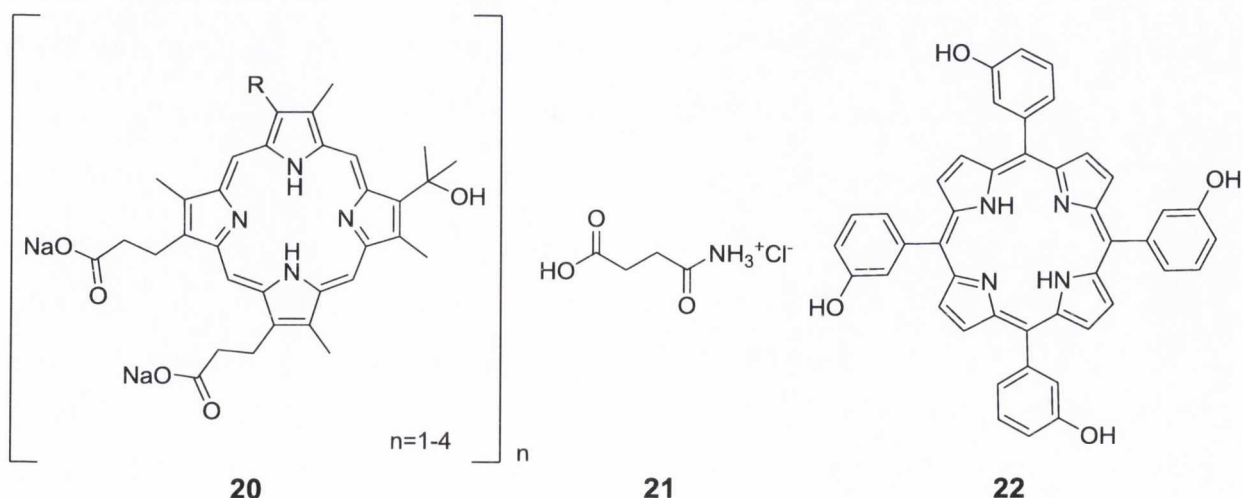


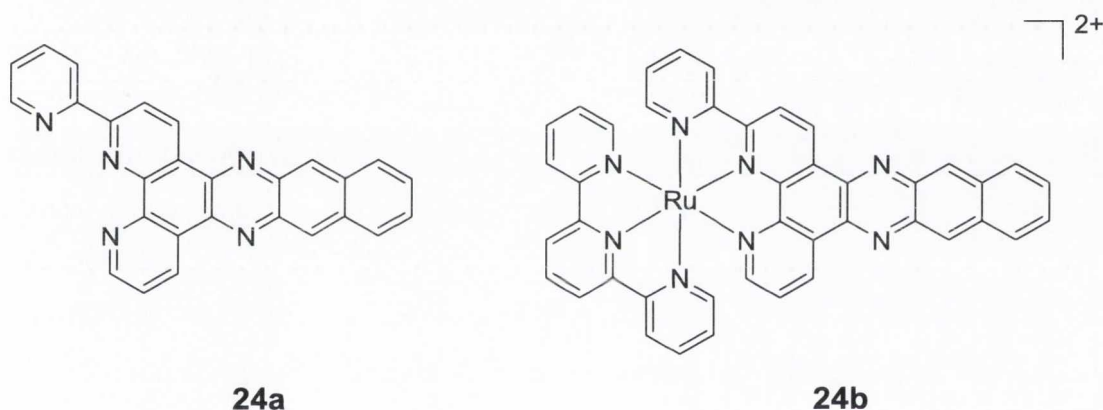
Figure 1.15. A simplified energy level diagram depicting the activation of a PDT agent, leading to the formation of singlet oxygen

The first PS for the treatment of cancers was approved in 1993 and was a purified hematoporphyrin derivative, Photofrin[®], **20** which was shown to preferentially localise in tumors and gave good tumoricidal effects when combined with red light.^{64,65} Within the US, 5-aminolevulinic acid (Levulan[®]), **21**, which acts a prodrug increasing porphyrin anabolism, is the only other approved chemical for use in PDT, gaining approval in 2000. Within the EU, Iceland and Norway a chlorin, Foscan[®], **22** has also been approved for the treatment of head and neck cancers, however, currently there are three other chromophores in clinical trials: metoxifin, tin ethyl etiopurpurin and mono-aspartyl chlorine.⁶⁶



1.6.1 Singlet Oxygen Sensitisation

While the $^1\text{O}_2$ quantum yields of $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ remain low,^{69,71} the photoredox processes of Ru(II) polypyridyl complexes, in general, can be tuned by changing the nature or combination of ligands surrounding the metal centre. The work of Turro *et al.*⁷² has utilised Ru(II) complexes possessing tridentate ligands with extended π systems such as 3-(pyrid-2'-yl)-4,5,9,16-tetraaza-dibenzo[a,c]naphthacene, pydppn, **24a**. The photophysical properties of a series of $[\text{Ru}(\text{tpy})_n(\text{L})_{2-n}]^{2+}$ (tpy = [2,2';6',2'']-terpyridine, L = pydppn, n = 0–2) complexes have revealed markedly different excited state behavior. Most interestingly, the lowest energy excited state is a ligand-centered $^3\pi-\pi^*$ state localised on the pydppn ligand itself with lifetimes of *ca.* 20 μs . Moreover, the $[\text{Ru}(\text{tpy})(\text{pydppn})]^{2+}$ complex, **24b** is able to generate $^1\text{O}_2$ with *ca.* 100% efficiency which exceeds that reported for Photofrin[®] and its derivatives, for which $\Phi_{1\text{O}_2}$ of 0.75 was reported.⁷³

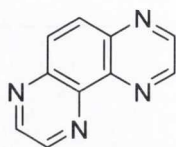
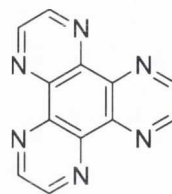


This complex also binds to DNA with a binding constant $K = 4.6 \times 10^6 \text{ M}^{-1}$ (5 mM Tris/HCl, 50 mM NaCl, pH 7.5), at the same time as causing efficient DNA cleavage. Such high quantum yields of $^1\text{O}_2$ photosensitisation and observed DNA photocleavage ability of Ru(II) complexes are another large step in the design of new systems for PDT.⁷²

An alternative approach to DNA photocleavage is that of direct reaction where the photosensitising species can form covalently attached photoadducts with DNA.⁶⁰ In order to achieve this, it is necessary to employ systems which are sufficiently strong oxidisers in their excited state. This can be achieved by using ligands which by their very nature are π -deficient and, thus, electron accepting resulting in properties that can be exploited in order to mimic endonuclease activity. A description of such compounds will be discussed in the following section.

1.6.2 π - Deficient Ru(II) Polyazaaromatic Complexes

Complexes that contain at least two π -deficient ligands, such as 1,4,5,8-tetraazaphenanthrene, **25** (TAP) or 1,4,5,8,9,12-hexaazatriphenylene, **26** (HAT) ligands have been shown to be extremely oxidising in their $^3\text{MLCT}$ excited state.⁷⁴⁻⁷⁶

**25****26**

As a result, two types of photophysical behaviour are generally observed in the presence of DNA (or oligonucleotides). In the first case, $[\text{Ru}(\text{TAP})_3]^{2+}$ mixed with $[\text{poly}(\text{dA-dT})]_2$, has been shown to exhibit an increase in the $^3\text{MLCT}$ lifetime along with an emission enhancement as a function of increasing amounts of polynucleotide.^{27,71} These effects are due to the protection of the excited state of the complex by the local DNA microenvironment from quenching caused by molecular oxygen and solvent molecules. In contrast, when the same system is mixed with $[\text{poly}[(\text{dG-dC})]_2$,⁷¹ the emission from the Ru(II) complex is efficiently quenched, and the lifetime significantly shortened. Subsequent experiments have shown that this quenching is caused by a photoinduced-electron transfer (PET) between *guanine* residues (the most easily oxidisable base among the four nucleotide bases)^{77,78} and the $^3\text{MLCT}$ state of the complex.^{66,67} The existence of such a charge transfer process followed by a back electron transfer (BET) has been observed by transient absorption spectroscopy when the electron donor can freely diffuse or when it is incorporated in DNA.⁷⁸⁻⁸⁰ Complexes of this type have also been shown to produce a PET process with amino acids (such as tryptophan and tyrosine).^{81,82} Although BET processes can occur between the excited complexes and biomolecules (with a *guanine* or tryptophan moiety), two other processes may compete with this back charge transfer process. It has been observed that with plasmid DNA, single-strand breaks followed by double-strand cleavages can occur and, moreover, the formation of covalent adducts between the complexes and *guanine* (or tryptophan residues) have also been detected.^{59,82} As mentioned above, several Ru(II) complexes have been shown to photo-induce a single-strand break (nick) of DNA plasmids, which transforms the covalently closed circular (CCC) form into the open circular (OC) form.^{77,83} It is thought that this strand break could originate from a 'leak reaction' in which the *guanine* radical, or radical cation, escapes from the BET process and reacts with a sugar moiety of the DNA.⁶⁰ This phenomenon has also been observed by atomic force microscopy (AFM) which clearly shows topological modifications of the plasmid DNA after such photoirradiation.⁸⁴

With *guanine* species a second reaction, in competition with the BET, has also been detected, corresponding to the formation of a photo-adduct between the π deficient Ru(II) complexes and GMP, or polynucleotides containing a *guanine*-unit.⁵⁹ In the case of a Ru(II) complex containing at least two TAP ligands, a photo-adduct originates from the formation of a covalent link between one chelated TAP ligand and the exocyclic nitrogen of the *guanine*-unit of DNA, as shown schematically in Fig. 1.16.⁸⁵

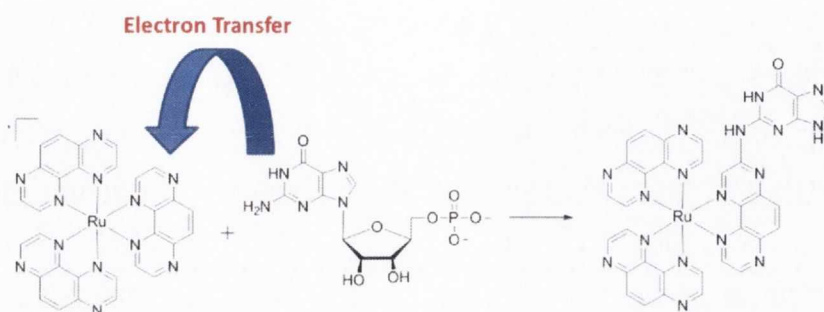


Figure 1.16 Structure of the photo-adduct formed upon illumination of $[\text{Ru}(\text{TAP})_3]^{2+}$ in the presence of GMP, after hydrolysis to remove the sugar–phosphate unit

It has been proposed that this adduct is formed from recombination of two radicals resulting from a proton coupled electron transfer (PCET) and re-aromatisation of the resulting adduct molecule (Fig. 1.17).⁸⁶ The presence of this photo-adduct between the TAP complex and a *guanine*-unit has been demonstrated by several techniques including the use of polyacrylamide gel electrophoresis, electrospray mass spectrometry (ESI/MS), dialysis (monitored by UV–Vis absorption spectroscopy) and steady-state illuminations followed by UV–Vis absorption spectroscopy experiments.^{77,86,87} Interestingly, the ET between the excited state of the Ru(II) complexes and tryptophan also leads to the formation of a photo-adduct in which the complex coordination sphere is not altered.⁸²

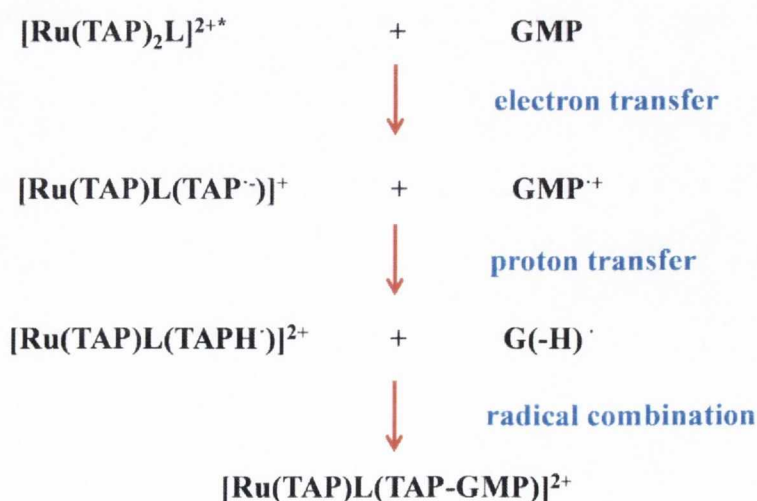
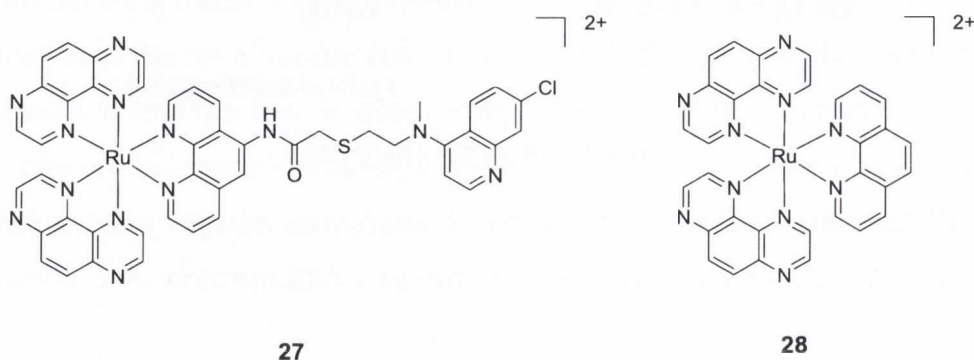


Figure 1.17 Schematic representation of the photo-electron transfer process between a GMP base and the excited state of the Ru(II) species under illumination

As mentioned in Section 1.3.4, the well-known cis-platin molecule, **5** is currently employed for the treatment of certain human cancers.⁸⁸ The biological activity of this compound is directly related to the formation of adducts with *guanine* bases yielding inter- or intra-strand crosslinks, giving rise to cancer cell death.⁸⁹ With π -deficient Ru(II) complexes, formation of the photo-adduct could be triggered exclusively under illumination, thus providing precise control over the reaction and subsequent cancer cell damage. The nature of this photo-adduct as described above is quite different from the adducts formed with cis-platin as the photoreactions between Ru(II) complexes and GMP do not disrupt the coordination sphere around the metal ion. Such useful photochemical properties make Ru(II) complexes extremely advantageous from the point of view of phototherapeutics. Moreover, their conjugation to appended functional species may provide a ‘double barrel’ approach, leading to modulations in their photochemical reactivity, in addition to conferring them with increased biological activity. The following section will discuss previous examples of bifunctional Ru(II) species from the literature.

1.6.3 Ru(II) Polyazaaromatic Complexes with Appended Functional Species

In general, it has been shown that the addition of an organic subunit to a metallic complex enhances the affinity of the resulting system for DNA compared to that of the unfunctionalised complex.⁹⁰ $[\text{Ru}(\text{TAP})_2(\text{POQ-Nmet})]^{2+}$ ($\text{POQ-Nmet} = 5\text{-}\{4\text{-}[\text{N-methyl-N-(7-chloroquinolin-4-yl)amino}]\text{-2-thiabutylcarboxamido}\}\text{-1,10-phenanthroline}$), **27** is an example of such a bifunctional complex which combines a high affinity for DNA with photoreactivity characteristic of TAP complexes.^{90,91} The photophysical characterisation of **27** revealed that the emission was strongly quenched in comparison to that of the parent complex, **28**. This indicates the presence of an efficient intramolecular quenching process which is restored upon protonation of the quinoline moiety.⁹⁰ This pH-dependent intramolecular quenching process was concluded to originate from a PET process from the non-protonated quinoline moiety to the excited $[\text{Ru}(\text{TAP})_2\text{phen}]^{2+}$ unit, **28**.^{90,92} However, in the presence of DNA, the emission from the Ru(II) centre was restored to varying degrees depending on the DNA sequence employed.⁹³ This was attributed to a “switching off” of the quenching pathway due to partial protonation of the quinoline in the local microenvironment of DNA.⁹⁴



Emission studies in the presence of different polynucleotides also revealed the particularly interesting photophysical behavior of **27**, where at pH 7.5, the quinoline unit was completely non-protonated thus efficiently quenching the emission of the Ru(II) unit. Upon addition of [poly(dA–dT)]₂ a 1.8-fold increase of the emission intensity was observed for **28**, whereas **27** exhibited a 100-fold increase. When bound to [poly(dA–dT)]₂, the quantum yield of emission and the excited state decay kinetics of both **27** and **28** we found to be similar, indicating that the addition of [poly(dA–dT)]₂ restores the luminescence of the Ru(II) unit which is efficiently quenched in bulk solution. The protonation of the quinoline subunit upon binding to polynucleotides accounts for the removal of the intramolecular photo-electron transfer quenching of the MLCT excited state of **27**. It is interesting to point out that **27** in the presence of [poly(dA–dT)]₂ acts as a molecular ‘light switch’ in the sense that the luminescence is ‘switched on’. While an emission enhancement was observed for **28** upon binding to [poly(dA–dT)]₂, its luminescence was quenched in the presence of DNA; originating, as previously demonstrated, from an electron transfer from *guanine* residues of DNA to the MLCT excited state of the complex.⁷⁷ In acidic conditions, the luminescence of **27** was also quenched upon addition of DNA and the binding isotherm displays a plateau value that was similar to that of **28**, although at lower [DNA]:[Ru(II)] ratios. It was therefore concluded that functionalisation of the Ru(II) unit in **27** does not affect the luminescence quenching efficiency with *guanine* residues of DNA, but at the same time the functionalisation resulted in an increase in binding affinity by one order of magnitude. In sharp contrast to the quenching of luminescence associated with DNA binding in acidic conditions, addition of DNA to **27** in buffer solution, at pH 7.5, induced a 16-fold increase of emission intensity. This behavior may be explained by protonation of the quinoline subunit upon DNA binding preventing the intramolecular quenching of the excited state of **27**. However, the restored luminescence is in turn quenched intermolecularly by *guanine* residues.⁹⁴

While **27** was shown not to exhibit sequence selectivity in binding, the nucleic base content of the polynucleotides profoundly affected the emission of the bound species. Studies were carried out with polynucleotides containing varying percentages of *guanine* residues and it was shown that the observed enhancement was dependent on the base content; the greater the percentage of AT base pairs, the greater the observed luminescence enhancement. Hence, **27** can be regarded as a luminescent sensor for base content, acting as a light switch for sequences possessing high AT content.⁹⁴ It has since been demonstrated that under photoirradiation, **27** dramatically reduces the transcription rate of RNA polymerase activity to around 50%, whereas [Ru(bpy)₂phen]²⁺ reduced it only to 20%. As a control, no inhibition

occurred in the dark and so this activity can be directly related to the photo-adduct formation where $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$ is unable to form a photo-adduct.⁹⁵ π -deficient Ru(II) complexes appended to functional units have thus been shown to be highly effective photoreagents for DNA. The effect of such photo-adduct formations on cellular function is clear and further conjugation to various other functional units opens a new approach to developing potential DNA probes, DNA photoreagents and PDT sensitiser drugs. A particular example of such a functional unit, with numerous advantageous properties is that of the 1,8-naphthalimide chromophore. The following section will give a brief overview of the properties of 1,8-naphthalimides and give a number of relevant examples from the literature.

1.7 1,8-Naphthalimides as DNA Photocleavage Agents

Due to its unique photophysical properties and DNA-intercalating ability, the 1,8-naphthalimide structure has found application in many areas of chemistry.⁹⁶⁻⁹⁸ Its absorption and fluorescence emission spectra lie within the UV and visible regions, and the various photophysical properties can be easily tuned through synthetic design. Structural modifications are readily achievable on either the aromatic moiety itself, or at the '*N*-diimide site', allowing for various functional groups and structural motifs to be incorporated (Figure 1.18).

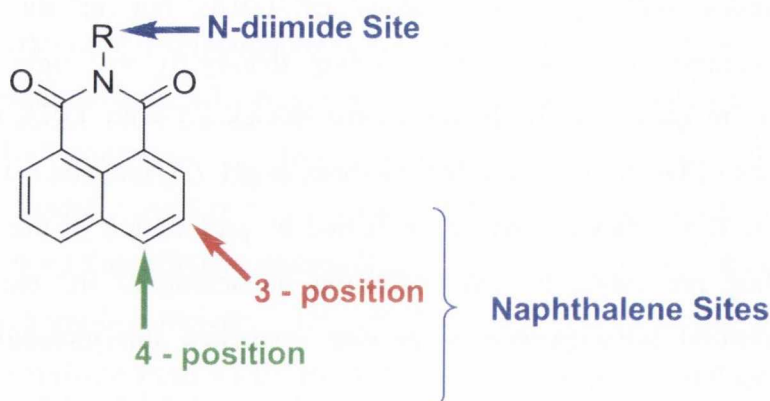
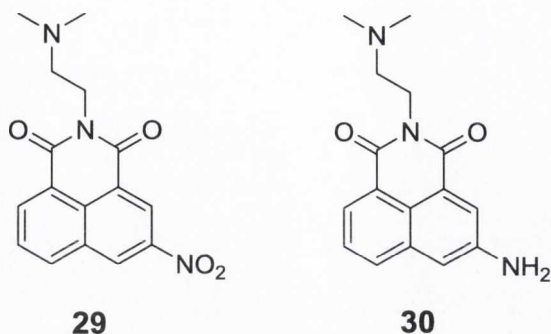


Figure 1.18 General structure of the naphthalimide chromophore showing the main positions of modification

Biological interest in 1,8-naphthalimides began in the 1970s when, in the search for new effective anti-tumour drugs, Brăna *et al.* designed and synthesised a series of imide derivatives of 3-nitro-1,8-naphthalic acid.⁹⁹ Waring *et al.* showed that compounds of this type bind to DNA, increase the length of sonicated DNA and cause unwinding of closed circular superhelical DNA, characteristics typical of intercalating agents.¹⁰⁰ The first drugs of this group that reached the clinical trial stage were mitonafide, **29** and amonafide, **30** which exhibited highly effective anti-tumor activity, primarily by interaction with the Topoisomerase

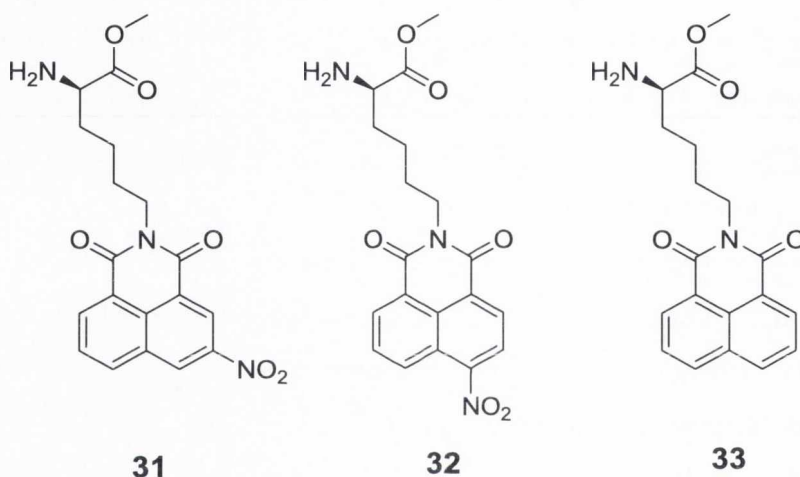
II enzyme resulting in inhibition of the replication process or an enhancement of Topoisomerase II mediated DNA cleavage.¹⁰¹ However, due to unexpected central neurotoxicity and limited efficiency, the clinical studies on these two drugs have been discontinued.¹⁰²



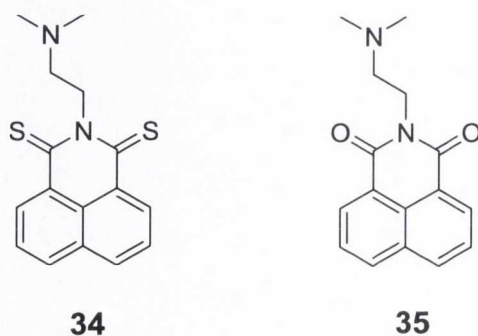
Since then a vast array of compounds have been generated in an attempt to further improve upon the DNA binding capacity and anti-tumor toxicity of 1,8-naphthalimide containing drugs. A large research effort has also been carried out by the Gunnlaugsson research group to this end, and as such the use of 1,8-naphthalimides as anti-cancer agents has already been reviewed extensively in previous theses from this research group including those of Frimansson,¹⁰³ Veale,¹⁰⁴ Gillespie,¹⁰⁵ Hussey¹⁰⁶ and Phelan.¹⁰⁷

In the 1990s, Saito *et al.* reported a family of nitro-substituted *L*-lysine-1,8-naphthalimide derivatives that make use of the inherent excited state reactivity of the 1,8-naphthalimide core displaying efficient DNA photocleavage properties.^{108,109} After irradiation and piperidine treatment, compound **31** was found to cleave duplex DNA with high selectivity for *thymine* residues. Irradiation of **31** in the presence of 3',5'-dibenzoyl protected thymidine led to oxidation of the *thymine* methyl group to an aldehyde (formyl-Uracil or fU) while prolonged irradiation resulted in decarbonylation, leaving *uracil* at that site. This demonstrates the ability of **31** to directly oxidise the nucleobase rather than (or in addition to) the sugar. The authors also investigated the ability of **31** to photocleave the single-stranded trimer 5'-d(ATA)-3'. Irradiation again led to oxidation of the *thymine* methyl group and piperidine treatment resulted in cleavage of the trimer at the oxidized T site. Finally, irradiation of poly(dA)-poly(dT) also led to formation of fU, indicating that **31** can oxidise the *thymine* methyl group in double-stranded as well as single-stranded DNA. The authors proposed that the oxidation reaction is due to photoinduced hydrogen abstraction from the methyl group by the excited state nitro group. Evidence in support of this hypothesis comes from the observation that **32** cleaves duplex DNA with significantly lower selectivity for *thymine* residues. If the two compounds bind to DNA, in similar orientations (by intercalation), then the nitro groups will point in different directions, leading to different H-abstraction

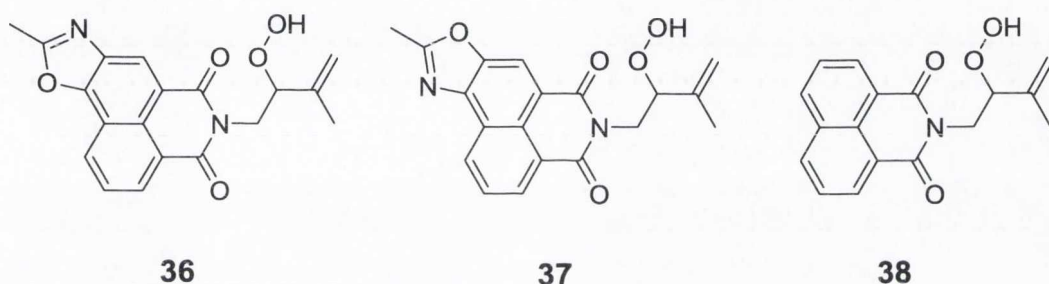
efficiencies. Significantly, compound **33** does not cleave at *thymine* residues, but rather at GG sites. Saito and co-workers performed mechanistic experiments in an effort to understand what factors led to cleavage at GG sites.¹⁰⁹ Laser spectroscopic experiments demonstrated production of an imide radical anion providing clear evidence for a photoinduced electron-transfer reaction. Subsequent *ab initio* calculations have also revealed that GG steps are the most electron donating and, thus, the most easily oxidized site for the B-form DNA conformation.¹¹⁰ These reports indicate that 1,8-naphthalimide derivatives are not only capable of cleaving DNA photochemically but that, depending on the substitution pattern, hydrogen abstraction atoms from deoxyribose, or from *thymine* methyl groups, can result in site selective photocleavage.



In more recent work Qian *et al.*¹¹¹ have developed a number of photocleavage agents exploiting the 1,8-naphthalimide core. The intercalation and photocleavage of DNA by **34** was seen to be extremely effective compared to that of the oxygen-containing counterpart **35**. Again, the photocleavage efficiency under UV light irradiation was proposed to proceed by electron transfer from the bases of DNA to the triplet excited state of the naphthalimides. In the same report the enhancement of the intercalation ability and the photocleavage of DNA was also observed for other compounds possessing a thiono or thio group compared with their oxygen-containing counterparts.¹¹¹



Another strategy employed by the same group involved the synthesis of a family of oxazolonaphthalimides which were capable of generating hydroxyl radicals.^{112,113} Using Electron Spin Resonance (ESR) they showed that they could efficiently generate hydroxyl radicals and photocleave supercoiled plasmid DNA while at the same time being tightly bound to DNA. Naphthalimide hydroperoxides **36** and **37** bearing a conjugated oxazole ring were designed and synthesised by the same group, and were shown to be more efficient than the non-substituted counterpart **38** in DNA photonicking. The authors suggested that the enlargement of the molecular planarity of compounds **36** and **37** by introducing the conjugated oxazole ring played an important role in the improvement of their DNA-intercalating capabilities, as well as in causing a bathochromic shift of their UV-absorption band, both factors, contributing to their enhanced DNA photocleaving capabilities.



In another study heterocyclic-fused naphthalimide intercalators with chiral amino side chains have been investigated in order to gain an understanding of the effect of chirality on DNA binding, photodamage and antitumor cytotoxicity (Figure 1.19).¹¹⁴ In general, it was reported that their side chains' chiral configuration was important in determining DNA binding affinities with preference being observed for *S*-enantiomers over both *R*-enantiomers and racemic mixtures of both species. In addition, their DNA photodamaging activities were in good agreement with their DNA binding constants calculated from fluorescence data; the *S*-enantiomers could photocleave circular supercoiled DNA more efficiently than their *R*-enantiomers. Even more importantly, almost all of these intercalators showed effective cytotoxicities against human lung cancer cells and murine leukemia cells. It is interesting to note that the racemic, *R*- and *S*-enantiomers all showed differing bioactivities. Although there was no obvious or direct relationship between their cytotoxicities and DNA binding affinities, it was noteworthy that the chiral amino side chains did play an important role in their antitumor cytotoxicities. *R/S*-enantiomers behaved quite differently in terms of cytotoxicity against different cell lines, suggesting that the chirality of amino side chains is an important consideration in developing novel naphthalimide based antitumor agents.

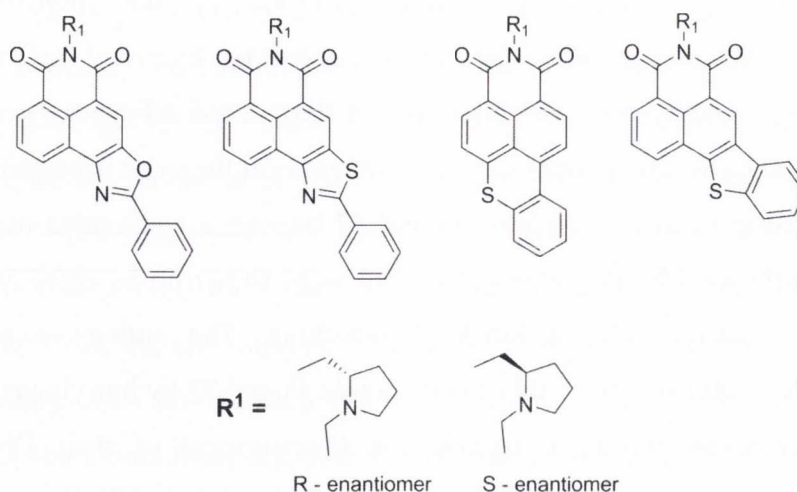


Figure 1.19 Chemical structures of heterocyclic-fused naphthalimide intercalators with chiral amino side chains

As a demonstration of their versatility, the naphthalimide derivative, **39** showed near-infrared emission and large Stoke's shifts ($\lambda_{\text{abs}} = 580 \text{ nm}$, $\lambda_{\text{em}} = 750 \text{ nm}$ in MeOH), showing its potential as a long-wavelength near-infrared (NIR) fluorescent imaging agent.¹¹⁵ After incubation with **39** for 2 hours at 37°C in V79 379A Chinese hamster cells, an intense intracellular red fluorescence was observed with excitation at 537 nm. The intense fluorescence observed after such a short time indicates that **39** could be potentially used as a biological label. Although the DNA binding ability of this compound was not evaluated one would expect, as has been demonstrated in the previous examples, with such a large planar aromatic area that this species would be a strong binder to DNA. With the added benefit of its excited state reactivity, **39** showed real potential as a dual imaging and DNA photodamaging agent for PDT. In addition to the examples given above many approaches are being utilised to create species that can selectively cleave DNA under light irradiation. Thiazonaphthalimides¹¹⁶ and sulphur substituted naphthalimides¹⁰² are just two other examples.

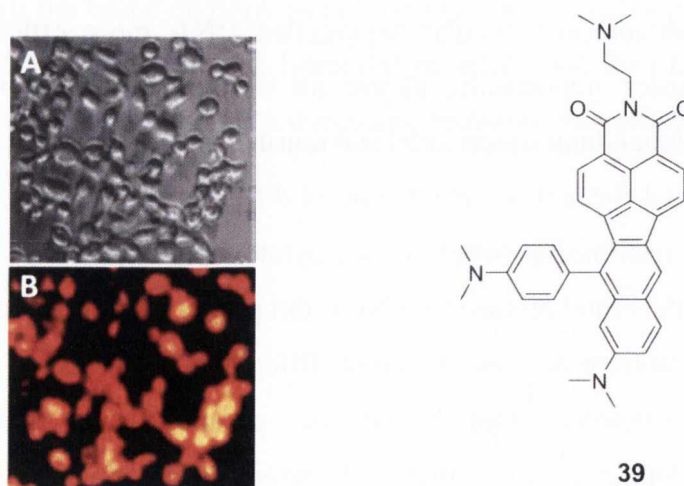


Figure 1.20 Fluorescence microphotographs of V79 cells incubated with **39** ($20 \mu\text{M}$).

(A) = bright field image, (B) = fluorescence after excitation at 537 nm

The outline of the preceding section shows the immense amount of work that has been undertaken in the area of naphthalimide chemistry. In addition, the versatility shown has ranged from DNA binding species to DNA photocleavers, antitumor agents and most recently NIR fluorescence imaging agents all of which make these species ideal for development as PDT photosensitisers. It also emphasises the potential importance that species containing this structure will have in the future. In particular, conjugation to other functional species will bring about novel structural probes or sequencing agents for DNA that may be activated in a controlled manner by irradiation. Increasingly, conjugation of active moieties to functional materials has been seen as a viable approach in many fields of chemical research. Gold nanoparticles (AuNPs), in particular, have seen a significant amount of research focus¹¹⁷ and their use, with particular attention to biological application will be reviewed in the following sections.

1.8 Gold Nanoparticles and Medical Application

In recent years, gold nanoparticle (AuNP) conjugates and their properties have led to new and exciting developments with enormous potential in biology and medicine.¹¹⁷⁻¹¹⁹ Recent studies by Mirkin *et al.*, as well as those of several other research groups, have shown AuNPs, when functionalised with appropriate surface moieties, can readily enter living cells, as shown in Figure 1.21.^{117,120}

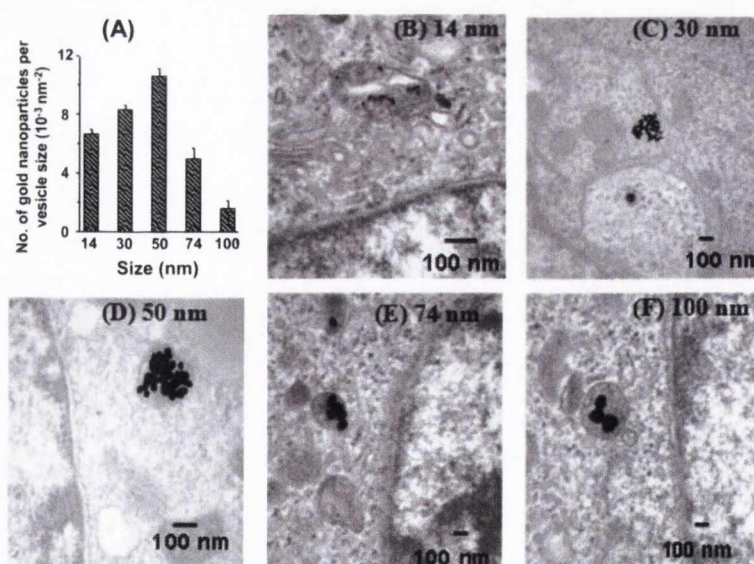
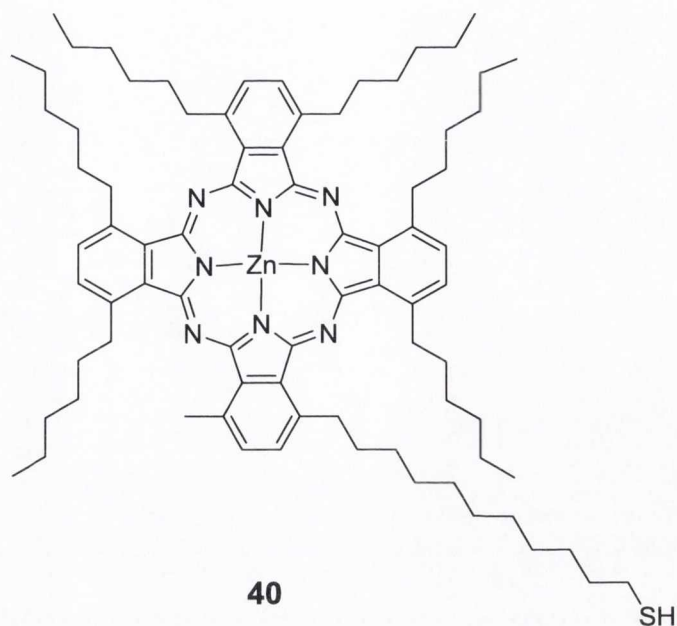


Figure 1.21 Transmission electron microscopy imaging and measurements of AuNPs in cells. (A) The graph of the number of AuNPs per vesicle diameter vs. AuNP size. (B-F) TEM images of AuNPs with sizes 14, 30, 50, 74, and 100 nm trapped inside vesicles of a HeLa cell, respectively

These developments have forged the way in nanoparticle research, leading to the broader use of AuNPs in cellular biology and the promise of their eventual use as therapeutic and diagnostic agents. Structures which behave as gene-regulating agents,^{121,122} drug carriers,^{123,124} imaging agents,¹²⁵⁻¹²⁷ and photoresponsive therapeutics^{128,129} have been developed and studied in the context of many different diseases. The highly tunable and multivalent surface architecture of AuNPs offers the potential to incorporate multiple therapeutic agents on the surface of a single nanoparticle. Coupled to their physical and chemical properties, which provide distinct advantages over molecule-based systems, AuNPs are expected to improve the delivery and efficacy of therapeutic payloads in cellular and medical applications.

1.8.1 Gold Nanoparticles Conjugates as PDT Agents

In the context of Chapter 1, AuNPs that have been conjugated to photoactive and photoresponsive species are of particular relevance. Studies undertaken by Hone *et. al.* have shown the synthesis of AuNPs stabilised with a phthalocyanine dye, **40** for the delivery of a photosensitiser for PDT.^{130,131} The AuNP conjugates were shown to have an average diameter of 2–4 nm after a synthetic strategy that resulted in a phase transfer reagent being interdigitated between the phthalocyanine molecules on the particle surface. This resulted in increased solubility of the hydrophobic photosensitiser in polar solvents enabling delivery of the AuNP conjugates to cells. The dye was shown to be present in the monomeric form on the AuNP surface, absorbing radiation at 695 nm resulting in the formation of singlet oxygen with high efficiency. Importantly the three-component (photosensitiser/gold/phase transfer reagent) AuNPs were shown to generate singlet oxygen with a $\approx 50\%$ enhancement of the quantum yield compared to that of the free dye suggesting that the phthalocyanine-AuNP conjugates are ideally suited for PDT. When the AuNP conjugates were incubated with a cervical cancer cell line, they were shown to be efficiently internalised, delivering the phthalocyanine photosensitiser directly into the cell interior. Subsequent irradiation of the AuNP conjugates within the cells induced substantial cell mortality, thought to be as a result of photodynamic production of singlet oxygen. Encouragingly, the PDT photocytotoxic efficiency of the AuNP conjugates was shown to be twice that obtained from using the free phthalocyanine dye, supporting the hypothesis of damage by singlet oxygen formation. These results suggest that AuNP conjugates are an excellent vehicle for the delivery of surface bound photosensitisers for efficacious PDT.



Using a slightly different approach Wang *et al.* have synthesised size-controlled supramolecular gold nanoparticles (Au-SNPs) using a supramolecular self-assembly approach, by combining cyclodextrin-grafted branched polyethylenimine (CD-PEI), adamantane functionalised polyethylene glycol (Ad-PEG) and 2 nm AuNPs.¹³² This work was based on the premise that the resulting Au-SNPs might exhibit enhanced photothermal effects over AuNPs alone.¹³³ The reported photothermal effects resulted from femtosecond laser excitation of AuNPs in an aqueous environment. This led to a transient heating of the nanoparticles, which decayed *via* heat transfer to the water phase where it was shown that the water temperature rose to near the critical temperature of the water which undergoes an explosive evaporation in the sub-nanosecond range.¹³⁴ The use of arginine–glycine–aspartic acid (RGD) peptides as targeting ligands displayed on the surface of the Au-SNPs saw successful selective photothermal treatment of a population of $\alpha_v\beta_3$ -positive cells with no damage to neighbouring $\alpha_v\beta_3$ -negative cells by laser-induced microbubble-generation (Figure 1.22).¹³²

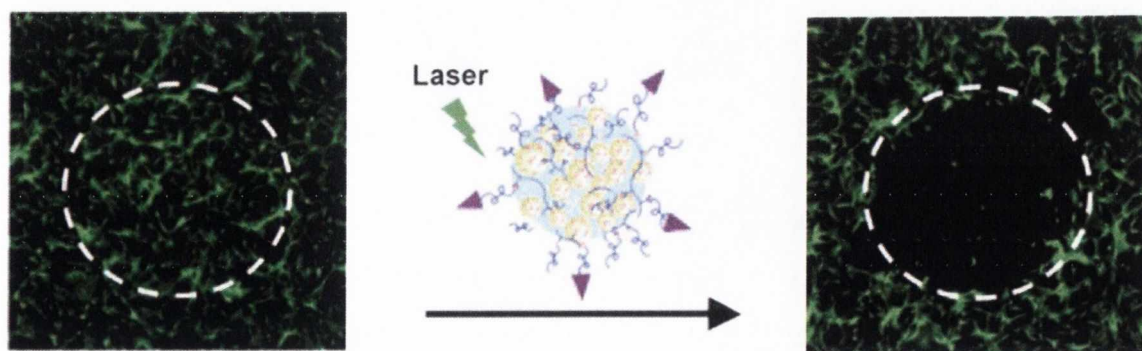
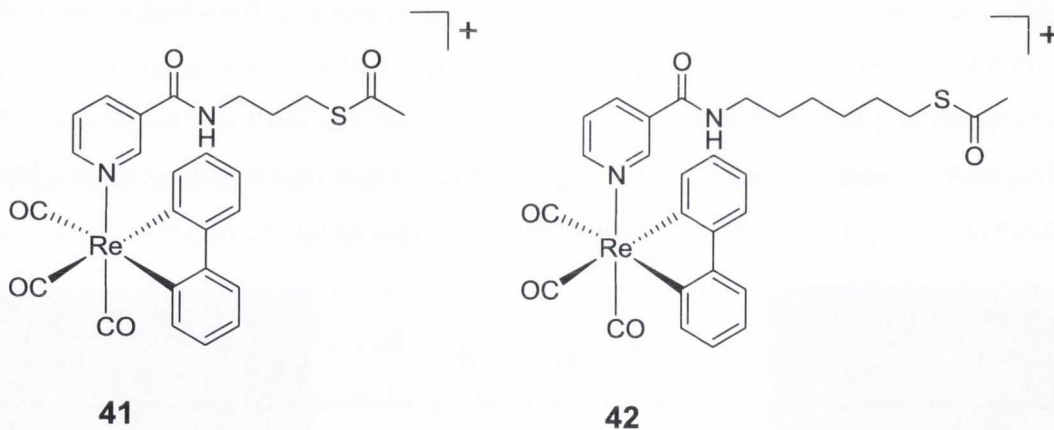


Figure 1.22 Fluorescence micrographs of $\alpha_v\beta_3$ -positive cells (green labelled) before and after treatment with 118 nm RGD-Au-SNPs and laser irradiation

The work discussed above further demonstrates the potential of AuNPs for use in alternative cancer therapies. Furthermore, the use of AuNP conjugates that incorporate a functional species that can act as both a targeting and a photoactivatable moiety will lead to further improved photoactivated therapies. As discussed in Section 1.6 transition metal complexes provide both DNA binding ability as well as photoreactivity and, as such, fulfil the requirements of being both targeted to, and capable of photoreacting with DNA. In addition, the rich photophysical properties of many metal complexes provide an alternative to traditional fluorescent imaging agents.

1.8.2 Transition Metal – Gold Nanoparticle Conjugates

Although still a relatively unexplored field, the coordination of transition metals to the outer surface of AuNPs has already been employed in diverse areas such as catalysis¹³⁵ and sensing.¹³⁶ Nanoparticle-based catalysis has taken advantage of their large surface area, solubility properties, robust nature and well-organised surface topography. Similarly, sensing applications have benefited from the pre-organisation of the metal–ligand unit on the surface leading to an amplified electrochemical response to anions and other analytes.¹³⁷ A major area in which metal functionalised nanoparticles have yet to be fully exploited is in the field of biomedical applications. Steps have been taken towards this end by the group of Pope *et al.* who have developed luminescent, water-soluble AuNPs functionalised with ³MLCT emitting rhenium complexes **41** and **42**.¹³⁸



The resulting hybrid conjugates were shown to be water-soluble and also displayed ³MLCT emission; two ideal characteristics that represent a useful approach for the future development of luminescent AuNP-derived materials. Materials of this type have the potential to be exploited in a number of applications including fluorescence microscopy and nanoelectronic devices.¹³⁸

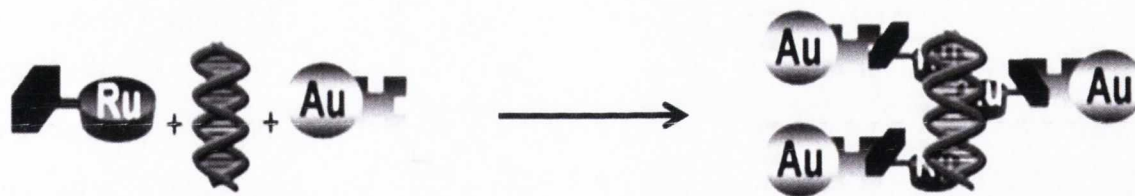
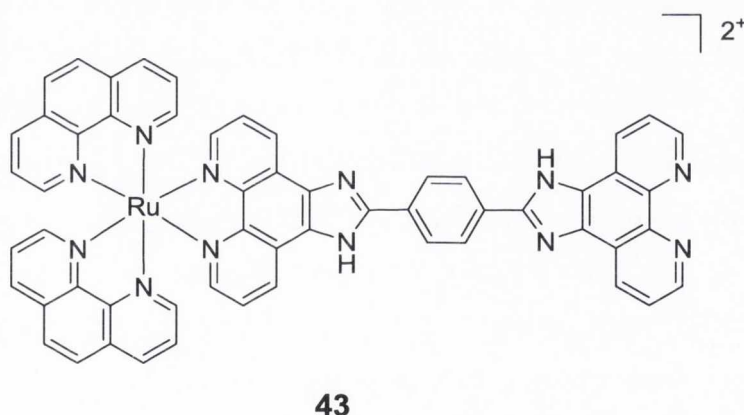


Figure 1.23 Schematic representation of the formation of AuNP-DNA assemblies by using a DNA-intercalating Ru(II) complex conjugated to a biotin unit crosslinker, AuNP-streptavidin conjugates, and a DNA template ¹³⁹

Sleiman *et al.* have developed an alternative approach by assembling discrete AuNP assemblies on a linear DNA template (Figure 1.23).¹³⁹ This work has made use of a DNA-intercalating Ru(II) complex conjugated to a biotin unit crosslinker, which shows very little luminescence in aqueous solution or when bound to streptavidin, and has its luminescence ‘switched on’ when bound to DNA.¹⁴⁰ The AuNP-DNA assemblies were then shown to form through mixing of the crosslinker and gold nanoparticle-streptavidin conjugates by TEM analysis. This fascinating work allows the simple and potentially selective assembly of a large number of readily synthesised streptavidin or biotin-labeled materials on DNA-AuNP conjugates. The use of such cross-linkers for the selective labelling of biologically important DNA structures (*e.g.*, mismatched DNA) with AuNPs and other functional components shows real promise for future biomedical application.



The 1,10-phenanthroline-terminated Ru(II) complex, **43** has also been used to stabilise and functionalise AuNPs using a terminal polypyridyl unit.¹⁴¹ The strong interaction between the nitrogen atoms of the polypyridyl ligand and the surface of AuNPs has allowed for the successful formation of Ru(II) stabilised AuNPs *via* a rigid phenanthroline type ligand allowing electronic communication throughout the assembly.¹⁴² Reverse phase transfer of these Au-NPs from water to acetonitrile by substituting the Cl^- counter anion with PF_6^- has also been demonstrated, thus allowing simple separation of the free and AuNP conjugated species. Importantly it has been demonstrated that Au surfaces often quench the fluorescence

of emissive species through charge or energy transfers,^{143,144} but fluorescence enhancement can also be observed depending on various factors such as the distance between the metallic NP and the fluorophore or the nature of the fluorophore itself.¹⁴⁵ The fluorescence spectra of the free and AuNP conjugated complex clearly showed a 33% quenching of emission from the grafted Ru(II) complex *vs.* the free species. Importantly, in this case, the emission maximum wavelength falls in the absorption band corresponding to the surface plasmon resonance, indicating that energy transfer is likely to be the major quenching process. Murray *et al.* have already reported such an energy transfer for $[\text{Ru}(\text{bpy})_3]^{2+}$ electrostatically associated to AuNPs.^{143,144} Nevertheless, due to the rigid and fully conjugated character of the linker, the authors surmised that electron transfer is the most likely contributor to the de-excitation of the fluorophore. The affinity of **43** for Au surfaces via its terminal phenanthroline pendant group offers new perspectives on a post-functionalisation conjugation approach to a large variety of metal complexes. Key parameters such as the anchoring point between the metal complex and the AuNP, the distance between the metal centre and the AuNP or the influence of a fully conjugated system compared with a non-conjugated system will be important factors to consider in order to modulate the interaction between the AuNPs and the metal complex. However, influence of such parameters on the luminescence and electrochemical properties of this family of AuNPs has not yet been reported.

The examples above serve to demonstrate the enormous potential of both organic and transition metal based AuNP conjugates. The properties of metal complexes self-assembled on AuNPs, in particular, leads to nano-sized bimetallic conjugates that will have a huge role to play in the future of many fields of research. Although largely unexplored to date their use in biomedical application, in particular, will involve cross-disciplinary research including synthetic chemists, materials scientists, biologists, engineers, and clinicians. The work carried out thus far provides only a glimpse of the wide range of potential applications for transition metal AuNP conjugates in biology and medicine.

1.9 Recent Advances within the Gunnlausson Group

The work of Gunnlausson *et al.* over the past number of years has spanned the various fields of chemistry described thus far. With extensive research undertaken in the field of naphthalimides as molecular sensors and reporters,¹⁴⁶⁻¹⁵¹ work has also focused on DNA targeting species derived from both the naphthalimide chromophore and Ru(II) conjugates. In addition, incorporation of lanthanide metal complexes on the surface of AuNPs is a burgeoning area in the field of supramolecular and lanthanide chemistry, work that has been pioneered by Gunnlausson *et al.*

Recent work has seen much research in the design and incorporation of nucleic acid recognition moieties into the 1,8-naphthalimide structure. To date numerous mono- and di-peptide-based 1,8-naphthalimide conjugates have been synthesised, using solution phase peptide chemistry. The basic structure comprises either an amino, nitro, chloro or bromo substituted naphthalimide species connected to a carboxylic anchor functionalised with a variety of α -amino acid/esters, examples of which are shown in Figure 1.24. Many of these compounds have shown sub 2 μ M IC_{50} (half maximal inhibitory concentration) values when tested against a series of leukaemia cell lines. The results of all of these studies are detailed in the Theses of Frimansson,¹⁰³ Veale,¹⁰⁴ Gillespie,¹⁰⁵ Hussey¹⁰⁶ and Phelan¹⁰⁷.

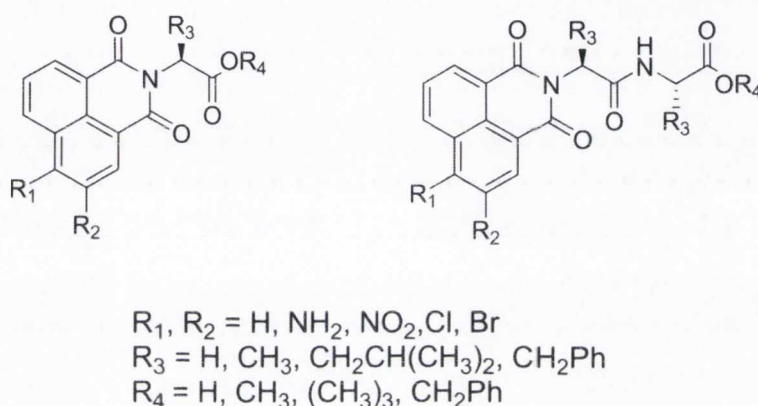


Figure 1.24 Examples of some of the mono- and di-peptide based naphthalimide derivatives developed by the Gunnlaugsson group

In addition to being a highly versatile structure for use in both cation and anion sensing,⁹⁶ the 1,8-naphthalimide structure has also been employed in the sensing of biomolecules, especially DNA. A series of 1,8-naphthalimide based Tröger's bases **44a-c** have been recently developed by Veale *et al.*, and were shown to bind strongly to DNA.^{152,153} These Tröger's base compounds have been shown to interact with DNA *via* electrostatic and hydrogen bonding interactions through the tertiary amines. These compounds also have the ability to bind through intercalation, and moreover, as demonstrated in Figure 1.25, these structures can also function as dual imaging–therapeutic agents. Confocal microscopy imaging studies have clearly shown that the structures accumulate within the nucleus and give rise to cellular death in drug-resistant K562 leukaemia cell lines. Interestingly the Tröger's base derivatives exhibited improved cytotoxicities over their 1,8-naphthalimide precursors.¹⁰³ With the observed increase in cytotoxicities over their 1,8-naphthalimide counterparts, ongoing research in this area is looking to develop mono- and di-peptide based Tröger's bases such as **45** and **46**, the results of which will be reported in the Thesis of Ms. Samantha Murphy.

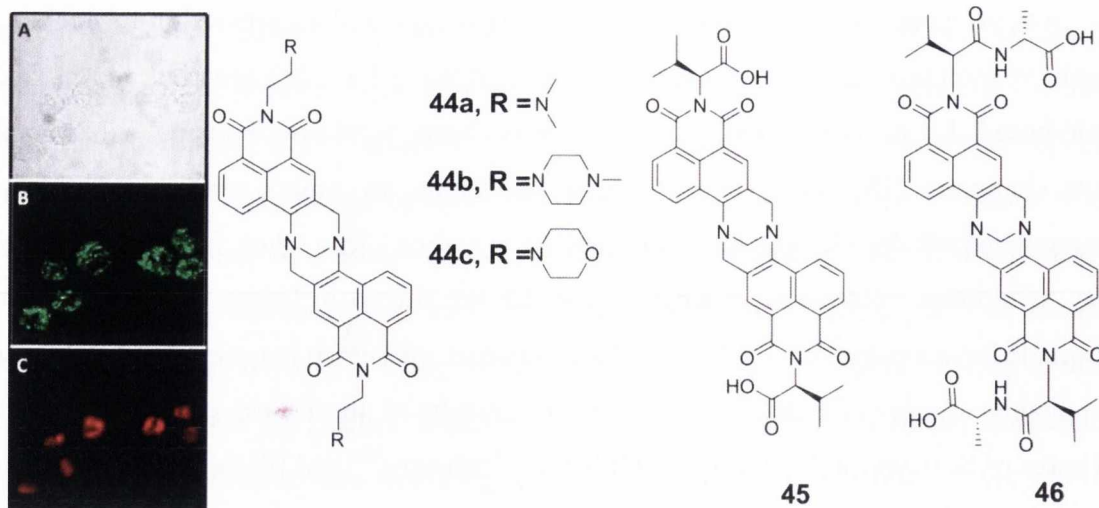
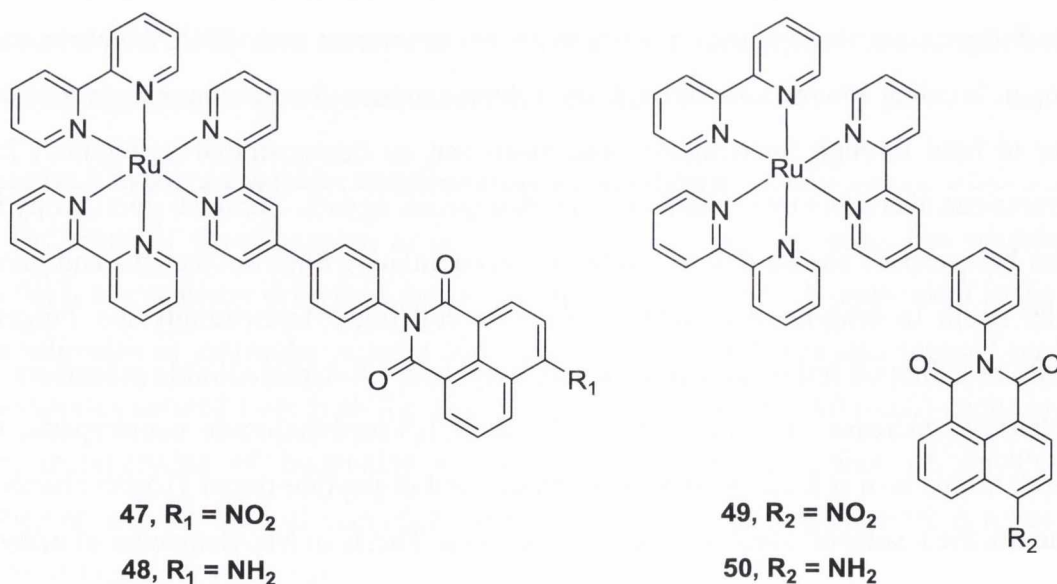


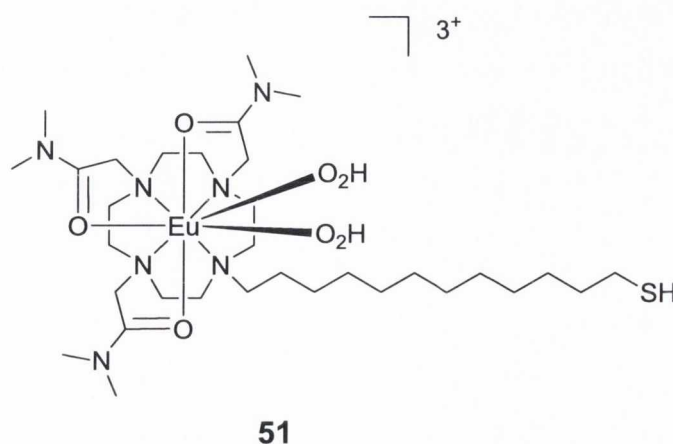
Figure 1.25 The 1,8-naphthalimide based Tröger's base and images showing the fluorescence arising from **44b** within cancer cells, imaged using confocal fluorescence microscopy: (A) bright field image, (B) fluorescence arising from **44b** (10 mM), (C) co-stained with propidium iodide

Ryan *et. al.* have developed an alternative approach to DNA targeting by the use of Ru(II) polypyridyl-1,8-naphthalimide conjugates **47-50**.^{154,155} In this work studies were conducted in order to understand the properties of bifunctional Ru(II)-1,8-naphthalimide conjugates, in particular the effect of variations in substitution and connectivity. Having synthesised various complexes containing both rigidly and flexibly linked 1,8-naphthalimide chromophores, the use of a more rigid aromatic group as the linking moiety was demonstrated, allowing for more precise control of the orientation of the two components as seen for **47** - **50**. A *meta* arrangement around the connecting ring was shown to result in greater affinity for DNA, and greater changes in the spectroscopic properties, due to greater complementarity in the shape of the complex to that of the DNA.



The nitro substituted complexes, **47** and **49**, were shown to undergo a DNA induced stacking process at low equivalents of DNA, with concomitant emission quenching. This was followed by an emission enhancement with further additions of DNA. This was suggested to be as a result of redistribution and protection from quenching in the local environment of the DNA. Amino complexes **48** and **50** possessed substantially different photophysical properties to their nitro analogues, being strongly emissive in solution. Furthermore, both displayed significant emission enhancements upon binding to DNA, an observation that was attributed solely to the protection of the complex from solvent quenching. An overall binding mode for conjugates **47** – **50** was proposed, which involves intercalation of the 1,8-naphthalimide into the helix with concomitant external groove association of the Ru(II) centre. The tight binding, typical of intercalative interactions, was apparent from thermal denaturation studies and ethidium bromide displacement assays, with further confirmation ascribed through circular dichroism, UV/Vis absorption and fluorescence emission titrations. Furthermore, all four complexes displayed efficient DNA photocleavage efficiency and were found to enter cervical cancer HeLa cells and localise within the nucleus, ascribed from flow cytometry experiments, and confocal microscopy experiments.¹⁵⁴

In the field of metal based AuNP assemblies Gunnlaugsson *et al.* have exploited emissive Eu(III)-AuNP conjugates within displacement arrays allowing phosphate anion detection in water.^{156,157} A heptadendate macrocyclic Eu(III) cyclen conjugate possessing an alkyl thiol group, **51** has been adsorbed onto the surface of AuNPs, forming water soluble AuNP conjugates. In aqueous solution, at pH 7.4, the AuNP conjugates are non-luminescent, however, upon addition of a β -diketone antenna the self-assembly formation results in a highly luminescent ternary complex upon excitation of the antenna. It was then demonstrated that these luminescent AuNPs can be used in the sensing of biologically relevant phosphates such as flavin monophosphate, through displacement of the β -diketone antenna (Figure 1.26).



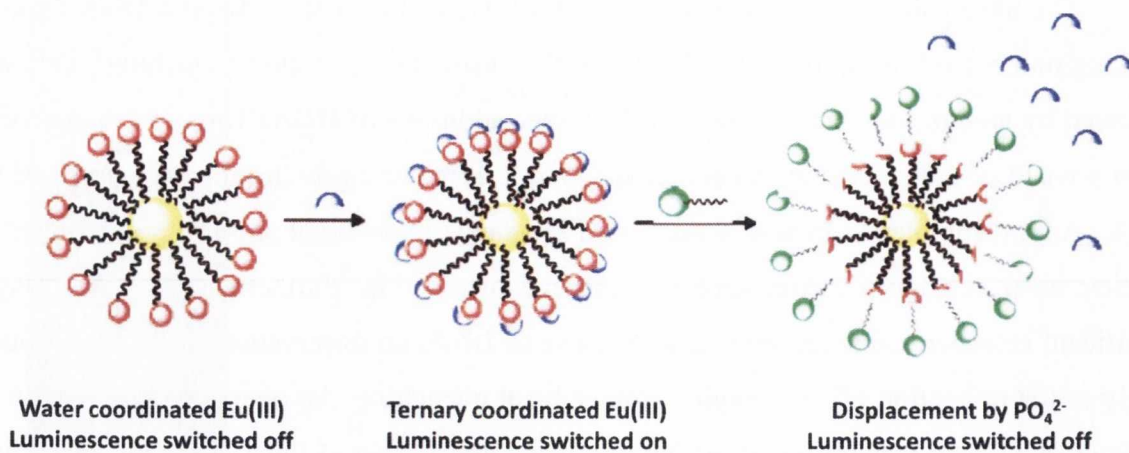


Figure 1.26 Schematic representation of self-assembly formation between the non-luminescent AuNP- Eu(III) conjugate and a β -diketone antenna, to give luminescent AuNP- Eu(III) conjugates. The sensing of flavin monophosphate occurs by the displacement of the antenna and the formation of the non-luminescent AuNP-Eu(III)-flavin conjugate ¹⁵⁶

Furthermore, this sensing mechanism was found to be highly selective for flavin mononucleotide which plays a key role in many electron transfer, oxidation, and dehydrogenation processes *in vivo*. Flavin mononucleotide was the only anion investigated that could effectively displace the β -diketone antenna from the luminescent ternary AuNP- Eu(III) conjugate leading to almost complete quenching of the Eu(III) emission. Phosphate containing molecules such as AMP, ADP, ATP, cyclic AMP, or NADP only quenched the Eu(III) luminescence between 20-55% after addition of 500 equivalents of these anions. Carboxylate containing creatine, coumaric, and panthotenic acid gave rise to minor luminescent changes while Na_2CO_3 and H_2PO_4 resulted in only 25% quenching. In a subsequent study, Bonnet *et. al.* studied the formation of the AuNP - Eu(III) - β -diketone self-assembly as a function of pH in water. This study revealed that the emission arising for the Eu(III) centre is significantly modulated as a function of pH, where within the physiological pH range, the emission is ‘switched on’. ¹⁵⁷

As can be seen from this brief review, the work of Gunnlaugsson *et al.* is vast and far ranging. The examples given above are just a small portion of work that has been undertaken and continues to evolve in the group. The work presented in this thesis will aspire to build upon the knowledge within the group by combining various aspects of chemical research with the aim of solving biological and medical problems.

1.10 Conclusion

The interaction of binding species with DNA has been the subject of intense investigation since the discovery of the double helical structure. An understanding of such interactions has been pivotal to the design and development of structural probes of DNA in

addition to novel chemotherapeutic agents. This chapter began with a brief discussion on the role of the cell cycle in cancer disease progression and how the targeting of DNA is such an important approach in chemotherapeutics.

The structure and modes of binding to DNA were also discussed with particular attention being drawn to intercalation. Intercalation is of particular importance due to its strength of binding and its ability to induce changes in the photophysical properties of the structure upon binding. This led to a discussion of transition metal species capable of binding to DNA, namely Ru(II) polypyridyl complexes. Ru(II) complexes show an inherent ability to interact with DNA, and furthermore, by varying the ligands around the metal centre it is possible to modify the interaction with nucleic acids in order to take advantage of their characteristic photostability and water solubility as well as their useful photophysics and photochemistry. As a result their use as spectroscopic probes for DNA was discussed.

As well as being utilised as spectroscopic probes for DNA, there has also been an increased focus on the use of Ru(II) complexes as DNA photoreagents due to their excited state reactivity. This led to a brief introduction to Photodynamic Therapy (PDT), an alternative approach to traditional chemotherapy, which takes advantage of the excited state properties of photosensitisers for medicinal application. In the context of Ru(II) complexes, particular attention was drawn to the utility of polyazaaromatic Ru(II) species due to their π -deficiency and strongly oxidising excited state reactivity; properties particularly well suited to species for application in phototherapeutics.

The discovery and progression of the 1,8-naphthalimide chromophore as a potential anticancer agent was also discussed. The purpose of this section was to show the versatility of this species. Examples were detailed that ranged from DNA binding species to DNA photocleavers, antitumor agents and most recently NIR fluorescence imaging agents. Many of the properties described show that this structure, with its excited state reactivity, is also ideal for incorporation into species that may be used as PDT agents.

A further functional material detailed in this introduction was that of gold nanoparticles (AuNPs) and their use in medical application. The examples given demonstrate the enormous potential of both organic and transition metal based AuNP conjugates for targeted drug delivery as well as light activated therapies. Although largely unexplored to date the use of transition metal based AuNPs, in particular, will lead to a wide range of potential applications in biology and medicine.

The final section dealt with recent scientific advances within the Gunnlaugsson group. This research represents an important contribution to the design of potent therapeutic agents, with the development of a large family of peptide-based 1,8-naphthalimide derivatives, many

of which have exhibited potent cytotoxic activity in leukaemia cell lines. DNA targeting agents based on Ru(II)- polypyridyl-1,8-naphthalimide conjugates as well as novel 4-amino-1,8-naphthalimide-derived Tröger's bases were also discussed. A final example of a heptadendate macrocyclic Eu(III) cyclen conjugate possessing an alkyl thiol group adsorbed onto the surface of AuNPs was given, forming water soluble AuNP conjugates. These AuNP conjugates were used in displacement assays allowing phosphate anion detection in water. This particular example emphasised the potential of metal based AuNP conjugates for the formation of functional species.

Building on work already carried out within the Gunnlaugsson group, the aim of the work discussed in this thesis will incorporate many different aspects of the research described in the previous sections. Agents based on combining photoactive Ru(II) complexes, the 1,8-naphthalimide structure and AuNPs may show potential as biomedical DNA probes, photoagents and eventually photoactivated therapeutics.

1.11 Work Described within this Thesis

This research project has been concerned with the development of a range of novel Ru(II) conjugates as bimodal probes and photoreactive reagents for DNA. The overall aim of the work discussed will be to combine the advantageous properties of Ru(II) polypyridyl complexes with those of various other functional species.

In *Chapter 2*, a series of Ru(II) complexes based on a new ligand, pyrazino[2,3-*h*]dipyrido[3,2-*a*:2',3'-*c*]phenazine, or pdppz, combining, in a single structure, two well-known polypyridyl ligands, dipyrido[3,2-*a*:2',3'-*c*]phenazine (dppz) and 1,4,5,8-tetraazaphenanthrene (TAP), are described. It was anticipated that further extension of the known, flat, planar dppz structure would increase DNA binding ability. Furthermore, it was envisaged that incorporation of a 'TAP like' moiety on the ligand in tandem with variation of the ancilliary ligands would infer both increased DNA binding and photocleavage ability with possible formation of DNA – photoadducts as has been seen previously with Ru(II) complexes containing TAP ligands. The synthesis of each derivative is discussed, in addition to their photophysical and DNA binding properties.

In *Chapter 3*, the ability of an organic quaternerised derivative of pdppz to bind to DNA was investigated. Due to its extended aromatic surface, in concert with its cationic character, it was foreseen that it would be an ideal candidate as a DNA intercalator. Furthermore, it was anticipated that this DNA targeting species should also possess rich photophysical properties that could be monitored to evaluate *in vitro* DNA binding

interactions, using various spectroscopic techniques. The synthesis, photophysical evaluation, DNA photocleavage ability as well as its cellular uptake and cytotoxicity are described.

In *Chapter 4*, Ru(II)(TAP)₂ - 1,8 naphthalimide conjugates with a rigid arrangement of the subunits will be discussed. The effect of inclusion of π -deficient TAP ancilliary ligands on the photophysical and DNA binding properties of these conjugates will be explored. In addition, their excited state reactivity with DNA and their preliminary cellular localistaion will be discussed.

In *Chapter 5*, a discussion of a Ru(II)(bpy)₃-1,8 naphthalimide conjugate as a fluorescent anion sensor is given. The spectroscopic effects on the Ru(II) excited state as well as preliminary NMR binding data is detailed.

In *Chapter 6*, two bis-Ru(II)(bpy)₂-bis-1,8-naphthalimide containing Tröger's base derivatives will be described. The effect the substitution pattern around the Ru(II) centre has on the synthesis and photophysical properties of these moieties will be explored, in addition to a preliminary description of their DNA binding ability and applicability to biological imaging.

In *Chapter 7*, a series of Ru(II) stabilised water-soluble, luminescent AuNPs are described. The synthesis, physical and photophysical evaluation of each conjugate will be discussed. In addition, preliminary DNA binding studies as well as cellular uptake studies will be detailed.

Finally, in *Chapter 8*, general experimental procedures are outlined and the synthesis of each of the compounds discussed is given.

Chapter 2:

A Family of Ru(II) Complexes Based on a Novel Extended Aromatic Polypyridyl Ligand

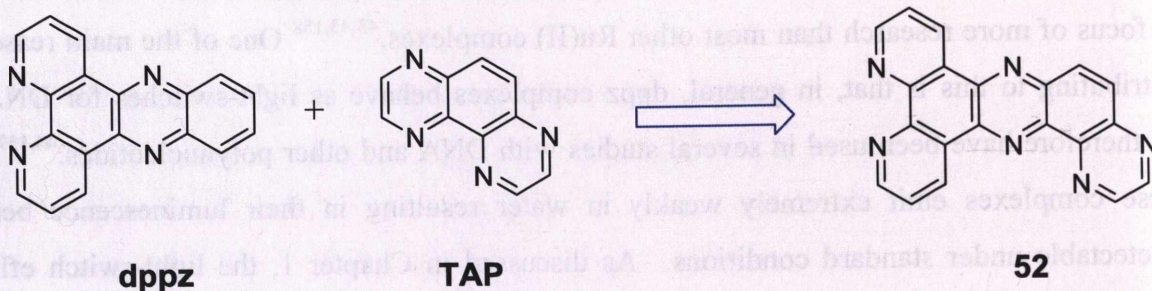
2.1 Introduction:

Since the first synthesis of $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ in 1985,³⁸ dppz complexes have been the focus of more research than most other Ru(II) complexes.^{43,45,158} One of the main reasons contributing to this is that, in general, dppz complexes behave as light-switches for DNA³⁵ and therefore have been used in several studies with DNA and other polynucleotides.^{44,159,160} These complexes emit extremely weakly in water resulting in their luminescence being undetectable under standard conditions. As discussed in Chapter 1, the light-switch effect with DNA has been attributed to the protection of the dppz ligand by insertion between the DNA base pairs, providing shelter from the aqueous solvent molecules.¹⁶¹ $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ is the classical example whereby it exhibits striking behaviour in aqueous solution; its luminescence lifetime is very short (*ca.* 250 ps) and its quantum yield of emission extremely low.^{162,163} Although the nature of the lowest excited states in $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ is still unclear, interaction of the excited state by H-bonding with water molecules is generally accepted to cause the short-lived emitting state in water.¹⁶² The origin of the interaction with DNA of Ru(II)(dppz) complexes stems from their extended aromatic heterocyclic ligands which, due to their large, planar surface area allows them to intercalate between the bases. Moreover, this facilitates their use for recognition and sensing of DNA owing to their luminescent properties. Additionally, they offer several advantages over existing organic based luminescent DNA markers due to characteristic photostability, water solubility, large Stokes shifts, effective DNA binding as well as useful photophysics, all combining to make them excellent candidates as spectroscopic probes for biomolecules.

Simultaneously, research on Ru(II) complexes that combine the use of π - deficient, electron accepting ligands, such as tetraazaphenanthrene (TAP), **25** has shown that the presence of these ligands confers a strongly oxidising excited state on the complex.^{60,164} This state results in a photoinduced-electron transfer (PET) between *guanine* and the ³MLCT state of the complex, which can result in both single-strand breaks followed by double-strand cleavages, as observed with plasmid DNA. Additionally, the formation of covalent adducts between the complexes and *guanine* or tryptophan residues have also been reported.^{59,81,82} Thus, Ru(II) complexes containing polyaaromatic ligands are highly photoreactive species capable of causing selective damage to biomolecules under illumination, a highly desirable characteristic from the point of view of photoactivated therapeutics.⁶⁰

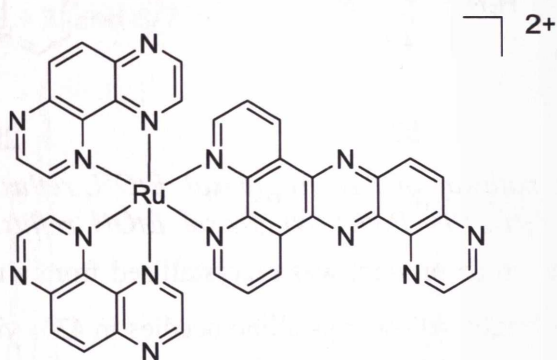
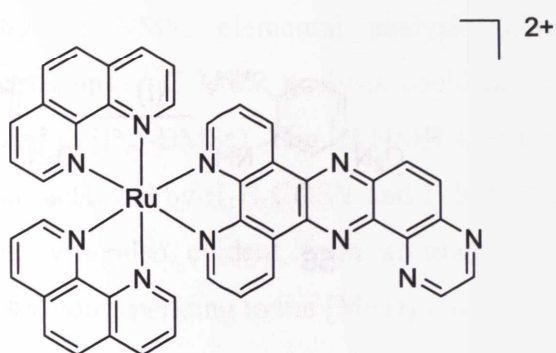
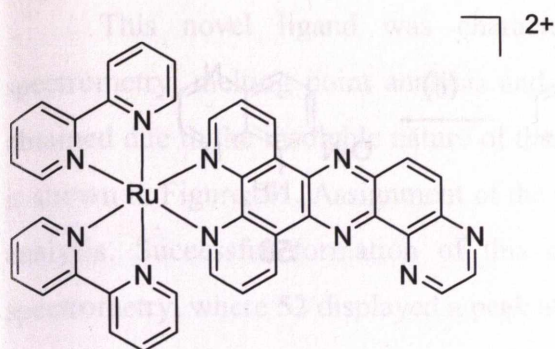
The objective of the research presented in this Chapter will be the development of a series of Ru(II) polypyridyl complexes based on a novel extended, aromatic, polypyridyl ligand, pyrazino[2,3-*h*]dipyrido[3,2-*a*:2',3'-*c*]phenazine, or pdppz for short, **52**. This new

ligand is based on combining, in a single structure, both dppz and TAP and has not, to the best of our knowledge, been synthesised before.



In the design of **52** it was anticipated that further extension of the flat, planar dppz structure would infer both increased DNA binding and photocleavage ability with possible formation of DNA photoadducts, as has been previously seen with Ru(II) complexes containing TAP.^{86,87} In order to establish the effect of variation of the ancilliary ligands around the Ru(II) centre, three species will be synthesised utilising bipyridine (bpy), phenanthroline (phen) and tetraazaphenanthrene (TAP) in order to provide a set of known photophysical properties that can be used to monitor the binding process. The nature of the binding interaction of each complex with DNA will be an important consideration in this context as the position of the photoactive species with respect to the DNA bases is likely to have a major effect on the efficiency of any photoreactions that occur.¹⁶⁵ The preorganised position of the complex in the DNA helix may lead to photoadduct formation and/or a reaction with the sugar moiety causing efficient DNA photocleavage. In any case, reaction of compounds with DNA can produce dramatic effects on DNA functions *in vivo*, and can disturb gene expression, disrupting the binding of enzymes involved in the replication processes. This is indeed of great interest for the inhibition of gene expression in cancerous cells with huge potential in cancer chemotherapy.²⁹

The overall aim of the work presented in this Chapter is to identify the potential of these systems as spectroscopic probes for DNA and also as possible DNA photocleavage agents for use in PDT. Firstly, a description of the synthesis and characterisation of each of the systems **53** – **55** is given. This is followed by a discussion of the photophysical properties of each complex and their DNA binding behaviour was then established using a range of spectroscopic techniques. Finally, biological studies will be described detailing DNA photocleavage ability, cellular uptake and localisation as well as cellular phototoxicity studies.

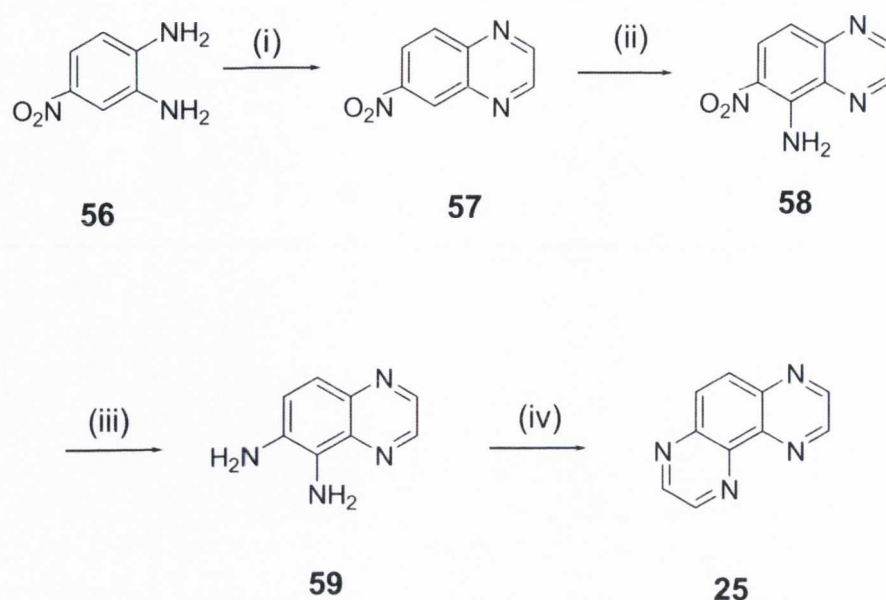


2.2 Synthesis

The target compounds **52** - **55** were prepared from commercially available starting materials. The same procedure was applied to the synthesis of all analogues and full experimental details are provided in Chapter 8.

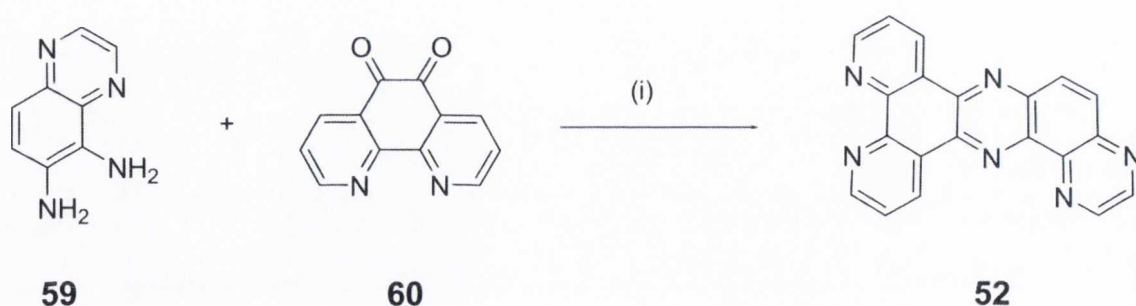
2.2.1 Synthesis of TAP, 25 and pdppz, 52

The synthesis of pdppz was achieved using an intermediate in the synthetic pathway of TAP as outlined in Scheme 2.1. The synthesis of TAP firstly involved condensation of an aqueous solution of glyoxal with 4-nitrobenzene-1,2-diamine **56** by heating under reflux in EtOH for 2 hrs. Removal of the solvent under reduced pressure, followed by recrystallisation from isopropanol, yielded the desired product **57** as golden needles in 83% yield. Direct amination of **57**, using the initial TAP procedure, which used hydroxylamine in the presence of NaOH, gave poor yields, in the range of 5-8%. An adapted method to that originally described was utilised involving direct amination using hydroxylamine in the presence of NaOMe.¹⁶⁶



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.

After 90 mins reflux, the crude product was recrystallised from a mixture of acetic acid and water (3:1) to yield **58** as bright yellow crystalline needles in 43% yield. Subsequent reduction of the nitro group of **58** using hydrazine in the presence of 10% Pd/C, gave the pure diamine **59** as a red solid in 98% yield. Condensation of an aqueous solution of glyoxal with **59** was achieved by heating under reflux in EtOH for 2 hrs. Removal of the solvent under reduced pressure, followed by recrystallisation from isopropanol, yielded the desired product **25** as golden needles in 72% yield. This compound was fully characterised by ^1H NMR, ^{13}C NMR, HRMS and IR spectroscopy which agreed with literature values.¹⁶⁷



Scheme 2.2: Synthetic pathway of **52** (i) glyoxal, EtOH, reflux.

The synthesis of the pdppz ligand, **52** was then achieved by condensation of diamine **59** with 1,10-phenanthroline-5,6-dione **60** as outlined in Scheme 2.2. The dione **60** was synthesised by oxidation of 1,10-phenanthroline using H_2SO_4 and HNO_3 in the presence of KBr, according to a literature procedure.¹⁶⁸ Condensation of **59** with dione **61** by reflux in EtOH yielded pure pdppz **52** as a brown solid in 95% yield.

This novel ligand was characterised by ^1H NMR, elemental analysis, mass spectrometry, melting point analysis and IR spectroscopy. ^{13}C NMR analysis could not be obtained due to the insoluble nature of the compound in $[\text{D}_6]\text{-DMSO}$. The ^1H NMR spectrum is shown in Figure 2.1. Assignment of the peaks was achieved by H-H COSY and C-H COSY analysis. Successful formation of this compound was also evident from accurate mass spectrometry, where **52** displayed a peak at 335.1048, corresponding to the $[\text{M}+\text{H}]$ ion.

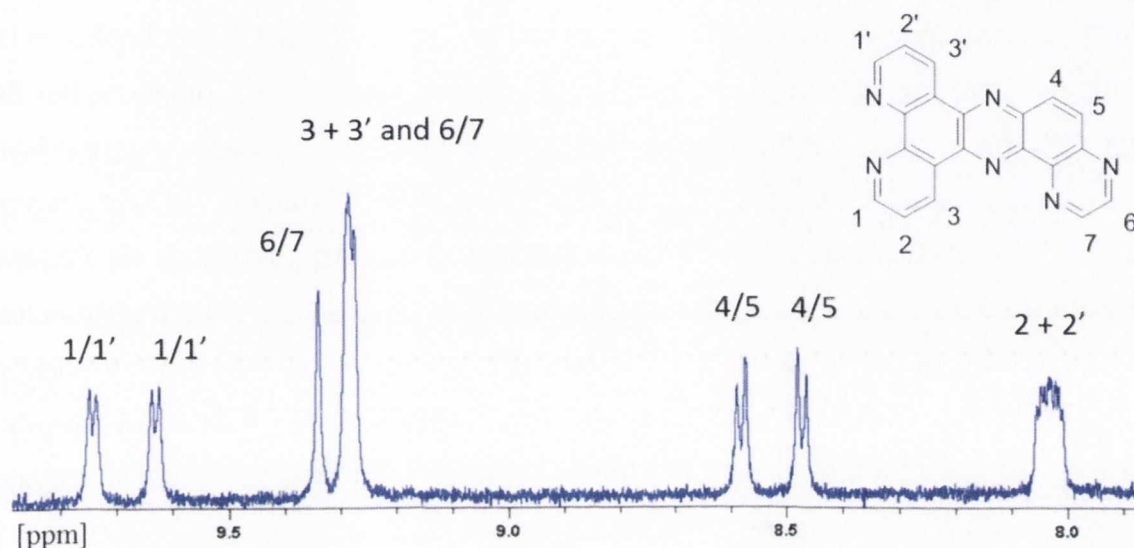


Figure 2.1: The ^1H NMR spectrum of **52** (600 MHz, $\text{DMSO-}d_6$)

2.2.2 Microwave Assisted Synthesis of Complexes 53-55

Previous syntheses of mixed ligand Ru(II) polypyridyl complexes have been undertaken using a procedure by Sullivan *et al.*¹⁶⁹ This was typically carried out by refluxing $\text{cis-Ru(L-L)}_2\text{Cl}_2$ (where L-L is one bidentate polypyridyl ligand) with the desired third polypyridyl ligand in solvent systems such as ethylene glycol or ethanol/water mixtures. In the case of the systems described herein, these solvents were not appropriate due to poor solubility. Another approach that has been developed within the Gunnlaugsson group involves the use of a DMF/water mixture to exploit the increased solubility of the reactants in DMF and also the solubility of the products in water. Although successful for formation of previously synthesised complexes, this method required numerous purification steps and subsequently had an impact on the product yields.

These problems of purification may be due to the reaction of Ru(II) with the degradation products of DMF. In general, the thermal degradation of DMF results in the formation of dimethylamine and carbon monoxide (CO). Since CO is an excellent ligand for Ru(II)-centres, carbonyl complexes may be formed as side products under these conditions.¹⁷⁰ With this in mind we set about to investigate more efficient methods for the synthesis of Ru(II) complexes. A report by Rau *et al.*¹⁷¹ described a highly efficient preparation of Ru(II)

polypyridyl complexes using a microwave activated reaction in more traditional solvent systems than those described above. However, this system has not yet been applied to complexes containing the TAP ligand or derivatives of TAP.

Microwave-enhanced chemistry is based on the efficient heating of materials by “microwave dielectric heating” effects. This phenomenon is dependent on the ability of a specific material (solvent or reagent) to absorb microwave energy and convert it into heat. An electromagnetic field causes heating by two main mechanisms: dipolar polarisation and ionic conduction. Irradiation of the sample at microwave frequencies results in the dipoles or ions aligning in the applied electric field. As the applied field oscillates, the dipole or ion field attempts to realign itself with the alternating electric field and, in the process, energy is lost in the form of heat, through molecular friction and dielectric loss. The amount of heat generated by this process is directly related to the ability of the matrix to align itself with the frequency of the applied field. From a practical and synthetic point of view, microwave irradiation leads to direct activation of either solvent or reactant molecules in solution by localised superheating. The transfer of microwave energy is rapid and direct with any absorbing material and creates high instantaneous temperatures (T_i). It is these high T_i that activate a high proportion of reacting species above the required activation energy. In comparison, with traditional conductive heating, energy is transferred from a heat source to an oil bath, which subsequently heats the outside surface of the reaction flask. Energy transfers through the vessel and is then dissipated throughout the reaction mixture; all in all an extremely inefficient process leading to long reaction times and side reactions (Figure 2.2). Not only is direct microwave heating able to reduce chemical reaction times from hours to minutes, but it is also known to reduce side reactions, increase yields, and improve reproducibility.¹⁷²

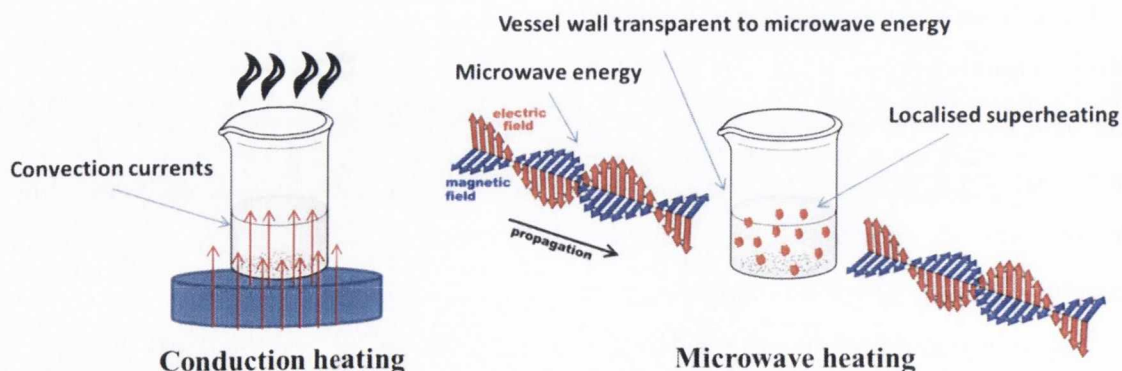
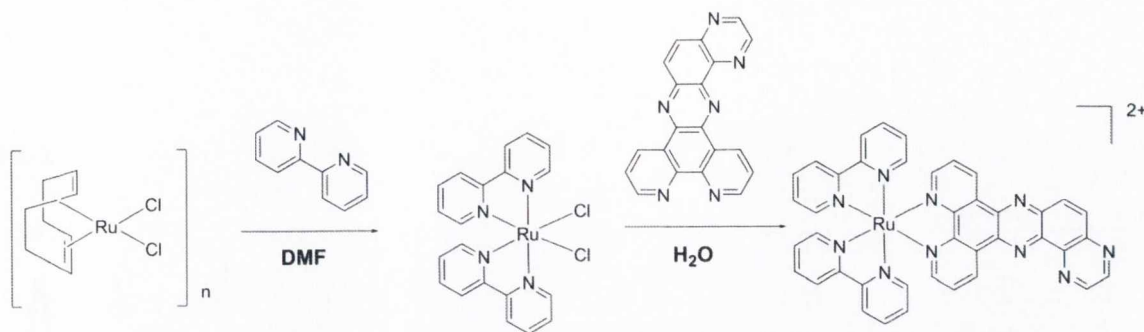


Figure 2.2: Schematic representation of the comparison between traditional conductive heating and microwave assisted heating

Taking into consideration the solubility of both starting materials and products, a slightly modified procedure was undertaken for the synthesis of complexes **53**, **54** and **55**. Scheme 2.3 shows the general method of complexation taking the synthesis of **53** as an example.



Scheme 2.3: Method of complexation of Ru(II) polypyridyl complexes using a microwave activated reaction.

The microwave-assisted synthesis of the $\text{Ru}(\text{L-L})_2\text{Cl}_2$ derivatives in DMF, where L-L is bpy, phen and TAP respectively, was achieved by reaction with $\text{Ru}(\text{cod})\text{Cl}_2$. Ligand **52** was subsequently complexed with each bis $\text{Ru}(\text{L-L})_2\text{Cl}_2$ precursor using degassed water as the sole solvent in the second complexation step. It was envisioned that the high solubility of the products in water would drive the reaction to completion even though the solubility of both starting materials was low. Upon 30 minutes irradiation in the microwave at 140°C the reaction mixtures were observed to change colour from dark purple to orange, evidence for the formation of the tris-polypyridyl Ru(II) complex. Subsequent precipitation of the tris complexes as their PF_6 salts was achieved by addition of a concentrated ethanolic solution of NH_4PF_6 . Isolation by suction filtration afforded the products **53**, **54** and **55** as red/brown solids in 83%, 69% and 52% yield respectively after purification by column chromatography [flash silica; 40:4:1 $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{NaNO}_3$ (sat)]. The main impurities present in each of the reaction mixtures were $\text{Ru}(\text{L-L})_2\text{Cl}_2$, $\text{Ru}(\text{L-L})_3^{2+}$, and a highly coloured green/blue compound, possibly a Ru(III) species formed by oxidation. The chloride salt of the complex was re-formed by dissolution in methanol followed by stirring with Amberlite IRA-400 ion exchange resin (Cl⁻ form) for 1 hour.

These novel complexes were characterised by ^1H NMR, ^{13}C NMR, elemental analysis, HRMS, melting point analysis and IR spectroscopy. The ^1H NMR spectra (600 MHz, CD_3CN) of **53** is shown in Figure 2.3 with that of **54** and **55** given in the appendix. Assignment of the peaks was achieved by 2-D H-H and C-H COSY analysis.

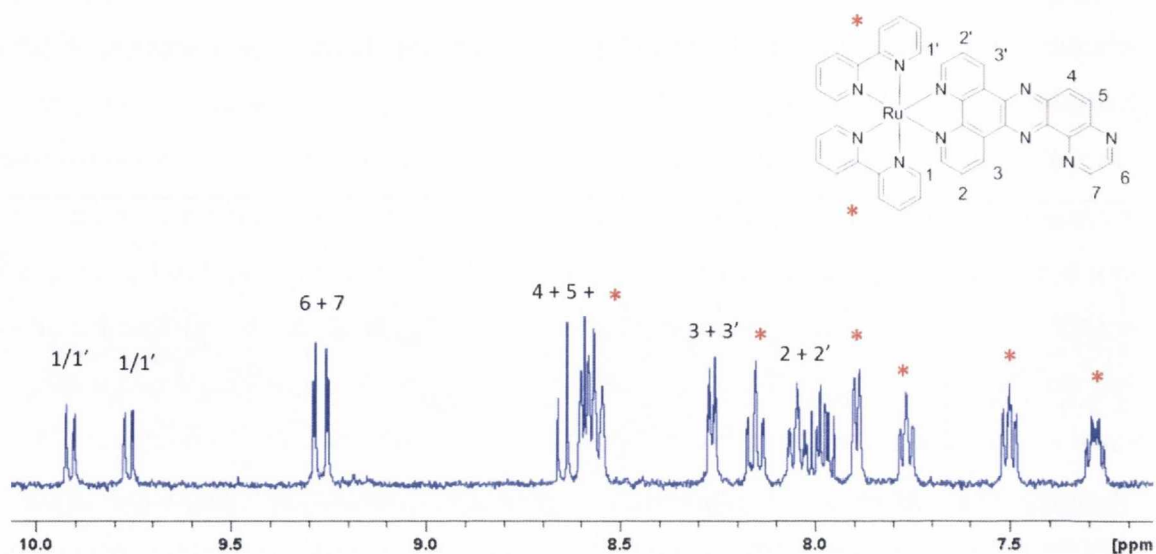


Figure 2.3: ^1H NMR spectra of **53** (600 MHz, $\text{DMSO}-d_6$)

Successful formation of the complexes was also evident from matrix assisted linear desorption ionisation (MALDI) mass spectrometry, where **53**, **54** and **55** displayed peaks at 893.0339, 941.1065 and 945.0837 respectively, corresponding to their $[\text{M} + \text{PF}_6]^+$ ions. A comparison between the estimated and actual isotopic distribution pattern is shown for **53** in Figure 2.4 with those for **54** and **55** shown in the Appendix. Synthesised as their chloride salts, all of the complexes were water-soluble, and their photophysical properties were investigated in 10 mM phosphate-buffered aqueous solutions at pH 7.4.

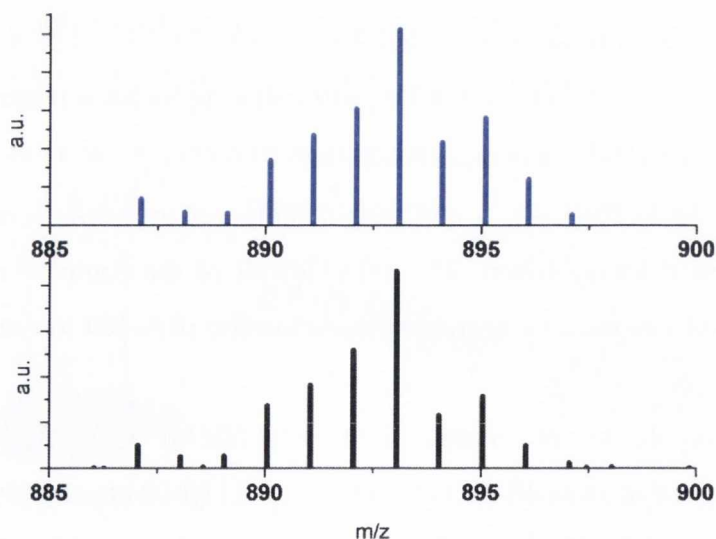


Figure 2.4: Comparison between the calculated (blue) and obtained (black) isotopic distribution pattern for **53** from high resolution mass spectrometry analysis

2.3 Photophysical Characterisation

Characterisation of the photophysics of both ligand **52** and the respective complexes **53**, **54** and **55** was undertaken in order to fully understand their photophysical behaviour; necessary to monitor the interaction of such systems with DNA.

2.3.1 Photophysical Characterisation of Ligand **52**

The UV/Vis absorption, emission and excitation spectra of ligand **52** in MeOH are shown in Figure 2.5.

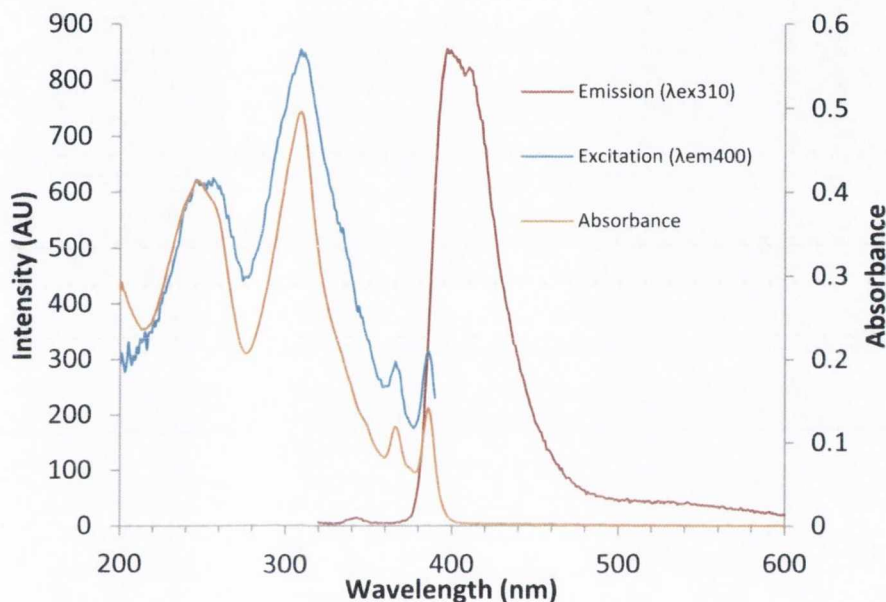


Figure 2.5: UV/Visible, excitation and emission spectra of **52** (10 μ M) in MeOH

Four different absorption maxima are shown in the UV/Vis absorption spectrum at 244 nm, 310 nm, 365 nm and 385 nm. The band at 310 nm is attributed to π - π^* transitions within the ligand with less intense bands at 365 nm and 385 nm being attributed to either n- π^* transitions¹⁷³ or to π - π^* transitions within the phenazine part of the ligand.²⁶⁶ Excitation of **52** at 310 nm resulted in emission with a λ_{max} centred at 400 nm assigned to π - π^* transitions within the ligand. Furthermore, from examination of the excitation spectrum it was apparent that maximum emission was observed from **52** when excited at 310 nm, however, excitation into the other peaks resulted in a similar emission profile. Spectra were also measured in MeCN and MeOH/H₂O solutions, however, no significant changes were observed.

2.3.2 Photophysical Characterisation of Complexes **53**, **54** and **55**

The absorption, excitation and emission spectra of **54** and **55**, recorded at pH 7.4 in 10 mM phosphate buffer are shown in Figure 2.6a and 2.6b, respectively. The spectra of **53** are shown in Appendix 2.

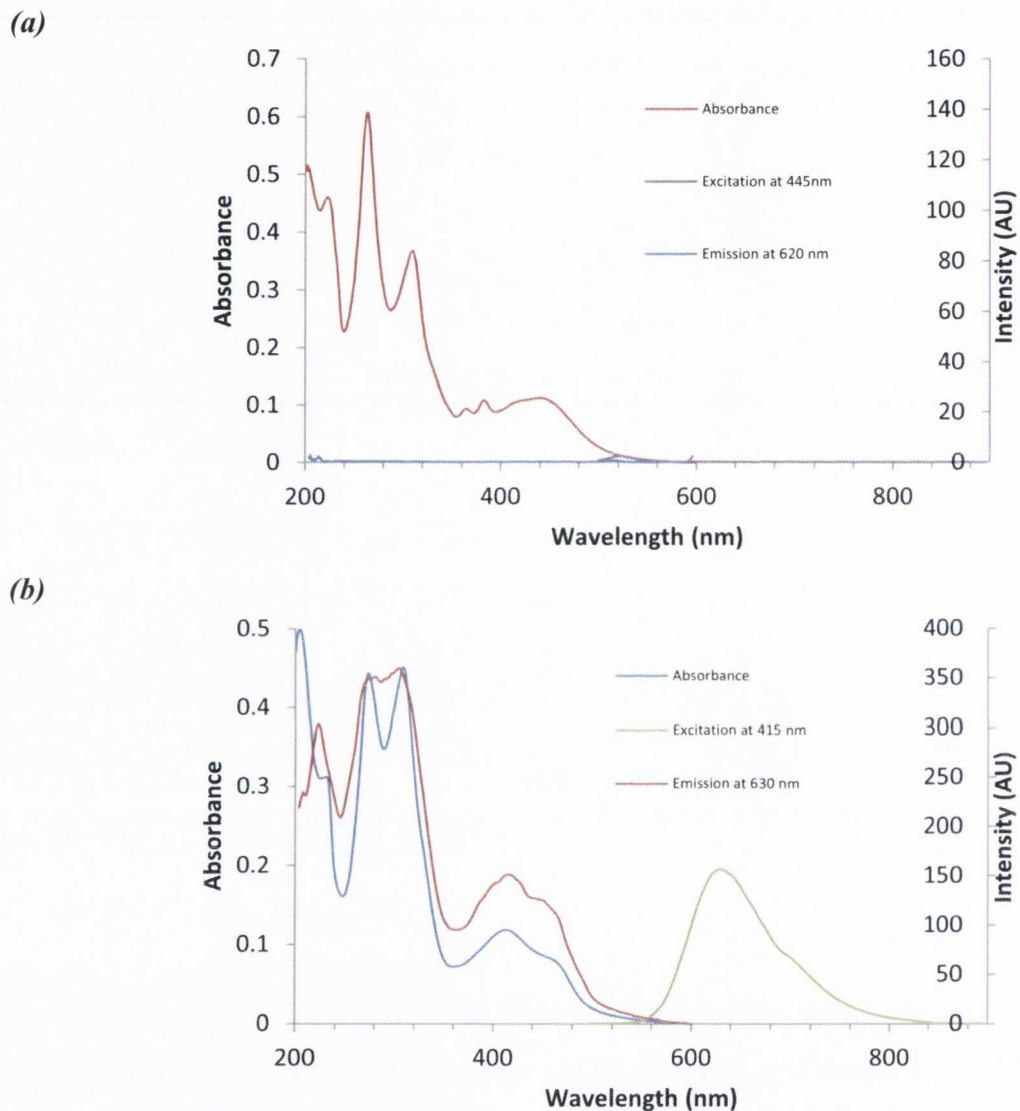


Figure 2.6: The UV/Visible, excitation and emission spectra of (a) **54** and (b) **55** (10 μ M) in 10 mM phosphate buffer at pH 7.4

The UV/Vis absorption spectrum of **53** shows λ maxima at *ca.* 290 nm, 310 nm, 365 nm, 385 nm and 445 nm. The band at 290 nm was characteristic of π - π^* intra-ligand transitions of the ancilliary bpy ligands while the band at 310 nm was attributed to π - π^* transitions within ligand **52**. Less intense bands at 365 nm and 385 nm were attributed to transitions within the phenazine part of the ligand as described above. The broad band centred at 445 nm can be assigned to the MLCT transitions within the Ru(II) polypyridyl centre. Similarly, the absorption spectrum of **54** showed absorbance maxima at *ca.* 225 nm, 265 nm, 310 nm, 365 nm, 385 nm and 445 nm. The bands at 225 nm and 265 nm are, again,

characteristic of $\pi\text{-}\pi^*$ intra-ligand transitions of the ancilliary phenanthroline ligands while the intense band at 310 nm is attributed to $\pi\text{-}\pi^*$ transitions within ligand **52** with less intense bands at 365 nm and 385 nm attributed to $n\text{-}\pi^*$ transitions within the phenazine part of the ligand. The broad band centred at 445 nm is characteristic of the MLCT transitions of the Ru(II) centre. The absorption spectra of **55** showed a less complex absorbance spectrum with three strong absorption bands at 275 nm, 310 nm and 415 nm. Again these bands correspond to $\pi\text{-}\pi^*$ intra-ligand transitions, $\pi\text{-}\pi^*$ transitions within ligand **52** and an MLCT band corresponding to the Ru(II) centre. A summary of the absorption properties of these systems as well as their respective molar absorptivities is given in Table 2.1.

Table 2.1: Absorption properties of **53**, **54** and **55** in 10 mM phosphate buffer, at pH 7.4

Complex	λ_{max} (nm) [$\epsilon(\text{M}^{-1} \text{cm}^{-1})$] in phosphate buffer ($\pm 10\%$)		
	$\pi\text{-}\pi^*$ IL	$\pi\text{-}\pi^*$ pdppz	MLCT
53	290 [66500]	310 [55700]	445 [14500]
54	265 [91000]	310 [55200]	445 [17500]
55	275 [66600]	310 [68300]	415 [17900]

Upon excitation into the MLCT bands of each complex, markedly different behaviour was observed. Both complexes **53** and **54** exhibited no detectable emission while complex **55** gave rise to an intense MLCT based emission with λ_{max} at 630 nm. The emission exhibited by **55** was similar to that observed for $[\text{Ru}(\text{TAP})_2(\text{dppz})]^{2+}$ where the lowest excited $^3\text{MLCT}$ state of this complex is localised on one of the TAP ligands.¹⁵⁸ Photophysically this causes one of the TAP ligands to act as an electronic ‘sink’ and relaxation to the ground state occurs solely *via* that TAP ligand. Thus, emission always takes place from the $^3\text{MLCT}$ state of TAP while no inter ligand charge transfer to the pdppz ligand is observed.¹⁷⁴ The emission of both **53** and **54** was expected to originate from the respective MLCT Ru–pdppz transitions in which the excited state is localised on the pdppz ligand. Evidence points to hydrogen bonding and/or excited-state proton transfer to the phenazine nitrogens as the mechanism of deactivation of the complex’s excited state as discussed in Chapter 1.⁴⁰ This type of behaviour is responsible for the ‘light switch’ effect of such dppz complexes and has been observed in related systems leading to emission quenching by polar solvent molecules. Similarly, excitation of **53** and **54** ($\lambda_{\text{em}} = 620 \text{ nm}$) did not give rise to a detectable excitation spectrum whereas excitation of **55** gave rise to an excitation spectrum that was structurally identical to the recorded absorption spectrum.

In a similar manner to Ru(II) complexes of dppz such as $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ the excited state properties of **53** – **55** were expected to be very sensitive to their local environment.³⁵ In order to further characterise this the fluorescence quantum yield (Φ_F) of each complex was determined in MeCN using the optically dilute method¹⁷⁵ and utilising $[\text{Ru}(\text{bpy})_3]^{2+}$ as a reference compound.¹⁷⁶ As both **53** and **54** were shown to be non - emissive in aqueous solution, the quantum yields were not obtained in H₂O. Emission from each complex is observed at *ca.* 600 nm in MeCN, characteristic of radiative deactivation from the ³MLCT state as detailed in Chapter 1. The emission of **55** was found to be slightly blue shifted in MeCN compared to that in H₂O which may be due to the more polar nature of H₂O having a stabilising effect on the excited state of **55**. As expected the emission intensity of each complex was increased upon degassing of the solution due to sensitivity of the ³MLCT to dissolved oxygen in solution. For these complexes Φ_F values of 0.070, 0.073 and 0.144 were obtained in MeCN for **53**, **54** and **55** respectively and are all significantly higher than those measured for the reference $[\text{Ru}(\text{bpy})_3]^{2+}$ (0.059).¹⁷⁶

Excited state lifetime measurements (τ_{em}) were also recorded in order to further characterise the excited state properties of **53** – **55** in MeCN using time correlated single photon counting (λ_{ex} = 444 nm, with absorbance at 444 nm of 0.15). Taking the average of four measurements for each complex, in all cases the data was best fitted to a single exponential decay function, with R^2 values in the range 0.98 – 0.99. Here, τ_{em} values of 165 ns and 170 ns were calculated for **53** and **54** respectively, which are of a similar magnitude to the related complex $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$, which has a τ_{em} value of 177 ns in aerated MeCN solution.¹⁷⁷ In contrast, **55** exhibited a much longer lifetime of τ_{em} values of 557 ns and 665 ns in MeCN and H₂O respectively. These values are slightly smaller than the corresponding values for $[\text{Ru}(\text{TAP})_2(\text{phen})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{dppz})]^{2+}$ determined by Kirsch-De Mesmaeker *et al.* as 690 ns and 820 ns respectively in H₂O¹⁵⁸ but indicate that the excited state character of **55** is similar to that of $[\text{Ru}(\text{TAP})_2(\text{phen})]^{2+}$ and $[\text{Ru}(\text{TAP})_2(\text{dppz})]^{2+}$. Similarly, argon saturated solutions of all three complexes resulted in an increase in excited state lifetime due to a reduction in excited state quenching caused by dissolved oxygen. A summary detailing the excited state properties of **53** – **55** is given in Table 2.2.

Table 2.2: Emission properties of **53** - **55** at 298K

Complex	$\lambda_{\text{emission}}$	Φ_f ($\pm 10\%$)	τ_{em} (ns)	τ_{em} (ns)	τ_{em} (ns)	τ_{em} (ns)
	(nm)		MeCN	MeCN	H ₂ O	H ₂ O
	(in MeCN)		(air)	(Ar)	(air)	(Ar)
53	630	0.070	165	364	-	-
54	625	0.073	170	411	-	-
55	615	0.144	557	914	655	813

In summary, **53** – **55** display varied and potentially useful photophysical properties in both aqueous solution and aprotic solvents. They possess multiple absorption bands which are expected to be sensitive to their environment and, thus, are expected to change upon interaction with DNA. Tight binding of the Ru(II) polypyridyl centre will be observed as modulations in the MLCT band at *ca.* 450 nm and as changes in the π - π^* transitions within ligand **52**. Furthermore, the complexes **53** and **54** are non-emissive in aqueous solution and as such it is anticipated, that the interruption of the quenching process upon binding to nucleic acids may lead to the ‘light switch’ effect being exhibited. Moreover, any changes in the observed emission from **55** will give important information with regard to any photo-induced electron transfer (PET) behaviour between the DNA nucleobases and the π deficient Ru(II) complex. The nature of their interaction with DNA will be discussed in the following sections and will form the basis of this study of a novel set of luminescent probes for targeting DNA.

2.4 DNA Binding Interactions

There are a number of literature reports documenting the use of Ru(II) complexes as luminescent probes for biomolecules, especially DNA.^{30,33,178} Complexes **53-55** were designed to interact with DNA through a combination of electrostatic and π -stacking interactions, with the extended heteroaromatic structure of ligand **52** expected to bind through intercalation causing modulations in their photophysical properties. Behaviour such as this is especially pronounced for Ru(II) complexes with extended aromatic ligands, as in the case of dppz (see above discussion).³⁵ Complexes of this type show little or no photoluminescence at ambient temperature, however, upon addition of double helical DNA intense photoluminescence is observed, *ie.* the ‘light switch’ effect. This is comparable to introducing the complex into a local organic solvent that shields the ring nitrogens on the intercalating ligand from the bulk solvent. From these modulations information on the equilibrium binding constant, host-drug stoichiometry and binding site size can be determined by fitting the changes in the ground and excited state spectra.¹⁷⁹ Hence, spectroscopic titrations were carried out **53** - **55** to investigate

the nature and binding affinity of these complexes with DNA. The results are presented and discussed in the following sections.

2.4.1 Spectroscopic Titrations of 53-55 with DNA

2.4.1.1 UV/Vis Absorption Titrations of 53-55 with DNA

The interaction of **53** - **55** with double stranded DNA was firstly examined using UV/Vis absorption spectroscopy. The titrations were carried out by addition of small aliquots of salmon testes DNA (stDNA) to a 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in the absorbance was reached. The changes in the $^3\text{MLCT}$ band and the $\pi\text{-}\pi^*$ bands were monitored for each complex. The ratios of concentrations of stDNA to each of the complexes were calculated upon addition of stDNA and represented as 'P/D ratio' (nucleotide Phosphate to Dye ratio). All titrations were repeated a number of times to ensure reproducibility. The overall changes in the ground state of **55** are shown in Figure 2.7 with those of **53** and **54** shown in the Appendix.

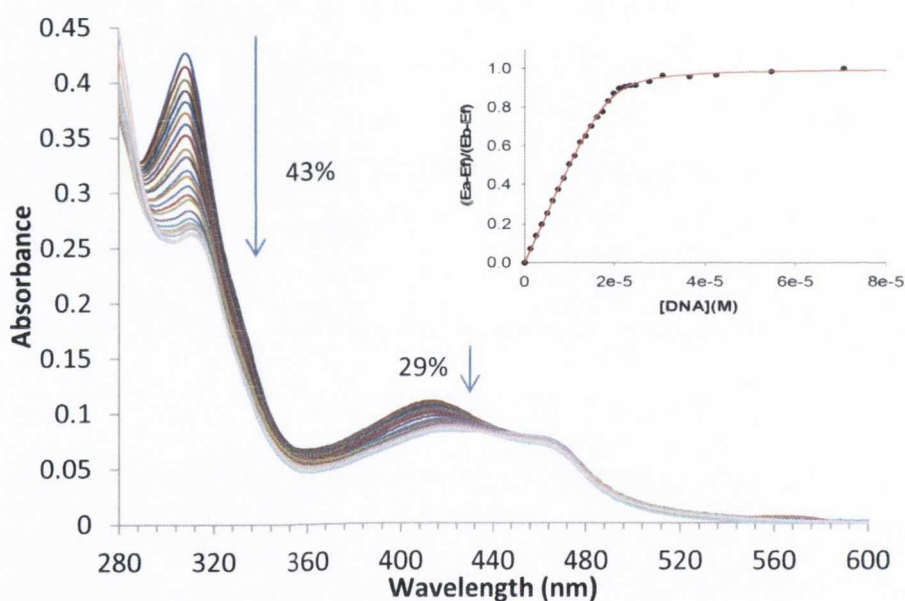


Figure 2.7: The changes in the UV/Visible absorption spectrum of **55** (6.3 μM) with increasing additions of stDNA (0-122 μM) at pH 7.4 (10mM phosphate buffer). Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}] (\text{M}^1, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.

The titration of **53** - **55** with stDNA resulted in significant changes to their absorption spectra. Complex **53** exhibited a 51% decrease in absorbance at 310 nm with a concomitant decrease of 18% in the MLCT band at 445 nm. In the case of **54**, a 53% hypochromism was observed for the pdppz band at 310 nm, while the MLCT band also experienced a 30% hypochromism. Complex **55** exhibited a hypochromism of 43% in the pdppz band and a further hypochromism of 29% in the MLCT band.

These results are similar to those obtained previously for the addition of DNA to $[\text{Ru}(\text{bpy})_3]^{2+}$, the observed changes in the $^3\text{MLCT}$ band having been attributed to electrostatic interactions between the complex and the phosphate backbone of DNA.¹⁸⁰ The defined hypochromicities at 310 nm would indicate intercalation of the pdppz ligand between the stacked bases (as expected with such an extended planar structure) and the magnitude of such changes reflects the high binding ability of each complex to DNA.

The data from the UV/Visible absorption titrations were next analysed in order to obtain a binding affinity for DNA. The intrinsic binding constant K_b , and binding site size n were determined by fitting the observed changes in the ground state absorption spectra using the model of Bard *et al.*¹⁸¹ (equations (1) and (2)), from a plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}]$. ϵ_a , ϵ_f , ϵ_b correspond to the apparent extinction coefficient, the extinction coefficient for the free Ru(II) complex, and the extinction coefficient for the Ru(II) complex in the fully bound form respectively, while C_t is the total complex concentration, $[\text{DNA}]$ is the DNA concentration, and n is the binding site size. The data was fit to this model using non-linear regression analysis with Sigmaplot 11.0.

$$\frac{(\epsilon_a - \epsilon_f)}{(\epsilon_b - \epsilon_f)} = \frac{[b - (b^2 - 2K_b C_t [\text{DNA}]/n)^{1/2}]}{(2K_b C_t)} \quad (1)$$

$$b = 1 + K_b C_t + K_b [\text{DNA}]/2 \quad (2)$$

As expected a range of binding constants and binding site sizes were determined for the various complexes. A representative plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}]$ and the corresponding best fit of the data to the Bard eqn. for **55** are shown as an inset in Figure 2.7 and a summary of the results obtained are detailed in Table 2.3.

Table 2.3: DNA binding parameters from fits to absorbance data

Complex	$\lambda(\text{pdppz})$ Hypochromism	$\lambda(\text{MLCT})$ Hypochromism m	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
53	51%	18%	$4.9 \times 10^6 (\pm 0.7)$	2.41 (± 0.05)	0.99
54	53%	30%	$1.2 \times 10^7 (\pm 0.3)$	1.45 (± 0.03)	0.99
55	43%	29%	$5.4 \times 10^6 (\pm 0.5)$	1.55 (± 0.02)	0.99

The results obtained confirmed that all of the complexes had high affinity for DNA. **53** showed binding with $K_b = 4.9 \times 10^6 (\pm 0.7)$ and $n = 2.41 (\pm 0.05)$, while **54** was shown to have double the affinity for DNA compared to **53** with $K_b = 1.2 \times 10^7 (\pm 0.3)$ and $n = 1.45 (\pm 0.03)$ while **55** had an intermediate $K_b = 5.4 \times 10^6 (\pm 0.5)$ and $n = 1.55 (\pm 0.02)$. The strong, well defined hypochromism of the band at 310 nm coupled with the observed high binding affinities of **53** – **55** may point to an intercalative mode of interaction. Similarly, the observed higher binding ability of **54** can be attributed to the ancilliary phen ligands lying in the grooves of the DNA helix; an effect that has been observed in the parent $[\text{Ru}(\text{phen})_3]^{2+}$ complex.³⁷ This enables the flat, planar pdppz ligand to intercalate further between the base pairs and also allows closer association of the positive complex with the negatively charged DNA backbone, causing a larger hypochromism of the ³MLCT absorption band. These results are, in general, readily compared to that of $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ which shows binding constants of *ca.* 10^6 M^{-1} in 50 mM NaCl buffer.³⁵

In conclusion, all systems **53** – **55** possess binding constants of the order of $10^6 - 10^7 \text{ M}^{-1}$, which are in keeping with those previously determined for $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$.^{35,43} In addition large changes in both the pdppz and the ³MLCT absorption bands indicate a strong association of each of the complexes with DNA. This strong association is most likely due to the bifunctional nature of the complexes; the Ru(II) centre providing initial electrostatic attraction to the DNA helix which places the pdppz ligand in an ideal environment for high affinity interaction through intercalation as had been anticipated in our initial design. In agreement with the ground state studies, significant changes were also observed in the excited state properties of **53** – **55** and these results will be presented in the next section.

2.4.1.2 Fluorescence Emission Titrations of 53-55 with DNA

As discussed in Section 2.3.2 the ³MLCT emission of **53** and **54** was quenched in aqueous solution due to interaction of the excited state with dissolved oxygen and water molecules with the phenazine ring nitrogens of the pdppz ligand, **52**. It was anticipated that addition of DNA would lead to sheltering of these ring nitrogens in the 'organic' duplex micro-environment causing the ³MLCT emission to be significantly enhanced. In contrast, **55** was shown to exhibit fluorescence in aqueous solution and as a result perturbations of its emission would be more likely to be as a result of photo-electron transfer processes occurring in solution. The excited state titrations were carried out in parallel with the UV/Visible absorption titrations by addition of small volumes of stDNA to a solution of each complex in phosphate buffer at pH 7.4, until a plateau in emission was reached.

Upon titration of **53** with stDNA large enhancements were observed with $^3\text{MLCT}$ emission centred at 630 nm evolving, reaching a maximum at a phosphate to dye ratio (P/D) = 4, and subsequently decreasing to a plateau value at P/D = 30 (See Appendix 2). Similarly intense luminescence was seen to evolve from a Ru(II) centred MLCT emission band at 625 nm for **54** (Figure 2.8). The initial addition of stDNA (P/D 0 $\rightarrow$ 3.5) resulted in an immediate fluorescence enhancement, with further additions of stDNA (P/D 3.5 $\rightarrow$ 30) resulting in the emission decreasing to a plateau in a similar manner to that seen for **53**. This suggests a biphasic interaction of these complexes with different distributions of the complex on the polynucleotide, where the luminescence at the plateau value would correspond to that of the isolated complex bound to DNA. The extra enhancement of luminescence might originate from closely bound metal complexes which are initially efficiently packed along the DNA helix providing increased shelter from solvent quenching. This geometry would provide further protection of the complex from water as compared to the isolated species on the double helix. Similar behavior has also been reported by Norden *et al.* for $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ and by Kirsch de Mesmaeker *et al.* for $[\text{Ru}(\text{phen})_2(\text{PHEHAT})]^{2+}$ and was attributed as described above in both cases.^{43,49}

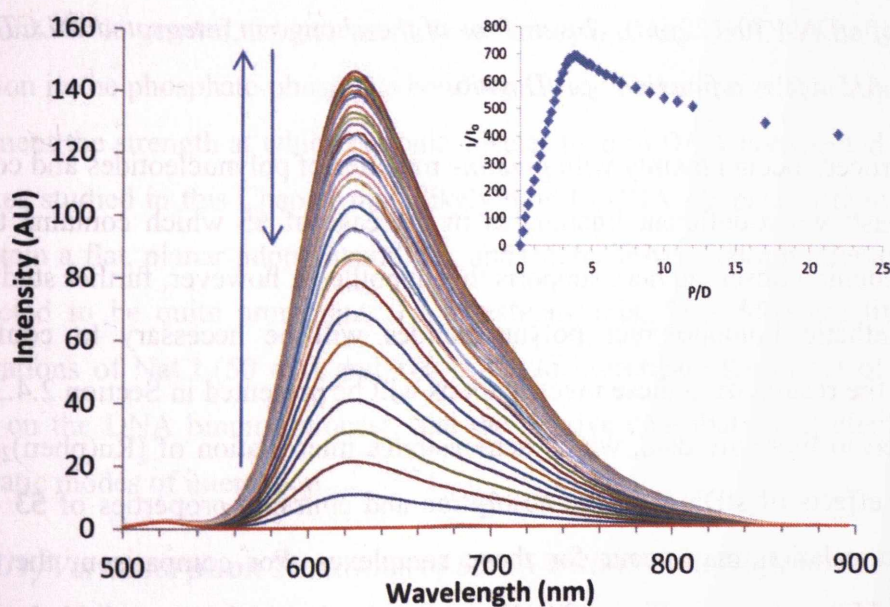


Figure 2.8: Changes in the emission spectrum of **54** ($6.3\mu\text{M}$) (λ_{ex} 445 nm) with increasing concentration of stDNA (0-122 μM). Inset: Plot of the change in integrated MLCT emission intensity at $\lambda = 625$ as a function of P/D ratio.

Conversely, **55**, which exhibited $^3\text{MLCT}$ based emission in aqueous solution was shown to have its excited state effectively quenched by 86% upon addition of stDNA (P/D 0 $\rightarrow$ 30) with the main changes occurring between P/D 0 $\rightarrow$ 3.5 (Figure 2.8). It has been shown experimentally that this quenching is due to a PET between *guanine* residues and the $^3\text{MLCT}$ state of the complex.^{32,79,158,182}

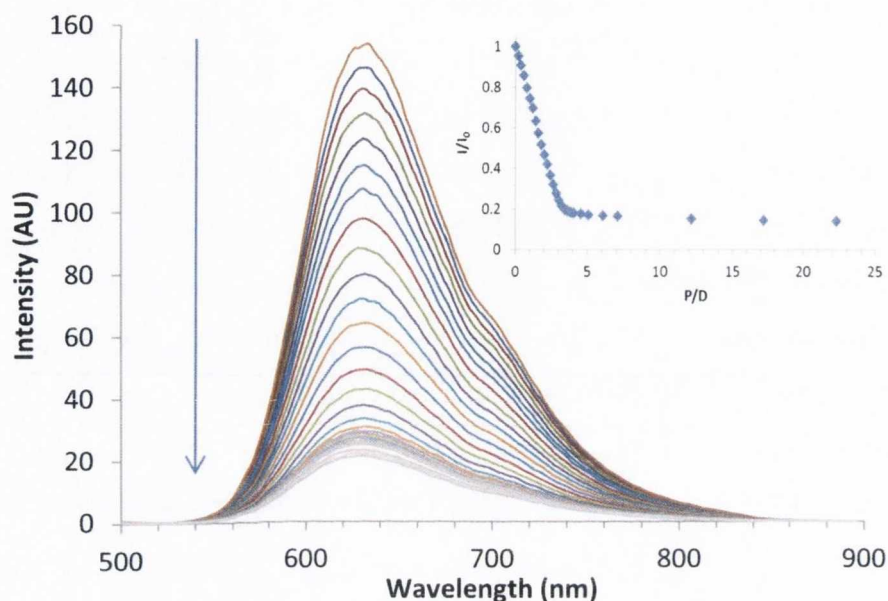


Figure 2.8: Changes in the emission spectrum of **55** ($6.3\mu\text{M}$) (λ_{ex} 415 nm) with increasing concentration of stDNA (0-122 μM). Inset: Plot of the change in integrated MLCT emission intensity at $\lambda = 635\text{ nm}$ as a function of P/D ratio.

Such a process occurs mainly with *guanine* moieties of polynucleotides and complexes containing at least two π -deficient ligands, as in the case of **55** which contains two TAP ligands. The quenching observed here supports this hypothesis, however, further studies in the presence of synthetic homopolymer polynucleotides will be necessary to confirm this observation and the results from these investigations will be presented in Section 2.4.3.2.

Compared to literature data, which demonstrates intercalation of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ in to DNA, the effects of stDNA on the absorption and emission properties of **53** - **55** also suggest that intercalation may occur for these complexes. For comparison, the emission profiles for **53** - **55** are shown in Figure 2.9. From examination of these overlaid changes, it is quite clear that the emission enhancements for **53** and **54** are of a similar order of magnitude. It is also apparent from Figure 2.9 that all three of the complexes display a similar maximum change culminating in a plateau occurring at a P/D = 4. Such low equivalents of DNA necessary to reach a plateau in the emission spectra further reflects the high affinity of these systems for DNA.

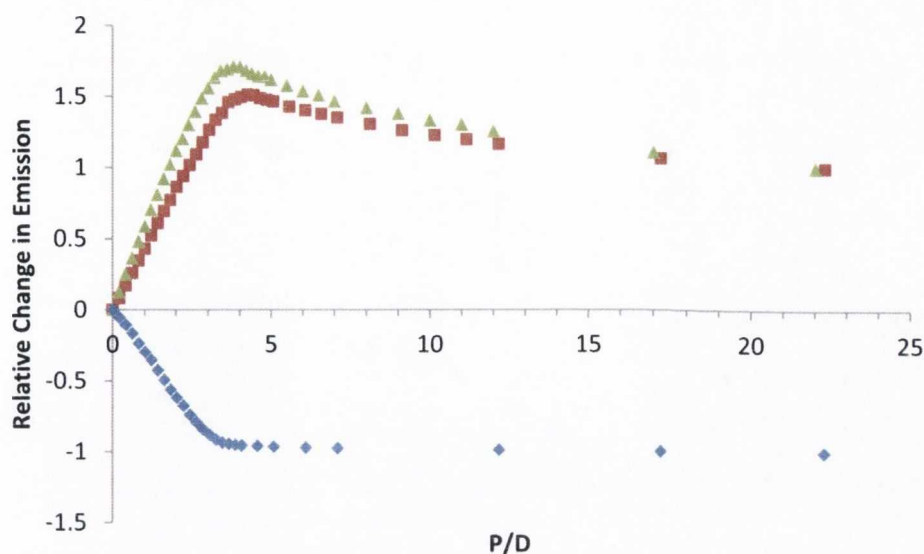


Figure 2.9: Comparison curve showing the relative changes in the emission intensity of **53** (■) (λ_{ex} 440 nm), **54** (▲) (λ_{ex} 440 nm) and **55** (◆) (λ_{ex} 415 nm) upon addition of stDNA in 10 mM phosphate buffer, at pH 7.4

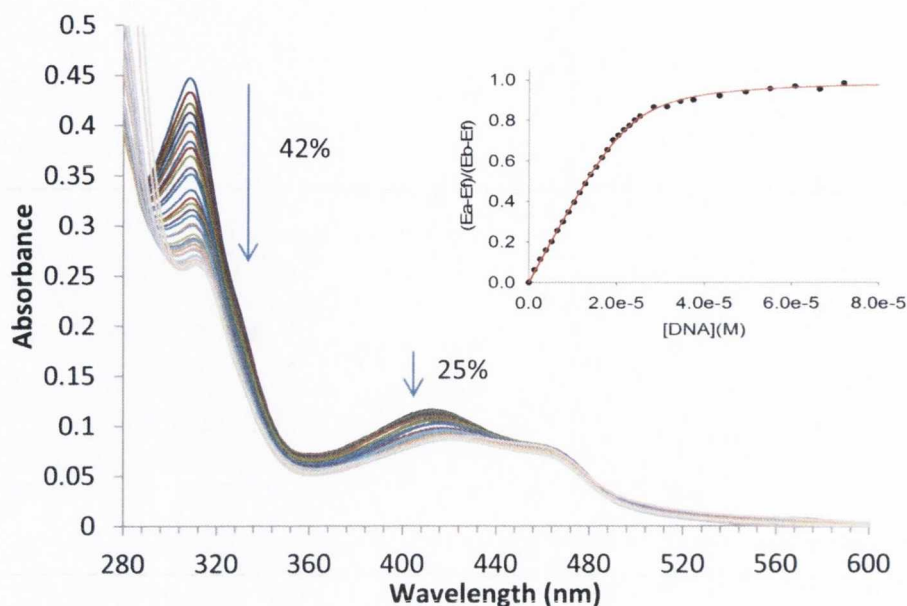
2.4.2 Spectroscopic Titrations of 53-55 with DNA at High Ionic Strength

Many DNA targeting Ru(II) complexes show poor affinity for DNA at high ionic strengths, as binding of many of these systems is driven primarily by electrostatic interactions.¹⁸⁰ In general, an increase in ionic strength causes the DNA helix to shrink due to a reduction in the phosphate-phosphate bond repulsion. Therefore, within a high ionic strength environment the strength at which cationic species bind to DNA is expected to decrease. The complexes studied in this Chapter most likely bind to DNA *via* electrostatic interactions, but also contain a flat, planar pdppz ligand, **52**, and intercalative stacking interactions would also be expected to be quite prominent. To investigate this, **53** - **55** were titrated at varying concentrations of NaCl (50 mM and 100 mM), to investigate the effect of increasing ionic strength on the DNA binding process, and the relative contribution of electrostatic vs. non-electrostatic modes of interaction.

2.4.2.1 UV/Vis Absorption Titrations of 53-55 with DNA at High Ionic Strength

The interaction of **53** - **55** with DNA at varying ionic strengths was examined using UV/Vis absorption spectroscopy in a manner similar to that described in section 2.4.1.1 involving the addition of small aliquots of stDNA to a 50 mM or 100 mM NaCl solution of 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached.

(a)



(b)

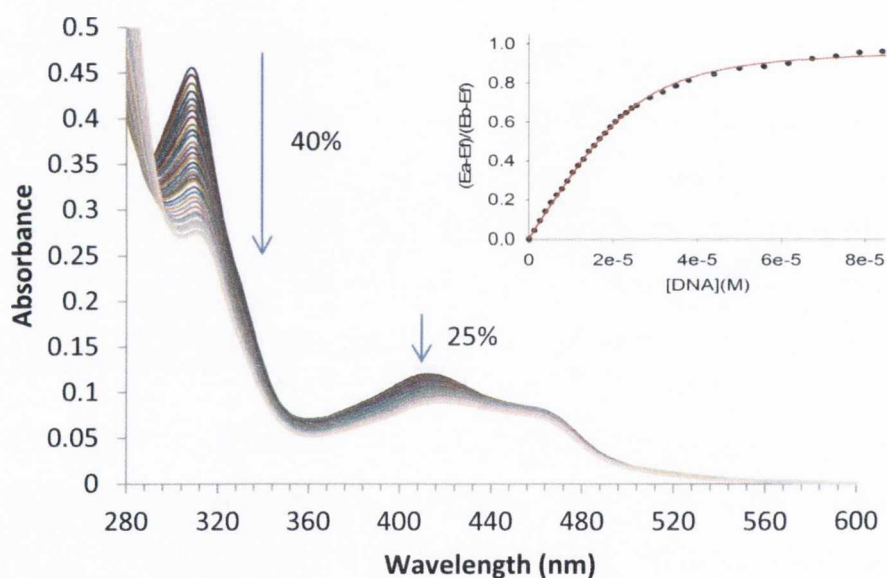


Figure 2.10: Changes in the UV/Visible spectrum of **55** ($6.5\mu\text{M}$) with increasing concentration of stDNA ($0\text{--}122\mu\text{M}$) at (a) 50mM and (b) 100mM NaCl concentration. **Inset:** Plots of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](\text{M})$, P using data with a P/D between $0\text{--}12$ and the best fit of the data (---) using the Bard Eqn.

The changes in the ground state of **55** at both 50mM and 100mM NaCl concentration are shown in Figure 2.10 (a) and Figure 2.10 (b) respectively, with those of **53** and **54** shown in the Appendix. The titration of **53** - **55** with stDNA at both 50mM and 100mM NaCl concentration, again, resulted in significant changes to their respective absorption spectra. At 50mM NaCl concentration, **53** exhibited a 49% decrease in absorbance at 310nm with a concomitant decrease of 24% in the MLCT band at 445nm . Similar changes were observed in 100mM NaCl solution with a 49% decrease at 310nm and a further decrease of 25% in the

MLCT band. In the case of **54**, at 50 mM NaCl concentration, a 54% hypochromism was observed for the pdppz band at 310 nm, with the MLCT band showing a 29% hypochromism. Similarly at 100 mM NaCl concentration, a 54% hypochromism was observed at 310 nm, while the MLCT band also experienced a 31% hypochromism. At 50 mM NaCl concentration, **55** exhibited a hypochromism of 42% in the pdppz band and a further decrease of 25% in the MLCT band with these changes being 40% and 25% for the band at 310 nm and the MLCT band, respectively, at 100 mM NaCl concentration.

The overall changes in these UV/Vis ground state titrations did not show a large dependence with increasing NaCl concentration showing only minor changes in the degree of hypochromism observed. However, the shapes of the binding profiles were significantly affected and, as such the values obtained for K_b revealed a more obvious trend with regard to the effect of NaCl concentration. The data was, again, fit to the model of Bard *et al.* using non-linear regression analysis with Sigmaplot 11.0. A summary of the results obtained are presented in Table 2.4.

Table 2.4: DNA binding parameters from fits to absorbance data at varying NaCl concentrations

Complex	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
53 + stDNA	4.9×10^6 (± 0.7)	2.14 (± 0.05)	0.99
53 + stDNA (50mM NaCl)	4.2×10^6 (± 0.4)	2.35 (± 0.03)	0.99
53 + stDNA (100mM NaCl)	3.8×10^6 (± 0.4)	1.93 (± 0.03)	0.99
54 + stDNA	1.2×10^7 (± 0.3)	1.45 (± 0.03)	0.99
54 + stDNA (50mM NaCl)	6.8×10^6 (± 0.9)	1.97 (± 0.02)	0.99
54 + stDNA (100mM NaCl)	4.6×10^6 (± 0.5)	1.96 (± 0.03)	0.99
55 + stDNA	5.42×10^6 (± 0.5)	1.55 (± 0.02)	0.99
55 + stDNA (50mM NaCl)	1.69×10^6 (± 0.1)	1.62 (± 0.03)	0.99
55 + stDNA (100mM NaCl)	8.35×10^5 (± 0.9)	1.93 (± 0.08)	0.99

The results obtained confirmed that all of the complexes retained their high affinity for DNA, even at very high NaCl concentrations and the study revealed an expected trend in binding affinities of these complexes for DNA with K_b at 10 mM phosphate buffer ≥ 50 mM NaCl ≥ 100 mM NaCl showing an obvious effect of NaCl concentration on the binding process. Further confirmation of the effect of NaCl on binding was obtained by carrying out

salt back titrations for **53** - **55**, in which each complex bound to DNA was displaced upon titration with NaCl. The titration profiles for **53** - **55** are shown in Figure 2.11 and clearly demonstrate that the interaction of **53** - **55** with DNA is dependent on the ionic strength of the medium, however, as none of the complexes are fully displaced after the addition of approximately 400 mM concentration of NaCl solution it can be concluded that, although electrostatic interactions have a role to play in the binding process, it is the ability of the flat, planar pdppz ligand **52** to stack between the bases that governs the overall association of these

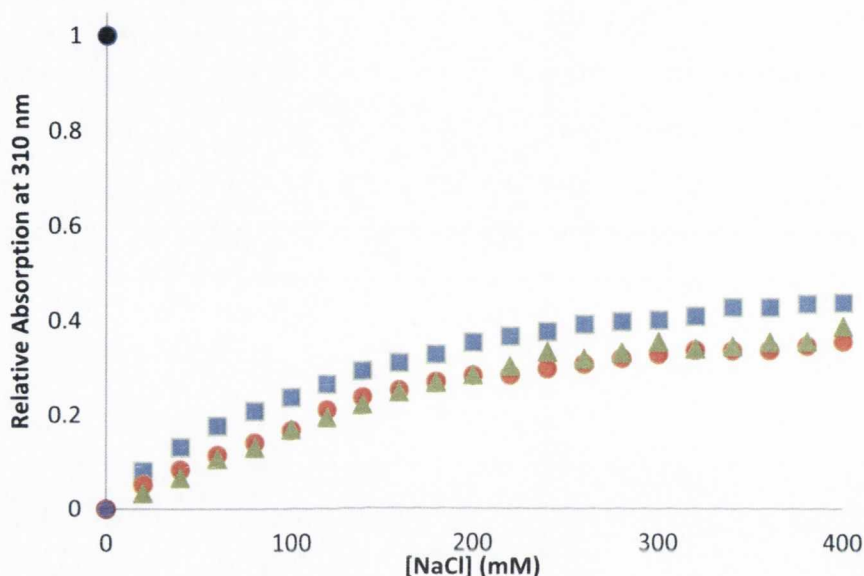


Figure 2.11: UV/Vis absorption NaCl back titration profiles of **53** - **55**. Absorption of unbound complex (●), fully bound complex (●) and bound **53**(■), **54**(●) and **55**(▲) in the presence of increasing concentration of NaCl

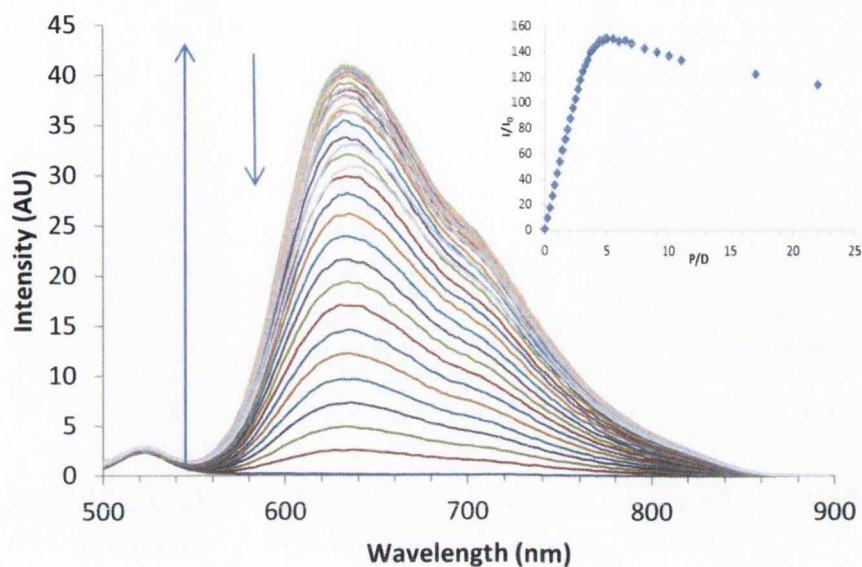
complexes with DNA.

2.4.2.2 Fluorescence Emission Titrations of **53-55** with DNA at High Ionic Strength

The results obtained in the UV/Vis absorption titrations were further confirmed by similar changes in the fluorescence emission spectra of complexes **53** - **55** at varying salt concentrations. The changes in the excited state of **53** at both 50 mM and 100 mM NaCl concentration are shown in Figure 2.12 (a) and Figure 2.12 (b), respectively. Upon titration with stDNA, **53** and **54** underwent significant changes, being ‘switched on’ on both occasions giving rise to a ³MLCT emission band centred at *ca.* 630 nm. For **53** at 50 mM NaCl concentration the point of maximum emission occurred at P/D *ca.* 5, while at 100 mM NaCl the largest emission intensity increase was found at P/D *ca.* 7. For **54** in 50 mM NaCl solution the the point of maximum emission occurred at P/D *ca.* 5, while at 100 mM NaCl concentration, again, the maximum emission intensity was shifted to P/D *ca.* 6. Both **53** and **54** showed a decreased emission enhancement in the presence of NaCl. Additionally, the rate

at which the enhancement occurred lessened, resulting in larger equivalents of DNA being required to reach a situation in which the complexes were fully bound.

(a)



(b)

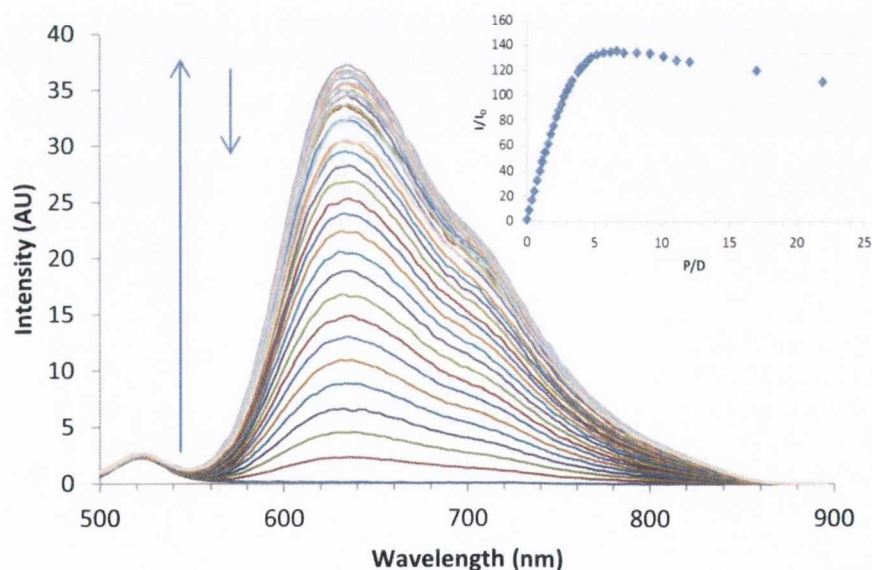


Figure 2.12: Changes in the fluorescence emission spectrum of **53** ($6.5\mu\text{M}$) at 630 nm ($\lambda_{\text{ex}} 445\text{ nm}$) with increasing concentration of stDNA ($0\text{--}125\mu\text{M}$) at (a) 50mM and (b) 100 mM NaCl concentration. Insets: Plots of the change in integrated MLCT emission intensity as a function of P/D ratio.

This further suggests that electrostatic binding of the metal centre to the DNA backbone does play a role in determining the overall affinity of **53** and **54** for DNA. An example of the effect that added NaCl has on the emission response is given in Figure 2.13, where the profiles for **53** under different ionic strength conditions are presented.

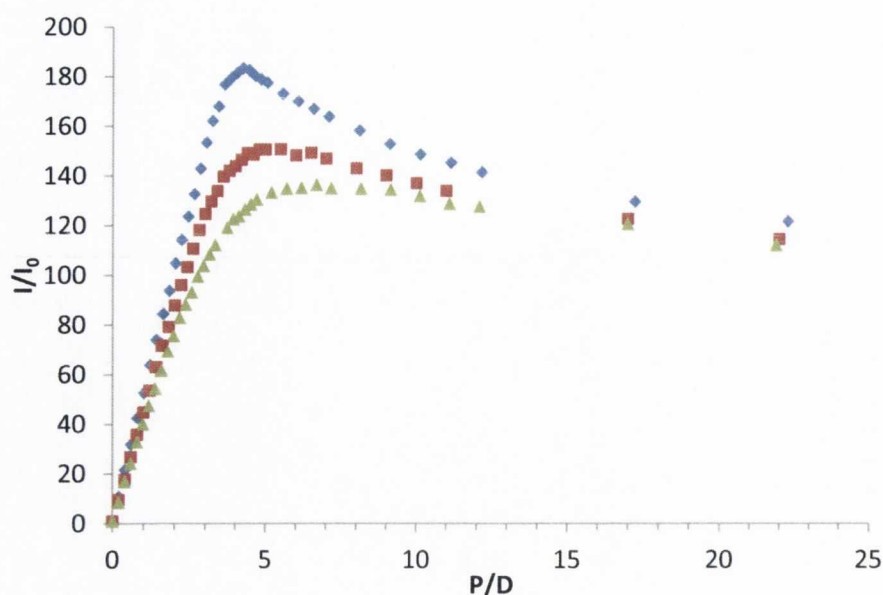


Figure 2.13: Relative changes in the integrated emission intensity of **53** ($6.5 \mu\text{M}$) at 630 nm ($\lambda_{\text{ex}} 445 \text{ nm}$) with increasing concentration of stDNA ($0 - 125 \mu\text{M}$), in 10 mM phosphate buffer ($\blacklozenge$), 10 mM phosphate buffer + 50 mM NaCl ($\blacksquare$) and 10 mM phosphate buffer + 100 mM NaCl ($\blacktriangle$).

The effect that added NaCl has on the emission response of **55** was not as pronounced (see Appendix) as that of **53** and **54**. However, by carrying out salt back titrations for **53** – **55** further confirmation that DNA binding strength is dependent on the ionic strength of the medium for all species was revealed, with none of the complexes being fully displaced (Figure 2.14).

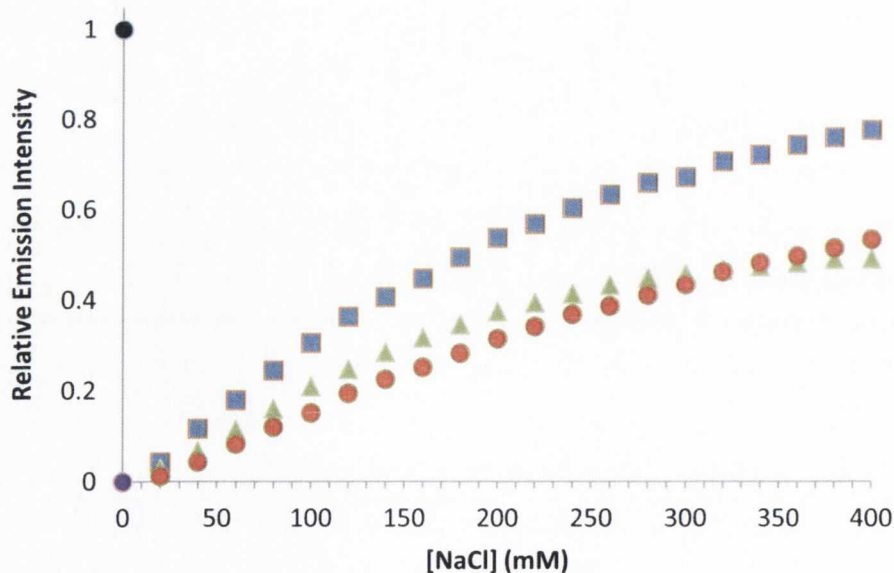


Figure 2.14: Fluorescence emission NaCl back titration profiles for **53** – **55**. Emission of unbound complex ($\bullet$), fully bound complex ($\bullet$) and bound **53** ($\blacksquare$), **54** ($\bullet$) and **55** ($\blacktriangle$) in the presence of increasing concentration of NaCl

In summary, the above NaCl dependence studies have shown that each of the complexes **53** - **55** remain bound to DNA at high concentrations of NaCl. Furthermore, a shift in equilibrium from bound complex was evident from both the smaller emission enhancement, and the DNA pair equivalents needed the maximum emission enhancement. From these studies it may be concluded that electrostatic binding of the metal centre to DNA does play a role in determining the overall DNA binding affinity. However, the fact that **53** - **55** remain bound at high ionic strength, clearly indicates that electrostatic interactions are important but most likely are not the predominant mechanism of interaction between these complexes and DNA.

2.4.3 Spectroscopic Titrations of **53-55** with Synthetic Homopolymer Polynucleotides

In a manner similar to that described above, **53** - **55** were titrated with synthetic polynucleotides, [poly(dAdT)]₂ and [poly(dGdC)]₂, in order to investigate their photophysical properties to determine if any sequence specific effects were observed.

2.4.3.1 UV/Vis Absorption Titrations of **53-55** with Synthetic Homopolymer Polynucleotides

The interaction of **53** - **55** with both [poly(dAdT)]₂ and [poly(dGdC)]₂ was firstly examined using UV/Vis absorption spectroscopy. These titrations were also carried out in a manner similar to that described in section 2.4.1.1.

The changes in the ground state of **54** in the presence of [poly(dAdT)]₂ and [poly(dGdC)]₂ are shown in Figure 2.15 (a) and Figure 2.15 (b) respectively, with those of **53** and **55** shown in the Appendix. The titration of **53** - **55** with both [poly(dAdT)]₂ and [poly(dGdC)]₂ resulted in significant changes to their absorption spectra all of which are summarised in Table 2.5. With [poly(dAdT)]₂, **53** exhibited a 55% decrease the λ_{max} at 310 nm with a concomitant decrease of 32% in the MLCT band at 445 nm. Similar changes were observed for [poly(dGdC)]₂ with a 51% decrease at 310 nm and a further decrease of 32% in the MLCT band. In the case of **54** with [poly(dAdT)], a 51% hypochromism was observed for the pdppz band at 310 nm, with the MLCT band showing a 28% hypochromism. Similarly with [poly(dGdC)]₂ a 55% hypochromism was observed at 310 nm, while the MLCT band also experienced a 24% decrease. In the presence of [poly(dAdT)]₂, **55** exhibited a hypochromism of 40% in the pdppz band and a further decrease of 26% in the MLCT band with these changes being 43% and 24% for the band at 310 nm and the MLCT band, respectively, upon titration with [poly(dGdC)]₂.

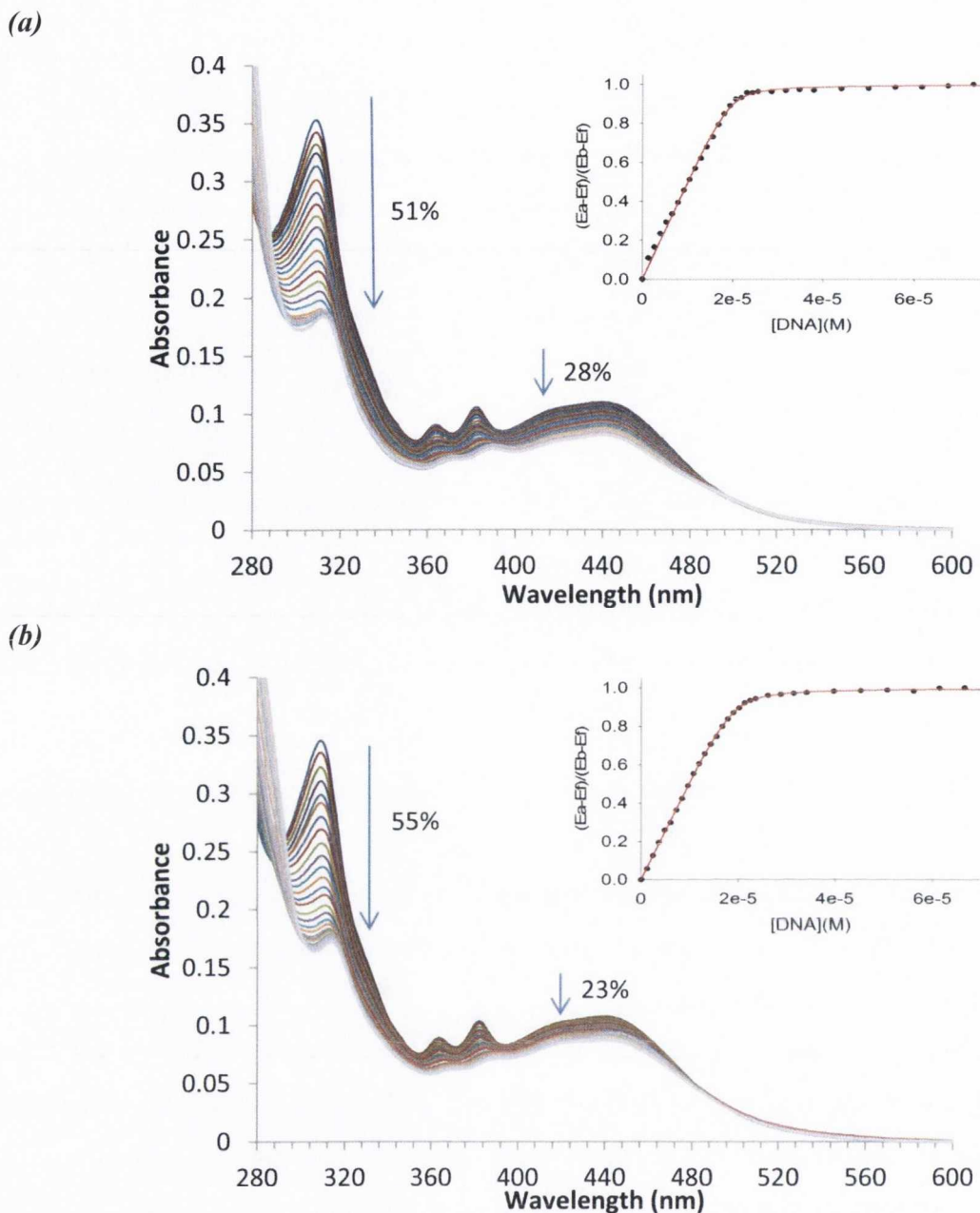


Figure 2.15: Changes in the UV/Visible spectrum of **54** (6.4 μM) with increasing concentration of (a) $[\text{poly(dAdT)}]_2$ (0–100 μM) and (b) $[\text{poly(dGdC)}]_2$ (0–90 μM) at pH 7.4. **Insets:** Plots of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](\text{M})$, P using data with a P/D between 0–12 and the best fit of the data (---) using the Bard Eqn.

In general, the changes in the UV/Vis ground state titrations observed above did not show a large degree of specificity for either $[\text{poly(dAdT)}]_2$ or $[\text{poly(dGdC)}]_2$. Minor differences were observed in the degree of hypochromism exhibited upon titration with the different polynucleotides. The comparison curve of **55** which exemplifies the changes in the $\pi - \pi^*$ absorption at 310 nm (Figure 2.16) demonstrates a slight preference for $[\text{poly(dGdC)}]_2$.

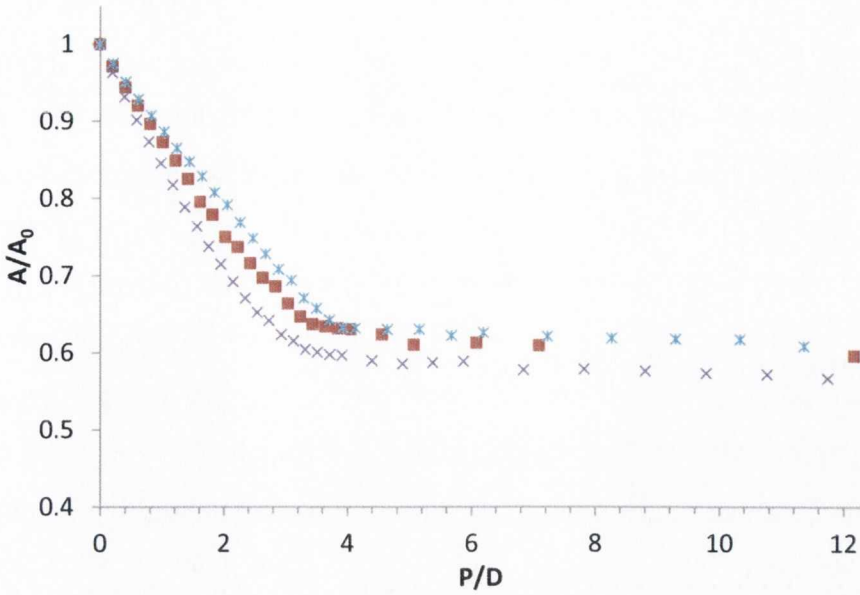


Figure 2.16: Changes in $\pi - \pi^*$ pdppz absorption of **55** ($6.5 \mu\text{M}$) in 10 mM phosphate buffer, at pH 7.4 upon addition of stDNA(■), [poly(dAdT)]₂ (*) and [poly(dGdC)]₂ (×).

These minor differences were reflected in the shapes of the binding profiles and as such the values obtained for K_b , from fits to the absorbance data using the model of Bard *et al.*, were in agreement with differences in the binding of **53** – **55** for [poly(dAdT)]₂ and [poly(dGdC)]₂. A summary of the results obtained are presented in Table 2.5.

Table 2.5: DNA binding parameters from fits to absorbance data in the presence of [poly(dAdT)]₂ and [poly(dGdC)]₂

Complex	$\lambda(\text{pdppz})$ Hypo- chromism	$\lambda(\text{MLCT})$ Hypo- chromism	Binding constant $K_b \text{ (M}^{-1}\text{)}$	Binding site size n (base pairs)	R^2
53 + stDNA	51%	18%	$4.9 \times 10^6 (\pm 0.7)$	2.14 (± 0.05)	0.99
53 + [Poly(dAdT)] ₂	55%	32%	$1.1 \times 10^7 (\pm 0.2)$	1.45 (± 0.02)	0.99
53 + [Poly(dGdC)] ₂	51%	32%	$6.9 \times 10^6 (\pm 0.9)$	1.24 (± 0.02)	0.99
54 + stDNA	53%	30%	$1.2 \times 10^7 (\pm 0.3)$	1.45 (± 0.03)	0.99
54 + [Poly(dAdT)] ₂	51%	28%	$1.8 \times 10^7 (\pm 0.4)$	1.71 (± 0.02)	0.99
54 + [Poly(dGdC)] ₂	55%	24%	$8.0 \times 10^6 (\pm 0.4)$	1.54 (± 0.08)	0.99
55 + stDNA	43%	29%	$5.4 \times 10^6 (\pm 0.5)$	1.55 (± 0.02)	0.99
55 + [Poly(dAdT)] ₂	40%	26%	$1.1 \times 10^7 (\pm 0.2)$	1.85 (± 0.02)	0.99
55 + [Poly(dGdC)] ₂	43%	24%	$3.8 \times 10^6 (\pm 0.2)$	1.33 (± 0.01)	0.99

The results obtained confirmed that all of the complexes retained high affinity for both [poly(dAdT)]₂ and [poly(dGdC)]₂ and revealed slight variations in binding affinities of these complexes with each of the homopolymers. For all complexes in the presence of [poly(dAdT)]₂ and [poly(dGdC)]₂ a trend was seen with K_b in the presence of [poly(dAdT)]₂ $\geq$ stDNA $\geq$ [poly(dGdC)]₂ (Table 2.5), for example in the presence of [poly(dAdT)]₂ **53** exhibited a $K_b = 1.1 \times 10^7 (\pm 0.2)$ and $n = 1.45 (\pm 0.02)$ while with [poly(dGdC)]₂ **53** had $K_b = 6.9 \times 10^6 (\pm 0.9)$ and $n = 1.24 (\pm 0.02)$, showing a slight preference for [poly(dAdT)]₂ tracks. Although the changes observed are minor, a possible explanation may be derived from the structural differences between [poly(dAdT)]₂ and [poly(dGdC)]₂, where for instance the grooves in AT rich regions are narrower than those of GC rich regions.¹⁸³ This may give rise to the Ru(II) centre not fitting into the groove in the same manner, which can result in differing binding affinities for the differing homopolymers. In addition, electrostatic calculations on nucleic acids suggest that the minor groove in AT-rich segments of nucleic acids have the highest negative electrostatic potential leading to highly cationic molecules showing a degree of AT specificity irrespective of their DNA binding capabilities.¹⁸⁴ However, it must be noted, that these are tentative explanations and further structural and photophysical studies are necessary in order to gain a more complete insight into the processes occurring upon binding to [poly(dAdT)]₂ and [poly(dGdC)]₂. In agreement with the ground state studies, changes in the excited state properties of **53** – **55** were also examined and these results will be presented in the next section.

2.4.3.2 Fluorescence Titrations of **53-55** with Synthetic Homopolymer Polynucleotides

As has been seen above, in general, the binding of a luminescent compound to polynucleotides leads to an increase in both the emission intensity and excited state lifetimes due to the rigidity of the DNA microenvironment and protection from solvent quenching.³² Moreover, two behaviours are observed for Ru(II) complexes: if the complex contains less than two oxidising ligands (*eg.* TAP or HAT), the emission increases with increasing DNA concentration, whereas if the complex contains two or three oxidising ligands, the emission is quenched. In the case of luminescence enhancement, the complexes are protected by the DNA double helix, thus, oxygen and solvent dependent deactivation processes are less efficient. In contrast, for complexes containing at least two π deficient ligands, the ³MLCT states are quenched by the nucleobases.^{59,78,85} As a result, fluorescence emission studies have been performed with synthetic polynucleotides, [poly(dAdT)]₂ and [poly(dGdC)]₂, to investigate the excited state properties of **53** – **55** in the hope of revealing similar sequence specific effects.

The changes in the excited state of **54** upon titration with $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ are shown in Figure 2.17 (a) and Figure 2.17 (b) respectively with those of **53** shown in the Appendix. The results obtained in the fluorescence emission titrations correlated well with the changes observed above in the UV/Visible absorption spectra of complexes **53** and **54** in the presence of $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$.

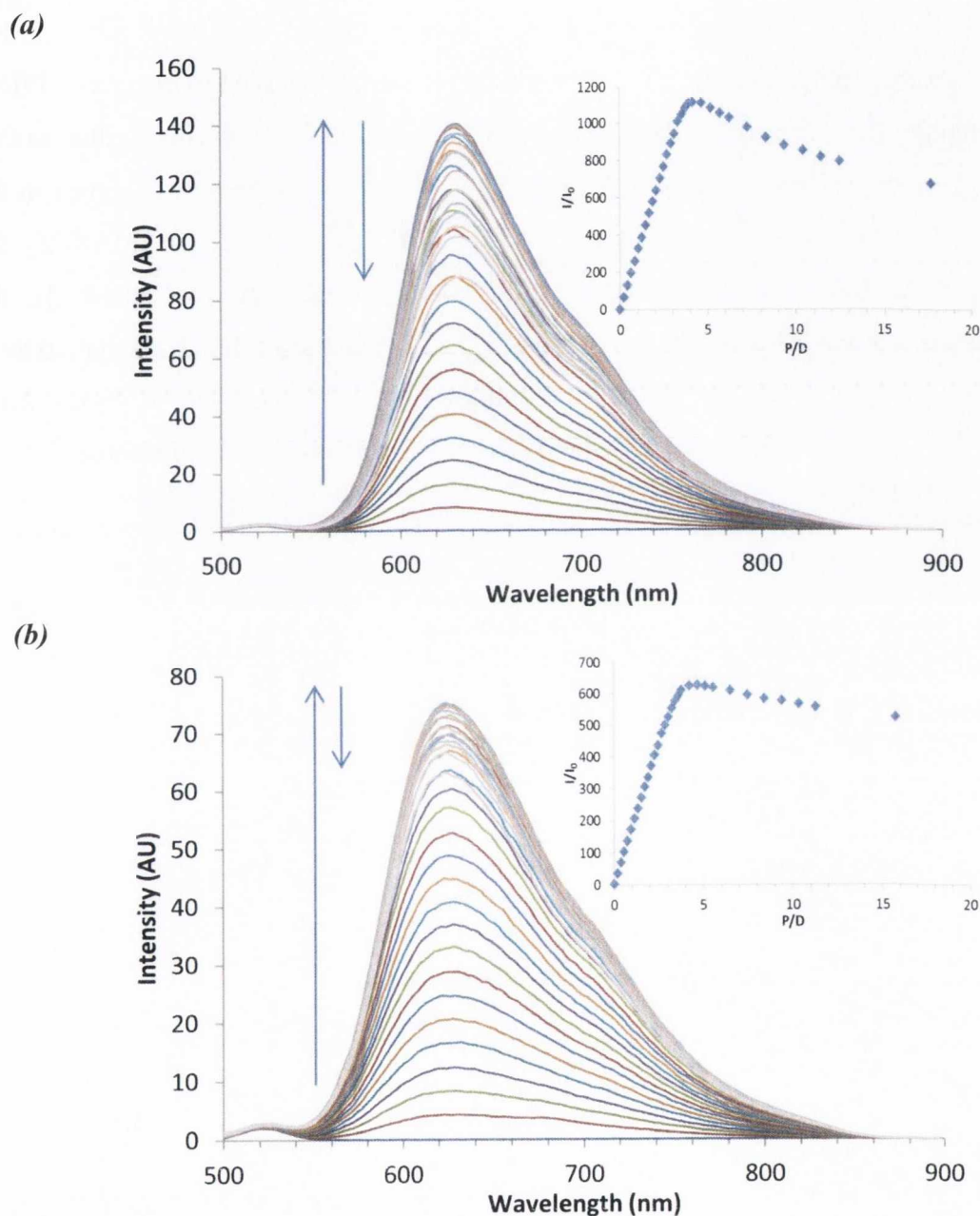


Figure 2.17: Changes in the fluorescence emission spectrum of **54** (6.4 μM) (λ_{ex} 445 nm) with increasing concentration (a) $[\text{poly}(\text{dAdT})]_2$ (0–100 μM) and (b) $[\text{poly}(\text{dGdC})]_2$ (0–90 μM) at pH 7.4 **Insets:** Plots of the change in integrated MLCT emission intensity at 625 nm as a function of P/D ratio.

Both **53** and **54** showed the same trend as observed in the UV/Vis absorption titrations with a slight preference seen for AT rich tracks resulting in fewer eq. needed to reach a maximum emission intensity for [poly(dAdT)]₂. Furthermore, the relative emission increase was largest in the presence of [poly(dAdT)]₂ and that of stDNA was shown to be close to that of [poly(dGdC)]₂. The changes observed upon addition of [poly(dAdT)]₂ and [poly(dGdC)]₂ to **53** and **54** culminated in the ³MLCT emission being ‘switched on’ as a function of increased polynucleotide concentration resulting in an emission band centred at 630 nm. For **53** in the presence of [poly(dAdT)]₂ a sharp maximum emission was seen to occur at a P/D = 2.5, while upon titration with [poly(dGdC)]₂ the maximum emission was shifted further to P/D = 6. This reflects the higher binding affinity of **53** for AT rich sequences as observed in the UV/Vis ground state titrations. Similarly, for **54** in the presence of [poly(dAdT)]₂ the maximum emission was slightly shifted to a P/D = 4, while with [poly(dGdC)]₂, the maximum emission was shifted further to (P/D) = 5.5. An example of the effect that stDNA, [poly(dAdT)]₂ and [poly(dGdC)]₂ have on the emission response is given in Figure 2.18, where the profiles for **54** in the presence of the different polynucleotides are presented.

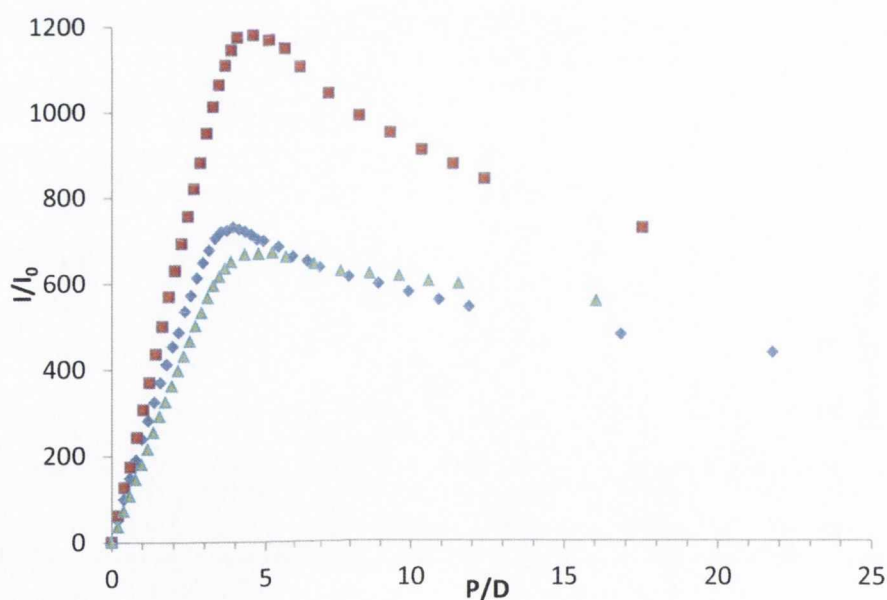


Figure 2.18: Relative changes in the integrated emission intensity of **54** (6.4 μM) (λ_{ex} 445 nm) with increasing concentration of stDNA (♦), [poly(dAdT)]₂ (■) and [poly(dGdC)]₂ (▲) in 10 mM phosphate buffer at pH 7.4.

In the case of **55** (Figure 2.19 (a) and 2.19 (b)), which ordinarily shows $^3\text{MLCT}$ based emission in aqueous solution, the emission was seen to increase by 57% in the presence of $[\text{poly}(\text{dAdT})]_2$ with main changes occurring between P/D 0 $\rightarrow$ 3.5. Conversely, upon addition of $[\text{poly}(\text{dGdC})]_2$, the emission of **55** was effectively “switched off” being quenched by *ca.* 98% between P/D 0 $\rightarrow$ 3.

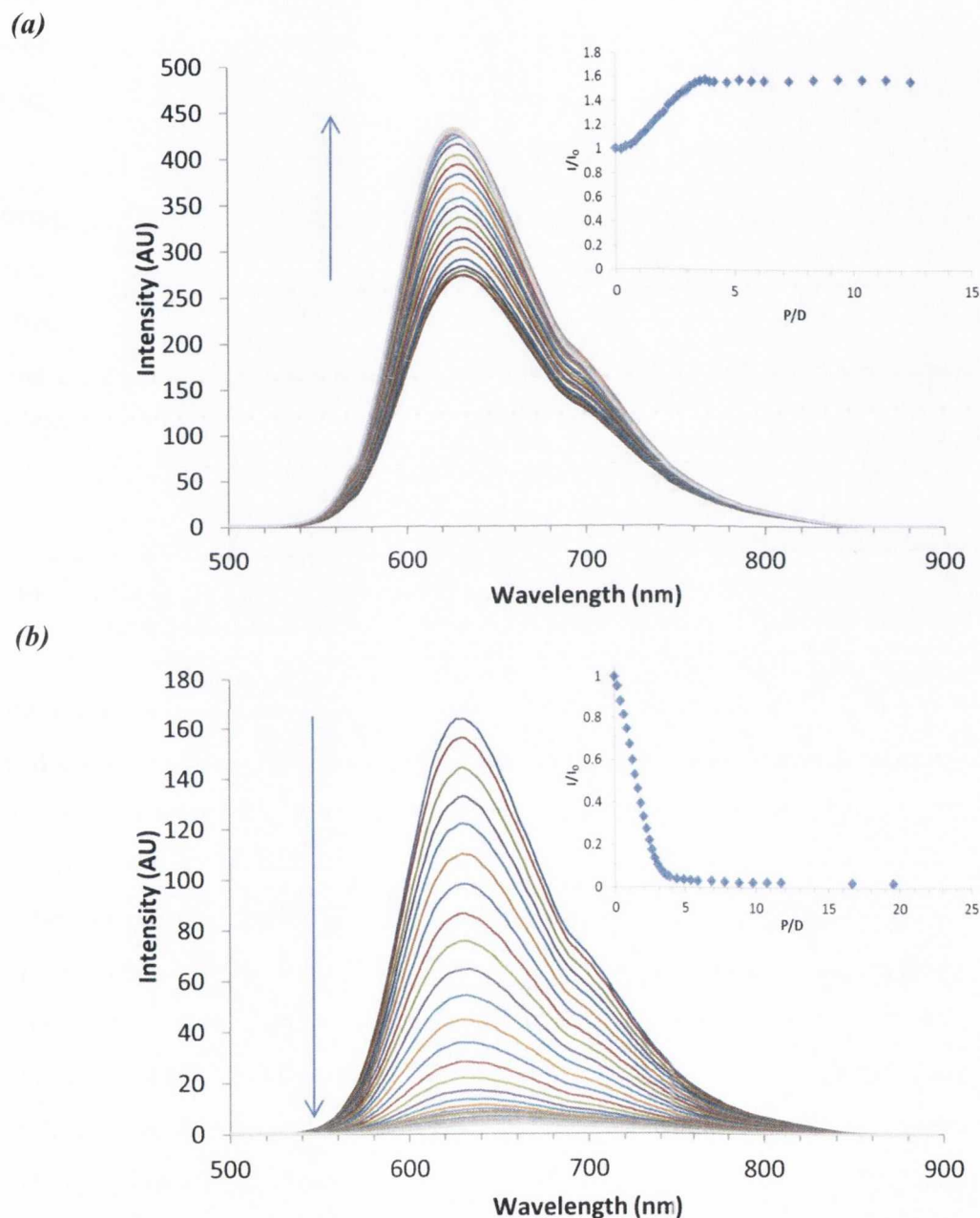


Figure 2.19: Changes in the fluorescence emission spectrum of **55** (6.5 μM) (λ_{ex} 415 nm) with increasing concentration (a) $[\text{poly}(\text{dAdT})]_2$ (0–75 μM) and (b) $[\text{poly}(\text{dGdC})]_2$ (0–118 μM) at pH 7.4 **Insets:** Plots of the change in integrated MLCT emission intensity at 635 nm as a function of P/D ratio.

This phenomenon can be explained as above, whereby the luminescence enhancements observed in the presence of $[\text{poly}(\text{dAdT})]_2$ result from a decrease of the efficiency of the non-radiative deactivation processes of the $^3\text{MLCT}$ excited state of these

complexes induced by the double helix microenvironment. Similarly, the large decrease in emission in the presence of $[\text{poly}(\text{dGdC})]_2$ is consistent with a PET process occurring from the guanine nucleobases to the Ru(II) polypyridyl centre. For comparison, the emission profiles for **55** in the presence of stDNA, $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ are shown in Figure 2.20.

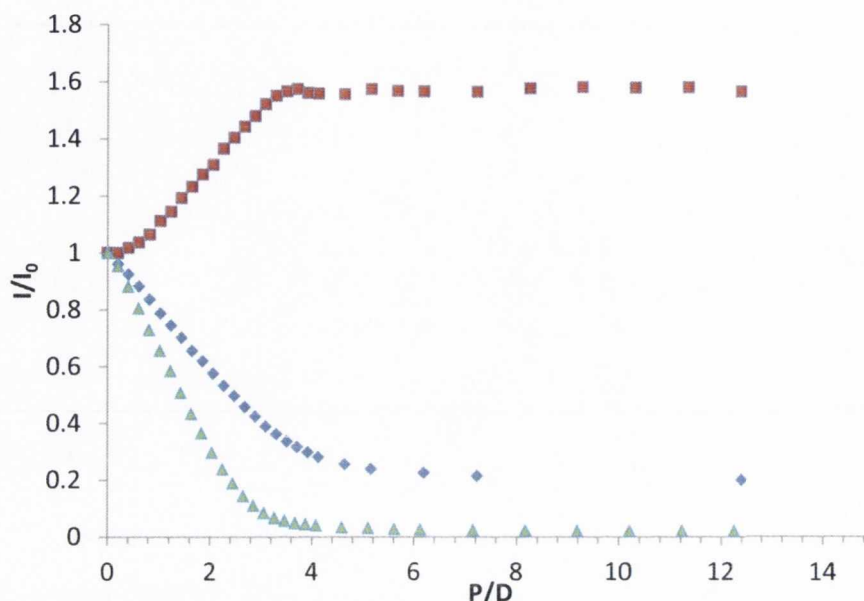


Figure 2.20: Relative changes in the integrated emission intensity of **54** ($6.4\mu\text{M}$) (λ_{ex} 445 nm) with increasing concentration of stDNA(♦), $[\text{poly}(\text{dAdT})]_2$ (■) and $[\text{poly}(\text{dGdC})]_2$ (▲) in 10 mM phosphate buffer at pH 7.4.

In summary, the above study has revealed modest sequence specific recognition with **53** – **55** all showing preferential binding to $[\text{poly}(\text{dAdT})]_2$ in the ground state. This has been tentatively attributed to structural differences between the helices of $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$. Furthermore, the general trend observed in the UV/Vis absorption titrations correlated well with the findings of the fluorescence emission titrations, where, **53** and **54** ‘switched on’ their emission in the presence of both polynucleotides, due to shelter by the DNA duplex affording a decrease of the efficiency of deactivation processes of the excited state. These changes occurred to a greater extent in the presence of $[\text{poly}(\text{dAdT})]_2$. Simultaneously **55** showed more complex behaviour whereby its emission was ‘switched on’ in the presence of $[\text{poly}(\text{dAdT})]_2$ but ‘switched off’ in the presence of $[\text{poly}(\text{dGdC})]_2$. This behaviour is consistent with the photo-electron transfer process from guanine nucleobases, observed for complexes containing at least two π deficient ligands. Subsequent sections will attempt to further quantify the binding affinity and mode of binding of **53** – **55** for DNA.

2.4.4 Ethidium Bromide Displacement Assays of **53** – **55**

Ethidium bromide (EtBr) binds avidly to DNA and, as a result, can be used as a fluorescent probe to investigate the binding interaction of small molecules with DNA. The method of EtBr displacement assays has thus been used to evaluate the DNA binding efficiency of both intercalative and non-intercalative.¹⁸⁵ When bound to DNA, EtBr is strongly emissive, however, upon displacement by a competitive DNA binding molecule, the relative decrease in fluorescence can be directly related to the extent of binding of the particular species under investigation.

As exemplified in Figure 2.21, EtBr is weakly emissive when free in solution and strongly emissive when bound to stDNA. The addition of complexes **53** – **55** caused the emission intensity to decrease dramatically, indicating their ability to displace EtBr at molar ratios of one or less than one, *i.e.* they displayed equal or greater affinity for DNA than EtBr. This confirms the high affinity of these complexes for DNA, which correlates well with the results obtained from UV/Visible absorption titrations discussed in Section 2.4.1.1.

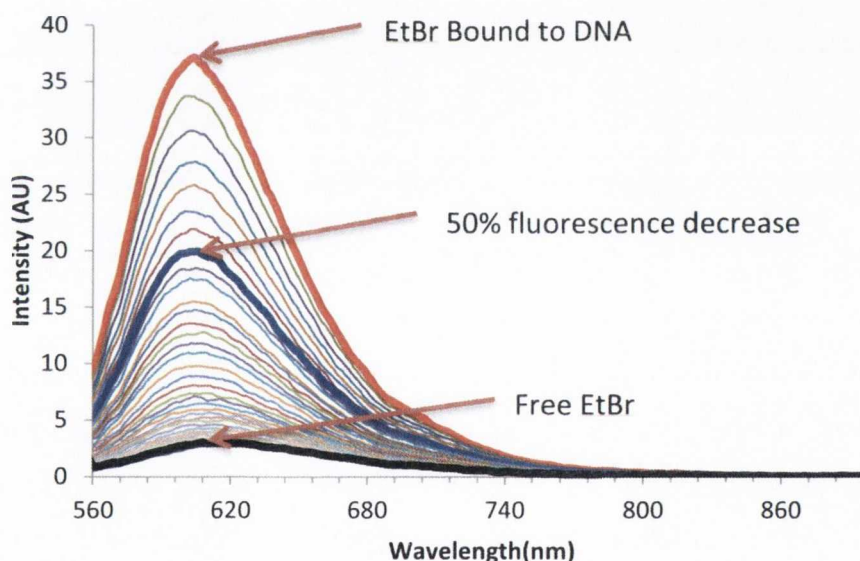


Figure 2.21: Emission changes observed for DNA bound EtBr ($\lambda_{ex}=545$ nm) with increasing concentration of **53** at pH 7 in 10 mM phosphate buffer solution

The apparent binding constants K_{app} derived from the titration, data were calculated according to the competitive binding model described by Boger *et al.*¹⁸⁵ using equation (3):

$$K_{app} = K_{EB} [EB] / [agent] \quad (3)$$

where K_{EB} is the binding constant for EtBr (1×10^6 M⁻¹),¹⁸⁶ $[EB]$ is the concentration of EtBr and $[agent]$ is the complex concentration at 50% of the fluorescence. It is clear from the

displacement profiles shown in Figure 2.22 that each complex **53** – **55** displace EtBr very effectively. A summary of binding constants calculated from eqn. (3) is listed as an inset in Figure 2.22. These results clearly show that **53** – **55** effectively displace EtBr when bound to stDNA, which gives rise to quenching of the EtBr emission. The displacement capability is in the order of **54** > **55** > **53** and this is reflected in the values obtained for the binding constants. The values obtained indicate a binding affinity almost 10 times that of EtBr.

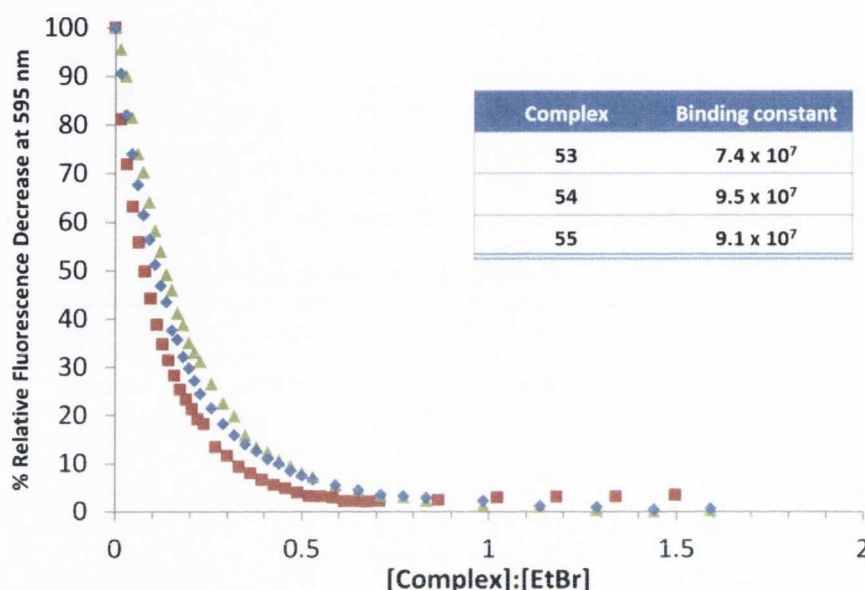


Figure 2.22: Change in fluorescence of DNA bound EtBr at 595 nm upon addition of **53** ($\blacktriangle$), **54** ($\blacksquare$) and **55** ($\blacklozenge$) in 10 mM phosphate buffer, pH 7.4. **Inset:** Table of binding affinities of **53** – **55** calculated from EtBr displacement assays

2.4.5 Thermal Denaturation Studies of **53** – **55**

DNA denaturation, also called DNA melting, is the process by which double-stranded DNA unwinds and separates into single-stranded DNA through the breaking of hydrogen bonding between the bases.¹⁸⁷ When duplex DNA molecules are subjected to conditions of pH, temperature, or ionic strength that disrupt hydrogen bonds, the strands are no longer held together, *i.e.* the double helical strands separate. The course of this dissociation can be followed spectrophotometrically due to the relative absorbance of the DNA solution at 260 nm which increases as the bases unstack. The melting temperature (or T_m) marks the midpoint of the melting process where half of the nucleic acid exists in the helical state and the other half exists in the single-stranded state. 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 . DNA denaturation measurements in the presence of DNA binders provide valuable information on the degree of stabilisation of the double stranded form and are a rapid method of identifying whether a molecule is binding to the DNA helix.

The thermal denaturation studies of stDNA (150 μ M) alone and in the presence of **53** – **55** were conducted at pH 7.4 in 10 mM phosphate buffer at a P/D ratio of 10, using degassed solutions. The absorbance at $\lambda_{\text{max}} = 260$ nm was then monitored as the temperature was gradually increased from 30 $\rightarrow$ 90 $^{\circ}\text{C}$ (1 $^{\circ}\text{C}$ per minute).

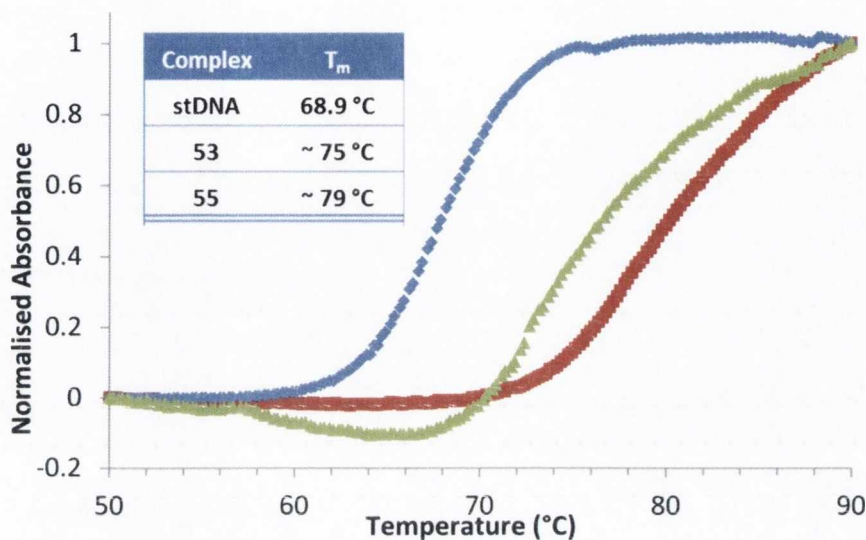


Figure 2.23: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacklozenge$) and presence of **53** ($\blacksquare$) and **55** ($\blacktriangle$) at P/D = 10. **Inset:** The T_m values from first derivative plots of the melting curve.

In the absence of metal complex, the T_m value for stDNA was determined to be 69 $^{\circ}\text{C}$ with significant modulations being measured in the presence of **53** and **55**. The resulting melting curves are shown in Figure 2.23 and demonstrate that the melting transition had not gone to completion at 90 $^{\circ}\text{C}$ and, consequently, an accurate T_m value could be not obtained. The value from the mid-point of the T_m curve, however, indicates approximate values of 75 $^{\circ}\text{C}$ and 79 $^{\circ}\text{C}$ for **53** and **55** respectively. It was not possible to obtain a true melting curve of **54** due to the strong absorption of this compound at 260 nm.

A T_m measurement was thus undertaken with [poly(dAdT)]₂ in the presence of **53** and **55**, again at a P/D ratio of 10. [poly(dAdT)]₂ has a lower T_m value due to A:T pairs being held together by two H-bonds whereas G:C pairs are held by three H-bonds; this results in a less stable helix and thus a lower T_m value. In the absence of metal complex, the T_m value for [poly(dAdT)]₂ was found to be 45.9 $^{\circ}\text{C}$. A substantial increase in T_m was again observed in the presence of **53** – **55** (Figure 2.24). The values determined from the first derivative plots of the absorbance are 58.2 $^{\circ}\text{C}$ and 53.2 $^{\circ}\text{C}$ for **53** and **55** respectively. This would suggest a significant stabilisation of the [poly(dAdT)]₂ structure upon binding.

In summary, **53** and **55** were both found to stabilise stDNA and $[\text{poly}(\text{dA-dT})]_2$ using thermal denaturation studies. Unfortunately an accurate T_m could not be calculated for stDNA due to the melting process not being fully completed at 90 °C but could be estimated as being greater than 75 °C indicating large stabilisation of the helix. Overall, these results support the results observed thus far from photophysical studies, however, it must be noted that these thermal denaturation studies do not provide evidence for a particular binding mode, as changes may arise from both groove binding¹⁸⁸ and intercalative interactions.¹⁸⁹ The preceding two sections will attempt to elucidate the particular binding mode of this family of complexes to DNA using spectroscopic techniques.

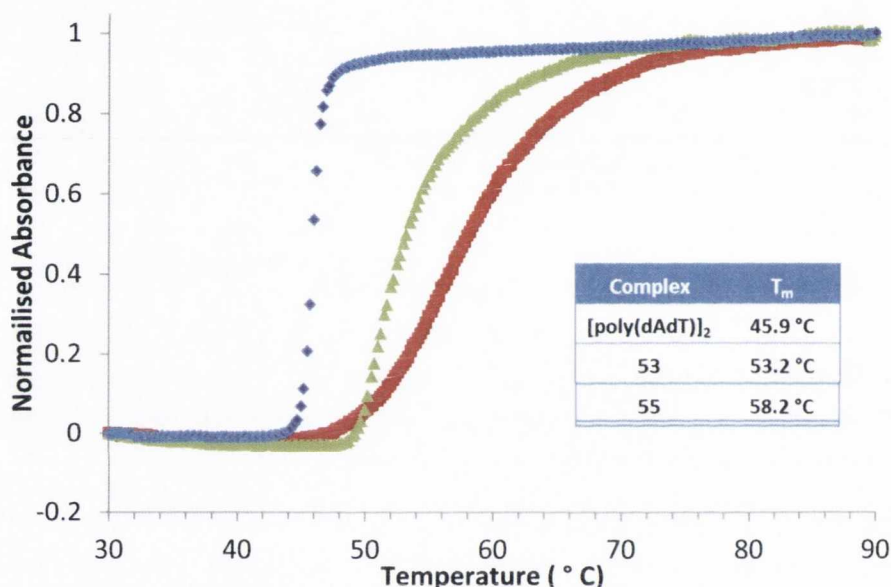


Figure 2.24: Thermal denaturation curves of $[\text{poly}(\text{dAdT})]_2$ (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacklozenge$) and presence of **53** ($\blacksquare$) and **55** ($\blacktriangle$). **Inset:** The T_m values from first derivative plots of the melting curve.

2.4.6 Circular Dichroism Studies of **53** – **55** in the Presence of DNA

Circular dichroism (CD) is a spectroscopic technique that can be used to study the structure and interactions of small molecules with DNA in solution.¹⁹⁰ CD probes the asymmetry of a system, by measuring the absorption of incident left and right circularly polarised light, and is defined as the difference between the two.¹⁹¹ As DNA is a chiral molecule the characteristic spectrum of B-DNA gives rise to two bands: a positive band at 275 nm, due to interaction between the $\pi\text{-}\pi^*$ transitions of the stacked bases, and a negative band at 248 nm, due to right handed helicity. When ligands bind to DNA, the ligand will alter the chirality of DNA and conversely the DNA may change the characteristic absorbance of the ligand, hence an induced CD (ICD) signal of the ligand can be acquired. Although an assignment of the binding mode of a ligand cannot be assigned definitively using CD, the

presence of an ICD signal is an immediate indication of ligand-DNA interaction. Small changes in the CD spectrum outside of the DNA absorption region are, generally, an indication of intercalation while larger changes are usually due to groove binding.¹⁹¹ The CD studies were carried out by keeping the concentration of stDNA constant at 150 μ M (OD = 1, at 260 nm) while varying the concentration of **53** - **55**. To avoid dilution affects, a range of solutions containing stDNA and each complex in 10 mM phosphate buffer were prepared at P/D ratios of 50, 20, 10 and 5.

The CD spectra resulting from the addition of **54** to stDNA are shown in Figure 2.25 with those of **53** and **55** shown in Appendix 2. The data obtained for **53** shows CD spectra with significant changes in the DNA absorption region. Evolution of a strong negative band at 285 nm corresponds to π - π^* intra-ligand transitions of the ancilliary bpy ligands and immediately implies strong association of **53** with the DNA helix. At high loading (P/D = 5) there was also the appearance of a small ICD signal at 320 nm corresponding to the main absorption band of the pdppz ligand. For **54** (Figure 2.25), a strong negative band was also observed evolving at 270 nm and shifting to 285 nm at P/D = 5 also implying strong association of **54** with DNA. Similarly, at high loading (P/D = 5) there is a stronger evolution of a positive ICD signal at 330 nm and a further weak, positive ICD signal at 470 nm. In the case of **55**, a similar trend was observed as for **53** and **54**, with another strong negative band evolving to 290 nm, however, with the ICD signal of the π - π^* intra-ligand pdppz transitions being less obvious.

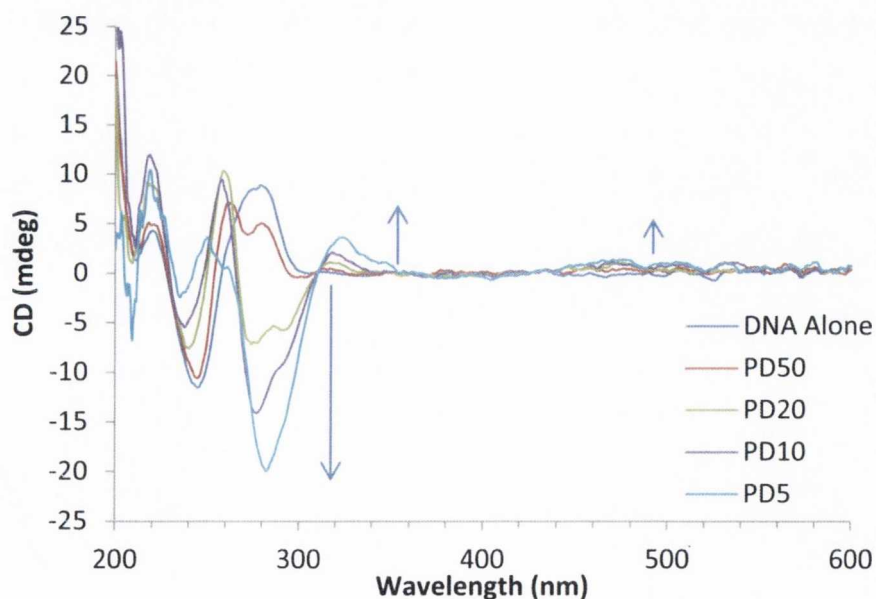


Figure 2.25: The circular dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **54** at varying P/D

From the observed changes it can be concluded that each of the Ru(II) complexes bind DNA and, in doing so, experience the helicity of the DNA duplex. The small positive signal,

observed in each case at *ca.* 310 nm, is indicative of an intercalator with a transition moment oriented along its long axis sitting parallel to the pseudo-dyad axis with the red-shift observed each time being typical for stacking interactions with the base pairs.^{43,190,191} Furthermore, the strong negative signal at *ca.* 285 nm may be attributed to groove binding of the ancilliary ligands. The intensity and sign of the ICD signal for a ligand bound in the grooves is more variable due to the greater number of possible orientations permitted by the width of the grooves. The orientation of the long wing of the pdppz ligand between the base pairs would put the ancilliary ligands in such a position to make it favourable for them to lie in the grooves and this behaviour has also been observed for complexes such as $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$.⁴⁴ The overall results point to a binding mode in which the metal centre is tightly associated with the helix, thus experiencing its chirality. Considered with the other spectroscopic studies presented so far, it is likely that the DNA binding involves insertion of the pdppz ligand into the helix, with tight association of the metal centre, through external binding or partial insertion into the grooves. The proceeding section will attempt to further characterise the orientation of the complexes **53** – **55** within the helix by carrying out linear dichroism (LD) studies.

2.4.7 Linear Dichroism Studies of **53** – **55** in the Presence of DNA

Linear dichroism (LD) spectroscopy is a technique that uses linear polarised light in one dimension and can be defined as the difference in absorption of light polarised parallel and perpendicular to an orientation axis. The polarised light produces an oscillation in the parallel and perpendicular planes resulting in it being possible to derive how much energy is absorbed in one dimension relative to the other. DNA itself is ideally suited to linear dichroism as DNA molecules tend to be long and very thin, making them easy to align in flow.¹⁹⁰ Once aligned the direction of polarisation in the molecule can be assigned relative to the orientation of the molecule (Figure 2.26 (a)).

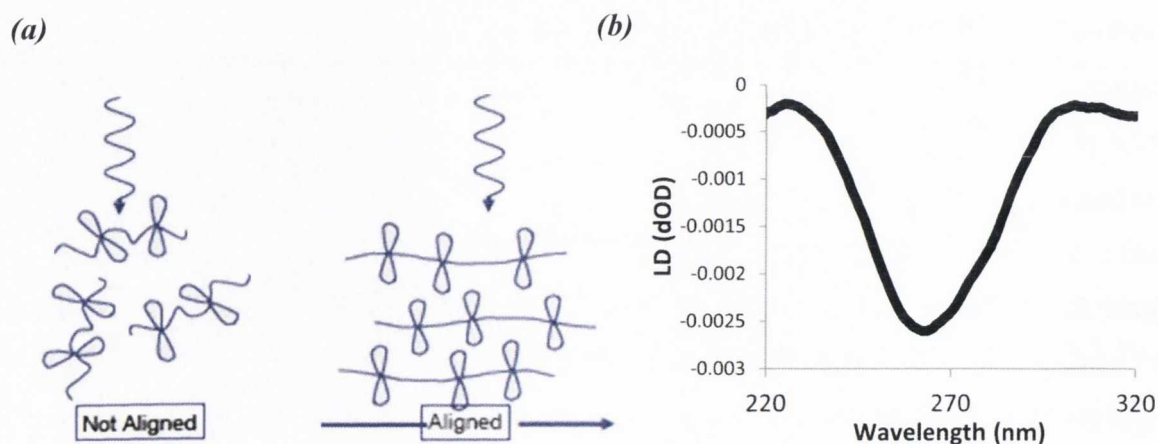


Figure 2.26: (a) Schematic representation of DNA molecules orientated in flow. (b) The characteristic negative LD signal of B DNA aligned in solution

DNA itself shows a strong negative signal at 260 nm due to the absorbance of the DNA bases giving rise to the electronic transitions being polarised perpendicular to the DNA helix axis as expected for B-DNA (Figure 2.26 (b)). Should the aligned DNA be treated with a DNA binding species that species should also become orientated in space and as a result the electronic transitions within the molecule itself can be observed to absorb more strongly in either one plane or another. LD provides important information regarding the binding event very quickly. For instance, existence of LD in the region outside of the DNA base absorption immediately tells us that the drug interacts with DNA and becomes orientated and similarly absence of an LD signal tells us that no binding is occurring. In addition, more precise structural information can be provided by the sign and magnitude of the signal. In general, if $LD > 0$, the transitions can be said to be absorbing more light parallel to the DNA helix axis and are bound in the grooves of DNA. Alternatively, if $LD < 0$, the transitions can be said to be absorbing light perpendicular to the DNA helix axis and are thus stacked between the bases, *i.e.* intercalated.

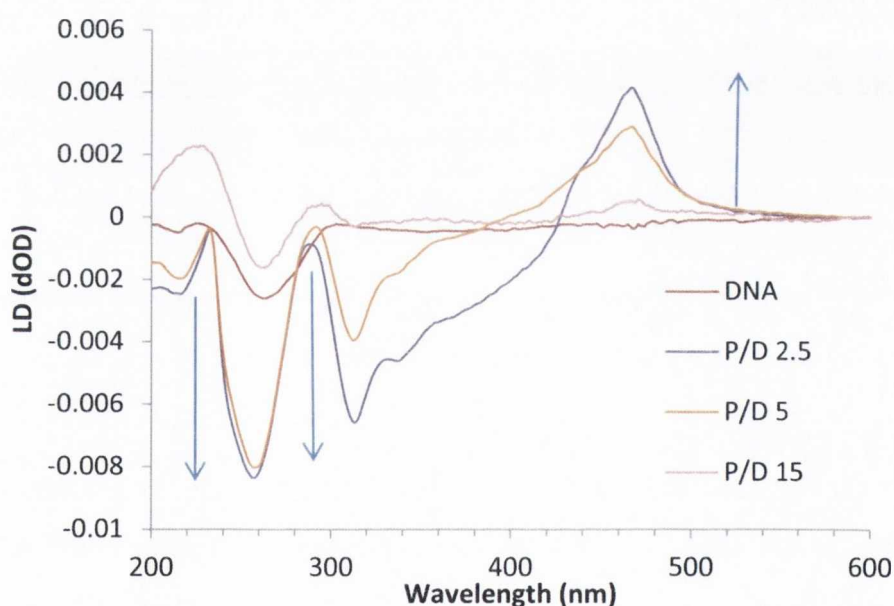


Figure 2.27: The changes in the linear dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the presence of **55** at varying P/D

The LD studies were carried out in a manner similar to the CD titrations by keeping the concentration of stDNA constant at 150 μ M (OD = 1, at 260 nm) while varying the concentration of **53** - **55**. The LD spectra resulting from the addition of **55** to stDNA are shown in Figure 2.27 with those of **53** and **54** shown in Appendix 2. It is clear from these large changes that each of these complexes interact with DNA and have become orientated, confirming the results from the previous sections. For **53**, a strong negative band at 320 nm and two weaker negative bands at 370 nm and 390 nm were seen which correspond to the π -

π^* transitions and $n - \pi^*$ transitions within the phenazine part of the pdppz ligand, respectively. Moreover, large changes were observed in the MLCT absorption region with a positive band at *ca.* 440 nm and a negative band at *ca.* 480 nm. In the case of **54**, a strong negative band at *ca.* 320 nm was also observed ($\pi - \pi^*$ transitions). As seen for **53**, large changes were exhibited in the MLCT absorption region with a large positive band seen at *ca.* 420 nm and a smaller negative band seen at *ca.* 490 nm. Similarly, **55** showed strong negative absorption with maximal peaks found at *ca.* 320 nm and 345 nm and a very pronounced positive band at 370 nm (see Appendix 2).

From this investigation it can be concluded that the existence of an LD signal in the region outside of the DNA base absorption tells us that these complexes associate with DNA. Moreover, the strong negative band at *ca.* 310 nm is an indication that the $\pi - \pi^*$ transitions of pdppz are lying perpendicular to the DNA helix, strongly supporting an intercalative mode of DNA interaction. Furthermore, large changes in the MLCT based absorption also provide evidence for strong DNA binding with the complexes being tightly associated with the DNA backbone. Firm conclusions, however, cannot be drawn in this region due to the presence of a racemic mixture (Δ and Λ) of the complexes. It has been demonstrated by Hiort *et al.* that different enantiomers demonstrate specific changes in this region which vary in magnitude depending on which enantiomer is binding.⁴³ This behaviour has been attributed to binding geometry varying somewhat between different enantiomers, which can be related to the fact that the dissymmetric pair of non-intercalated ancilliary ligands interact differently with the helical DNA strands surrounding the groove in which the complex sits.⁴³

2.4.8 Summary of DNA Binding Studies

In this Chapter the DNA-binding affinities of **53** – **55** in aqueous solution have been explored, using stDNA, [poly(dA-dT)]₂ and [poly(dG-dC)]₂. The ground state interactions of **53** – **55** with stDNA were firstly evaluated and showed distinct spectral changes that enabled the determination of binding constants K_b of $4.9 \times 10^6 \text{ M}^{-1}$, $1.2 \times 10^7 \text{ M}^{-1}$ and $5.4 \times 10^6 \text{ M}^{-1}$ for **53**, **54** and **55** respectively derived from the Bard equation.¹⁸¹ Such binding constant determinations are common for complexes of this type and, as result, give a method of comparison between the complexes reported here and others in the literature. For example, the equilibrium binding constant of [Ru(bpy)₂(dppz)]²⁺ to calf thymus DNA in 50 mM NaCl/5 mM Tris, pH 7.0, at ambient temperatures is $> 10^6 \text{ M}^{-1}$ based upon absorption titrations and equilibrium dialysis experiments.³⁵ It must be noted, however, that despite the excellent fits obtained for the titrations of **53** – **55** such values should be taken just as an estimate of the effective binding constant, as the concentrations used are high compared to the binding

constant and that the interaction of these complexes with DNA is quite complex and may involve varying modes of interaction. As such, caution must be exercised when taking such values into consideration. In addition to the ground state studies excited state studies for **53** and **54** were shown to exhibit the 'light switch effect' showing striking emission enhancements upon interaction with DNA. Conversely, the emission of **55** was shown to decrease in the presence of DNA and was attributed to the occurrence of a photoinduced electron transfer process between the TAP containing complex and guanine nucleobases. Studies in the presence of DNA at high ionic strength were shown to affect the binding strength of **53** – **55**; but the complexes were seen to remain bound even at high ionic strength ($[\text{NaCl}] > 400 \text{ mM}$). Studies in the presence of the homopolymers $[\text{poly}(\text{dA-dT})]_2$ and $[\text{poly}(\text{dG-dC})]_2$ revealed modest specificity with **53** – **55** all showing preferential binding to $[\text{poly}(\text{dAdT})]_2$ in the ground state. Evidence for the photoinduced electron transfer process occurring with **55** was supported by the fact that the fluorescence emission was quenched by 98% in the presence of $[\text{poly}(\text{dG-dC})]_2$ while a 57% emission enhancement was observed in the presence of $[\text{poly}(\text{dA-dT})]_2$.

EtBr displacement assays were also utilised to show that **53** – **55** were capable of displacing EtBr effectively when bound to stDNA. By comparison with the binding ability of EtBr, binding constants of $7.4 \times 10^7 \text{ M}^{-1}$, $9.5 \times 10^7 \text{ M}^{-1}$ and $9.1 \times 10^8 \text{ M}^{-1}$ were calculated for **53** – **55**; in general, a one order of magnitude increase over those derived from the Bard model. It is difficult to account for such variation, however, it must be noted that these titrations merely give an indication of binding to DNA and a large variation in quoted binding constants of EtBr make comparisons using this method difficult.^{185, 186} Moreover, the apparent existence of more than one mode of interaction of **53** – **55** with DNA further complicates the calculation of such values; a point that is largely ignored in the literature. T_m experiments found that all complexes were capable of stabilising the helical structures of stDNA and $[\text{poly}(\text{dA-dT})]_2$; both methods confirming the strong association of each complex with DNA. CD studies were employed to further investigate the mode of binding of **53** – **55** to DNA with these studies recognising that each of the Ru(II) complexes bind to DNA and, in doing so, experience the chirality of the DNA structure. Finally, LD studies confirmed that the long axis of the pdppz ligand was lying perpendicular to the DNA helix axis and was, thus, most likely intercalated between the base pairs. Without the use of NMR spectroscopy or X-ray crystal diffraction methods this method is as close to definitive evidence as possible.

The main aims of this Chapter were to identify the potential of a family Ru(II) complexes as spectroscopic probes for DNA and also as possible DNA photocleavage agents for use in PDT. With this in mind we have designed and synthesised three compounds, **53** -

55, which show promise as *in vitro* molecular probes for DNA. Moreover, **55** has the added advantage of both binding to DNA as well as having pronounced photoreactivity. The remainder of this Chapter will now deal with preliminary biological studies. These sections will detail singlet oxygen production efficiency, DNA photocleavage ability, cellular uptake and localisation as well as cellular phototoxicity studies.

2.5 Singlet Oxygen Sensitisation Ability of **53** - **55**

As discussed briefly in Chapter 1, singlet oxygen ($^1\text{O}_2$) sensitisation is one of the main causes of DNA photocleavage and also the mechanism by which many PDT agents exert their activity. Electronically excited compounds with long lived triplet states and sufficiently high triplet energy can generate $^1\text{O}_2$ by energy transfer to molecular O_2 . $^1\text{O}_2$ is a highly reactive species that preferentially adds to guanine with the resulting oxidised guanine inducing a strand break.⁸³ It is known that both $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ cause photocleavage of DNA, with $^1\text{O}_2$ sensitisation thought to be one of the main processes by which it occurs.^{27,69,71} This section will deal with the evaluation of the $^1\text{O}_2$ production ability of **53** – **55**, and their ability to cause DNA photocleavage *via* $^1\text{O}_2$.

A photo-oxidation study using **53** - **55** was undertaken by monitoring the light induced degradation of 1,3-diphenylisobenzofuran (DPBF) with generated $^1\text{O}_2$. The mechanism by which the light induced degradation of DPBF occurs is outlined in Chapter 8.¹⁹²

This was achieved following a modified procedure of Frimansson *et al.* by irradiating solutions containing **53** - **55** (1×10^{-8} M) and DPBF (initial concentration 5×10^{-5} M) and following the disappearance of its absorbance band at 415 nm over a time period of 180 min.¹⁹³ Filtered light of wavelength 470 nm was chosen for excitation of **53** – **55** as well as $[\text{Ru}(\text{bpy})_3]^{2+}$ in order to irradiate the complexes selectively over the DPBF. $[\text{Ru}(\text{bpy})_3]^{2+}$ (1×10^{-8} M) was chosen as a comparative reference from which relative rates of reactive oxygen species (ROS) production could be determined because of its known singlet oxygen quantum yield (Φ_Δ) (0.86 in oxygen-saturated MeOH at 1 atm).¹⁹⁴ As shown in Figure 2.28, **53** and **54** showed very little observable $^1\text{O}_2$ generation during these experiments. This is not so unexpected as neither of these compounds exhibit strong fluorescence and as such excited state mediated $^1\text{O}_2$ production should not be detected. In contrast, **55** was shown to be a producer of $^1\text{O}_2$ with a comparable DPBF degradation profile to that of $[\text{Ru}(\text{bpy})_3]^{2+}$ (Figure 2.28). The relative rates of DPBF degradation under these conditions were determined by linear regression and were found to be in a ratio of 1:1.07 for $[\text{Ru}(\text{bpy})_3]^{2+}$: **55** (Appendix 2).

Considering the established ability of Ru(II) complexes to photodamage DNA and the observed $^1\text{O}_2$ sensitisation ability of **55**, it was subsequently decided to undertake a study of the potential of **53** – **55** as DNA photocleavage agents.

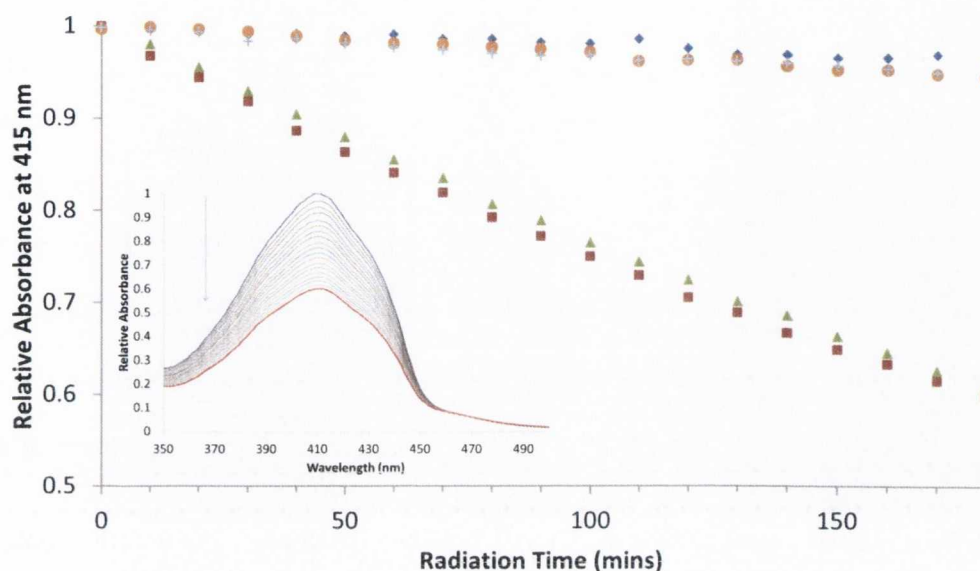


Figure 2.28: Comparative reactive oxygen species generation rates plot for $[\text{Ru}(\text{bpy})_3]^{2+}$ (▲), **53** (●), **54** (+), **55** (■) and no photosensitizer (◆). **Inset:** Observed absorbance decrease of DPBF in the presence of **55** over 180 minutes. Concentration of $[\text{Ru}(\text{bpy})_3]^{2+}$, **53**, **54** and **55** at 1×10^{-8} M and DPBF initially at 5×10^{-5} M in MeOH.

2.6 DNA Photocleavage efficiency of **53** – **55**

Photocleavage of nucleic acids typically involves an initial oxidative reaction with either a nucleobase or sugar residue.⁸³ The damaged nucleotide then degrades yielding shorter DNA strands as well as small molecule by-products, such as sugar fragments or nucleobases. The nucleic acid fragments are generally separated using electrophoresis through agarose or polyacrylamide gels. The information obtained from such gels can be useful in determining the mechanism by which the photocleavage agent reacts with the nucleic acid.¹⁹⁵ Electrophoresis itself involves separation of the products of cleavage according to their ability to migrate through the various types of gel. The simplest of these techniques and the one described here involves circular segments of DNA from bacteria known as plasmids.

In its normal undamaged state, plasmid DNA is supercoiled and referred to as **Form I**. Single strand (ss) cleavage (*i.e.* “nicking”) converts the supercoiled DNA to a relaxed, circular form (**Form II**) while double-strand (ds) cleavage produces linear DNA (**Form III**). The three forms are readily separated on an agarose gel and detected by fluorescent staining. This assay is highly sensitive since a single ss or ds cleavage event is sufficient to cause the change to

circular or linear forms. Moreover, analysis and comparison of the relative amounts of each form gives an indication of the efficiency of DNA cleavage, and whether single or double strand breaks have occurred. The cleavage processes involved and the differences in migration of the different forms on an agarose gel are shown schematically in Figure 2.29.¹⁵⁴

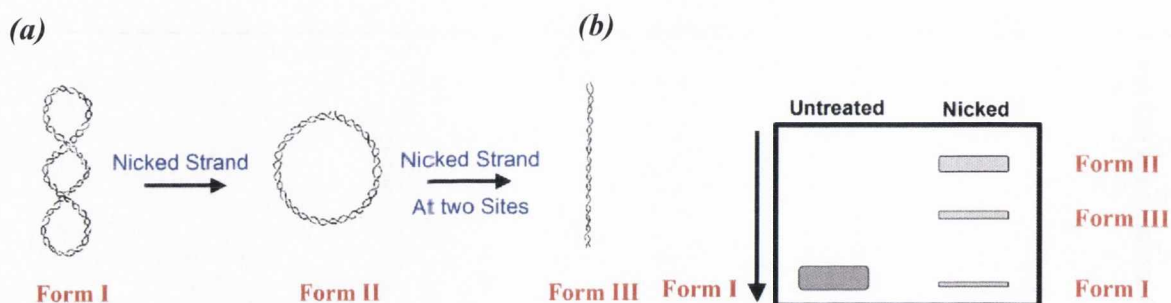


Figure 2.29: (a) Single and double strand cleavage of plasmid DNA (**Form I**) yielding circular (**Form II**) and linear (**Form III**) forms. (b) The differences in migration of the different forms of plasmid DNA as analysed by agarose gel electrophoresis.

The DNA photocleavage studies of **53** – **55** were carried out by treating pBR322 plasmid DNA (1 mg/ml) with each of the complexes at varying P/D ratios in 10 mM phosphate buffer solution. The samples were then subjected to light irradiation of 2 J/cm² using a Hg-Xe lamp, equipped with a > 390 nm NaNO₂ filter, before being separated using horizontal agarose gel electrophoresis in TBE buffer.

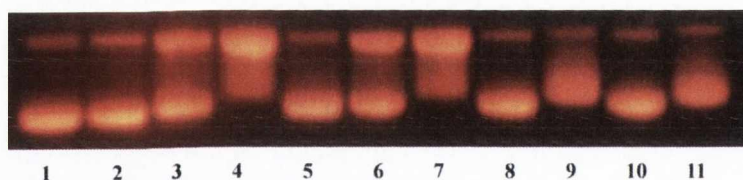


Figure 2.30: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after irradiation (2 J cm⁻²) at $\lambda > 390$ nm in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: [Ru(bpy)₃]²⁺ (P/D 100); Lanes 3-4: **55** (P/D 100, 50); Lane 5 : **55** in the dark (P/D 100); Lanes 6-7: **55** + 10mM NaN₃ (P/D 100, 50); Lanes 8-9: **54** (P/D 100, 50) ; Lanes 10-11: **53** (P/D 100, 50).

Shown in Figure 2.30 are the results obtained from the DNA photocleavage study of **53** – **55**. The complete Figures and corresponding tables for **53** and **54** are contained in Appendix 2. When incubated in the dark **53** – **55** showed no DNA cleavage. Similarly, **53** and **54** showed negligible photocleavage even at high P/D loading of the complexes. However, as can be seen in Figure 2.30 (Lanes 9 and 11) irradiation and subsequent electrophoresis of pBR322 DNA in the presence **53** and **54** at a P/D ratio = 50 showed clear evidence of a low mobility ‘dragging’ of the DNA. In contrast, **55** showed efficient photocleavage after irradiation under aerobic conditions at a P/D ratio of 100, with 38% cleavage of the plasmid

DNA being observed (Lane 3). Furthermore at P/D = 50, **55** showed complete conversion of the supercoiled DNA into both nicked and linear forms (Lane 4). This compares to just 14% cleavage in the presence of the known DNA photocleaver $[\text{Ru}(\text{bpy})_3]^{2+}$ (Lane 2). Moreover, the presence of ‘dragging’ was also observed after the main cleavage band. When irradiated in the presence of NaN_3 , a known singlet oxygen scavenger, it was observed that the photocleavage efficiency of **55** remained the same with 41% cleavage at P/D = 100 and with complete cleavage seen at P/D = 50 (Lanes 6 and 7). The relative amounts of supercoiled vs. open form DNA are summarised in Table 2.6 and a representative graph is shown in Figure 2.31.

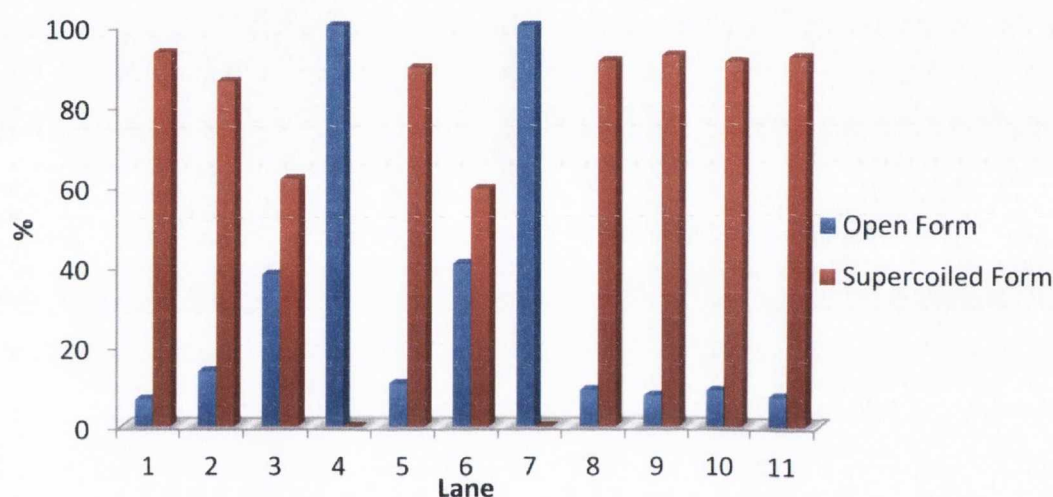


Figure 2.31: Relative percentages of supercoiled vs. open form pBR322 plasmid DNA from the photocleavage study of **53** - **55**

Table 2.6: Summary of percentages of supercoiled vs. open form pBR322

Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	6.7	93.3	7	100	0
2	13.8	86.2	8	8.9	91.1
3	38.1	61.9	9	7.5	92.5
4	100	0	10	8.8	91.2
5	10.5	89.5	11	7.5	92.5
6	40.6	59.4			

From these studies it is clear that complexes **53** and **54** display DNA photocleavage with efficiencies that are less than that of $[\text{Ru}(\text{bpy})_3]^{2+}$; most likely due to their inefficiency to produce $^1\text{O}_2$ in solution. Furthermore, when bound to DNA, emission of these complexes is localised on the pdppz ligand which is intercalated between the DNA bases. This tight association with the nucleic acid (where access to molecular oxygen is restricted), may limit the amount of $^1\text{O}_2$ generated, thereby also contributing to their inefficiency in causing DNA cleavage. The appearance of ‘dragging’ on the gel may further indicate the binding of **54** to DNA as its accumulation on the DNA molecules increases the overall positive charge of the species resulting in the mobility of the DNA through the gel being inhibited. Another explanation may be due to the formation of adducts with DNA but this was not investigated here.⁸⁶ In comparison, **55** showed greater photocleavage efficiency than $[\text{Ru}(\text{bpy})_3]^{2+}$, even though their $^1\text{O}_2$ quantum yields are comparable (Section 2.5). It is expected that this is due to the high binding affinity of **55** for DNA in addition to its ability to photoreact with *guanine* nucleobases.⁶⁰ The damage to DNA caused by **55** does not seem to involve $^1\text{O}_2$ formation due to its efficiency not being affected in the presence of NaN_3 . Photo adduct formation is also a likely occurrence as ‘dragging’ of the DNA is again observed and this is a phenomenon well documented for complexes containing at least two TAP ligands.^{59,85,196}

Taking into account the observed DNA binding of **53** and **54** and, especially, the DNA binding ability and DNA photoreactivity of **55** the next logical step is to establish the cellular uptake properties of these systems. Knowledge of the uptake of these complexes into biological systems will give an initial insight into their potential application as diagnostic or therapeutic agents.

2.7 Cellular Uptake and Localisation Studies of **54** and **55**

Sub-cellular uptake and localisation studies of **54** and **55** in HeLa cells (cervical cancer cell line) were undertaken using a variety of experimental techniques such as confocal microscopy, transmission electron microscopy (TEM) and subcellular fractionation experiments. Discussion here will focus on the preliminary data obtained through collaboration between the Gunnlaugsson Lab and the Williams research group in the School of Biochemistry and Immunology at Trinity College Dublin. The work discussed here has been undertaken jointly with Marialuisa Erby and Suzanne M. Cloonan from the Williams research group. The results of detailed studies undertaken hereafter will be reported in the thesis of Ms. Erby.

2.7.1 Confocal Microscopy Studies of **54** and **55**

Confocal microscopy experiments were carried out in order to provide visual evidence of the localisation of **54** and **55** in live cells. Populations of live HeLa cells (0.5×10^5) were incubated with either **54** or **55** (100 μ M) at 37 °C for 4 hrs, 8 hrs and 24 hrs, respectively before being treated with the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope with a 60 $\times$ oil immersion lens.

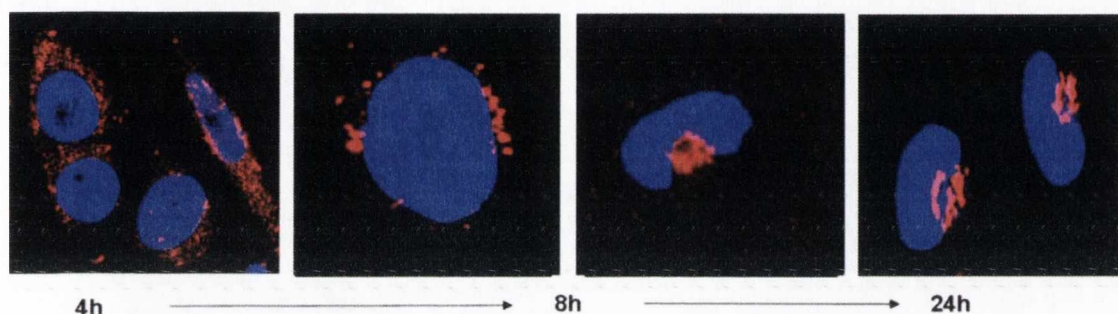


Figure 2.32: Confocal microscopy study showing the sub-cellular localisation of **55** (red) co stained with DAPI (blue) after 4h, 8h, 24h in HeLa cells.

The results obtained from these experiments provided evidence that both **54** and **55** show time dependant cellular uptake where discrete ‘packets’ of fluorescence were initially observed in the cytoplasm of the HeLa cells at 4 hr time points (Figure 2.32). Incubation of 8 hrs led to localisation of both complexes around the nuclear envelope, with some cells exhibiting a large single intense fluorescent spot associated around the nucleus. Concomitant with this location was the observation of a concave or ‘bean-shaped’ nucleus; an effect that was more pronounced after 24 hrs (Figure 2.32).

The tight association of the fluorescence emitted by these complexes around the nucleus and the appearance of co-localisation with the nuclear stain DAPI imply that these compounds eventually target nuclear DNA. Quantitative analysis illustrating the percentage of complex in the cytoplasm, at the nucleus edge and inside the nucleus using **55** as a representative showed that these complexes appear to move from the outside of the cell towards the nucleus in a time-dependent manner over 24 hrs (Figure 2.33 and Appendix 2). Such results were repeated numerous times and were fully reproducible.

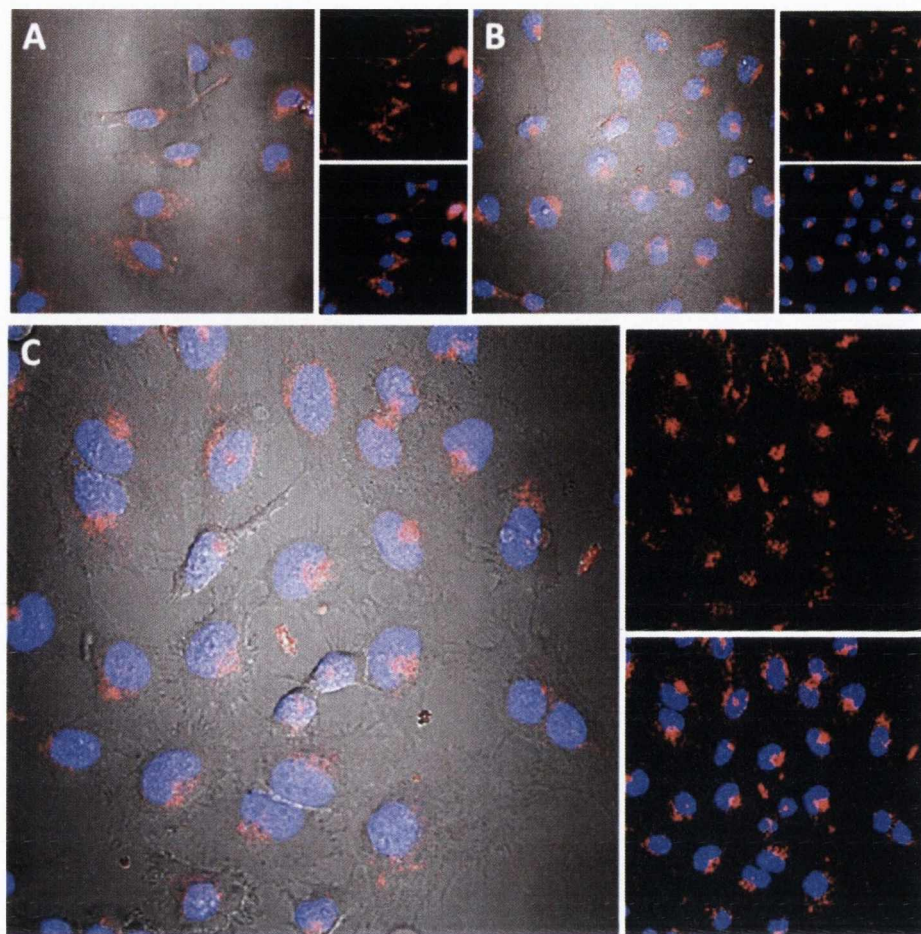


Figure 2.33: Confocal microscopy study of **55** in HeLa cells. (A), (B) and (C) represent cell populations after 4 hrs, 8 hrs and 24 hrs respectively. Shown are the images obtained with **55** (red), **55** + DAPI nuclear co-stain (blue) and an enlarged optical image of the cells (grey) overlaid with **55** (red) and DAPI (blue).

In order to shed more light upon the results obtained with confocal microscopy, several purified fractions of the cellular material were isolated by differential centrifugation of homogenised HeLa cells (5×10^6). This resulted in mitochondrial, nuclear and cytosolic enriched fractions from which the fluorescence of each was measured. Fluorescence emitted at 635 nm (indicative of the presence **55**) of the mitochondrial, cytosolic and nuclear enriched fractions treated with 100 μ M of **55** (24 hrs) revealed that approximately 54% of the fluorescence was found to be localised to the mitochondrial fraction, 5% to the cytosolic fraction and 34% to the nuclear fraction compared to untreated HeLa cells. Repeated three times this experiment gave fully reproducible results and further supports the observation that **55** may localise to the mitochondria and nucleus of HeLa cells, both of which contain DNA (Figure 2.34 (a)).

Furthermore, temperature dependent confocal microscopy studies with HeLa cells (1×10^6) incubated with **55** (100 μ M) were carried out for 4 hrs at 37 °C or 4 °C in order to ascertain the mechanism of cellular uptake of these complexes. The percentage of cells exhibiting fluorescence between 600 nm – 700 nm in the cytoplasm was expressed over the total amount of cells in a field of view (approximately 200 cells). It was found that cellular uptake was temperature-dependent with less than 10% of cells showing localisation of **55** at 4 °C compared to almost 80% uptake at 37 °C after the same amount of time. This result implies that these compounds are not membrane-permeable through passive diffusion and require active or facilitated uptake in order to be localised within these cells (Figure 2.34 (b)).

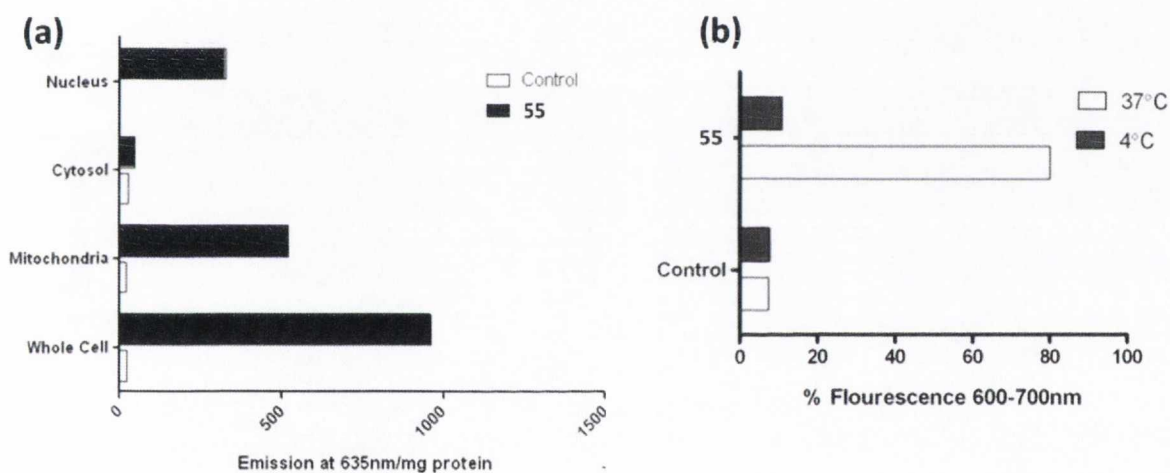


Figure 2.34: (a) Relative emission intensity measured at 635 nm from mitochondrial, nuclear and cytoplasmic enriched fractions of HeLa cells treated with **55** (100 μ M) for 24 hrs. (b) Fluorescence intensity measured between 600 nm-700 nm in HeLa cells expressed over the total amount of cells (approximately 200) per field of view after incubation with **55** (100 μ M) for 4 hrs at 37 °C or 4 °C.

2.7.2 Transmission Electron Microscopy Studies of **55**

Studies were also undertaken by using transmission electron microscopy (TEM) of HeLa cells. Cells were fixed, dehydrated, embedded in epoxy resin, ultra-sliced, mounted on a copper grid and stained with uranyl acetate and lead citrate before examination on a JEM-2100 transmission electron microscope. These experiments were conducted with the aim of further determining the sub cellular localisation of **55** and the phenomenon of ‘bean shaped’ nuclei by observing any structural differences between treated and untreated cells. Cells treated with **55** (100 μ M for 24 hrs) revealed the apparent clustering of mitochondria to one side of the nuclear envelope co-localised with the development of nuclear invaginations (Figure 2.35). This type of behaviour has been associated with a phenomenon termed ‘perinuclear mitochondrial clustering’ and has been linked to the initiation of a cell-death response.¹⁹⁷ Although an interesting result, firm conclusions cannot be made at this stage and the utilisation of more detailed confocal co-localisations studies as well as cell death mechanistic studies are necessary to investigate this phenomenon further.

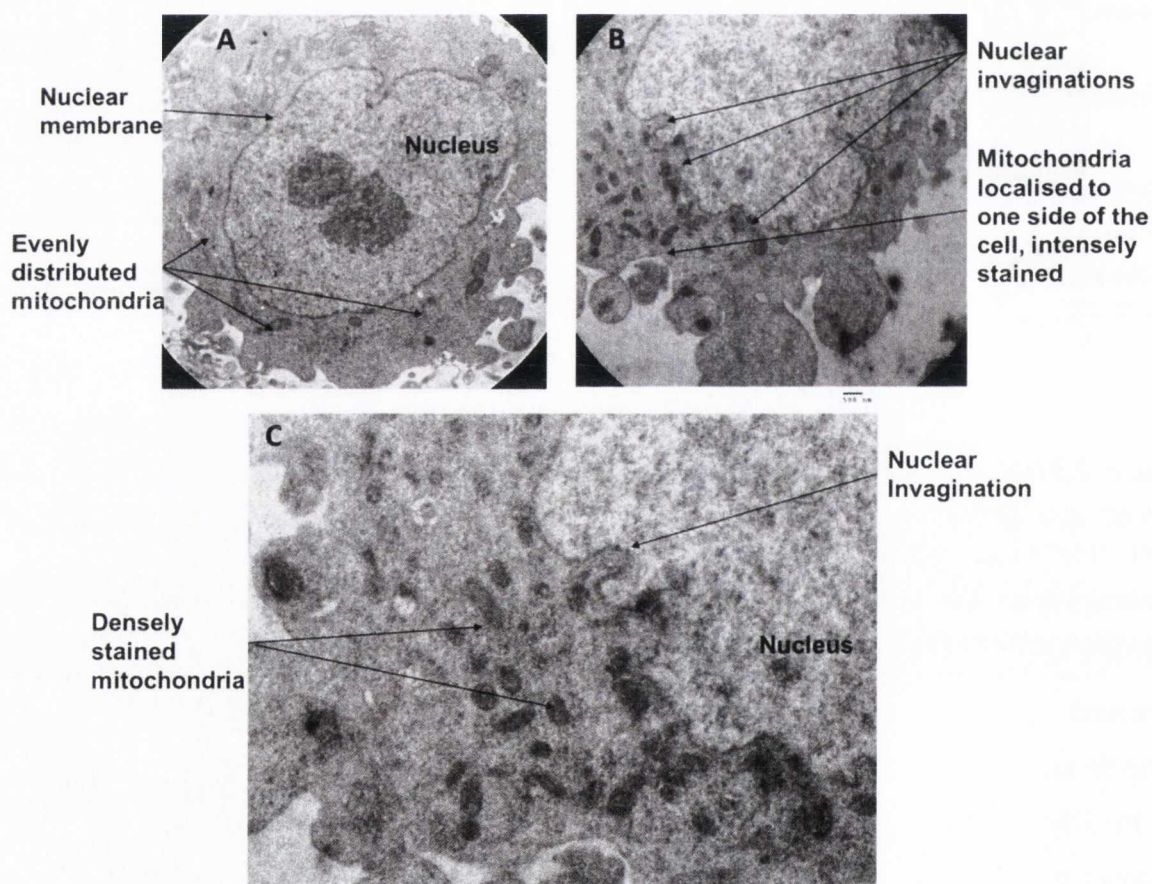


Figure 2.35: Transmission electron microscopy images of (A) untreated HeLa cells and (B) cells treated with **55** (100 μ M). (C) is a close up image of a section of (B).

Although the sub cellular location of **54** and **55** is still under question it is clear from all of the above studies that localisation of both complexes inside the cytoplasm is occurring with a time dependant movement towards nuclear DNA. With access to the nucleus the photoactivity of **54** and **55** becomes particularly important from the point of view of novel PDT agents. The next section will attempt to investigate the phototoxicity profile of both **54** and **55** cancer cell lines.

2.8 Cellular Phototoxicity Studies of **54** and **55**

As described in Chapter 1, the development of non-porphyrin based PDT agents is desirable to overcome many of the side effects associated with porphyrin based species. Based on the results presented in this Chapter we anticipated that while **54** did not show desirable characteristics, **55** may show potential as an ideal Ru(II) based PDT candidate due to its ability to target DNA with high binding affinity, be internalised inside cancer cells and its inherent excited state reactivity with biomolecule substrates.

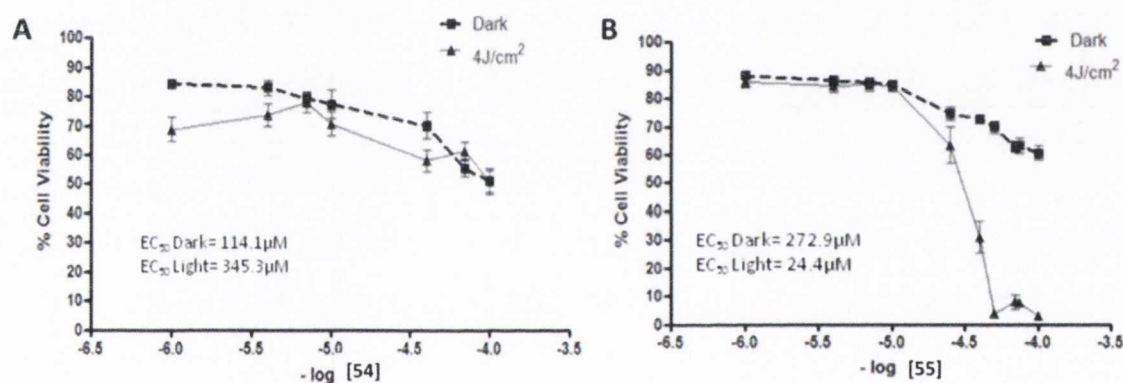


Figure 2.36: The cytotoxic effects of **54** and **55** on HeLa cells with or without light activation. Cells were seeded in a 96-well plate and treated with the respective complex for 24 hrs followed by irradiation with a light dose of 4 J cm^{-2} .

For comparison purposes the cytotoxic potential of both **54** and **55** was evaluated by conducting cytotoxicity assays in HeLa cells, in collaboration with Ms. Erby, using an alamar blue cytotoxicity assay. Such assays rely on the non-fluorescent resazurin dye, which is reduced to the pink coloured and highly red fluorescent resorufin dye; a process that can be directly related to cell viability. Both complexes were also ‘photoactivated’ ($\lambda \geq 400 \text{ nm}$), using a Hg-Xe lamp (4 J/cm^2) after incubation for 24 hrs and the cytotoxicity was again assessed. The 4 J/cm^2 light dose was employed in order to reduce the amount of time that the cells were exposed to room temperature. In comparison to clinically available compounds such doses are especially low with light doses of between 100 and 200 J/cm^2 being utilised for tumor control in clinical settings.⁶¹ As shown in Figure 2.36 the use of **54** under these

conditions did not result in any significant light dependent cytotoxicity at high concentrations. In contrast, **55** was shown to display a strong degree of light-dependent cytotoxicity in a concentration dependent manner (Figure 2.36 (b)). The EC_{50} value (half maximal inhibitory concentration) for **55** was found to be 24 μM while an EC_{50} value of 273 μM was determined in the absence of light irradiation.

The ability of **55** to induce light-dependant cytotoxicity may be due to its ability to photoreact with biomolecules (Section 2.6). Such cytotoxic effect may be mediated by DNA strand cleavage or through photoreaction with tryptophan containing protein residues due to its π deficient nature.⁸¹ Alternatively, as shown in Section 2.5, **55** causes $^1\text{O}_2$ sensitisation which may provide an alternative mechanism for cell cytotoxicity.¹⁹⁸ These results suggest that complex **54** may be more suited for use as an *in vitro* fluorescent DNA probe whereas complex **55** may have more applications as a dual therapeutic/diagnostic probe. More detailed biochemical data relating to **54** and **55** will be presented in the Thesis of Ms. Erby

In order to further confirm the ability of **55** to photoinduce cell death, photoactivation experiments were conducted using confocal fluorescence microscopy imaging. Irradiation of **55** (100 μM) *in vitro* was achieved using a 488 nm Argon laser diode attached to a real-time confocal microscope. After 30 minutes light exposure, images were taken every 10 mins over a 12 hr period and changes in the cell population morphology were observed. Representative video files of all three experiments are included as a CD attached to this Thesis. Control experiments were performed where the HeLa cells were irradiated in the absence of **55** (Video 1) and similarly where HeLa cells treated with **55** were not irradiated (Video 2) and observed over a 12 hr period. The resultant control cell populations were seen to behave normally, whereby cells were seen to continue to divide and multiply with a negligible amount of ‘background apoptosis’ (commonly associated with such rapidly dividing cancer cell lines). Conversely, the cells treated with **55** and irradiated exhibited an apoptotic phenotype and membrane ‘blebbing’ after approximately 4 hrs with continuing damage occurring until the 12 hr experiment had concluded (Video 3 and Figure 2.37).

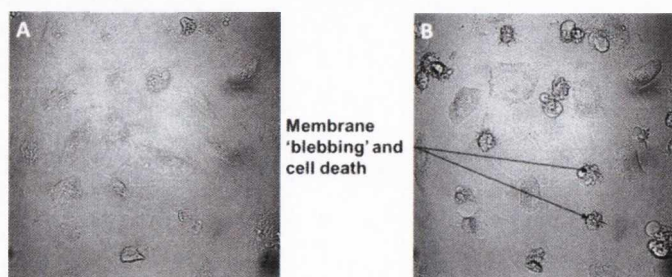


Figure 2.37: The cytotoxic effect of **55** on HeLa cells, observed through time resolved confocal fluorescence microscopy. (A) A healthy population of cells treated with **55** (100 μM) prior to irradiation with an Argon 488 nm laser. (B) The same population pictured 12 hours post irradiation.

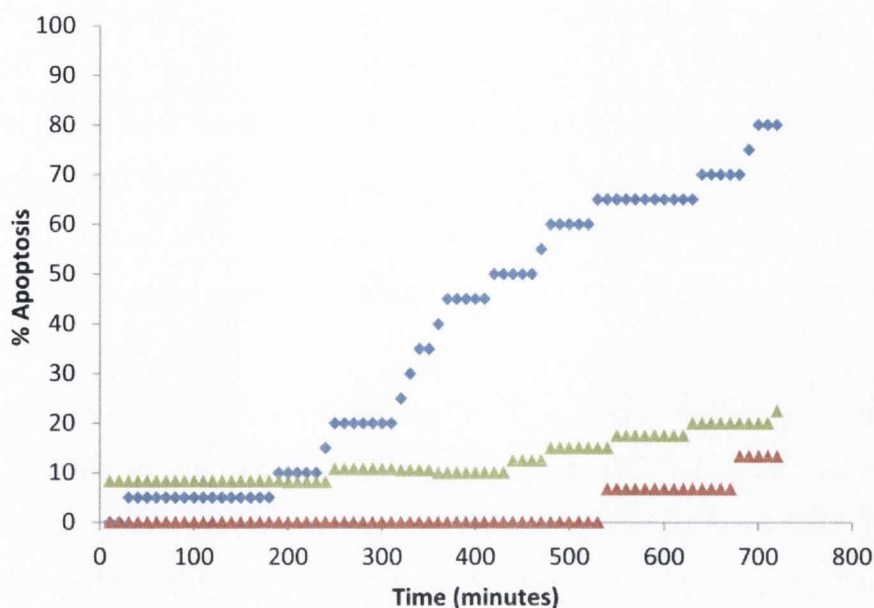


Figure 2.38: A comparison graph of induced apoptosis by **55** alone (▲), irradiation alone (▲) and **55** + irradiation (◆).

It is thus, clear from these investigations that even though **53** - **55** display spectroscopic responses signifying their binding to DNA, it is only **55** that possesses an efficient ability to photocleave DNA; a process occurring with an efficiency greater than that of $[\text{Ru}(\text{bpy})_3]^{2+}$. Both **54** and **55** were found to be taken up by cervical cancer HeLa cells and localise (in a time dependent manner) towards the nucleus structure. Data ascribed from cell fractionation experiments, TEM analysis and confocal microscopy experiments all supported this observation. In a manner similar to its DNA photocleavage efficiency, **55** was also shown to display a strong light dependent phototoxic effect towards cancer cells with an EC_{50} value of $24 \mu\text{M}$ being measured after a $4 \text{ J}/\text{cm}^2$ light dose. These findings were visually confirmed by time resolved confocal microscopy analysis of HeLa cell populations where cells incubated with **55**, following 30 mins irradiation, were observed to undergo cell death over a 12 hr period. Observed collectively, such results have been a further step towards the development of Ru(II) based complexes for use in light activated cancer therapies.

2.9 Conclusions and Future Perspectives

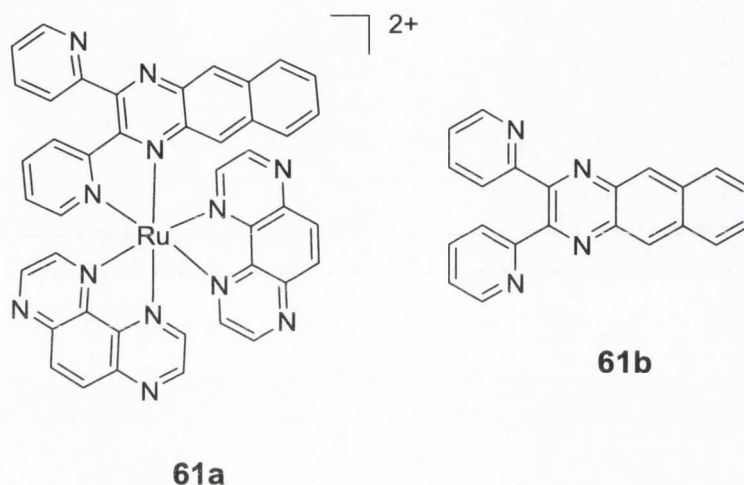
The initial rationale behind the study discussed in this Chapter was to develop novel Ru(II) based complexes that would intercalate into DNA and provide species with potential as phototherapeutic agents. Herein, the design, synthesis and characterisation of three novel Ru(II) polypyridyl complexes, **53** – **55**, and their various photophysical properties have been presented. Their DNA-binding affinities in aqueous solution have also been explored through a variety of techniques such as UV/Vis absorption and fluorescence titrations as well as EtBr displacement assays, T_m experiments and CD and LD studies all of which have helped in our

understanding of the nature of their DNA-binding interactions. Analysis of the ground state data allowed for the calculation of DNA binding constants (K_b) of the order of $10^6 - 10^7 \text{ M}^{-1}$, with fluorescence studies showing striking emission modulation upon interaction with stDNA and the homopolymers [poly(dA-dT)]₂ and [poly(dG-dC)]₂. Both EtBr displacement assays and T_m experiments, in addition to LD and CD studies confirmed that **53** – **55** are capable of binding with high affinity to DNA, with strong evidence for an intercalatory mode of interaction.

We were subsequently able to identify that both **53** and **54** were unable to sensitise $^1\text{O}_2$ where **55** was capable of $^1\text{O}_2$ production with a comparable profile to that seen for [Ru(bpy)₃]²⁺. DNA photocleavage experiments were also undertaken using **53** – **55** with a similar trend seen in which **53** and **54** showed negligible photocleavage while **55** showed highly efficient cleavage. Interestingly this process was unaffected by NaN₃, implying a non-oxygen dependent pathway was possibly involved in this process. Both **54** and **55** were subsequently chosen as candidates for preliminary biological testing where it was demonstrated that these complexes were readily taken up by HeLa cells and, over a period of 24 hrs, accumulate at the nucleus. As **55** also combines the use of π -deficient TAP ligands, its photoreactive nature was thought to offer potential as a novel PDT agent. An assessment of the antiproliferative abilities of both **54** and **55** in the presence and absence of light activation in malignant HeLa cells was undertaken and revealed that **55** showed potent light-dependent toxicity ($\text{EC}_{50} = 24 \text{ }\mu\text{M}$) whereas **54** did not. Consequently, **55** was shown to induce dose and time dependent apoptosis in HeLa cells. We speculate that subcellular location of **55** before activation results in accumulation in mitochondria before it is shuttled to the nucleus where it binds to DNA. Indeed, as mitochondria also contain DNA, mitochondria may perform this shuttling function for **55**. However, more detailed studies are ongoing in order to fully determine and understand this process. Subcellular fractionation studies revealed that the Ru(II) based fluorescence, attributed to **55**, was found mainly in mitochondrial and nuclear enriched fractions after 24 hrs of treatment. TEM studies of HeLa cells treated with **55** show densely stained mitochondria which appear to cluster around the nucleus.

Future perspectives of this work will focus on the development of a series of analogues of **55** such as **61a** which may possibly be photoactivated by light absorption above 600 nm due to the dpq ligand, **61b** having a delocalized π -system which may allow for better tissue penetration.¹⁹⁹ In addition, further studies with **55** will provide a more detailed view of the subcellular localisation of this species. Studies examining the co-localisation of **55** with mitochondrial, endoplasmic reticulum, golgi apparatus and lysosomal markers will shed light on its precise localisation. Furthermore, from the point of view of cytotoxic effect, **55** will be

subjected to further investigations into the mechanism by which its cytotoxicity occurs. Analysis of this mechanism will be revealed by propidium iodide (PI) fluorescent activated cell sorter (FACS) analysis which should detect the formation of apoptotic bodies in a dose-, time- and light- dependent manner. Moreover, using the antioxidant N-acetyl cysteine (NAC) the involvement of reactive oxygen species in the photo-induced cell death mechanism will give rise to valuable information on the involvement of reactive oxygen species towards cell death.

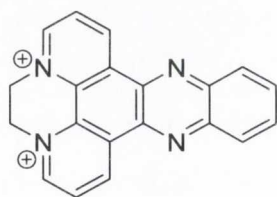
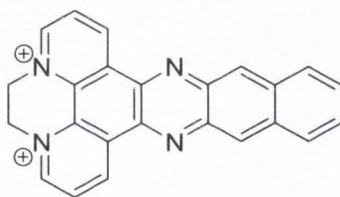
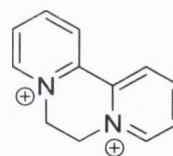


Chapter 3:

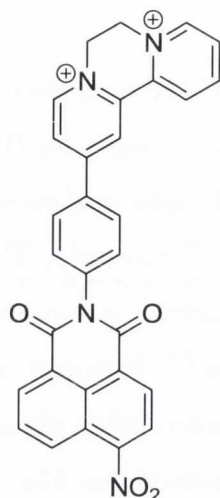
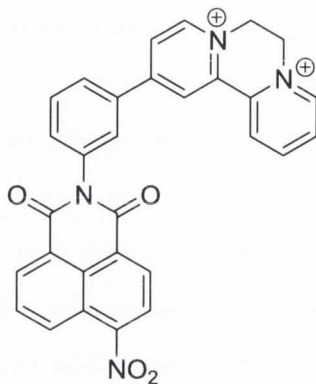
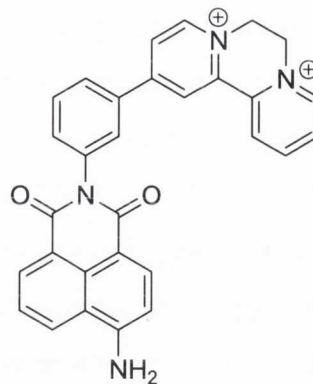
An Organic pdppz Derivative as a DNA Probe and Photocleavage Agent

3.1 Introduction

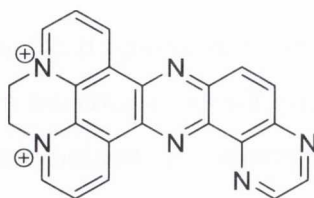
As mentioned in Chapter 2, dppz complexes of ruthenium have been the focus of much research due to their fascinating photophysical properties. Excellent affinity for DNA has been observed for many of these systems, signalled by significant changes in their spectroscopic properties, particularly the Ru(II) based MLCT emission.^{43,45,158} Recently, Thomas *et al.* reported on the photophysical and DNA binding properties of a cationic water-soluble organic derivative of dppz, ethylene-bipyridyldiylm-phenazine, **62a**.²⁰⁰ Previous studies have revealed that, despite structural resemblance to the well-known weed killer, diquat, **63**, **62a** is surprisingly non-toxic; at least to plants.²⁰¹ Moreover, it was observed that **62a** showed good water solubility and distinctive photophysical properties that allowed the nature of the interaction of **62a** with DNA to be probed as an isolated dppz unit, at relatively long wavelengths, without the need for the Ru(II) centre. It was found that the DNA binding affinity of **62a**, while comparable to that of many metal complexes of dppz,³³ was slightly lower than that of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ ¹⁷⁸ and it was thought that this may be explained by a consideration of the additional contribution of groove binding by the $[\text{Ru}(\text{phen})_2]^{2+}$ moiety for the metal complex.²⁰⁰

**62a****62b****63**

A subsequent synthetic, biophysical and photophysical study revealed that cationic derivative **62b**, exhibited an enhanced affinity for DNA with a binding constant calculated to be comparable to that of mononuclear $[\text{Ru}(\text{II})(\text{dppz})]$ complexes.²⁰² Furthermore, **62b** showed a two orders of magnitude preference for GC sequences over AT tracks, at the same time as showing mixed luminescence responses; a luminescence increase in the presence of $[\text{poly}(\text{dAdT})]_2$ and a luminescence decrease in the presence of $[\text{poly}(\text{dGdC})]_2$. This behaviour was rationalised by luminescence quenching due to redox processes occurring between the internal charge transfer (ICT) excited state of **62a** and *guanine* sites with the ICT state of **62a** deemed to be insufficiently oxidising to be quenched by *adenine* sites. This was confirmed by cyclic voltammetry results which revealed that the reduction potential of **62a** was indeed lower than that of *adenine*.²⁰²

**64****65****66**

Within the Gunnlaugsson research group a family of rigid 1,8 naphthalimide-diquat conjugates **64** – **66** have recently been synthesised and studied in the presence of DNA.¹⁵⁴ Using various techniques such as UV/Vis absorption and fluorescence titrations, T_m measurements as well as CD and isothermal calorimeter these compounds have all been shown to bind DNA with high affinity with binding constants, $K \approx 10^6 - 10^7 \text{ M}^{-1}$. These systems also had their emission efficiently quenched in the presence of DNA with the *meta* substituted amino species **66** showing the most efficient interaction with the duplex. This was proposed to be most likely due to a more cooperative mode of binding between the diquat and the 4-amino or 4-nitro-1,8 naphthalimide components. The study of these conjugates demonstrated that a charged organic unit can perform the same function as a Ru(II) complex in terms of DNA binding, in providing both electrostatic attraction to the helix and associating in the grooves.

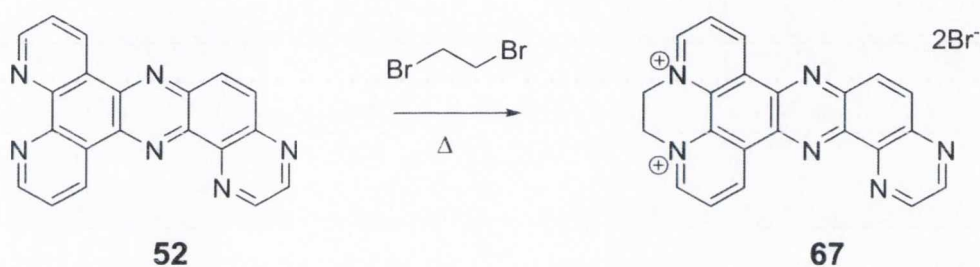
**67**

Taking into consideration the promising results obtained for the family of complexes **53** – **55** described in Chapter 2 it was decided to undertake a similar study utilising a cationic derivative of pdppz, **67**. This Chapter will deal with the synthesis and characterisation of **67** in addition to an evaluation of its photophysical properties and interaction with DNA, [poly(dAdT)]₂ and [poly(dGdC)]₂ under various experimental conditions. These interactions will, again, be evaluated using a range of spectroscopic and biophysical techniques before the excited state reactivity of **67** with DNA will be considered using agarose gel electrophoresis.

The final section of this Chapter will detail some preliminary biological data obtained in order to evaluate the potential of **67** to initiate light dependent cellular toxicity.

3.2 Synthesis and Characterisation of **67**

The synthesis of **67** was achieved by quaternerisation of pdppz as outlined in Scheme 3.1. This involved refluxing **52**, in dibromoethane, in which it was sparingly soluble at room temperature, but dissolved fully as the reaction reached reflux temperature. A precipitate formed as the reaction progressed which was isolated by suction filtration after 48 hours. This precipitate was redissolved in MeOH and reprecipitated by slow diffusion of Et₂O before isolation by suction filtration to give the product as a brown solid in 78% yield. The chloride salt of the cationic derivative was formed by dissolution in methanol followed by stirring with Amberlite ion exchange resin (Cl⁻ form) for 1 hr.



Scheme 3.1: The synthesis of cationic pdppz derivative **67**

Compound **67** was characterised by ¹H NMR, ¹³C NMR, elemental analysis, HRMS, melting point analysis and IR spectroscopy. The ¹H NMR spectrum (600 MHz, MeOD) of **67** is shown in Figure 3.1, with the assignment of the peaks being achieved by H-H and C-H COSY analysis. Product formation was clear from the appearance of a single resonance in the ¹H NMR spectrum at approximately 5.85 ppm, integrating for 4 protons, corresponding to the two CH₂'s of the bridge. Successful formation of **67** was also evident from accurate mass spectrometry, where it displayed a peak at 393.1446, corresponding to its mass having lost a Cl⁻ ion and gained an MeO⁻ ion. Synthesised as its chloride salt, **67** was water-soluble, and its photophysical properties were investigated in 10 mM phosphate-buffered aqueous solutions at pH 7.4.

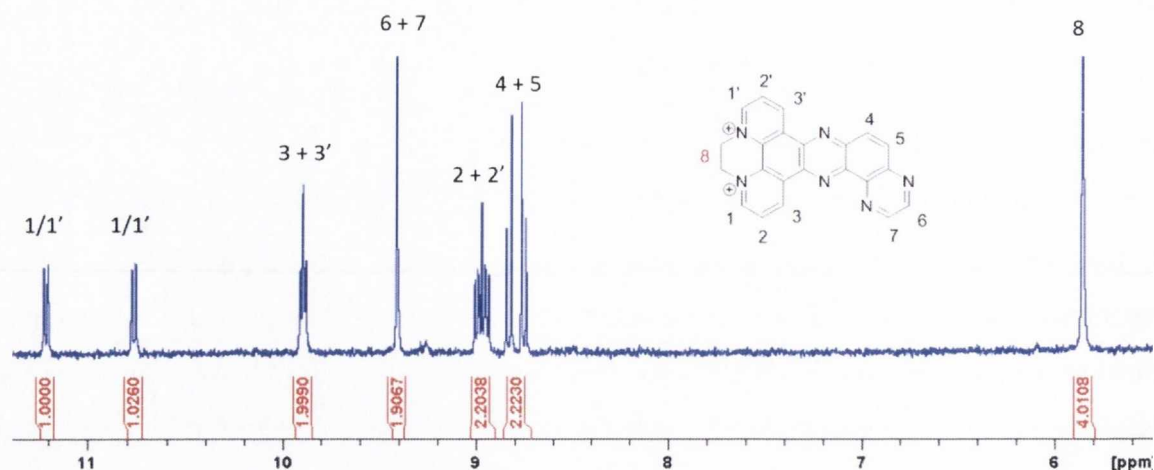


Figure 3.1: The ^1H NMR spectrum of **67** (600 MHz, MeOD)

3.3 Photophysical Characterisation of **67**

The photophysical behaviour of **67** was evaluated in aqueous solution to make it possible to better understand its spectroscopic interaction with DNA. The UV/Vis absorption, emission and excitation spectra of **67** in 10 mM phosphate-buffer solution are shown in Figure 3.2. The absorption spectrum of **67** shows absorbance maxima at *ca.* 285 nm, 310 nm, 370 nm, 390 nm along with a broad shoulder centred at 460 nm. The band at 285 nm is characteristic of $\pi-\pi^*$ transitions of the phen portion of the compound while the band at 310 nm is attributed to $\pi-\pi^*$ transitions within the phenazine portion. The bands at 370 nm and 390 nm are attributed to $n-\pi^*$ transitions within the phenazine part of the ligand and are similar to those seen for the ligand **52**.

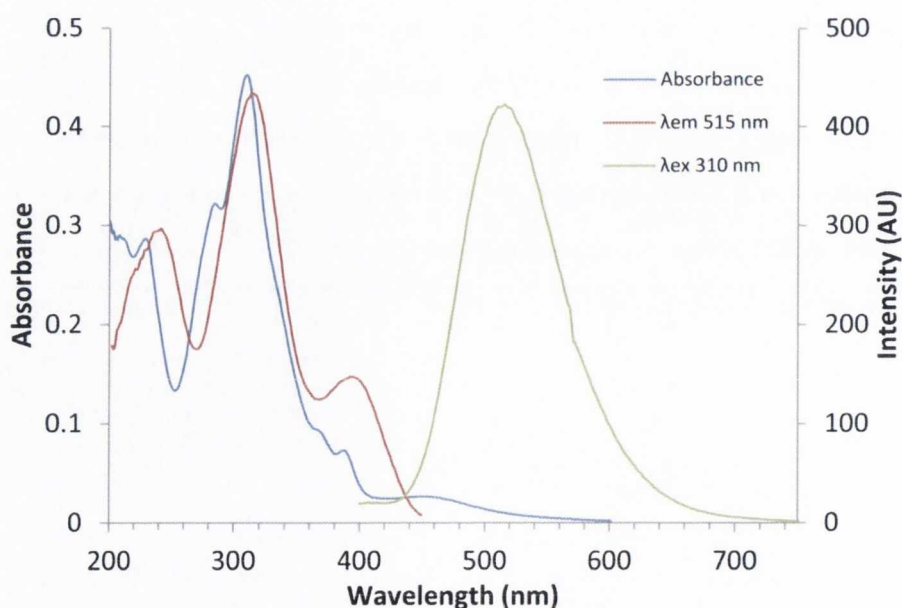


Figure 3.2: The UV/Visible, excitation and emission spectra of **67** (10 μM) in 10 mM phosphate buffer solution at pH 7.4

A summary of the absorption properties of **67** as well as the respective molar absorptivities of each band are given in Table 3.1.

Table 3.1: Absorption properties of **67** in 10 mM phosphate buffer, at pH 7.4

Compound	$\lambda_{\max}$ (nm) [ϵ (M ⁻¹ cm ⁻¹)] in phosphate buffer ($\pm 10\%$)		
	π - π^* phen	π - π^* phenazine	n- π^* phenazine
67	285 [31400]	310 [44100]	390 [6900]

Upon excitation into each band, **67** gave rise to intense emission with $\lambda_{\max}$ at 515 nm exhibiting a red shift of 115 nm from the parent pdppz ligand which showed emission with $\lambda_{\max}$ at 400 nm. This observation is consistent with previous density functional theory (DFT) calculations on the structurally related compound **62a**, that attribute the emission to an ICT state.²⁰⁰ These calculations reveal that the ICT involves a HOMO isolated on the phenazine unit and a LUMO centred on the diquat region of the cation. A Φ_F value of 0.053 was calculated using quinine sulphate in 0.1 N H₂SO₄ with a quantum yield of 0.542 as a reference.²⁰³ Similarly, measurement of the emission of **67** at 515 nm yielded a fluorescence excitation spectrum structurally identical to its absorption spectrum.

Due to its extended aromatic surface, in concert with its cationic character, it was expected that that **67** would be an ideal candidate as a DNA intercalator. Moreover, with its cationic nature furnishing **67** with good aqueous solubility and having demonstrated its advantageous photophysical properties, **67** shows potential for use as a spectroscopic probe for DNA without the need for a Ru(II) centre. As seen in Chapter 2, modulations of these photophysical properties are expected upon interaction with DNA and as a result spectroscopic titrations were carried out to investigate the nature and binding affinity of **67** with DNA. The obtained results are presented and discussed in the following sections.

3.4 DNA Binding Interactions

3.4.1 UV/Vis Absorption Titrations of **67** with DNA

The interaction of **67** with DNA was firstly examined using UV/Vis absorption spectroscopy. The titrations were carried out in a manner similar to that described in Chapter 2 by addition of small aliquots of stDNA to a 10 mM phosphate buffer solution of **67** at pH 7.4, until a plateau in absorbance was reached. Changes in the π - π^* band at 310 nm were monitored spectroscopically and all titrations were repeated a number of times to ensure reproducibility.

The changes in the ground state of **67** are shown in Figure 3.3. The titration of **67** with stDNA resulted in significant changes over the entire absorbance wavelength range with a 51% decrease in absorbance at 310 nm being observed. Concomitantly, decreases were also seen at 370 nm, 390 nm and in the long wavelength shoulder at 460 nm.

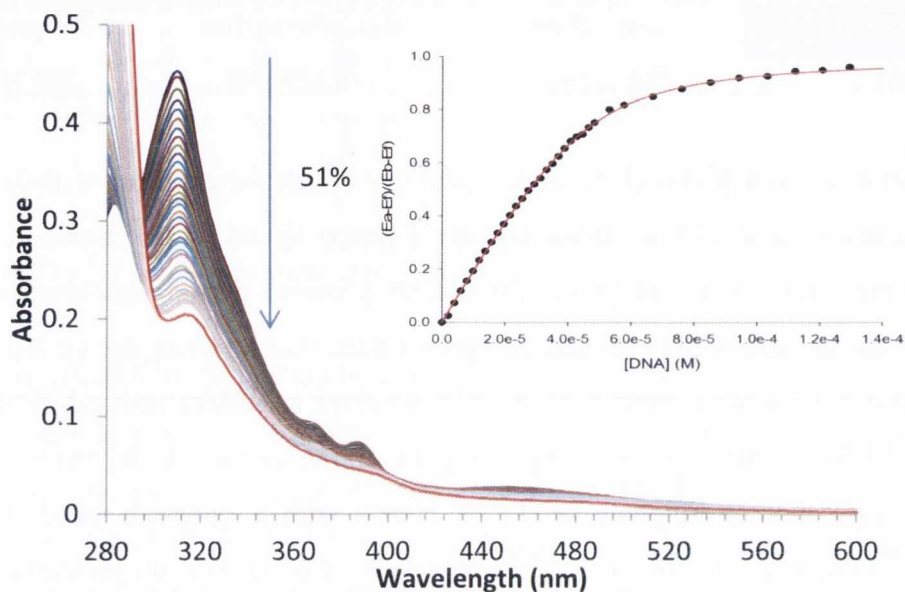


Figure 3.3: Changes in the UV/Visible absorption spectrum of **67** (10 μM) with increasing additions of stDNA (0-170 μM) in 10 mM phosphate buffer, pH 7.4. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[DNA](M^1, P)$ using data with a P/D between 0-20 and the best fit of the data (---) using the Bard Eqn.

These results are similar to those obtained previously for the addition of DNA to **56**, the observed changes being attributed to strong interactions between the cation and the helical structure of DNA in the ground state.²⁰⁰ The large degree of hypochromicity seen at 310 nm could indicate intercalation of **67** between the stacked bases, as expected with such an extended aromatic structure. In order to quantify the interaction of **67** with DNA, the data from the UV/Visible absorption titration was analysed to obtain a binding affinity for DNA. The intrinsic binding constant, K_b , and binding site size, n , were determined using the model of Bard *et al.* as described in Chapter 2. The results obtained confirmed that **67** had high affinity for DNA, showing strong binding with $K_b = 6.5 \times 10^5 (\pm 0.5)$ with a binding site size, $n = 2.60 (\pm 0.03)$. This value is comparable to that seen for many transition metal based intercalators, and is of similar magnitude to that reported by Thomas *et al.* for **62a** and **62b**.^{200,202} This strong association of **67** is most likely due to the bifunctional nature of this structure; the positively charged diquat portion providing initial electrostatic attraction to the DNA helix which places the extended aromatic surface in an ideal environment for high affinity interaction through intercalation. In order to further confirm this interaction, changes

were also monitored in the excited state properties of **67** and these results will be presented in the following section.

3.4.2 Fluorescence Emission Titrations of **67** with DNA

Given the excited state character of **67** it was anticipated that addition of DNA would lead to a modulation of the emission response, thus providing more information on the processes occurring upon binding to DNA. The subsequent titrations were carried out in parallel with the UV/Visible titrations as described in Chapter 2.

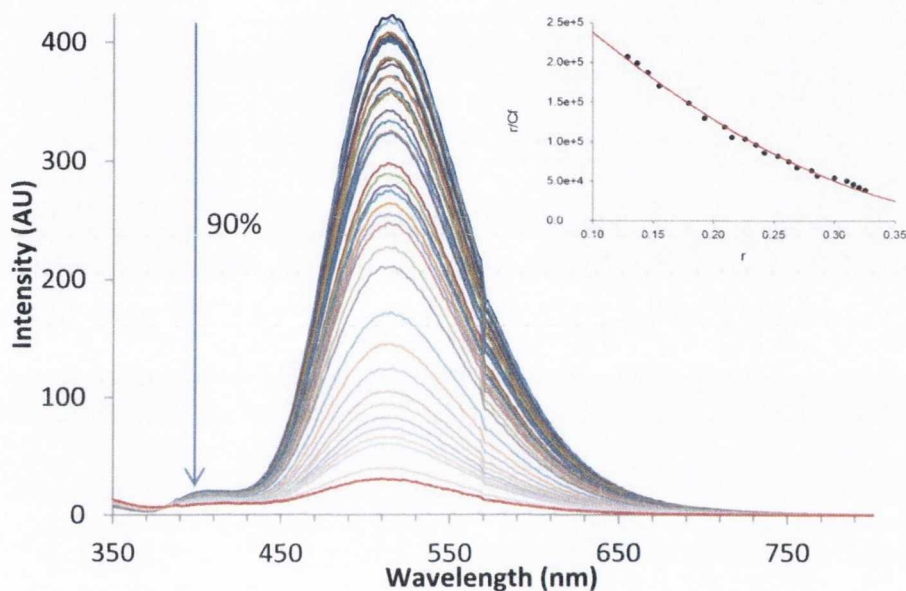


Figure 3.4: Changes in the fluorescence emission spectrum of **67** ($10\mu\text{M}$) (λ_{ex} 310 nm) with increasing concentration of stDNA ($0\text{--}170\mu\text{M}$). Inset: Plot of r/C_f vs. r and the best fit of the data (---) using the McGhee Von Hippel equation.

The ICT based emission of **67** in aqueous solution was shown to have its excited state effectively quenched by *ca.* 90% upon addition of stDNA (P/D $0 \rightarrow 30$) with the main changes occurring between P/D $0 \rightarrow 15$ (Figure 3.4). Compounds containing diquat functionality are very well known oxidizing agents, and therefore the observed emission quenching is likely due to electron transfer from the DNA bases.²⁰⁴ This quenching has also been observed for both **62a** and **62b** above and was also attributed to redox processes in both cases.^{200,202} Furthermore, quenching of this magnitude implies close association of **67** with the nucleotides, in a manner that allows for electron transfer from the bases to **67** to take place. The observation of 90% fluorescence quenching may indicate that **67** is sufficiently reactive to oxidise both *adenine* and *guanine* sites; behaviour previously seen for **62a**, but not for **62b** which was only sufficiently oxidising to react with *guanine* sites. Studies in the presence of

homopolymer polynucleotides [poly(dAdT)]₂ and [poly(dGdC)]₂ described in Section 3.4.3 will attempt to further support this hypothesis.

In order to further confirm the association of **67** with DNA, binding parameters were also estimated by fitting the changes in the excited state titrations to the McGhee–von Hippel model, equation (6).²⁰⁵ The concentration of the bound compound C_b , and free compound C_f , at a given concentration were firstly determined from equations (4) and (5):

$$C_b = \frac{I_f - I}{I_f - I_b} \cdot C \quad (4)$$

$$C_f = C - C_b \quad (5)$$

Here I_f and I_b are the fluorescence intensities of the free and bound compound, I is the fluorescence response and C is the dye concentration. Data analysis was then carried out as described in equation (6), where, $r = C_b/[DNA]$, ($[DNA]$ is the concentration of DNA expressed in base pairs). Analysis was achieved using the plot of r/C_f versus r and the curve was fit to equation (6) using Sigmaplot 11.0.

$$\frac{r}{C_f} = K_b(1 - nr) \left(\frac{1 - nr}{1 - (n-1)r} \right)^{n-1} \quad (6)$$

The results obtained confirmed that **67** retained high affinity for DNA as calculated from the excited state titrations. In the presence of stDNA **67** exhibited a $K_b = 3.55 \times 10^5 (\pm 0.2)$ and $n = 4.05 (\pm 0.05)$ showing good agreement with the values obtained from the ground state titrations. Moreover, the results are also in agreement with the values obtained for **62a** and **62b** which were calculated as being 1.8×10^5 with $n = 3.10$ and 1.33×10^6 with $n = 3.15$ respectively.

For **67**, electrostatic binding to DNA was expected to play a significant role in the binding process due to its cationic nature. To investigate this hypothesis and provide further information on the binding mode, a salt back titration was carried out, in which **67** bound to DNA was displaced upon titration with NaCl. The profile resulting from this titration is shown in Figure 3.5.

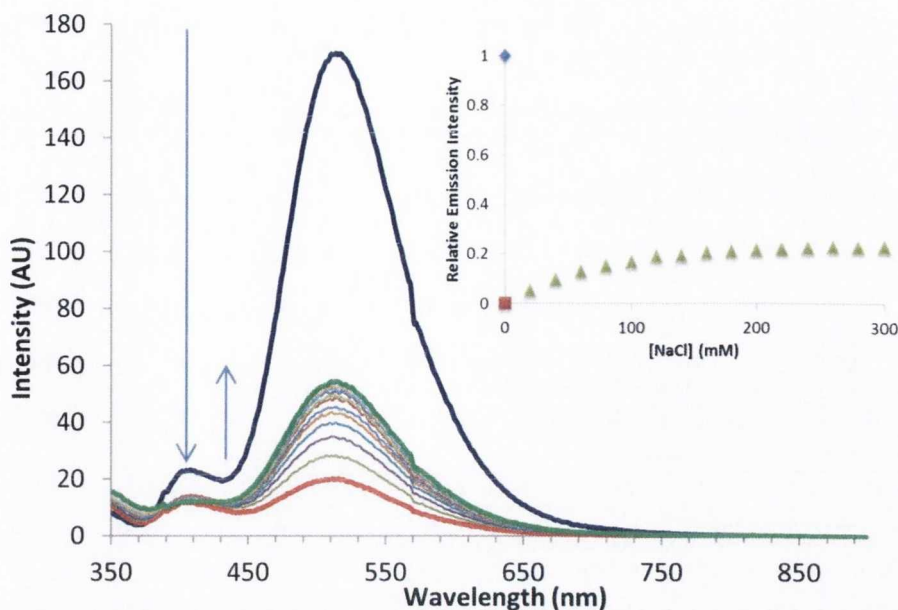


Figure 3.5: Fluorescence emission NaCl back titration of **67**. Inset: Emission of unbound compound (◆), fully bound compound (■) and bound compound at 515 nm in the presence of increasing concentration of NaCl(▲)

For **67** the fluorescence emission is seen to be only marginally affected by increasing NaCl concentration, gradually increasing towards that of the free compound, before reaching a plateau at *ca.* 180 mM NaCl, with about a 20% increase towards that of the free species. No further changes occurred with the addition of up to 300 mM NaCl, indicating that the majority of the dication remains bound to DNA at high ionic strength. This study indicates that non-electrostatic modes of interaction are contributing to the binding of **67** to DNA, however, it is likely that the ability of **67** to stack between bases governs its overall association with DNA.

In summary, from the spectroscopic titrations of **67** performed thus far, it is clear that significant changes have been seen in both the ground and excited state titrations. Such large and well defined hypochromicities are indicative of a strong association with the DNA helix and are likely to be as a result of intercalation. Binding constants calculated from these titrations were also in agreement, giving $K_b \approx 10^5 \text{ M}^{-1}$ from both UV/Vis and fluorescence data. The large fluorescence decrease observed upon titration with DNA has been attributed to redox processes occurring between the excited state of **67** and the nucleobases; a phenomenon observed for structurally related species.^{200,202} Moreover, from the NaCl dependence studies, it is clear that **67** remains bound to DNA even at high concentrations of NaCl. This evidence points to the conclusion that electrostatic binding of the cationic portion does play a role in determining the overall binding affinity, however, given the fact that **67** remains bound at high ionic strength, electrostatic interactions are not likely to be the predominant contributor to its overall affinity. Subsequent studies in the presence of homopolymer polynucleotides [poly(dAdT)]₂ and [poly(dGdC)]₂ will attempt to reveal any sequence specific effects for **67**.

3.4.3 UV/Vis Absorption Titrations of **67** with Synthetic Homopolymer Polynucleotides

Studies in the presence of both $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ were also carried out with **67**, with the aim of establishing any sequence selectivity in its binding. This was initially examined using UV/Vis absorption spectroscopy in a manner similar to that employed in Chapter 2. The changes in the ground state of **67** in the presence of $[\text{poly}(\text{dAdT})]_2$ are shown in Figure 3.6 with that of **67** in the presence of $[\text{poly}(\text{dGdC})]_2$ shown in Appendix 3.

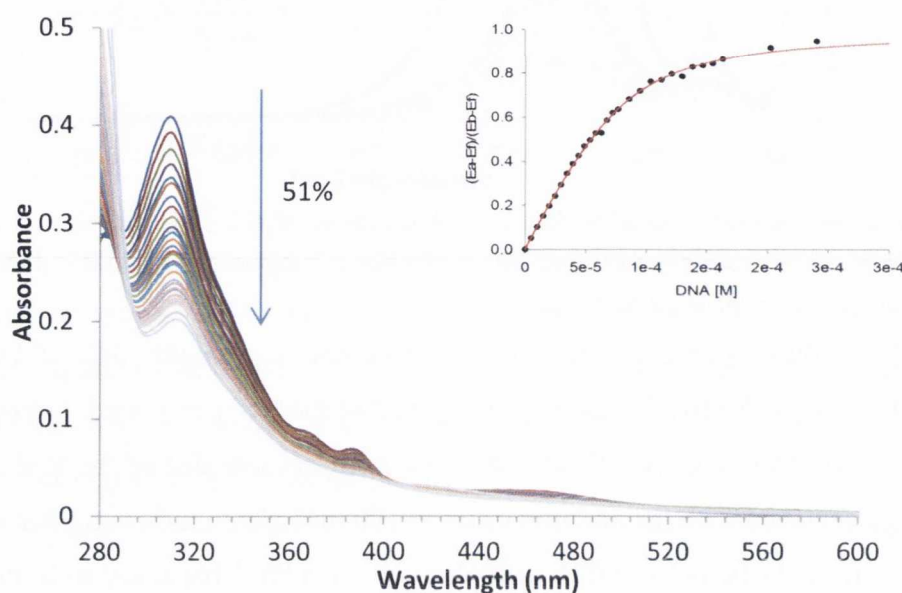


Figure 3.6: Changes in the UV/Visible spectrum of **67** (10 μM) with increasing additions of $[\text{poly}(\text{dAdT})]_2$ (0–200 μM) in 10 mM phosphate buffer, pH 7.4. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}] (M^1, P)$ using data with a P/D between 0–20 and the best fit of the data (---) using the Bard Equation.

The titration of **67** with $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ resulted in significant changes to its absorption spectrum. With $[\text{poly}(\text{dAdT})]_2$, **67** exhibited a 51% decrease in its absorbance at 310 nm with concomitant decreases in all of its other absorbance bands. Similar changes were observed for $[\text{poly}(\text{dGdC})]_2$ with a 54% decrease at 310 nm. The changes in the UV/Vis ground state titrations show some degree of specificity for $[\text{poly}(\text{dGdC})]_2$ over $[\text{poly}(\text{dAdT})]_2$. The differences are exhibited in the degree of hypochromism resulting from titration with the different polynucleotides. The comparison curve of **67**, which exemplifies the changes in the $\pi - \pi^*$ absorption at 310 nm demonstrates this preference for $[\text{poly}(\text{dGdC})]_2$ (Figure 3.7). Furthermore, the values of K_b obtained from the ground state titrations of both polynucleotides confirm that **67** shows a preference for $[\text{poly}(\text{dGdC})]_2$ with $K_b = 1.35 \times 10^7 (\pm 0.6)$ and $n = 1.67 (\pm 0.05)$ in comparison to $K_b = 4.85 \times 10^5 (\pm 0.33)$ and $n = 3.91 (\pm 0.01)$ for

[poly(dAdT)]₂. The values obtained for [poly(dAdT)]₂ and [poly(dGdC)]₂ with **67** are presented and compared with those calculated for stDNA in Table 3.2.

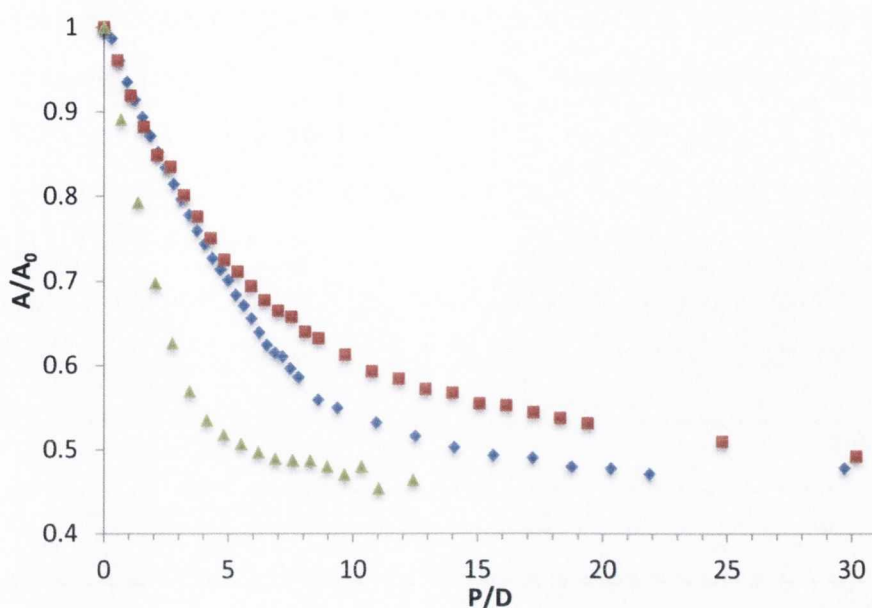


Figure 3.7: Relative changes in the absorbance of **67** (10 μ M) with increasing concentration of stDNA (0–172 μ M) ($\blacklozenge$), [Poly(dAdT)]₂ (0–175 μ M) ($\blacksquare$), and [Poly(dGdC)]₂ (0–63 μ M) ($\blacktriangle$) in 10 mM phosphate buffer.

Table 3.2: DNA binding parameters for **67** from fits to absorbance data in the presence of stDNA, [poly(dAdT)]₂ and [poly(dGdC)]₂

Complex	λ (pdppz) Hypo- chromism	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
67 + stDNA	52%	6.5×10^5 (± 0.5)	2.6 (± 0.03)	0.99
67 + [Poly(dAdT)]₂	51%	4.85×10^5 (± 0.3)	3.91 (± 0.01)	0.99
67 + [Poly(dGdC)]₂	54%	1.35×10^7 (± 0.6)	1.67 (± 0.05)	0.99

It has previously been claimed that the dppz ligand is structurally similar to the well-known minor groove intercalator actinomycin d.²⁰⁶ Given this structural similarity and the low steric demand of **67**, coupled with the proposed intercalative nature of binding, it seems highly likely that this compound could also bind to DNA from the narrower minor groove. Furthermore, the higher affinity of **67** for [poly(dGdC)]₂ is also consistent with this theory as it is known that actinomycin d binds preferentially to specific GC sequences.²⁵ These results are also in agreement with the studies of Thomas *et al.* who saw a preference for [poly(dGdC)]₂ over [poly(dAdT)]₂ for both **62a** and **62b**.^{200,202}

3.4.4 Fluorescence Emission Titrations of **67** with Synthetic Homopolymer Polynucleotides

Similar to its UV/Visible behaviour discussed above, **67** displayed marked differences in its fluorescence spectra upon binding to AT and GC rich DNA sequences. The changes in the excited state of **67** in the presence of [poly(dAdT)]₂ are shown in Figure 3.8 with that of **67** in the presence of [poly(dGdC)]₂ shown in Appendix 3. With [poly(dAdT)]₂, **67** exhibited a 71% quenching of its excited state over the entire range of the titration (PD 0 → 30). More dramatic changes were observed for [poly(dGdC)]₂ with a 95% decrease at 515 nm, primarily occurring between PD 0 → 5.

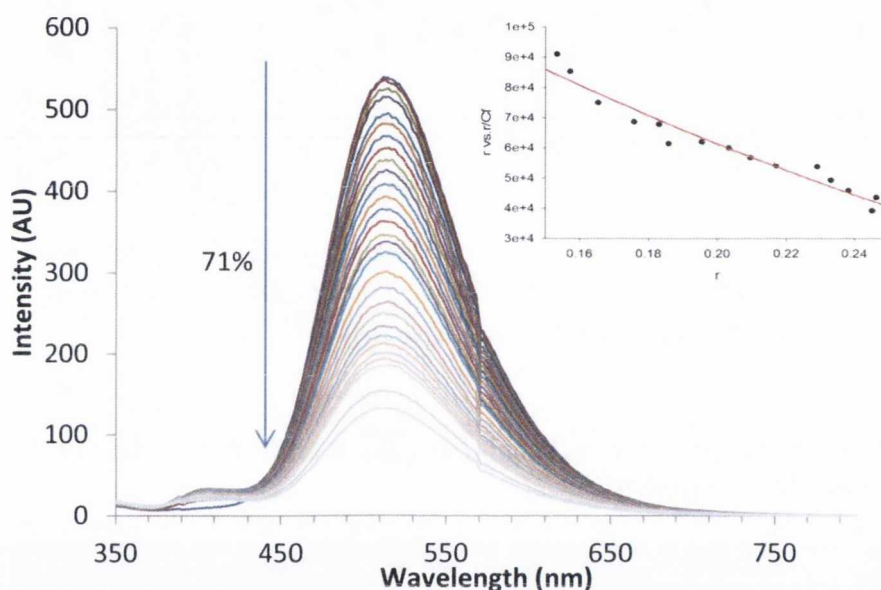


Figure 3.8: Changes in the fluorescence emission spectrum of **67** (10 μM) (λ_{ex} 310 nm) with increasing additions of [poly(dAdT)]₂ (0–200 μM) in 10 mM phosphate buffer, pH 7.4. Inset: Plot of r/C_f vs. r and the best fit of the data (---) using the McGhee Von Hippel equation.

It is clear from the above changes in luminescence that addition of [poly(dGdC)]₂ to aqueous buffer solutions of **67** resulted in changes that mirror those observed in analogous experiments with **56**, with ICT emission being fully quenched.²⁰⁰ This supports the observation that the excited ICT state of **67** is sufficiently oxidising to be quenched by redox processes involving *guanine* sites. Similarly, experiments involving [poly(dAdT)]₂ also result in ICT emission quenching, however, this process seemed to occur with less efficiency with the quenching process achieving just a 71% decrease at a P/D ratio of 30. These results suggest that, although occurring with reduced efficiency, cation **67** is also sufficiently oxidising to be quenched by *adenine* sites. The differences between the quenching efficiencies are exhibited in the comparison curve of **67** which summarises the changes observed at 515 nm and demonstrates the increase in quenching efficiency observed for [poly(dGdC)]₂ (Figure 3.9).

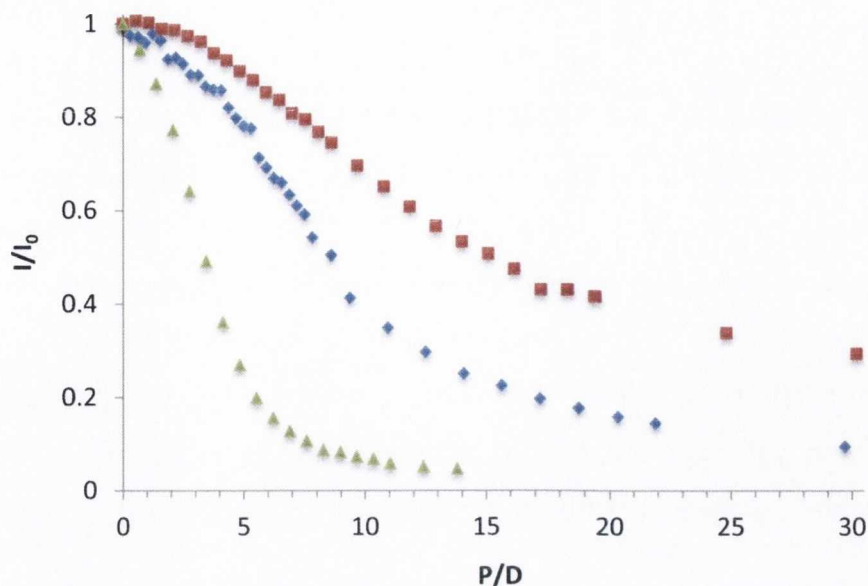


Figure 3.9: Relative changes in the fluorescence emission of **67** (10 μM) at 515 nm with increasing concentration of stDNA (0–172 μM) ($\blacklozenge$), [Poly(dAdT)]₂ (0–175 μM) ($\blacksquare$), and [Poly(dGdC)]₂ (0–63 μM) ($\blacktriangle$) in 10 mM phosphate buffer.

Using these DNA induced changes in the luminescence of **67**, it was possible estimate the binding variations between the interaction of **67** with [poly(dAdT)]₂ and [poly(dGdC)]₂ compared them with those calculated for stDNA. From Table 3.3 it can be seen that [poly(dGdC)]₂ exhibited a $K_b = 1.84 \times 10^7 (\pm 0.1)$ and $n = 1.35 (\pm 0.01)$ in comparison to $K_b = 5.60 \times 10^5 (\pm 0.27)$ and $n = 3.3 (\pm 0.05)$ for [poly(dAdT)]₂. This comparison of binding affinities revealed pronounced sequence selectivity as had been observed in the ground state titrations previously described. In contrast to **62a**, which shows only a slight preference for GC sites,²⁰⁰ **67** displays an almost two orders of magnitude preference for GC regions over AT steps. Many classical intercalators, such as EtBr and actinomycin D,²⁰⁷ can show selectivity for GC sites, although this preference is only around a single order of magnitude for EtBr.²³

Table 3.3: DNA binding parameters for **67** from fits to emission data in the presence of stDNA, [poly(dAdT)]₂ and [poly(dGdC)]₂

Complex	$\lambda(\text{ICT})$ Hypo- chromism	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
67 + stDNA	90%	$3.55 \times 10^5 (\pm 0.2)$	$4.05 (\pm 0.05)$	0.97
67 + [Poly(dAdT)] ₂	71%	$5.60 \times 10^5 (\pm 0.3)$	$3.3 (\pm 0.05)$	0.99
67 + [Poly(dGdC)] ₂	95%	$1.84 \times 10^7 (\pm 0.1)$	$1.35 (\pm 0.01)$	0.99

It is clear from the above studies that compound **67** displays significant emission responses upon binding to DNA, with 90% quenching being observed. The binding affinity determined for this system from UV/Visible absorption and emission titrations, suggest that **67** is closely associated with the DNA structure, in a manner that does not rely solely on electrostatic association. This is proposed to be due to binding of the planar extended structure of pdppz *via* intercalation, while the diquat portion remains externally bound to the phosphate backbone. This results in high DNA binding affinity, in addition to pronounced excited state quenching; most likely as a result of redox processes occurring between the excited state of **67** and the DNA nucleobases. Furthermore, studies in the presence of [poly(dAdT)]₂ and [poly(dGdC)]₂ revealed that **62** binds more strongly to [poly(dGdC)]₂, with a two orders of magnitude preference over [poly(dAdT)]₂. Binding constants derived from both UV/Vis absorption and fluorescence emission data confirmed this. The following sections of this Chapter will attempt to further quantify the binding affinity and mode of binding of **67** for DNA by utilising various other spectroscopic and biophysical techniques.

3.4.5 Ethidium Bromide Displacement Assays of **67**

As discussed in Chapter 2 the method of EtBr displacement assays can be used to evaluate the binding efficiency of DNA targeting molecules by its displacement by more competitive DNA binders. The addition of **67** to a mixture of EtBr and DNA was found to displace 50% EtBr at molar ratios less than one, showing that it displayed equal or greater affinity for DNA than EtBr. This confirms the high affinity of **67** for DNA, which correlates well with the results obtained from the UV/Visible absorption and emission titrations discussed in previous sections. As can be seen in Figure 3.10, the addition of **67** caused the emission intensity of intercalated EtBr to decrease dramatically exemplifying the ability of **67** to displace it from the DNA structure.

Furthermore, a binding constant derived from the displacement profile of **67** with DNA gave an apparent constant $K_{app} = K_{EB}[EB]/[67]_{50\%FI} = 1.26 \times 10^7$. The value obtained is considerably removed from that determined by UV/Visible and fluorescence data, being over an order of magnitude higher. It has already been indicated that the UV/Visible and fluorescence changes displayed by **67** are due primarily to interaction of the planar pdppz moiety which most likely involves an intercalative interaction with DNA. However, at high binding ratios a considerable degree of groove association of the diquat portion of the molecule may be occurring. In this way the varying modes of interaction may effectively displace DNA bound EtBr and thus the apparent affinity from the displacement assay is

higher. In any case, the results presented here represent further confirmation of the high degree of binding of **67** to DNA.

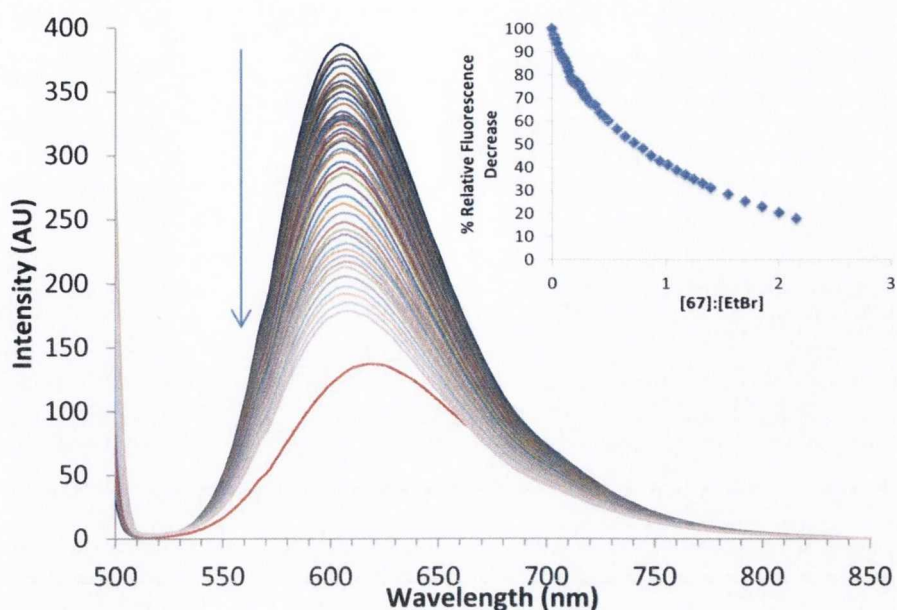


Figure 3.10: Emission changes at 610 nm for DNA bound EtBr ($\lambda_{\text{ex}} = 545 \text{ nm}$) with increasing concentration of **67** in 10 mM phosphate buffer at pH 7.4. Inset. Relative decrease in fluorescence of DNA bound EtBr upon addition of **67**.

3.4.6 Thermal Denaturation Studies with **67**

Thermal denaturation studies were also carried out to further investigate the interaction of **67** with stDNA (150 μM) in 10 mM phosphate buffer (pH 7.4) as described in Chapter 2. The melting curves observed in the presence of **67** are shown in Figure 3.11.

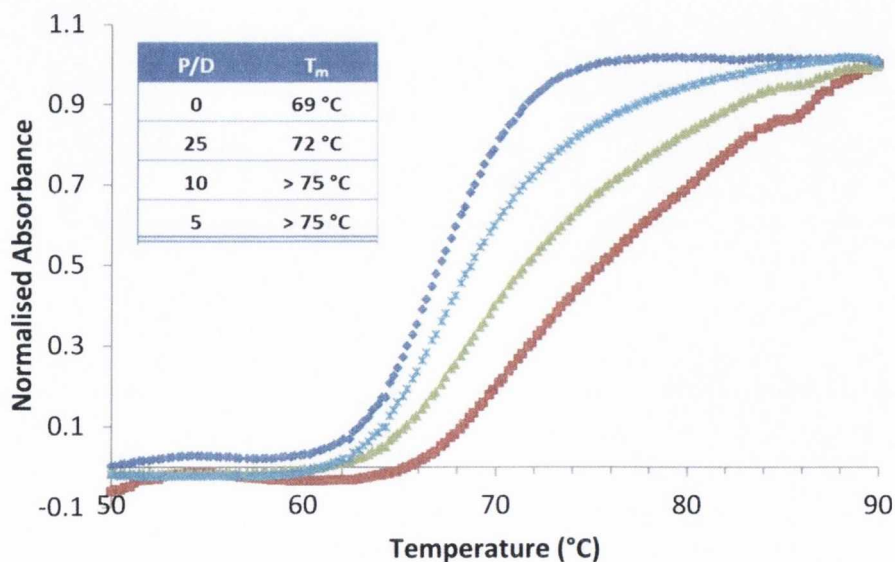


Figure 3.11: Thermal denaturation curves of stDNA (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence ($\diamond$) and presence of **67** at $P/D = 25$ ($*$) $P/D = 10$ ($\triangle$) and $P/D = 5$ ($\blacksquare$). Inset: The T_m values from first derivative plots of the melting curve.

In the absence of any DNA binding species, the T_m value determined for stDNA was 69 °C. This temperature was found to be modulated significantly in the presence of varying ratios of **67**; reflecting the stabilisation of the helix structure by **67**. At P/D = 25 the T_m value was shifted by 3 °C to 72 °C, while at P/D = 10 and 5 the melting transition had not gone to completion at 90 °C and consequently an accurate T_m value could be not obtained. The increase in T_m at P/D = 5 was especially large, again, demonstrating the high affinity of **67** for DNA.

3.4.7 Linear and Circular Dichroism Studies with **67**

Circular and Linear dichroism titrations were also carried out on **67**, where the concentration of DNA was kept constant, and that of the compound was varied. Very minor changes were seen upon addition of **67** to DNA at longer wavelengths of the CD spectra (P/D = 20 $\rightarrow$ 2.5) (see Appendix 3), with more obvious changes occurring in the DNA absorption region. These observations imply some sort of association of **67** with the DNA helix, however such results are unambiguous.

In contrast, LD studies revealed a similar behaviour to that seen for complexes **53** – **55** described in Chapter 2. Due to the existence of an LD signal in the region outside of the DNA base absorption it can be concluded immediately that **67** interacts with DNA and becomes orientated (Figure 3.12). Moreover, the formation of a strong negative band at 318 nm, resulting from the π - π^* transition of the phenazine part of the ligand, strongly supports an intercalative mode of DNA interaction for **67**. Furthermore the red-shift from 310 nm to 318 nm is typical for stacking interactions between the base pairs.^{43,190,191} Similarly, a large negative change in the DNA absorption band at 260 nm suggests that the alignment of DNA in flow is enhanced due to an increase in the flexibility of the helix. This behaviour is likely to be brought about by ‘kinks’ caused by an intercalating species, in this case **67**.

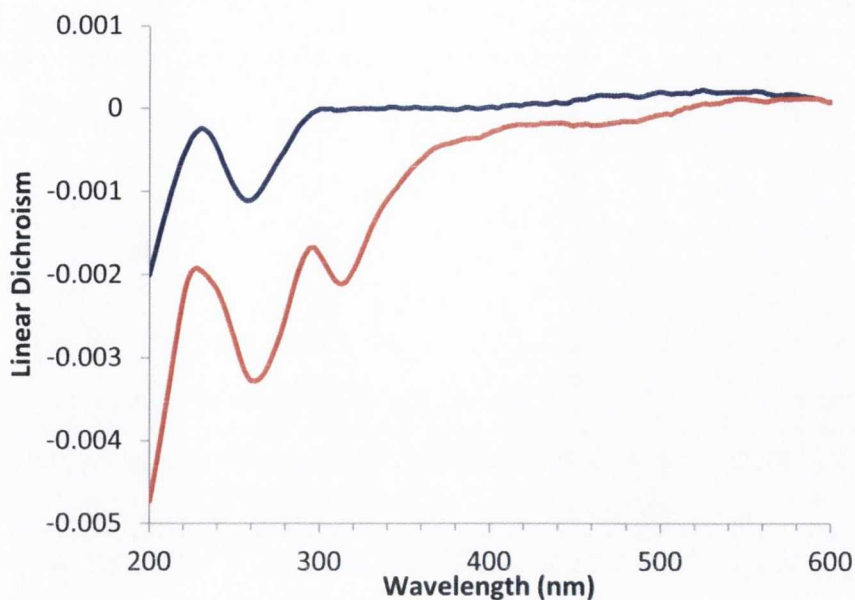


Figure 3.12: The linear dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **67** ($P/D = 2.5$).

The cationic species **67** described in this Chapter has not displayed significant CD changes, however, the changes in the LD spectra have been dramatic and have provided strong evidence to assign the nature of its interaction with DNA as intercalative. Moreover, coupled to the evidence already described in the previous sections where the strong binding affinity of **67** with DNA has been demonstrated, a strong body of data now exists which supports the potential of **67** as a DNA probe.

3.4.8 Summary of DNA Binding Studies of **67**

The DNA binding studies of **67** in aqueous solution have been explored in this Chapter thus far. The evaluations have been carried out using stDNA and the homopolymers, [poly(dA-dT)]₂ and [poly(dG-dC)]₂, using UV/Vis and fluorescence spectroscopy. EtBr displacement assays, T_m experiments, CD studies and LD studies were also undertaken to further aid in elucidating the specific mode of binding of **67** to DNA.

The ground state and the excited state interactions of **67** with stDNA revealed distinct spectral changes giving binding constants calculated from the Bard model ranging from $10^5 - 10^7 \text{ M}^{-1}$ for the various DNA sequences. Furthermore, **67** exhibited an emission decrease in the presence of the different types of DNA and this we attributed to a PET process occurring between **67** and the nucleobases. An NaCl back titration in the presence of DNA showed that high salt concentration was seen to only marginally affect the binding strength of **67** with the cationic species seen to remain bound even at high ionic strength (NaCl conc. > 300 mM).

Studies in the presence of the homopolymers [poly(dA-dT)]₂ and [poly(dG-dC)]₂ revealed sequence specific recognition of **67** for [poly(dG-dC)]₂ in both the ground and excited state data. This type of behaviour was attributed to **67** intercalating into DNA from the minor groove; a type of behaviour associated with other classical intercalating species such as actinomycin d.

An EtBr displacement assay also showed that **67** was capable of displacing EtBr effectively when bound to stDNA but showed a large variation from that calculated from the Bard model. As described in Chapter 2, such variation is common when using different techniques and such values should be treated with a certain degree of caution. T_m experiments found that it was capable of stabilising the DNA double helical structure to a large degree. While CD studies did not provide definitive evidence for a particular mode of DNA interaction, LD studies confirmed the perpendicular orientation of the pdppz portion of the compound relative to the DNA helix axis and is, thus, most likely intercalated between the base pairs. The remainder of this chapter will now deal with preliminary biological studies carried out in order to evaluate the DNA photocleavage ability, cellular uptake and localisation of **67**, as well as looking for a preliminary insight into its cellular phototoxic ability.

3.5 DNA Photocleavage Efficiency Studies of **67**

The efficiency of light sensitised DNA cleavage by **67** was investigated with a view to determining the extent of its excited state reactivity. It was anticipated that the tight binding of **67**, in addition to the observed photoredox processes occurring between its excited state and the DNA nucleobases, would be reflected in its photocleavage efficiency. Although studies of this type are well documented for Ru(II) complexes, photocleavage studies have not been reported for the related cationic organic derivatives **62a** and **62b**. In order to provide a reference point, the cleavage efficiency of **67** was compared to the known DNA photocleavage agent [Ru(bpy)₃]²⁺. DNA photocleavage studies were carried out by treating pBR322 plasmid DNA (1 mg/ml) with **67** at varying ratios in 10 mM phosphate buffer solution. The samples were then subjected to 2 J/cm⁻² using a Hg-Xe lamp equipped with either a > 320 nm or a > 390 nm cut off filter before being separated using horizontal agarose gel electrophoresis in TBE buffer. Shown in Figure 3.13 are the results of the DNA photocleavage studies.

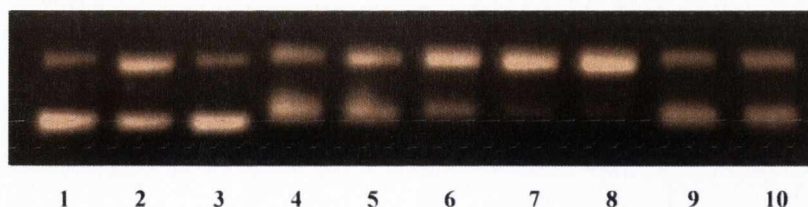


Figure 3.13: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after 30 min irradiation (2 J/cm^{-1}) in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: $\text{Ru}(\text{bpy})_3^{2+}$ (P/D 20); Lane 3: NaN_3 Control; Lane 4 : **67** in the dark (P/D 10); Lanes 5-6: **67** (P/D 20, 10) ($\lambda > 400 \text{ nm}$); Lanes 7-8: **67** (P/D 20, 10) ($\lambda > 320 \text{ nm}$); Lanes 9-10: **67** + 10mM NaN_3 (P/D 20, 10).

The relative intensity of the bands showed that the plasmid DNA was somewhat damaged to begin with, being comprised of 81% supercoiled and 19% open form DNA. The presence of $\text{Ru}(\text{bpy})_3^{2+}$ (P/D = 20) resulted in an increase to 55% open form after irradiation confirming its effectiveness as a DNA photocleaver. When incubated in the dark **67** showed a slight increase in DNA cleavage with 33% open form being observed. Moreover, as can be seen in Figure 3.13, lanes 5 and 6, irradiation and subsequent electrophoresis of pBR322 DNA in the presence **67** at P/D ratios = 20 and 10 showed clear evidence of DNA photocleavage, with an increase in open form to 46% and 74% respectively. Furthermore, when a lower wavelength cut off filter ($\lambda > 320 \text{ nm}$) was utilised the cleavage efficiency was increased further giving effectively quantitative cleavage with 90% and 96% open form observed for P/D = 20 and 10, respectively. When irradiated in the presence of the known singlet oxygen scavenger, NaN_3 (Lanes 9 and 10), it was observed that the photocleavage efficiency of **67** was reduced with only 36% cleavage seen at P/D = 20 and 46% cleavage seen at P/D = 10. The relative amounts of supercoiled vs. open form DNA are summarised in Table 3.4 and a representative graph is shown in Figure 3.14.

Table 3.4: Summary of percentages of supercoiled vs. open form pBR322

Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	19.2	81.8	6	73.9	26.1
2	54.8	45.2	7	90.4	9.6
3	18.5	81.5	8	96.1	3.9
4	33.1	66.9	9	35.9	64.1
5	45.7	54.3	10	45.8	54.2

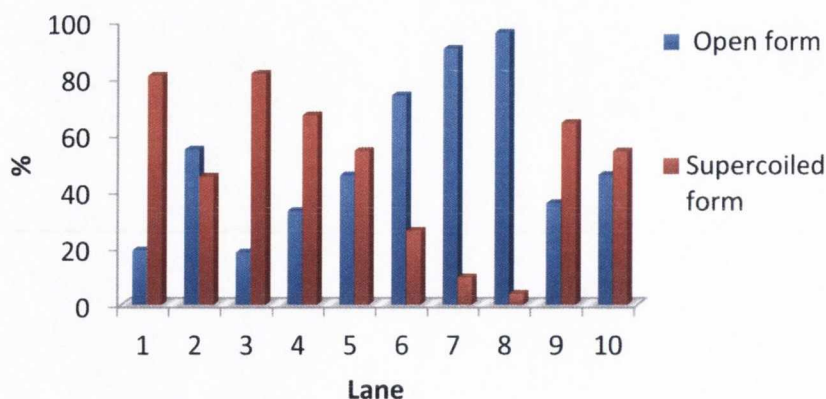


Figure 3.14: Relative percentages of supercoiled vs. open form pBR322 plasmid DNA from the photocleavage study of **67**

From the above results it is clear that cationic derivative **67** displays DNA photocleavage with efficiencies that are greater than that of $[\text{Ru}(\text{bpy})_3]^{2+}$. It is expected that this is due to the high binding affinity of **67** for DNA, which places the excited system in close proximity to the DNA helix, whereby it may effectively induce damage. In addition, it was observed that utilising different wavelength cut off filters had a dramatic effect on photocleavage efficiency. This observation can be rationalised in terms of the amount of light that **67** has the opportunity to absorb. Utilisation of a > 390 nm cut off filter allows light to be absorbed solely through its minor absorption shoulder at 460 nm; a band with a low extinction coefficient. Conversely, a > 320 nm cut off filter allows absorption through the bands at 370 nm and 390 nm as well as the shoulder at 460 nm resulting in more energy being absorbed; a process essential for DNA photocleavage to occur. Furthermore, the damage to DNA caused by **67** was also shown to involve $^1\text{O}_2$ formation whereby the presence of NaN_3 was shown to significantly inhibit the yield of open form DNA.

Having established the efficient excited state reactivity of **67** the next step will be to establish the cellular uptake properties of this system within target cells. The preceding section will attempt to reveal its cellular localisation.

3.6 Confocal Microscopy Cellular Localisation Study of **67**

Although previous cellular localisation studies had not been undertaken with analogues of **67** it was anticipated that **67** would be a likely candidate to enter cells due to its cationic nature. This would be a particularly important characteristic for **67**, as it was intended to investigate its ability to act as an *in vitro* biological probe and, in light of its observed excited state reactivity, to selectively kill cells upon light irradiation. Populations of live HeLa cells (0.5×10^5) were incubated with **67** (10 μM) at 37 $^\circ\text{C}$ for 24 hrs before being treated with

the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope as described in Chapter 2.

Figure 3.15 shows the fluorescence confocal laser scanning microscopy picture of the live HeLa cells after incubation with **67**. The image clearly demonstrates the localisation of **67** in all cells, with apparent localisation within the cytoplasm. Visualisation within the nucleus is detected to a lesser extent, as demonstrated in Figure 3.15C, a bright field image showing co-localisation of **67** (green) with the nucleic acid stain DAPI (blue). The observation of higher intensity fluorescence within the cytoplasm compared to the nucleus may be due to two reasons. Firstly, the compound may simply not localise to the same extent within the nucleus leading to reduced fluorescence in these areas. Alternatively, as seen in the fluorescence emission titrations, the excited state of **67** is quenched upon interaction with the nucleobases of DNA. It is possible, therefore, that **67** is indeed located in the nucleus but is no longer fluorescent due to the occurrence of redox quenching, resulting in the compound not being visible. Further studies are underway to further ascertain the exact localisation of **67** within cells and these results will be reported in the thesis of Ms. Luisa Erby in the School of Biochemistry and Immunology at Trinity College Dublin. In light of the observed cellular localisation of **67** in HeLa cells, its antiproliferative effects were assessed in a number of malignant cell lines by Ms. Erby. These results will be briefly summarised in the following section to highlight the biological application of **67**.

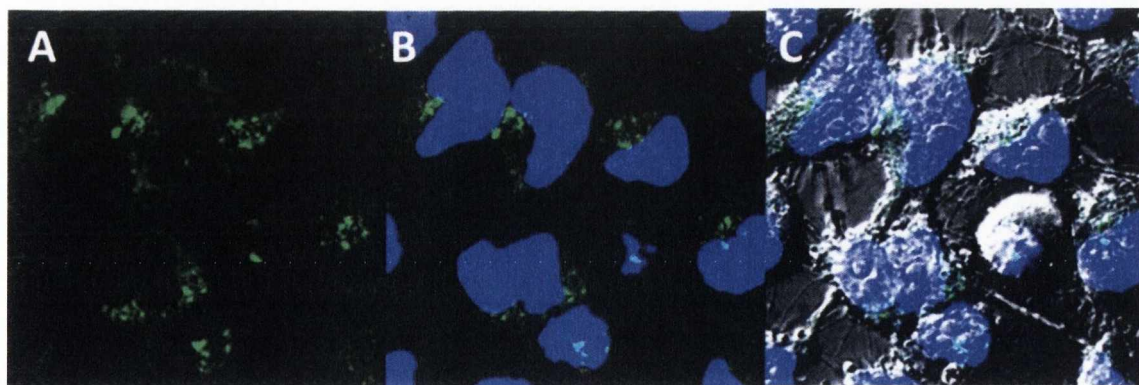


Figure 3.15: Confocal Laser Scanning Microscopy live cell images of **67** (10 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **67** (green), (B) overlay of **67** (green) and nuclear co-stain DAPI (blue) and (C) overlay of **67** (green) , nuclear co-stain DAPI (blue) and the bright field view after 24 hrs of incubation.

3.7 Preliminary Cellular Toxicity Studies with 67

To assess the anticancer potential of **67**, its antiproliferative effects were assessed using the alamar blue cytotoxicity assay, as described in Chapter 2, on a number of malignant cell lines, including HeLa cells, the mesothelioma cell lines ONE58 and CRL5915 and the Burkitt's lymphoma cell lines, DG-75 and MUTU-I. Figure 3.16A illustrates the results obtained showing that **67** (shown as 1 in Figure 3.16A) was found to reduce the viability of these malignant cells by approximately 50-60% in the dark and by 80-90% after light irradiation, indicating both a dark and a light-dependent cytotoxic death effect. This cytotoxicity occurs at a similar potency to the well-known platinum-based chemotherapeutic cis-platin (shown as C in Figure 3.16A). Further studies carried out by Ms. Erby using propidium iodide FACS (fluorescent activated cell sorter) analysis, which detects the proportion of apoptotic bodies in the pre-G1 phase of the cell cycle, also suggest that **67** induces light-dependent apoptosis in HeLa cells (Figure 3.16B). This indicates that cytotoxicity may involve a programmed cell death mechanism; a favourable outcome in cancer chemotherapies. Further studies with **67** are ongoing and will be reported by Ms. Erby.

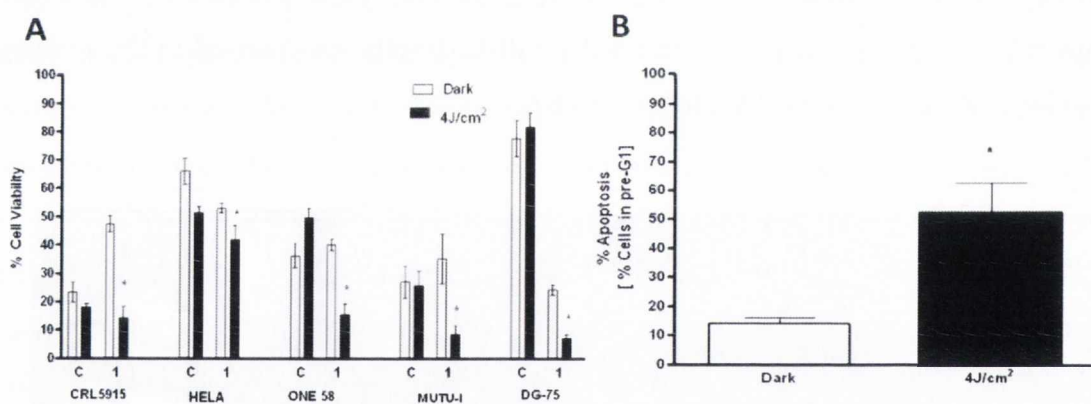


Figure 3.16: (A) The antiproliferative effects of **67** (100 μ M) on the malignant cell lines CRL5915, HeLa, ONE58, MUTU-I and DG-75. (B) Percentage of light induced apoptosis by **67** (100 μ M) as measured by FACS analysis.

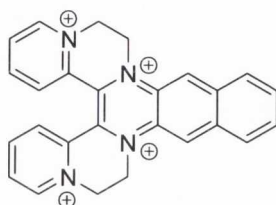
From the above biological results, **67** has been identified as having an efficient ability to photocleave DNA in addition to being able to localise within cancer cells. Furthermore it has been demonstrated that **67**, upon cellular uptake in a number of cancer cell lines, is capable of inducing cell death, which is further enhanced upon photoactivation. The results presented, therefore, clearly demonstrate that this small molecular weight agent has the potential to be used as a novel analogue to classical photodynamic therapeutic (PDT) drugs.

3.8 Conclusions and Future Perspectives

In this Chapter we have demonstrated that **67** shows good water solubility and a distinctive set of photophysical properties. This has allowed us to probe the nature of the interaction of a virtually isolated pdppz unit with duplex DNA, without the need for the Ru(II) centre. We have derived DNA binding affinities of **67** with stDNA, [poly(dA-dT)]₂ and [poly(dG-dC)]₂ where binding constants ranging from $10^5 - 10^7 \text{ M}^{-1}$ were calculated; a particular preference was seen for [poly(dG-dC)]₂ tracks from both ground and excited state titrations. Furthermore, we have seen that **67** is highly oxidising in its excited state whereby it undergoes a PET processes in the presence of both *adenine* and *guanine* sites on DNA. EtBr displacement assays, T_m experiments as well as CD and LD studies all provided further confirmation of the strong interaction of **67** with DNA. In particular, LD studies confirmed the perpendicular orientation of the pdppz portion of the compound relative to the DNA helix axis and pointed towards intercalation as the most likely mode of interaction of **67** with DNA.

In addition to its DNA binding parameters, we have demonstrated that **67** is an efficient photocleavage agent for DNA, and coupled to the observed high degree of selectivity, this becomes a highly desirable property. Confocal microscopy studies revealed the rapid cellular localisation of **67** within cancer cells where intense emission was observed in the cytoplasm. Finally, we have demonstrated the ability of **67** to induce cellular apoptosis in a range of cancer cells, with enhanced effectiveness after light irradiation. The work presented here, thus, represents an alternative strategy to Ru(II) complexes as DNA photocleavage agents and is a step towards the use of non-classical PDT agents.

Future investigations will look to evaluate a role for reactive oxygen species in the mechanism of cell death induced by **67**, and to determine its cellular localisation more precisely. Furthermore, synthetic studies will concentrate on the construction of systems with targeted photophysical properties and further enhancements in binding selectivity through variation of the chromophore. This will allow for modulation of the structure of the intercalative system allowing facile tuning of the luminescent and photoredox properties with a view to increasing DNA photocleavage efficiency at the same time as increasing the window between light and dark toxicity. Similarly, compounds such as **68**, with an increased cationic character may allow for a high degree of DNA binding affinity.



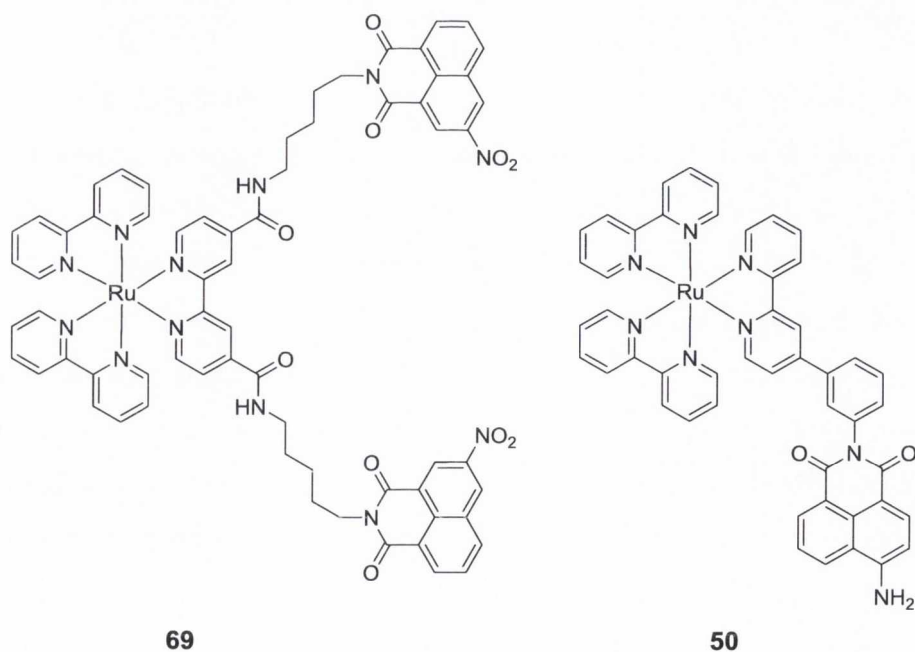
68

Chapter 4:

Π -Deficient Rigid Ru(II)-mono-1,8-Naphthalimide Conjugates

4.1 Introduction

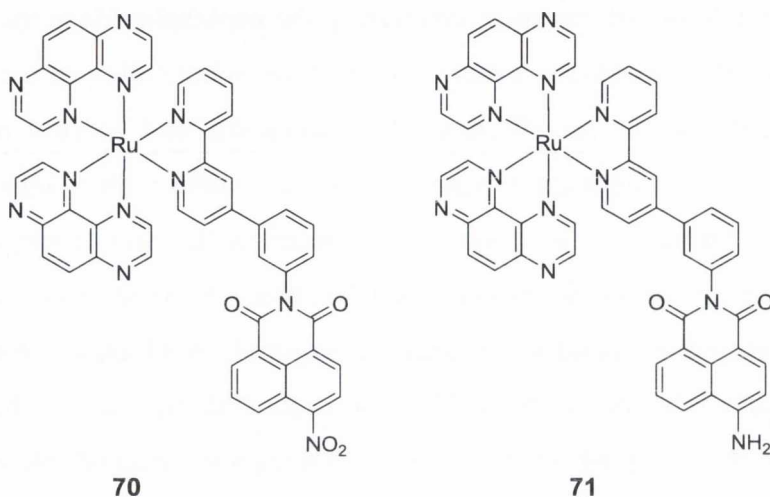
One of the main aims of the work carried out within the Gunnlaugsson group over the past number of years has been the conjugation of Ru(II) polypyridyl complexes to other functional species for use as bimodal probes and photoreactive reagents for DNA. One of the major bodies of research conducted towards this end has been the work of Dr. Gary Ryan who systematically designed, synthesised and photophysically evaluated various families of Ru(II) complexes containing the 1,8-naphthalimide chromophore.^{154,155} These systems were designed in the hope that the attached naphthalimide structure would serve to increase the DNA binding affinity while simultaneously displaying characteristic photophysical properties. Variation of the distance and flexibility of the linker connecting the naphthalimide to the Ru(II) centre, in addition to varying the substitution pattern on the chromophore allowed the establishment of important structural features for efficient DNA interaction and spectral response of these systems. The findings of this study revealed that those systems with a flexible chromophore-quencher arrangement such as, **69** should be regarded as the best strategy for DNA probe candidates, as large spectroscopic responses to DNA were observed. Conversely, for use as a DNA photoreagent, a more rigid arrangement is required, in which a non-quenching amino substituted 1,8-naphthalimide, such as **50**, is incorporated. Systems of this type result in tighter helix affinity coupled to the most advantageous excited state reactivity and, consequently, the most efficient DNA photocleavage ability.



Subsequent work in the group carried out by Dr. Daniel Frimansson set out to establish the biological utility of the Ru(II)-1,8-naphthalimide compounds synthesised by Dr. Ryan and

their potential as PDT agents.¹⁰³ Importantly, complex **50** and its nitro analogue were shown to selectively localise within the nucleus of cancerous HeLa cells giving them direct access to the cellular DNA. Consequently, the phototoxicity of the family of Ru(II) conjugates under investigation was shown to correlate well with DNA photocleavage studies carried out by Dr. Ryan whereby it was shown that the 4-amino derivative **50** was the most phototoxic and the most efficient DNA photocleaver.

In light of this work and the work described in Chapter 2, we set out to modify the structure of **50** in order to incorporate the π -deficient ligand TAP, to yield π -deficient rigid Ru(II)-mono-1,8-naphthalimide conjugates, **70** and **71**.



It was expected that the inclusion of two TAP ligands in their design would serve to increase the DNA cleavage efficiency of these systems. In the context of DNA binding the 1,8-naphthalimide was expected to contribute to high affinity binding through intercalation, while the Ru(II) centre should contribute to the interaction through electrostatic association with the double helix or insertion into the grooves of DNA. Similarly, the attached 1,8-naphthalimide structure should serve to further increase the excited state reactivity, while simultaneously providing a known set of photophysical properties that could be used to monitor the binding process. This design would make use of a rigid aromatic group as the linking moiety, allowing for control of the orientation of the components of the complex with respect to each other. It was also decided to prepare both the 4-nitro- and 4-amino-substituted species due to the amino analogue being easily prepared and having distinct photophysical properties from the nitro analogue. This was also seen to be an efficient method to investigate the effect of substitution on their DNA binding affinity. Finally, a *meta* arrangement around the connecting ring with respect to the Ru(II) centre was chosen as it had been shown by Dr. Ryan to result in greater affinity for DNA, and greater changes in the spectroscopic properties, due to complementarity between the shape of the complex and that of the DNA.

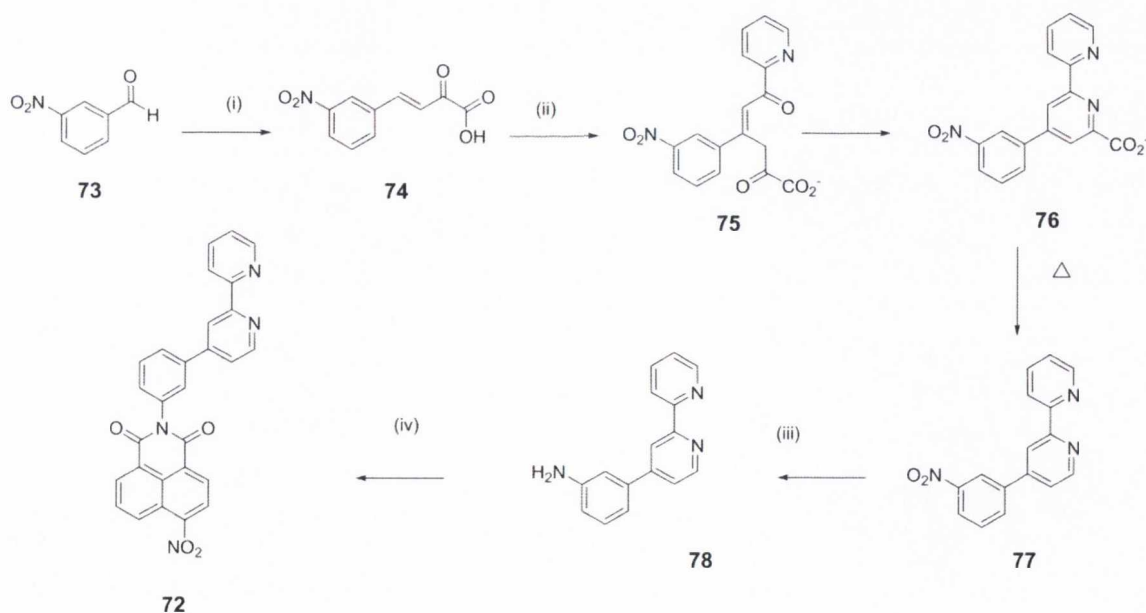
The work described in this Chapter aims to build upon the research already carried out by Dr. Ryan and Dr. Frimannsson by identifying systems with enhanced DNA photocleavage ability and, consequently, enhanced phototoxicity. By retaining direct access to cellular DNA it is hoped that these systems will be able to directly photoreact with DNA nucleobases, disrupting the gene regulation processes and having potential for use in cancer phototherapy. Firstly, a description of the synthesis and characterisation of both of the systems **70** and **71** is given. This is followed by a discussion of the photophysical properties of each complex and the DNA binding behaviour of each complex was then be established using a range of spectroscopic techniques. Finally, preliminary biological studies will be described detailing DNA photocleavage ability and cellular uptake and localisation.

4.2 Synthesis

The target compounds **70** and **71** were prepared from commercially available starting materials. The same procedure was applied to the synthesis of both analogues and full experimental details are provided in Chapter 8.

4.2.1 Synthesis of nitro-1,8-naphthalimide substituted bipyridine **72**

The conformationally restricted system **72** was synthesised as outlined in Scheme 4.1 according to a procedure initially reported by Johansson *et al.* and developed further by Dr. Ryan.^{154,208}



Scheme 4.1: (i) EtOH, sodium pyruvate, NaOH; (ii) ammonium acetate, H₂O; (iii) EtOH, N₂H₄, 10%Pd; (iv) EtOH, 4-nitro-naphthalic anhydride.

The first step of the synthesis involved the condensation of **73** with sodium pyruvate under basic conditions at 0 °C. Acidification of the reaction mixture with 0.1 M HCl yielded chalcone **74** as a yellow solid in 54% yield. Pyridacyl pyridinium iodide was refluxed in water with **74** to give intermediate **75**, incorporating the first pyridine moiety. NH_4OAc which was also present in the reaction mixture subsequently reacted at the two carbonyl groups to initiate the second ring closure and installed the second pyridine ring. This resulted in the carboxyl intermediate **76** which was heated strongly under high vacuum to cause decarboxylation and afford the desired nitro derivative, **77** in 51% yield. Subsequent reduction of the nitro group using hydrazine in the presence of 10% Pd/C catalyst gave the aniline functionalised bipyridine **78** as an off white solid in 94% yield. Following condensation of **78** with 4-nitro-1,8-naphthalic anhydride by heating under reflux for 24 hrs, a brown solid formed which was isolated by suction filtration affording **72** in 84% yield.

The ligand was fully characterised using ^1H NMR, ^{13}C NMR, IR and MALDI-ToF HRMS analysis. ^1H NMR peaks were assigned using 2-D C-H and H-H COSY analysis, the results of which correlated with those reported by Dr. Ryan.¹⁵⁴ The ^1H NMR spectrum (400 MHz, CDCl_3) of **72** is shown in Figure 4.1.

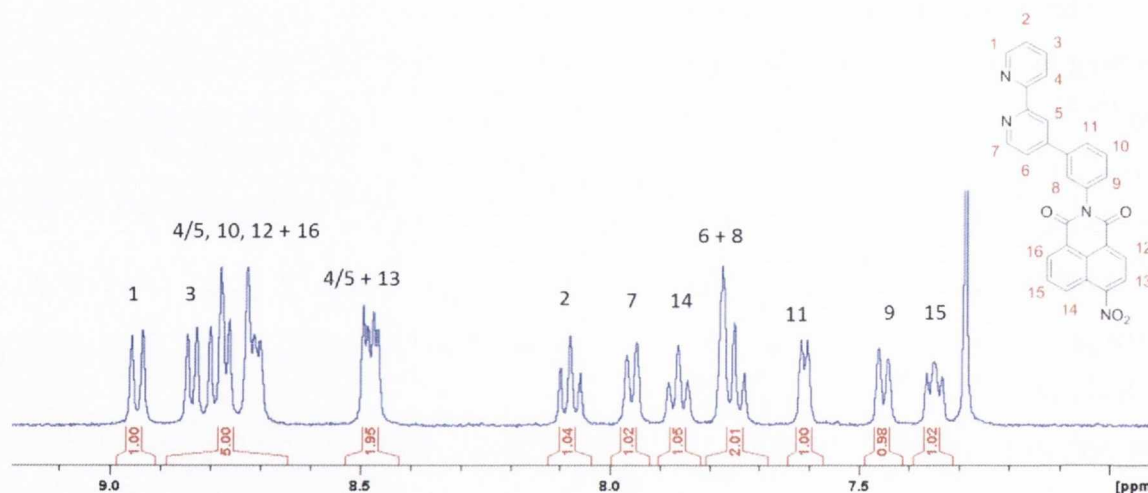


Figure 4.1: ^1H NMR spectrum of **72** (400 MHz, CDCl_3)

Resonances corresponding to both the 1,8-naphthalimide and bipyridine moieties are evident, in addition to those for the bridging aromatic ring. Assignment of the peaks was achieved by H-H COSY, C-H COSY and selective TOCSY analysis. Successful formation of the compound was also confirmed from accurate mass spectrometry, where **72** displayed a peak at 473.1233, corresponding to the $[\text{M}+\text{H}]$ ion.

4.2.2 Complexation of **72** with $\text{Ru}(\text{TAP})_2\text{Cl}_2$

Initial attempts to complex **72** to $\text{Ru}(\text{TAP})_2\text{Cl}_2$ were undertaken using the procedure of Sullivan *et al.* in the same manner as described in Chapter 2 using a DMF/water mixture.¹⁶⁹ Although effective for many Ru(II) systems, this approach proved to be extremely inefficient for the preparation of the $[\text{Ru}(\text{TAP})_2(\textbf{72})]^{2+}$ systems described herein.

The complexation reaction mixture was observed to change colour from purple to orange, upon 24 hrs reflux in DMF/ H_2O , evidence of the formation of the tris-polypyridyl Ru(II) complex. However, upon isolation of the product by precipitation of the PF_6 salt, the crude yields obtained were extremely poor, ranging from 5-15%. In addition, upon ^1H NMR analysis it was discovered that the product obtained was a complex mixture of many different products. Numerous purification methods were attempted, however, these were unsuccessful. The methods investigated are summarised in Table 4.1.

Table 4.1: Methods attempted for purification of Ru-1,8-naphthalimide conjugate **70**

Attempt	Method of purification	Result
1	Precipitation from diethyl ether	Impure
2	Sephadex LH-20 column (MeCN)	Impure
3	Silica column ($\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{Sat. NaNO}_3$ - 40:4:1)	Impure
4	Alumina column ($\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{Sat. NaNO}_3$ - 40:4:1)	Impure
5	Cation exchange CM Sephadex C-25 (0.1M aq NaCl)	Impure
6	Cation exchange SP Sephadex C-25 (0.1M aq NaCl)	Impure

In a manner similar to that described in Chapter 2, highly efficient microwave-assisted synthesis of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ in DMF was subsequently undertaken by reaction with $\text{Ru}(\text{COD})\text{Cl}_2$. Reaction at $140\text{ }^\circ\text{C}$, of the resultant $\text{Ru}(\text{TAP})_2\text{Cl}_2$ in the presence of **72** using a DMF/ H_2O mixture yielded a similar colour change from dark purple to orange, signifying the formation of a tris-Ru(II) complex. The mixture was brought to dryness under reduced pressure, redissolved in H_2O and filtered before precipitation of the crude complex as the PF_6 by addition of a concentrated ethanolic solution of sodium hexafluorophosphate. Analysis of the crude product by ^1H NMR immediately showed increased purity in comparison to the purification attempts outlined in Table 4.1 (Figure 4.2). Small impurities which remained were thought to be of $\text{Ru}(\text{TAP})_2\text{Cl}_2$, $[\text{Ru}(\text{TAP})_3]^{2+}$ or various oxidation products. These impurities were ultimately removed by slow crystallisation from a saturated CH_3CN solution placed in an ether-saturated closed vessel as reported by Rickling *et al.*²⁰⁹ giving **70** in 52% yield.

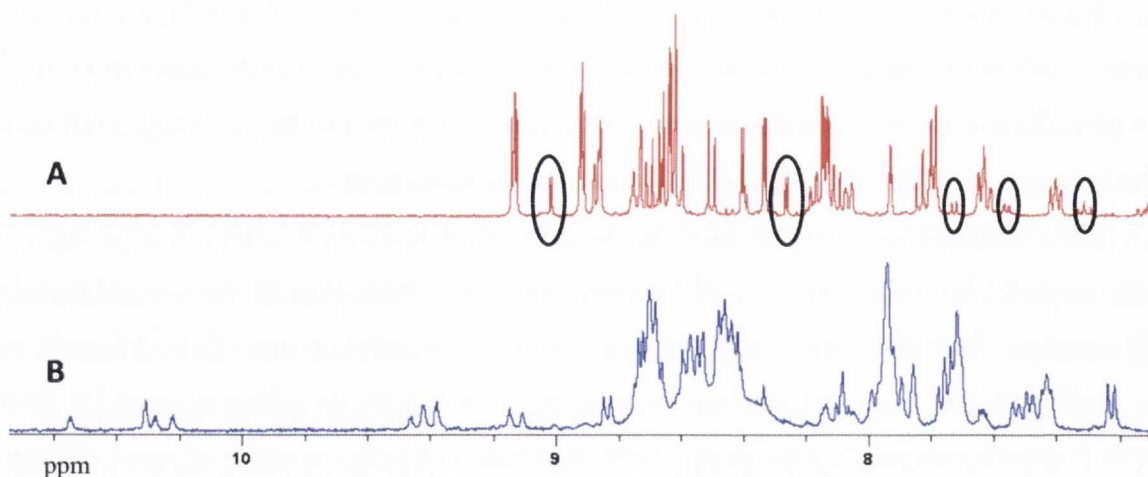


Figure 4.2: (A) ^1H NMR spectrum (400 MHz, CD_3CN) of crude **70** after 40 mins microwave irradiation at 140 °C in $\text{DMF}:\text{H}_2\text{O}$ (B) ^1H NMR spectrum (400 MHz, CD_3CN) of crude **70** after 24 hrs reflux at 100 °C in $\text{DMF}:\text{H}_2\text{O}$

The 4-nitro-1,8-naphthalimide based **70** was then reduced to its corresponding 4-amino-1,8-naphthalimide analogue **71**, in MeOH using 10% Pd/C, at 1 atm of H_2 , for 24 hours (Figure 4.3). Filtration of the reaction mixture through celite afforded the desired product **71** in high purity in 95% yield. These novel compounds were characterised using the full range of techniques described in Chapter 2, see Chapter 8 for further details. Due to the highly aromatic nature of both **70** and **71** the ^1H NMR spectra (recorded in CD_3CN) were observed to be extremely complicated exhibiting a large degree of overlap in the spectra of both complexes. Nonetheless, through the use of H-H COSY, C-H COSY and selective TOCSY analysis an assignment of all resonances was possible.

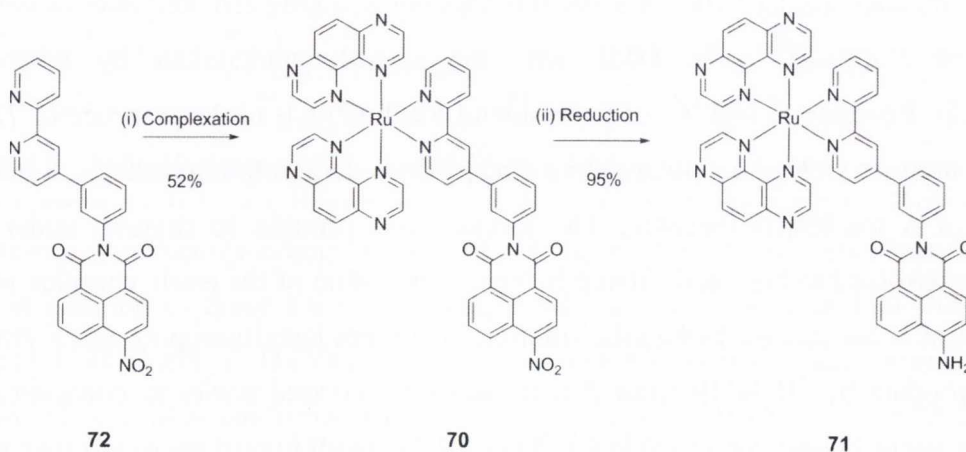


Figure 4.3: Complexation of ligand **72** with $\text{Ru}(\text{TAP})_2\text{Cl}_2$ and reduction of the resulting complex. Reagents and conditions: (i) $\text{Ru}(\text{TAP})_2\text{Cl}_2$, $\text{DMF}/\text{H}_2\text{O}$, argon, (ii) MeOH, Pd/C, 1 atm H_2 .

The ^1H NMR spectra (600 MHz, CD_3CN) of **71** is shown in Figure 4.4 with that of **70** given in Appendix 4. ^{13}C NMR spectra (recorded in CD_3CN) were also obtained, in which all of the expected resonances were visible.

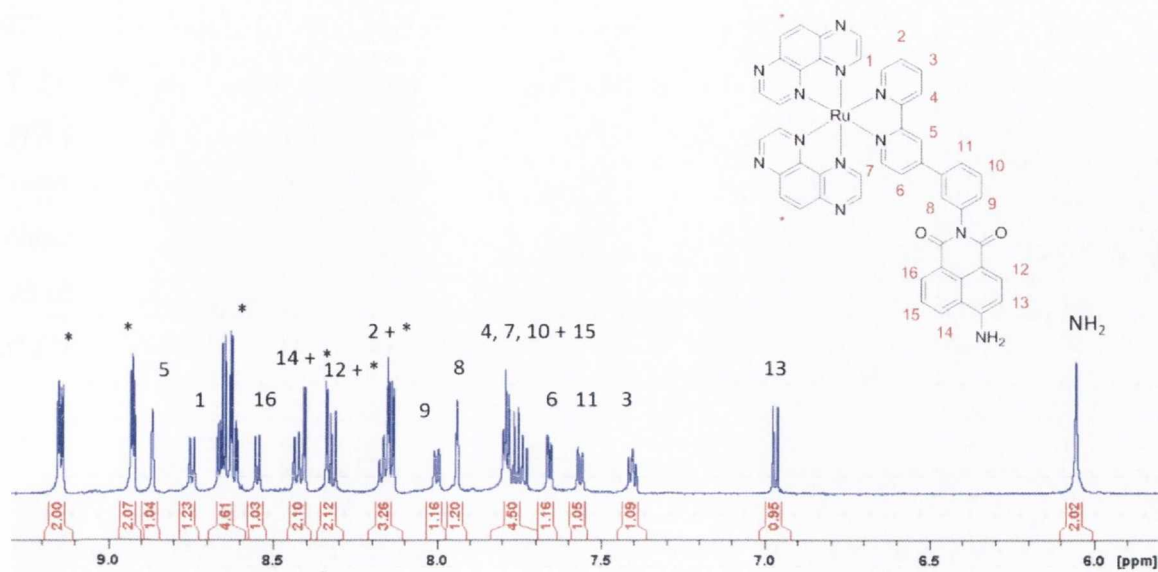


Figure 4.4: ^1H NMR spectra of **71** (600 MHz, CD_3CN)

The successful formation of the complexes was also clearly evident from accurate mass spectrometry, where **70** displayed a peak at 1083.1088, corresponding to its M^{2+} ion + PF_6 . Similarly, **71** displayed a peak at 1053.1266, also corresponding to its M^{2+} ion + PF_6 . A comparison between the calculated and observed isotopic distribution patterns are shown for **70** and **71** in Figure 4.5. Synthesised as their chloride salts, both complexes were water-soluble, and their photophysical properties were investigated in 10 mM phosphate-buffered aqueous solutions at pH 7.4.

(a)

(b)

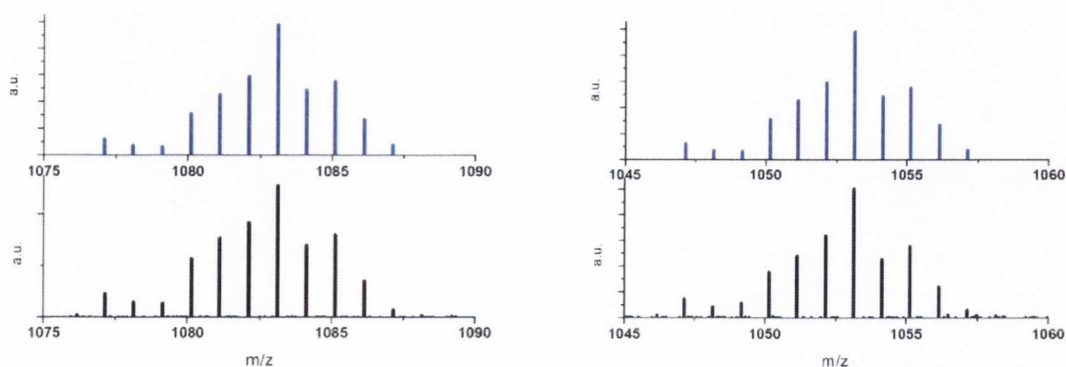
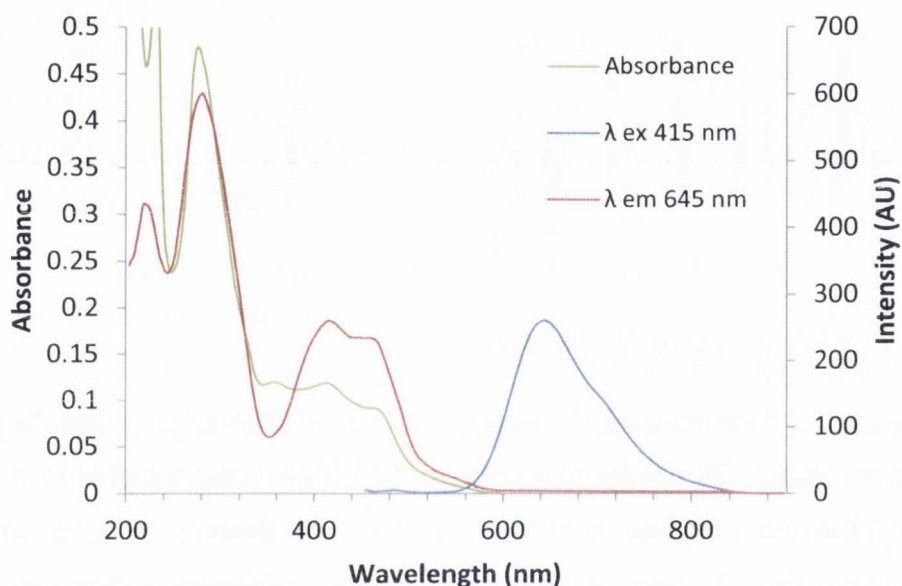


Figure 4.5: Comparison between the calculated (blue) and obtained (black) isotopic distribution pattern for (a) **70** and (b) **71** from high resolution mass spectrometry analysis

4.3 Photophysical Characterisation

Characterisation of the photophysics of both complexes **70** and **71** in solution was necessary as these compounds have been designed as binders of DNA. It was anticipated that both the substituents on the 1,8-naphthalimide ring and the incorporation of two TAP ligands would have a major effect on the photophysical properties of both of these systems. The absorption spectra with the fluorescence excitation and emission spectra of **70** and **71**, recorded in 10 mM phosphate buffer solution (pH 7.4) are shown in Figure 4.6 (a) and 4.6 (b), respectively.

(a)



(b)

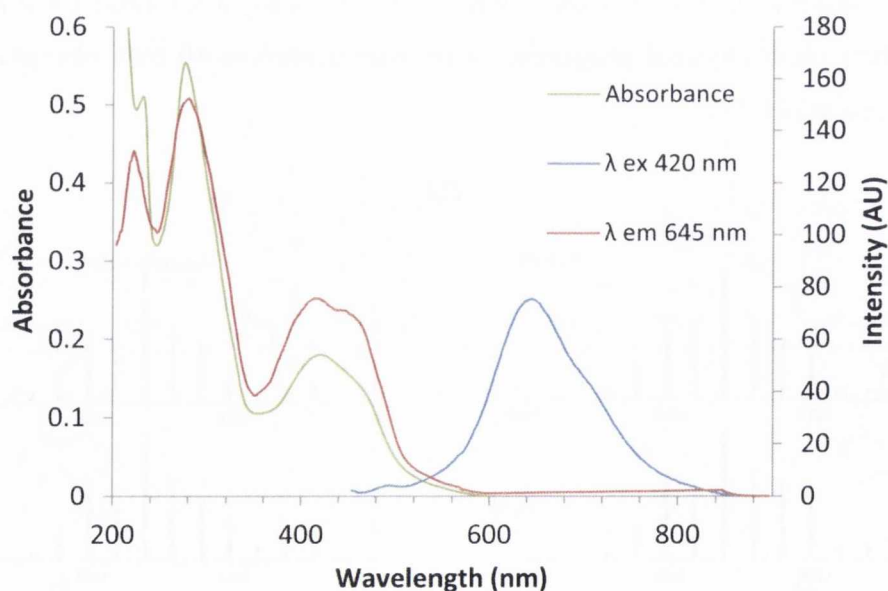


Figure 4.6: UV/Visible absorption and fluorescence excitation and emission spectra of (a) **70** and (b) **71** (10 μ M) in 10 mM phosphate buffer at pH 7.4

Examination of the UV/Vis absorption spectrum of **70** reveals bands characteristic of both the Ru(II) polypyridyl and 4-nitro-1,8-naphthalimide chromophores, where the intense band centred around 275 nm was attributed mainly to π - π^* intraligand transitions, the band at 358 nm to the 1,8-naphthalimide π - π^* transitions, and the band centred at 415 nm to the MLCT transitions of the Ru(II) polypyridyl centre. Importantly, bands characteristic of the 1,8-naphthalimide and the Ru (II) MLCT may be resolved, which will be useful when examining the interaction of both components upon binding of the complexes with DNA. The absorption properties of **71** were somewhat different. The band at 275 nm was still present, although its molar absorptivity was slightly increased compared to that of **70**. A single band was observed at 425 nm corresponding to both the 4-amino-1,8-naphthalimide and the Ru(II) MLCT transitions. A summary of these absorption properties is given in Table 4.2.

Table 4.2: Absorption properties of **70** and **71** in 10 mM phosphate buffer, at pH 7.4

Complex	$\lambda_{\max}$ (nm) [ϵ (M ⁻¹ cm ⁻¹)] in phosphate buffer ($\pm 10\%$)		
	π - π^* IL	π - π^* Naph	MLCT
70	275 [61250]	358 [15300]	415 [15000]
71	275 [65700]	-----	425 [22900]

Excitation of all absorption bands of the the 4-nitro-1,8-naphthalimide containing complex **70** resulted in essentially no emission from the 1,8-naphthalimide moiety, and MLCT based emission at around 645 nm. The absence of 1,8-naphthalimide associated emission suggested that the sensitisation of the MLCT excited state occurred through an energy transfer process, in a similar manner to the complexes discussed in the thesis of Dr. Ryan.¹⁵⁴ Excitation of an aqueous solution of **71** at 425 nm resulted in similar behaviour, in that no emission was observed from the 1,8-naphthalimide, but a band centred at 645 nm corresponding to MLCT emission was observed. The $\lambda_{\max}$, τ_{em} and Φ_{F} values for **70** and **71** are summarised in Table 4.3.

Table 4.3: Emission properties of **70** and **71** in phosphate buffer solution at pH 7.4 at 298K

Complex	$\lambda_{\text{emission}}$ (nm)	Φ_{f} ($\pm 10\%$)	τ_{em} (ns) (air)
70	645	0.022	437
71	645	0.004	166

The Φ_F value for **70** was found to be 0.022 ($\pm 10\%$), which is approximately half the value of that reported for $[\text{Ru}(\text{TAP})_2(\text{bpy})]^{2+}$ but higher than that reported for the $\text{Ru}(\text{bpy})_2$ analogue, **49** described by Ryan which was reported as 0.001.^{75,154} Conversely, the Φ_F value for **71** was found to be 0.004 ($\pm 10\%$), a value lower than that described for its $\text{Ru}(\text{bpy})_2$ analogue, **50** which was reported as 0.018. The reduction in the quantum yields, and the resulting weak emission displayed by these complexes, is attributed to an interaction between the 1,8-naphthalimide and the MLCT triplet state. As discussed in Chapter 1, similar phenomena have been observed for $\text{Ru}(\text{TAP})_2$ -aminoquinoline conjugates and were attributed to electron transfer processes between the $\text{Ru}(\text{TAP})_2$ centre (chromophore) and the organic moiety (quencher).²¹⁰ We anticipate that a similar process is likely to be occurring here, where the excited state of the metal complex is efficiently quenched by electron transfer to the 1,8-naphthalimide moiety. Interestingly, the quantum yield of amino substituted **71** is greatly reduced in comparison to the nitro derivative **70** suggesting that the amino naphthalimide is a more efficient quencher than its nitro counterpart. Excited state lifetime measurements (τ_{em}) followed the same trend where **70** and **71** showed τ_{em} values of 437 ns and 166 ns, respectively; the lower τ_{em} value being exhibited by **71** due to its ability to efficiently quench the MLCT based emission.

The photophysical properties described above clearly demonstrate the advantageous nature of the bifunctional approach employed in the design of **70** and **71** with bands characteristic of the 1,8-naphthalimide and the Ru (II) MLCT centre being observed. By changing the nature of the substituents at the 4-position of the appended 1,8-naphthalimide chromophore it has been possible to yield systems with varying photophysical properties. In addition, incorporation of π deficient TAP ligands has equipped these species with a highly oxidising excited state that is expected to have a profound effect on the nature of their interaction with DNA. Their ability to recognise DNA will be discussed in the following sections and the potential use of **70** and **71** as photoresponsive and photoreactive DNA targeting agents will be examined.

4.4 DNA Binding Interactions of **70** and **71**

As was the case in the previous two Chapters complexes **70** and **71** were expected to interact strongly with DNA resulting in significant modulations of their photophysical properties. Due to their bifunctional nature, it was expected that **70** and **71** would interact with DNA through a combination of electrostatic and π -stacking interactions, where the metal complex would be expected to bind externally to the phosphate backbone, while the 1,8-naphthalimide moieties would bind through intercalative or groove binding interactions.

To determine the nature of their binding affinity for DNA a number of spectroscopic titrations were carried out and these results are presented in the following sections.

4.4.1 Spectroscopic Titrations of **70** and **71** with DNA

4.4.1.1 UV/Vis Absorption Titrations of **70** and **71** with DNA

UV/Vis absorption spectroscopy was firstly utilised to probe the interaction of **70** and **71** with DNA. The titrations were carried out in a similar fashion to that described in previous chapters by addition of small aliquots of stDNA to a 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached. Changes in the Ru(II) complex $^3\text{MLCT}$ band and in the 1,8-naphthalimide $\pi\text{-}\pi^*$ band at 358 nm were monitored for **70** while those changes at the broad band centred at 425 nm was monitored in the case of **71**. All titrations were repeated a number of times to ensure reproducibility. The changes in the ground state of **70** are shown in Figure 4.7 with those of **71** shown in Appendix 4.

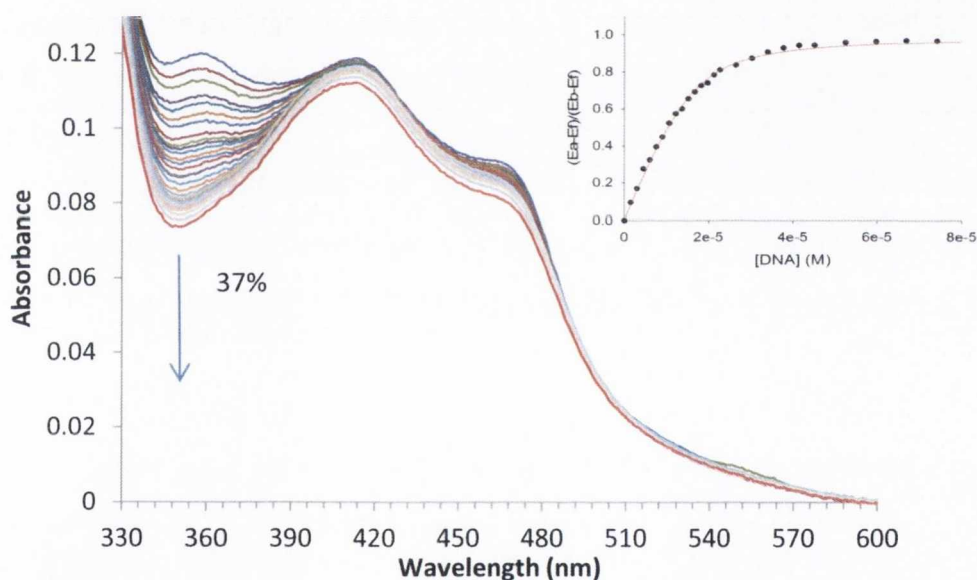


Figure 4.7: Changes in the UV/Visible spectrum of **70** ($8\ \mu\text{M}$) with increasing additions of stDNA ($0\text{--}210\ \mu\text{M}$) in 10mM phosphate buffer, pH 7.4). Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](\text{M})$, P using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.

The titration of **70** and **71** with stDNA resulted in significant changes to their absorption spectra. Complex **70** exhibited a 37% decrease in absorbance at 358 nm with a concomitant decrease of 6% in the MLCT band at 415 nm. The absorbance changes displayed by **71** were somewhat different but, nonetheless, culminated in a 22% hypochromism in the band centred at 425 nm.

The defined hypochromicities observed in the ground state spectra of both complexes immediately indicates strong interactions occurring with DNA. In the case of **70**, the large degree of hypochromicity seen at 358 nm would indicate intercalation of the 1,8-naphthalimide moiety between the stacked bases, as expected with such a planar structure. Similarly, the changes in the MLCT region confirm that the metal centre of **70** is tightly bound to DNA. Due to the overlap of the MLCT and naphthalimide bands of **71** it is more difficult to draw firm conclusions regarding the separate portions of this system. However, the large absorbance decrease observed at 425 nm was also coupled with a slight red shift of the absorbance maximum of *ca.* 4 nm; behaviour which is generally associated with classical intercalation.²¹¹

Analysis of the ground state titration data using the Bard equation, as described in the previous Chapters, confirmed that both **70** and **71** showed strong affinity for DNA (Table 4.4). Complex **70** showed strong binding with $K_b = 1.3 \times 10^6$ (± 0.1) and $n = 1.3$ (± 0.03), while **71** gave $K_b = 5.8 \times 10^6$ (± 1.2) and $n = 1.5$ (± 0.03). The lower affinity observed for complex **70** may be due to steric repulsion caused by the nitro group which may be twisted slightly out of plane due to steric repulsion from the neighbouring H-atom in the 5-position of the ring system, as represented schematically in Figure 4.8.²¹² The resultant diminished planarity makes intercalation or insertion into the DNA helix less favourable for the nitro-substituted species while the amino-substituted complex **71** does not experience this type of twisting, and as such its presence may allow for more complete insertion into the helix resulting in a stronger binding affinity.

Table 4.4: DNA binding parameters from fits to absorbance data

Complex	$\lambda(\text{Naph})$ Hypochromism	$\lambda(\text{MLCT})$ Hypochromism	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
70	37%	6%	1.3×10^6 (± 0.1)	1.3 (± 0.03)	0.99
71	----	22%	5.8×10^6 (± 1.2)	1.5 (± 0.03)	0.99

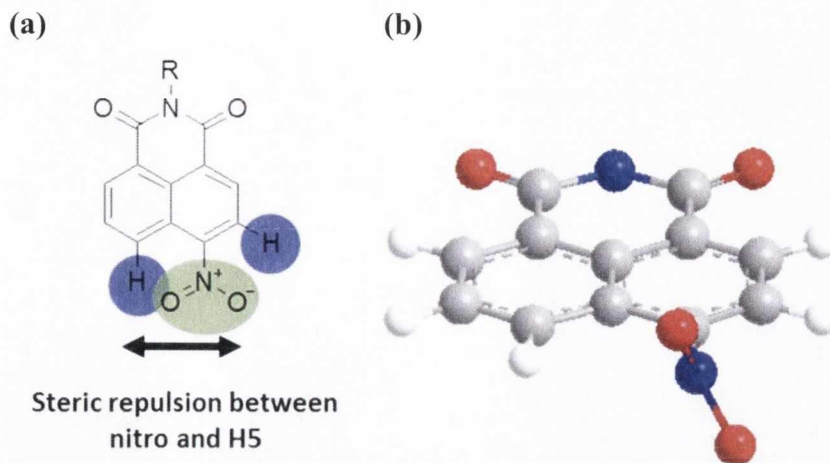


Figure 4.8 (a) Steric repulsion experienced by the nitro group when in the 4-position of the 1,8-naphthalimide chromophore. (b) Schematic representation of the 3D structure of 4-nitro-1,8-naphthalimide with nitro group twisted out of plane decreasing planarity and increasing steric bulk.

It is clear from the ground state titrations that both **70** and **71** show large spectroscopic changes in response to DNA. In addition, both species have been found to possess binding constants of the order of 10^6 M^{-1} ; values that are in keeping with those previously determined for complexes **49** and **50** reported by Dr. Ryan.¹⁵⁴ In order to further confirm this interaction and monitor any excited state redox processes, the excited state properties of **70** and **71** were also monitored as a function of added DNA concentration and these results will be presented in the following section.

4.4.1.2 Fluorescence Emission Titrations of **70** and **71** with DNA

Due to the incorporation of two π deficient TAP ligands in the structures of both **70** and **71** it was anticipated that perturbations of their emission would be likely as a result of photo-electron transfer processes occurring with DNA in solution as seen in Chapter 2. At the same time, the amino substituted derivative **71** was weakly emissive in solution, compared to emission from its nitro analogue **70** and it was expected that significant differences in spectral response to added DNA would be observed between both systems. The subsequent titrations were carried out in parallel with the UV/Visible titrations as described in previous Chapters.

The MLCT based emission of **70** in aqueous solution was shown to undergo excited state quenching with a *ca.* 30% decrease being observed upon addition of stDNA (P/D 0 $\rightarrow$ 30) with the main changes occurring between P/D 0 $\rightarrow$ 3 (Figure 4.9). Similarly, **71** was also shown to have its MLCT based emission quenched, but to a larger degree showing quenching of *ca.* 43% upon addition of stDNA (P/D 0 $\rightarrow$ 30) with the main changes occurring between a shorter P/D range of 0 $\rightarrow$ 2 (Appendix 4).

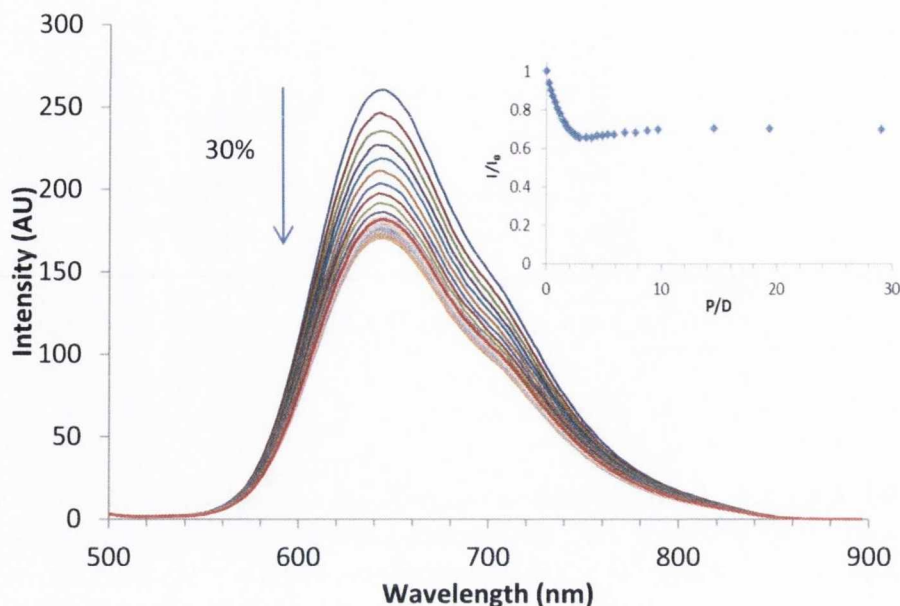


Figure 4.9: Changes in the fluorescence emission spectrum of **70** ($8\mu\text{M}$) ($\lambda_{\text{ex}} = 415\text{ nm}$) with increasing concentration of stDNA ($0\text{--}210\mu\text{M}$). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.

The observed quenching of the excited state is most likely due to a photo-electron transfer between *guanine* residues and the $^3\text{MLCT}$ state of the complexes as described in detail in Chapter 2 for systems containing two or more TAP ligands.⁵⁹ A biphasic binding profile, with an immediate fluorescence decrease followed by a more gradual increase to a plateau over a P/D range $3 \rightarrow 30$ was observed for both complexes. This suggests that these complexes distribute themselves differently on the polynucleotide depending on the availability of binding sites. As previously described in Chapter 2 the extra enhancement of luminescence quenching might originate from DNA induced stacking of the complexes.²¹³ For comparison, the emission profiles for each of the systems, **70** and **71** are shown in Figure 4.10. It is clear from these overlaid profiles that **71** experiences a larger initial emission decrease, reflecting the higher binding affinity of this system for DNA. This observation correlates well with the results seen with the UV/Vis absorption studies. It is also apparent from Figure 4.10 that **71** reaches a plateau in emission at lower P/D ratios compared to the nitro analogue **70**. Such low equivalents of DNA necessary to reach a plateau in emission further reflect the high DNA binding affinity of this system.

For determination of binding constants, such fluorescence data is often represented as a Scatchard plot and fitted to the model of McGhee and Von Hippel²⁰⁵, however, in this case the biphasic shape of the titration curves did not allow the accurate determination of binding data. Moreover, such data analysis often necessitates omission of points from the beginning and end of the titration and for this reason binding constants, K_b , were solely calculated from the Bard Model allowing a direct comparison with complexes **49** and **50** described by Ryan.¹⁵⁴

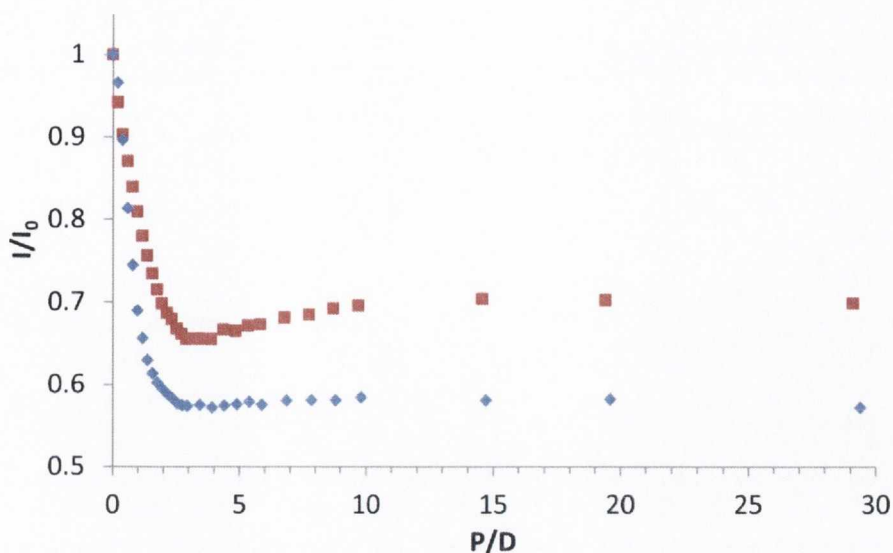


Figure 4.10: Relative changes in the emission intensity of **70** (■) and **71** (◆) ($\lambda_{\text{ex}} = 415 \text{ nm}$) upon addition of stDNA in 10 mM phosphate buffer, at pH 7.4

4.4.2 Spectroscopic Titrations of **70** and **71** with DNA at High Ionic Strength

The susceptibility of the binding affinity of **70** and **71** to ionic strength was next investigated by a series of titrations at varying concentrations of NaCl (50 mM and 100 mM) in the presence of DNA. As both species are cationic, electrostatic binding to DNA was expected to play a significant role in the binding process and it was expected that these titrations would reveal the relative contribution of electrostatic vs. non-electrostatic modes of interaction. The spectroscopic changes were monitored for both UV/Vis absorption and fluorescence emission titrations and these results are presented in the following sections.

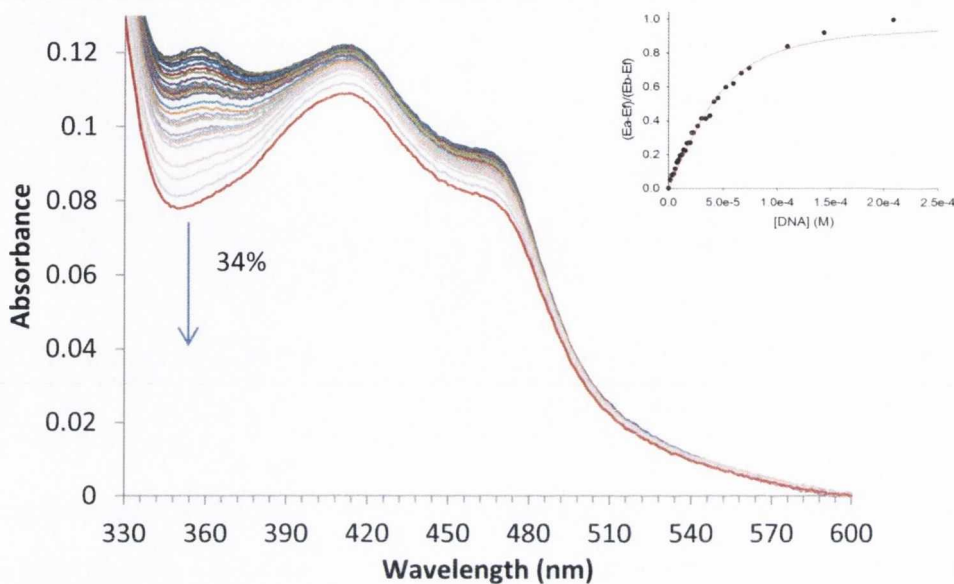
4.4.2.1 UV/Vis Absorption Titrations of **70** and **71** with DNA at High Ionic Strength

The interaction of **70** and **71** with DNA at varying ionic strengths was examined using UV/Vis absorption spectroscopy in a manner similar to that described previously involving the addition of small aliquots of stDNA to a 50 mM or 100 mM NaCl solution of 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached.

The changes in the ground state of **70** at both 50 mM and 100 mM NaCl concentration are shown in Figure 4.11 (a) and Figure 4.11 (b) respectively, with those of **71** shown in Appendix 4. The titration of **70** and **71** with stDNA at both 50 mM and 100 mM NaCl concentration, again, resulted in significant changes to their absorption spectra. At 50 mM NaCl, **70** exhibited a 32% decrease in its absorbance at 358 nm with a concomitant decrease of 10% in the MLCT band at 415 nm. Similar changes were observed at 100 mM NaCl with a

34% decrease in the 1,8-naphthalimide band at 358 nm and a further decrease of 10% in the MLCT band. In the case of **71**, at 50 mM NaCl concentration, an 18% hypochromism was observed at 425 nm with a slight red shift of *ca.* 4 nm again being exhibited. Similarly at 100 mM NaCl an 18% hypochromism was observed with a slight red shift of *ca.* 4 nm.

(a)



(b)

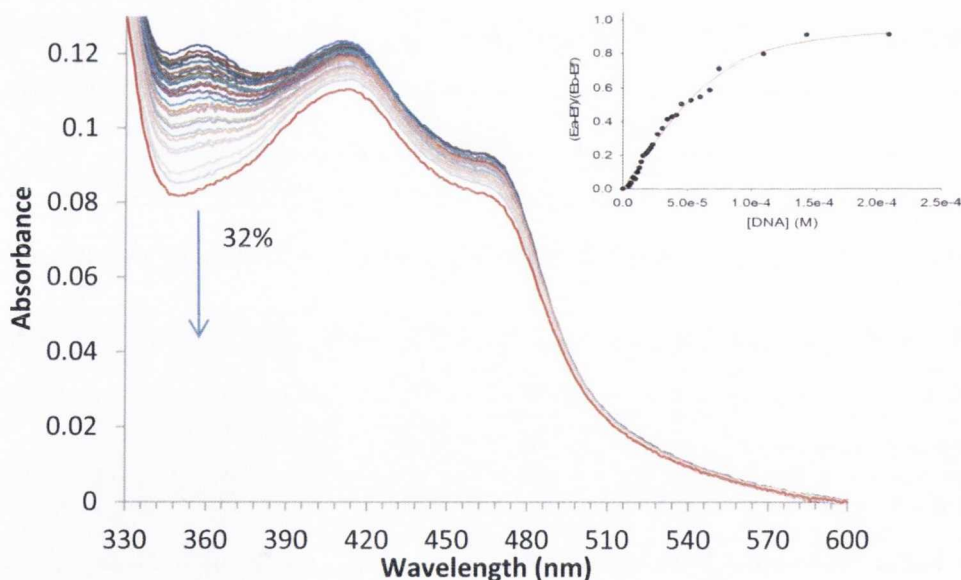


Figure 4.11: Changes in the UV/Visible spectrum of **70** (8 μ M) with increasing concentration of stDNA (0–210 μ M) at (a) 50 mM and (b) 100 mM NaCl concentration. **Insets:** Plots of $(\epsilon_a - \epsilon_b)/(\epsilon_b - \epsilon_f)$ vs. $[DNA] (M^1, P)$ using data with a P/D between 0–12 and the best fit of the data (---) using the Bard Eqn.

The overall changes in the UV/Vis ground state titrations observed above showed slight variations with increasing NaCl concentration where only minor changes in the degrees of hypochromism were observed. However, the shapes of the resultant binding profiles were different and as such the values obtained for K_b revealed a more obvious trend with regard to

the effect of NaCl concentration. The data was again fit to the model of Bard *et. al.* using non-linear regression analysis with Sigmaplot 11.0. A summary of the results obtained are presented in Table 4.5.

Table 4.5: DNA binding parameters from fits to absorbance data at varying NaCl concentrations

Complex	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
70 + stDNA	$1.3 \times 10^6 (\pm 0.1)$	1.3 (± 0.03)	0.99
70 + stDNA (50mM NaCl)	$7.4 \times 10^5 (\pm 0.9)$	3.1 (± 0.03)	0.99
70 + stDNA (100mM NaCl)	$4.3 \times 10^5 (\pm 1.8)$	4.45 (± 0.3)	0.99
71 + stDNA	$5.8 \times 10^6 (\pm 1.2)$	1.50 (± 0.03)	0.99
71 + stDNA (50mM NaCl)	$5.2 \times 10^5 (\pm 0.9)$	0.97 (± 0.07)	0.99
71 + stDNA (100mM NaCl)	$1.2 \times 10^5 (\pm 0.3)$	1.3 (± 0.2)	0.99

The results obtained confirmed that both of the complexes retained their high affinity for DNA, even at very high NaCl concentrations. For example, at 50 mM NaCl **70** exhibited a $K_b = 7.4 \times 10^5 (\pm 0.9)$ and $n = 3.1 (\pm 0.03)$ while at 100 mM NaCl **70** had $K_b = 4.3 \times 10^5 (\pm 1.8)$ and $n = 4.45 (\pm 0.3)$. The overall study revealed an expected trend in binding affinities of these complexes for DNA with K_b at 10 mM phosphate buffer $\geq$ 50mM NaCl $\geq$ 100 mM NaCl showing an obvious effect of NaCl concentration on the binding process.

It is clear from these results that the interaction of **70** and **71** with DNA is dependent on the ionic strength of the medium as the magnitude of the changes were decreased relative to that in 10 mM phosphate buffer alone. Nevertheless, as both complexes retain high affinity for DNA at 100 mM NaCl solution it can be concluded that, although electrostatic interactions have a role to play in the binding process, it is the bifunctional nature of these systems comprising an intercalating 1,8-naphthlimide and a cationic Ru(II) centre that governs the overall association of these complexes with DNA.

4.4.2.2 Fluorescence Emission Titrations of **70** and **71** with DNA at High Ionic Strength

The results obtained in the UV/Vis absorption titrations were further confirmed by similar changes in the fluorescence emission spectra of complexes **70** and **71** at varying salt concentrations. The changes in the excited state of **71** at both 50 mM and 100 mM NaCl concentration are shown in Figure 4.12 (a) and Figure 4.12 (b), respectively.

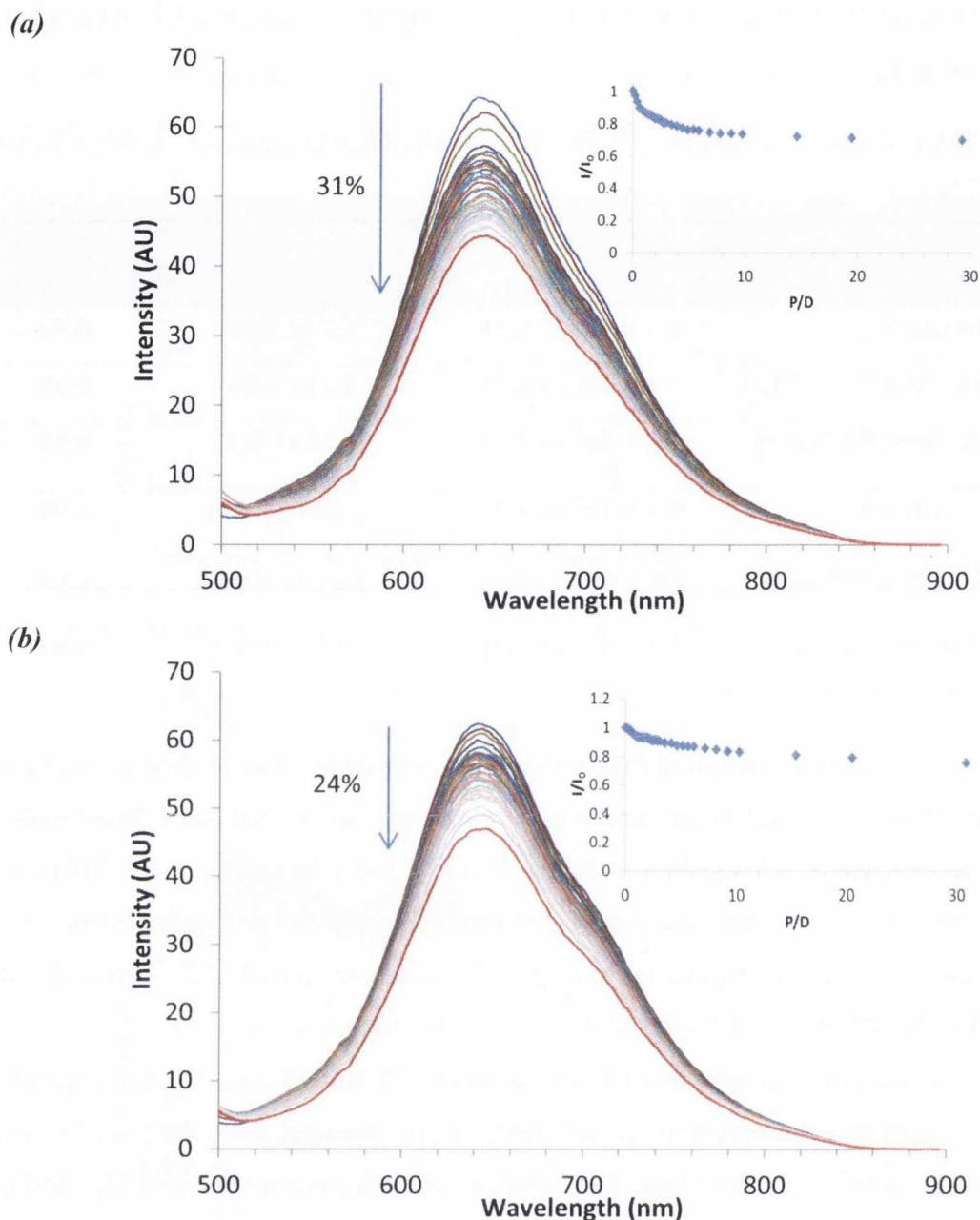


Figure 4.12: Changes in the fluorescence emission spectrum of **71** (8 μM) ($\lambda_{\text{ex}} = 425$ nm) with increasing concentration of stDNA (0-210 μM) at (a) 50 mM and (b) 100 mM NaCl concentration. **Insets:** Plots of the change in integrated MLCT emission intensity as a function of P/D ratio.

Upon addition of stDNA to complexes **70** and **71** the MLCT emission band centred at 645 nm was seen to decrease with increasing amounts of stDNA. For **70** at 50 mM NaCl the MLCT was quenched by 26% with the quenching process occurring over the entire P/D range from 0-30. Similarly, at 100 mM NaCl the MLCT was quenched by 24%. For **71** at 50 mM NaCl the band at 645 nm was seen to be quenched by 31% while at 100 mM NaCl, the MLCT

was seen to be quenched by 24% with the quenching process again occurring over the entire P/D range from 0 $\rightarrow$ 30 in both cases.

Both **70** and **71** showed a decreased degree of emission quenching in the presence of NaCl suggesting that neither complex is bound to DNA with the same affinity as was seen in 10 mM phosphate buffer alone. In addition, the biphasic binding profile that had been observed upon titration in 10 mM phosphate buffer was no longer evident. It is likely that the increase in ionic strength causes the DNA helix to shrink due to a reduction in the phosphate-phosphate bond repulsion. As a result, intramolecular luminescence quenching may not be as significant due to DNA induced stacking of the complexes being less favourable. These results further suggest that electrostatic binding of the metal centre to the DNA backbone does play a significant role in determining the overall affinity of **70** and **71** for DNA. An example of the effect that added NaCl has on the emission response is given in Figure 4.13, where the profiles for **71** under different ionic strength conditions are presented.

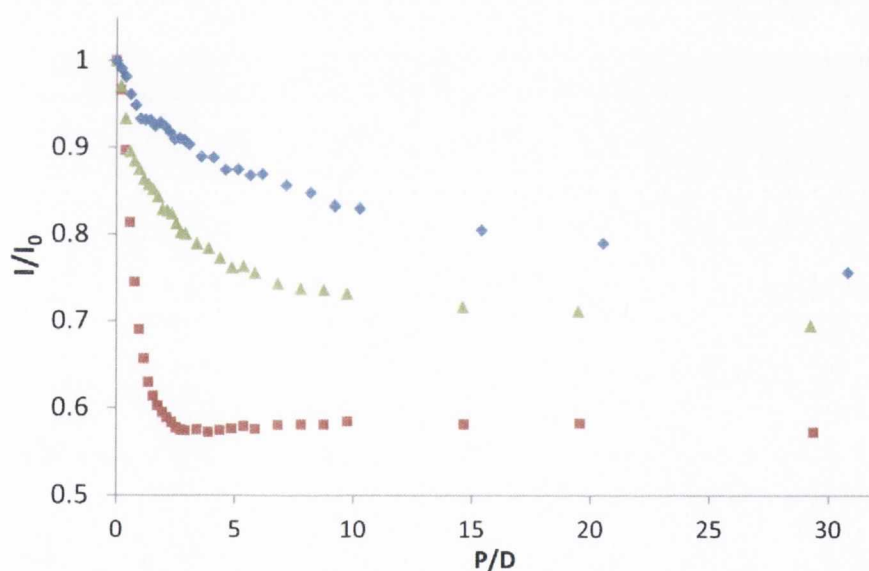


Figure 4.13: Relative changes in the integrated emission intensity of **71** (8 μ M) (λ_{ex} = 425 nm) with increasing concentration of st-DNA (0 – 210 μ M), in 10 mM phosphate buffer (■), 10 mM phosphate buffer + 50 mM NaCl (▲) and 10 mM phosphate buffer + 100 mM NaCl (◆).

Further confirmation of the effect of ionic strength was obtained from the emission response observed by carrying out salt back titrations for **70** and **71** as described in previous Chapters. The fluorescence emission was seen to be affected by increasing NaCl concentration, gradually increasing towards that of the free compound, but reaching a plateau at *ca.* 140 mM NaCl, with about a 40% increase towards that of the free species. No further changes occurred with the addition of up to 250 mM concentration of NaCl, indicating that the

majority of compound remains bound at high ionic strength. The results presented above have revealed further confirmation that DNA binding strength is dependent on the ionic strength of the medium for both species, but with neither of the complexes being fully displaced (Figure 4.14).

From the above results it is clear that both **70** and **71** remain bound to DNA at high concentrations of NaCl. As a result, it can be concluded that electrostatic binding of the metal centre to DNA does play a significant role in determining the overall affinity but due to the fact that **70** and **71** remain bound at high ionic strength it is likely that non-electrostatic interactions are not the most prominent in the binding of these complexes. This was anticipated in the initial design of these complexes; the 1,8-naphthalimide providing high affinity binding through intercalation into DNA, with the metal centre remaining externally bound through electrostatic attraction to the phosphate backbone.

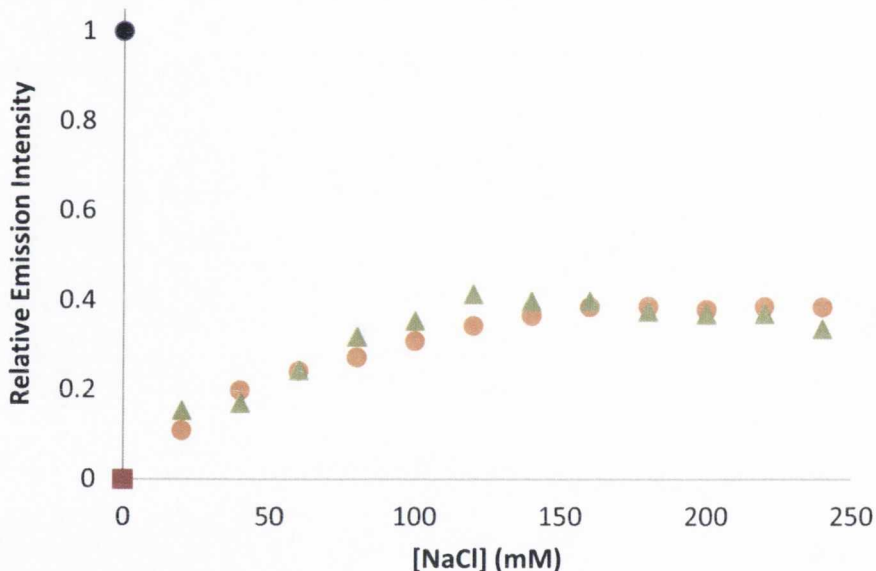


Figure 4.14: Fluorescence emission NaCl back titration profiles of **70** and **71** ($\lambda_{\text{ex}} = 415 \text{ nm}$). Emission of unbound complex (●), fully bound complex (■) and bound **70**(▲) and **71** (●) in the presence of increasing concentration of NaCl

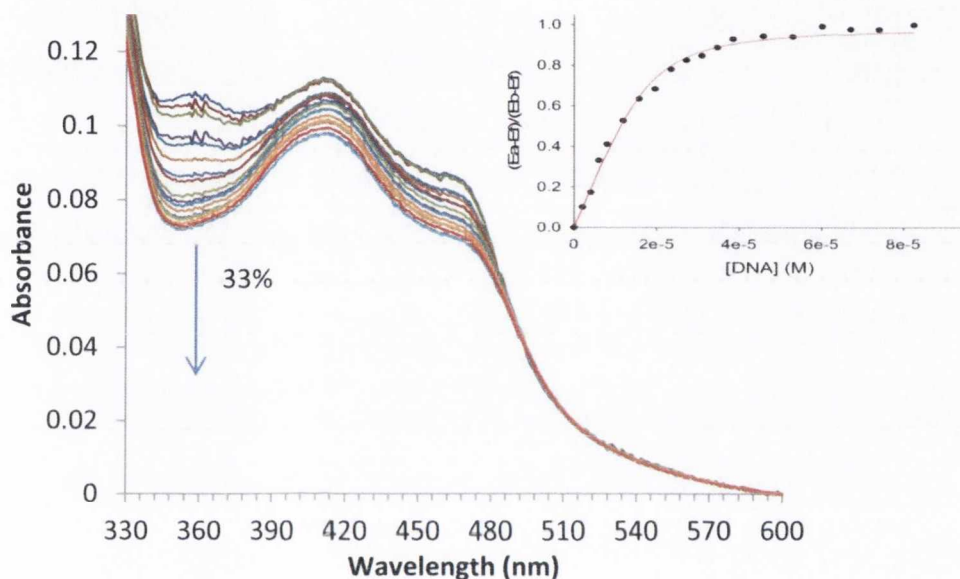
4.4.3 Spectroscopic Titrations of **70** and **71** with Synthetic Homopolymer Polynucleotides

In a manner similar to that described in Chapter 2, studies were also performed with synthetic polynucleotides, [poly(dAdT)]₂ and [poly(dGdC)]₂, in order to investigate their effect on the photophysical properties of **70** and **71**.

4.4.3.1 UV/Vis Absorption Titrations of **70** and **71** with Synthetic Homopolymer Polynucleotides

The interaction of **70** and **71** with both $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ was measured firstly using UV/Vis absorption spectroscopy and was carried out in a manner similar to that described in Chapter 2 involving the addition of small aliquots of the polynucleotide solution to a 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached.

(a)



(b)

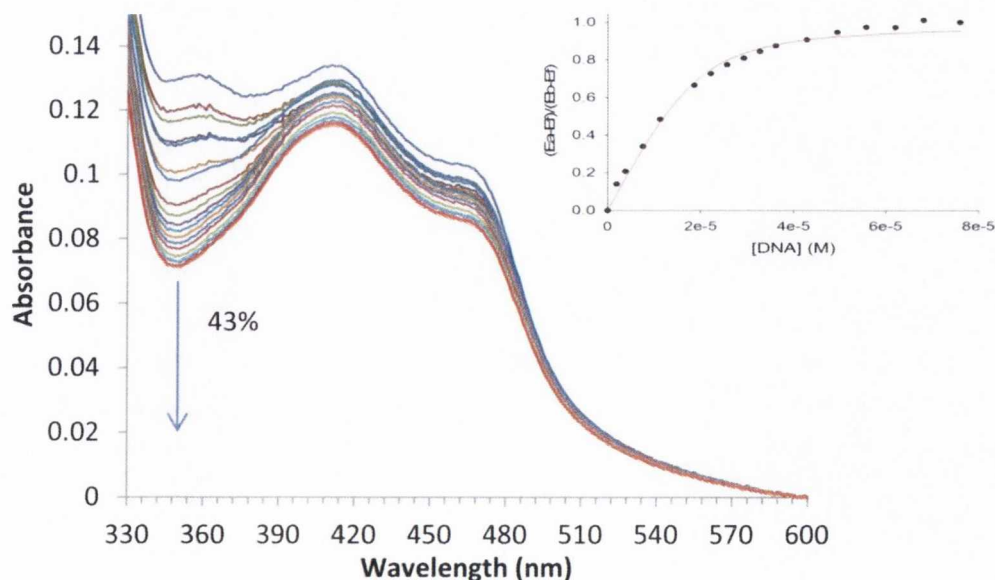


Figure 4.15: Changes in the UV/Visible spectrum of **70** (7.5 μM) with increasing concentration of (a) $[\text{poly}(\text{dAdT})]_2$ (0–83 μM) and (b) $[\text{poly}(\text{dGdC})]_2$ (0–76 μM) at pH 7.4. **Insets:** Plots of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}]$ (M^{-1} , P) using data with a P/D between 0–12 and the best fit of the data (---) using the Bard Eqn.

The changes in the ground state of **70** in the presence of $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$ are shown in Figure 4.15 (a) and Figure 4.15 (b) respectively, with those of **71** shown in Appendix 4. Upon binding to $[\text{poly}(\text{dAdT})]_2$, **70** exhibited a 33% decrease in absorbance at 358 nm with a concomitant decrease of 12% in the MLCT band at 415 nm. Similar changes were observed for $[\text{poly}(\text{dGdC})]_2$ with a 43% hypochromism at 358 nm and a further decrease of 14% in the MLCT band. In the case of **71**, upon binding to $[\text{poly}(\text{dAdT})]_2$ a 19% hypochromism was observed for the MLCT band while in the presence of $[\text{poly}(\text{dGdC})]_2$ a 23% hypochromism was observed at 415 nm.

These results did not show a large preference for either $[\text{poly}(\text{dAdT})]_2$ or $[\text{poly}(\text{dGdC})]_2$. Minor differences were observed in the degree of hypochromism exhibited upon titration with the different polynucleotides, as exemplified in the comparison curve of **70**, shown in Figure 4.16, which compares the changes in the naphthalimide absorption at 358 nm where a slightly larger decrease was seen upon titration with $[\text{poly}(\text{dGdC})]_2$ for both **70** and **71**.

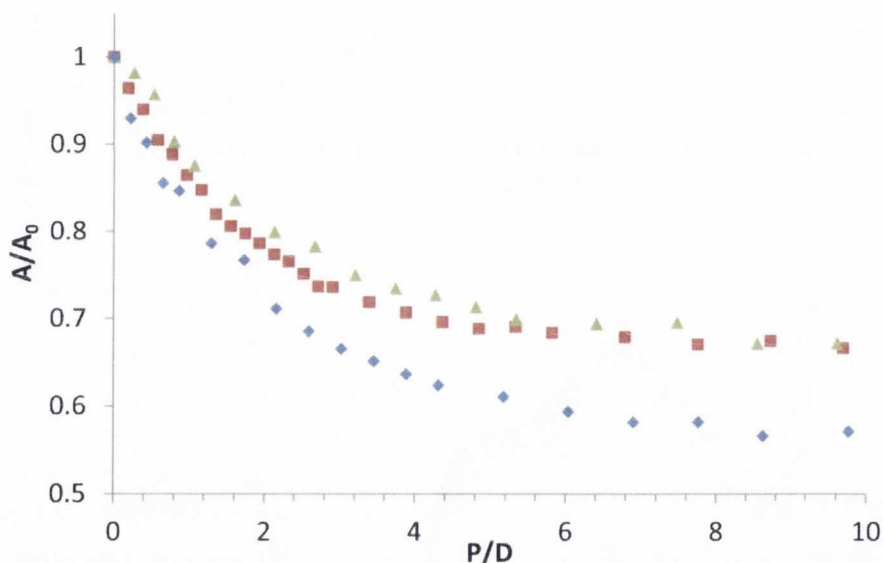


Figure 4.16: Changes in naphthalimide absorption at 358 nm of **70** (8 μM) in 10 mM phosphate buffer, at pH 7.4 upon addition of stDNA(■), $[\text{poly}(\text{dAdT})]_2$ (▲) and $[\text{poly}(\text{dGdC})]_2$ (◆).

Although the binding profiles of the different titrations were largely similar a range of values were obtained for K_b , from fits to the absorbance data using the model of Bard *et al.*, and were in agreement with differences in the binding of **70** and **71** for [poly(dAdT)]₂ and [poly(dGdC)]₂. A summary of the results obtained are presented in Table 4.6.

Table 4.6: DNA binding parameters from fits to absorbance data in the presence of [poly(dAdT)]₂ and [poly(dGdC)]₂

Complex	$\lambda(\text{Naph})$ Hypo- chromism	$\lambda(\text{MLCT})$ Hypo- chromism	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
70 + stDNA	37%	6%	$1.3 \times 10^6 (\pm 0.1)$	1.3 (± 0.03)	0.99
70 + [Poly(dAdT)]₂	33%	12%	$1.0 \times 10^6 (\pm 0.2)$	1.2 (± 0.1)	0.99
70 + [Poly(dGdC)]₂	43%	14%	$8.5 \times 10^5 (\pm 1.2)$	1.1 (± 0.1)	0.99
71 + stDNA	----	22%	$5.8 \times 10^6 (\pm 1.2)$	1.5 (± 0.03)	0.99
71 + [Poly(dAdT)]₂	----	19%	$1.8 \times 10^6 (\pm 0.7)$	1.3 (± 0.03)	0.99
71 + [Poly(dGdC)]₂	----	23%	$1.5 \times 10^6 (\pm 0.4)$	0.9 (± 0.06)	0.99

The results from this study have highlighted that each of the complexes **70** and **71** exhibited a strong binding affinity for both [poly(dAdT)]₂ and [poly(dGdC)]₂, as observed for stDNA. This behaviour is demonstrated by **70** in the presence of [poly(dAdT)]₂ where this complex exhibited a $K_b = 1.0 \times 10^6 (\pm 0.2)$ and $n = 1.2 (\pm 0.01)$ while with [poly(dGdC)]₂, $K_b = 8.5 \times 10^5 (\pm 1.2)$ and $n = 1.1 (\pm 0.01)$ were found. Similar behaviour was seen for **71** which also retained a high affinity for [poly(dAdT)]₂ and [poly(dGdC)]₂.

The UV/Vis absorption study in the presence of synthetic homopolymer polynucleotides has revealed very similar binding affinities of both **70** and **71** with [poly(dAdT)]₂ and [poly(dGdC)]₂ but with both of them binding slightly more strongly to stDNA. As was seen in Chapter 2 such minor preferences may be derived from structural differences between [poly(dAdT)]₂, [poly(dGdC)]₂ and stDNA, however, the degree of specificity is not significant enough to warrant the use of **70** or **71** as sequence specific recognition agents. Nevertheless, in order to probe the excited state interactions of **70** and **71** with the homopolymers, changes in the excited state properties of **70** and **71** were also examined in tandem with the ground state studies and these results will be presented in the preceding section.

4.4.3.2 Fluorescence Titrations of **70** and **71** with Synthetic Homopolymer Polynucleotides

As previously mentioned, due to the π deficient nature of the Ru(II) cores of **70** and **71** the observed quenching of the $^3\text{MLCT}$ states upon addition of stDNA is expected to be as a result of PET reactions with *guanine* nucleobases.^{59,78,85}

With this in mind, fluorescence emission studies were undertaken with the synthetic polynucleotides, $[\text{poly}(\text{dAdT})]_2$ and $[\text{poly}(\text{dGdC})]_2$. The resultant changes in the fluorescence emission spectrum of **70** are shown in Figure 4.17 (a) and Figure 4.17 (b), respectively with those of **71** shown in Appendix 4.

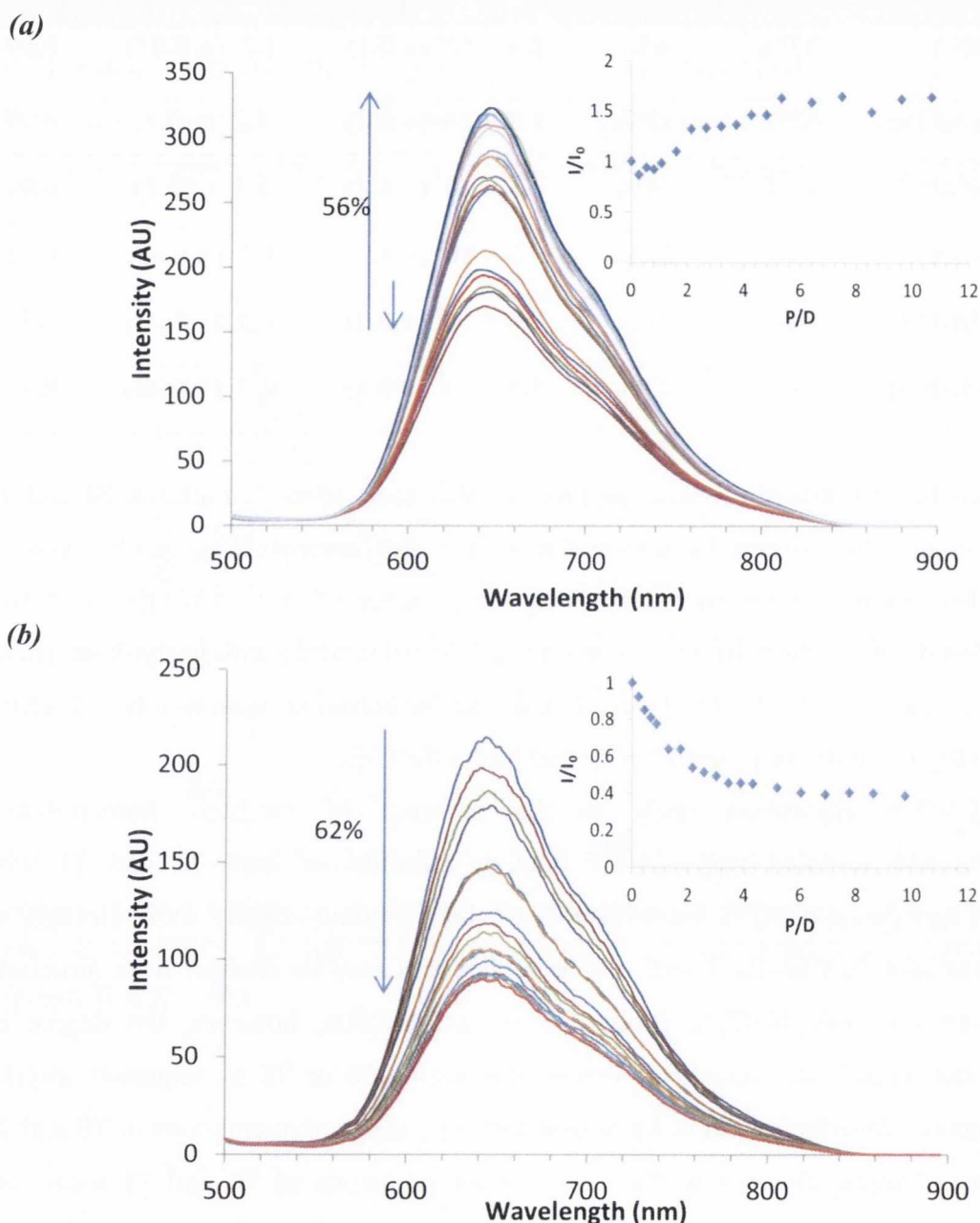


Figure 4.17: Changes in the fluorescence emission spectrum of **70** ($8\mu\text{M}$) ($\lambda_{\text{ex}} = 415\text{ nm}$) with increasing concentration (a) $[\text{poly}(\text{dAdT})]_2$ (0– $83\mu\text{M}$) and (b) $[\text{poly}(\text{dGdC})]_2$ (0– $76\mu\text{M}$) at pH 7.4 **Insets:** Plots of the change in integrated MLCT emission intensity as a function of P/D ratio.

Upon addition of $[\text{poly(dAdT)}]_2$ and $[\text{poly(dGdC)}]_2$ to complexes **70** and **71**, similar behaviour to that seen previously in Chapter 2 for complex **55** was observed, whereby the characteristic $^3\text{MLCT}$ emission bands centred at 645 nm were seen to experience an overall increase upon addition of $[\text{poly(dAdT)}]_2$ while, conversely, the emission was seen to be effectively quenched in the presence of $[\text{poly(dGdC)}]_2$. For **70** in the presence of $[\text{poly(dAdT)}]_2$ an initial slight decrease in emission was followed by a sharp 56% increase culminating in a plateau at $\text{P/D} = 10$, while upon titration with $[\text{poly(dGdC)}]_2$ the emission was efficiently quenched by 62% at $\text{P/D} = 10$. Similarly, in the case of **71** upon titration with $[\text{poly(dAdT)}]_2$, the observed fluorescence at 645 nm was seen to experience an initial slight decrease followed by a subsequent 5% overall increase with the main changes occurring between $\text{P/D } 0 \rightarrow 4$. In a manner similar to **70**, upon addition of $[\text{poly(dGdC)}]_2$, **71** was shown to have its excited state effectively quenched by 54% with the main changes occurring between $\text{P/D } 0 \rightarrow 2$.

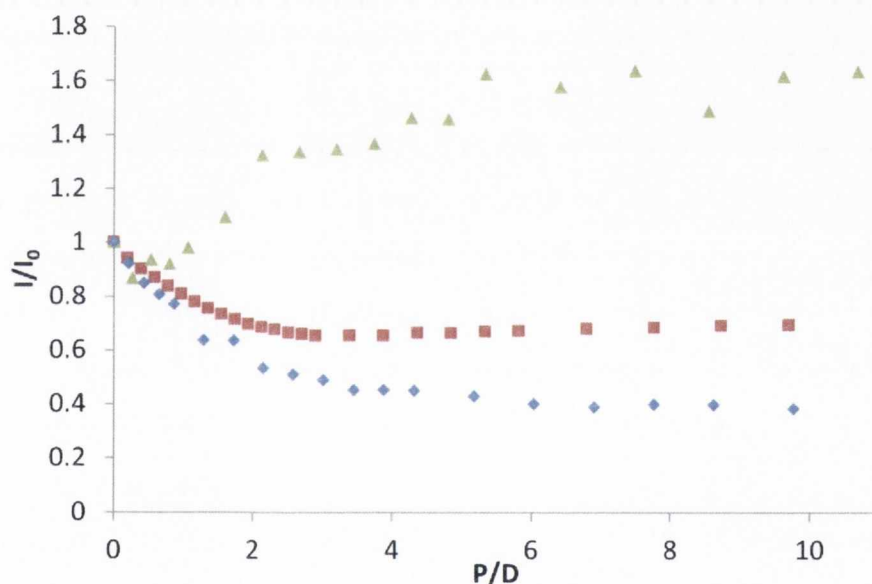


Figure 4.18: Relative changes in the integrated emission intensity of **70** ($8 \mu\text{M}$) ($\lambda_{\text{ex}} 415 \text{ nm}$) with increasing concentration of stDNA(■), $[\text{poly(dAdT)}]_2$ (▲) and $[\text{poly(dGdC)}]_2$ (◆) in 10 mM phosphate buffer at pH 7.4.

The observed behaviour can be explained in a similar manner to that described in Chapter 2, whereby a luminescence enhancement is observed in the presence of $[\text{poly(dAdT)}]_2$ for both **70** and **71**, most likely resulting from shelter from non-radiative deactivation processes of the $^3\text{MLCT}$ excited state afforded by the double helix microenvironment. Similarly, the large decreases of emission observed for **70** and **71** in the presence of $[\text{poly(dGdC)}]_2$ is consistent with the PET process to *guanine* nucleobases, documented for

complexes containing two TAP ligands. For comparison, the emission profiles for **70** in the presence of stDNA, [poly(dAdT)]₂ and [poly(dGdC)]₂ are shown in Figure 4.18.

To summarise, this study has revealed from UV/Vis titrations that both **70** and **71** show negligible sequence specific recognition with no great degree of preferential binding to either [poly(dAdT)]₂ or [poly(dGdC)]₂ in the ground state. Conversely, as expected, the excited state measurements carried out on **70** and **71** showed similar behaviour whereby their emission was slightly increased in the presence of [poly(dAdT)]₂ but decreased in the presence of [poly(dGdC)]₂, consistent with the PET process, observed for complexes containing at least two π deficient ligands. The preceding sections of this Chapter will further explore the binding affinity and modes of interaction of **70** and **71** for DNA through the use of various spectroscopic techniques.

4.4.4 Ethidium Bromide Displacement Assays of **70** and **71**

The binding efficiency of both **70** and **71** was also evaluated by displacement of DNA bound EtBr. The addition of **70** and **71** to a mixture of EtBr and DNA was found to displace 50% EtBr at molar ratios well below one, again, showing that they display much greater affinity for DNA than EtBr. This confirms the high affinity of both complexes for DNA and confirms the results obtained from UV/Visible absorption and emission titrations discussed in the previous sections. As can be seen in Figure 4.19, the addition of **70** and **71** caused the emission intensity of intercalated EtBr to decrease dramatically demonstrating their ability to competitively displace the known DNA intercalator.

The apparent binding constants K_{app} were again calculated according to the competitive binding model described by Boger *et. al.* as demonstrated in Chapter 2. A summary of binding constants derived from these titrations is displayed as an inset in Figure 4.19. From these results it can be concluded that **70** and **71** are capable of displacing EtBr effectively when bound to stDNA, which gives rise to quenching of the EtBr emission. The displacement capabilities correlate well with the behaviour observed in the UV/Vis and fluorescence titrations where the amino substituted complex **71** exhibits a higher affinity, with $K_{app} = 1.90 \times 10^7$, in comparison to its nitro substituted analogue **70** with $K_{app} = 1.66 \times 10^7$. However, such binding constants give only an indication of binding ability and should not be considered quantitative.

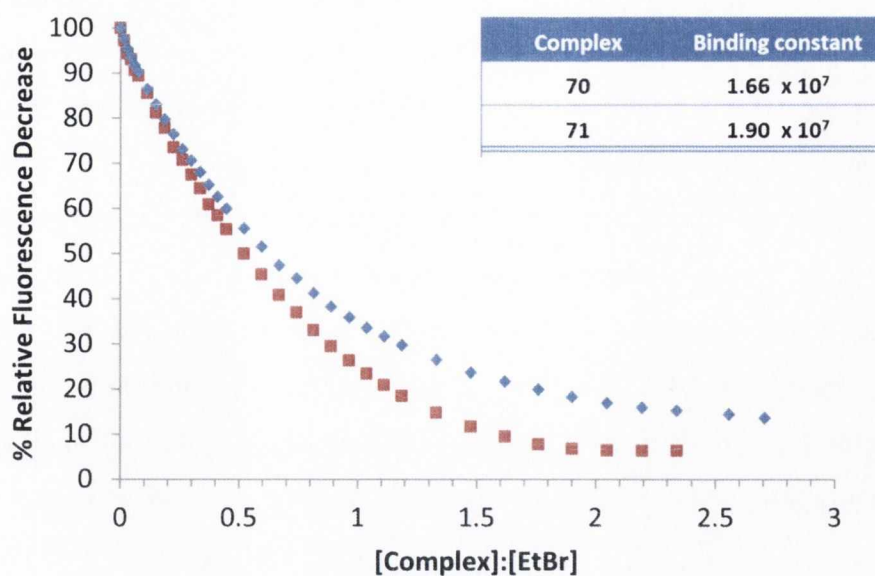


Figure 4.19: The changes in fluorescence of DNA bound EtBr ($\lambda_{ex} = 545$ nm) at 610 nm upon addition of **70** ($\blacklozenge$) and **71** ($\blacksquare$) in 10 mM phosphate buffer, pH 7.4. **Inset:** Table of binding affinities (K_{app}) of **70** and **71** calculated from EtBr displacement assays

4.4.5 Thermal Denaturation Studies of **70** and **71**

Thermal denaturation studies were carried out on **70** and **71** in the presence of stDNA, as in the previous Chapters. In the absence of metal complex the T_m value for DNA was determined to be 68 °C. The melting transition for DNA in the presence of both **70** and **71** showed large perturbations at all P/D ratios evaluated, as seen for **71** in Figure 4.17.

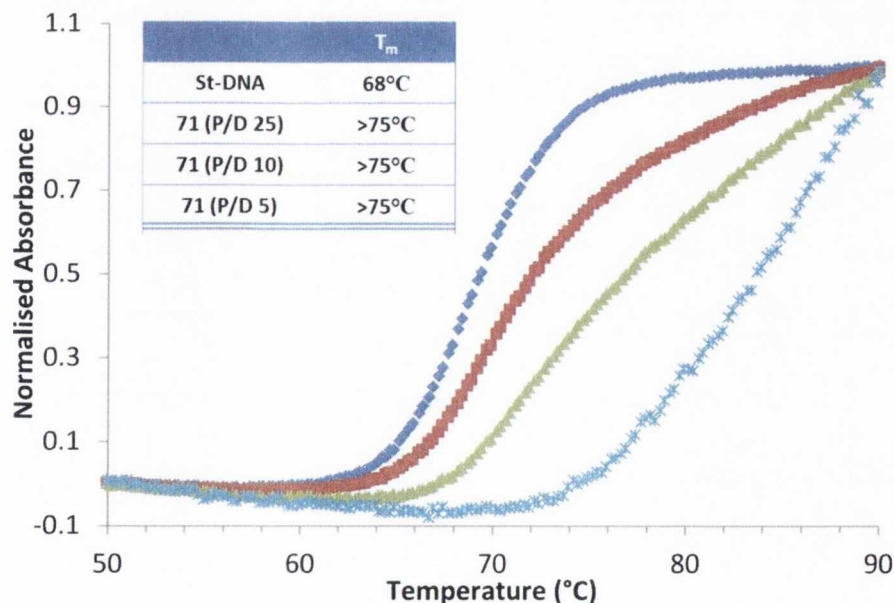


Figure 4.17: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacklozenge$) and presence of **71** at P/D = 25 ($\blacksquare$) P/D = 10 ($\blacktriangle$) and P/D = 5 ($\ast$). **Inset:** The T_m values from first derivative plots of the melting curve.

The changes reflected the stabilisation of the helix structure as a function of complex concentration. At $P/D = 25$, 10 and 5 the melting transition had not gone to completion at 90 °C and consequently, it was not possible to fit the data to a sigmoidal function and as such exact melting values could not be reported, but may be regarded as being greater than 75 °C (Figure 4.17). It is noteworthy that the different extent of the stabilisation between the nitro and amino substituted systems is apparent from these changes, where **71** resulted in a slightly greater shift to higher temperature in the melting profile (Appendix 4). This result is complementary to those results from the UV/Visible absorption and emission studies, and emphasises the higher affinity of this system in comparison to the nitro analogue. Overall these experiments have demonstrated significant stabilisation of the helix associated with binding of these systems and further emphasises the high affinity that they both possess for DNA.

4.4.6 Circular Dichroism Studies of **70** and **71** in the Presence of DNA

In an attempt to further probe the interaction of **70** and **71**, CD titrations were carried out in the same manner as described in previous Chapters, in which the concentration of DNA was kept constant and that of either complex was varied to give a range of P/D ratios. The spectra for **70** are shown in Figure 4.18 with those of **71** shown in Appendix 4. The spectra obtained for both complexes mirror each other and those that were seen for the bipyridine derivatives **49** and **50** studied by Dr. Ryan. Taking **70** as an example, examination of Figure 4.18 reveals a small negative ICD signal at long wavelength, corresponding to both the 1,8-naphthalimide and the MLCT absorption bands.

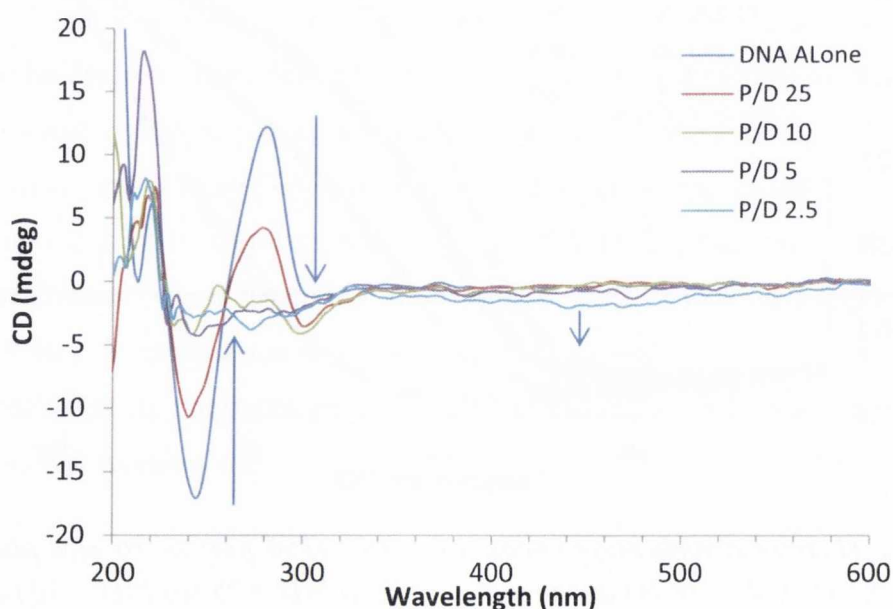


Figure 4.18: CD spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **70** at varying ratios.

Although small in magnitude compared to the complexes **49** and **50** described by Dr. Ryan, it is likely that these changes are due to close association of the metal centre with the grooves, resulting in a slight induced CD signal. Similarly, the CD spectrum of DNA is substantially affected with the characteristic DNA CD signal practically disappearing. As previously stated, this UV region is sensitive to changes in conformation resulting from complex binding, but interpretation of the CD signal in terms of structural changes is not unequivocal. It has been shown that the DNA CD spectrum is significantly altered upon binding of small molecules, possibly due to either the coupling of DNA and complex transitions, or changes in the DNA base coupling resulting from changes in DNA geometry following binding.¹⁹¹ As both complexes exhibit transitions in the UV region, which overlap with the DNA absorption bands, it is difficult to discern if these bands result from an ICD, or from structural changes in the DNA as a result of intercalator-induced unwinding or lengthening of the DNA.

The results from the above CD studies reveal further confirmation of the close association of these species with DNA. Furthermore, the appearance of an ICD signal points to a binding mode in which the metal centre is tightly associated with the helix, thus experiencing its chirality. Further LD studies are expected to yield additional information on the particular binding interaction of both **70** and **71** with DNA.

4.4.7 Linear Dichroism Studies of **70** and **71** in the Presence of DNA

As LD studies had provided strong evidence for the binding mode of species studied in the previous two Chapters it was also decided to undertake LD titrations with complexes **70** and **71** in order to gain a better insight into their specific interaction with the DNA helix. The behaviour of **70** was of particular interest as it showed distinct absorption bands for the 1,8-naphthalimide and Ru(II) polypyridyl portions. Consequently, it was expected that its LD behaviour would give valuable information on the binding geometry of each of the separate functional moieties. The LD studies were carried out in a manner similar to that described previously in Chapter 2 by keeping the concentration of stDNA constant at 150 μ M (OD = 1, at 260 nm) while varying the concentration of **70** and **71**.

The LD spectra resulting from the addition of **70** to stDNA and comparison with its absorption spectrum are shown in Figure 4.19 (a) with those of **71** shown in Figure 4.19 (b). Changes are immediately obvious outside of the DNA absorption region in the case of both complexes, further confirming their strong interaction with DNA in addition to giving information on the specific mode of interaction. In the case of **70**, when stDNA was treated with a low loading of the complex (P/D = 25) a small negative signal is evident at *ca.* 360 nm

suggesting that the 1,8-naphthalimide portion of the complex is lying perpendicular to the DNA helix axis, *i.e.* is intercalated. This observation is further emphasised upon higher loading of **70** ($P/D = 2.5$), whereby a more strongly negative signal is observed at *ca.* 365 nm, the maximum being slightly red shifted by 5 nm. More complex behaviour is seen in the MLCT absorption region of **70** where the broad band is seen to be split in two, with a positive band centred at *ca.* 415 nm and a negative band with a maximum at *ca.* 475 nm. This behaviour would suggest that the positive band at 415 nm is as a result of the MLCT from the Ru(II) centre to the ancilliary TAP ligands lying in the grooves of the DNA helix. Conversely, the negative MLCT band at 475 nm may be associated with an MLCT from the Ru(II) centre to the bipyridine fragment of the naphthalimide ligand, further confirming the proposed intercalative geometry of the 1,8-naphthalimide portion. Similar behaviour was seen for **71** whereby a negative signal is observed at 460 nm at $P/D = 25$ and this negative band exhibits a further decrease at $P/D = 2.5$. The spectra obtained for **71** do not show as structured results however, due to the overlap of both the 1,8-naphthalimide and the MLCT absorption bands. As a result firm conclusions cannot be drawn, however, the negative band centred at *ca.* 460 nm suggests that the 1,8-naphthalimide portion of this conjugate is most likely intercalated between the nucleobases as was seen for **70**.

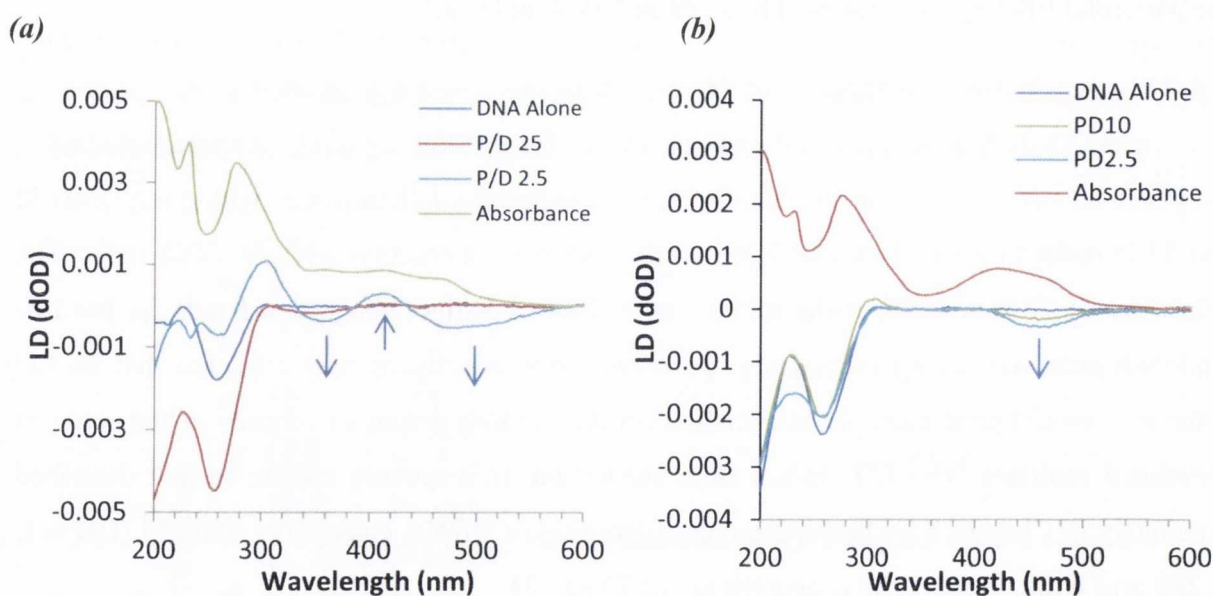


Figure 4.19: LD spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of (a) **70** and (b) **71** at varying ratios.

4.4.8 Summary of DNA Binding Studies of **70** and **71**

As this Chapter aims to identify species with DNA photoreactivity, the sections described have focused on the DNA binding capability of both **70** and **71** in solution. The investigations have been undertaken using stDNA and the homopolymers, $[\text{poly}(\text{dA-dT})]_2$ and

[poly(dG-dC)]₂, at various ionic strengths utilising UV/Vis and fluorescence spectroscopy and supplemented with EtBr displacement assays, T_m experiments, CD studies and LD studies.

The photophysical properties of both complexes were first evaluated in solution before perturbations in the ground and the excited state interactions in the presence of stDNA were observed and quantified. Analysis of the ground state data allowed the calculation of binding constants of the order of 10^6 M^{-1} for both complexes with the amino substituted complex **71** showing a slightly higher binding affinity. Furthermore, both **70** and **71** exhibited excited state quenching upon interaction with DNA attributed to the occurrence of a PET process between the TAP containing complexes and *guanine* nucleobases. Studies in the presence of DNA at high ionic strength also added to the investigation where high salt concentration was seen to affect the binding strength of **70** and **71** but the complexes were seen to remain bound even at high ionic strength ([NaCl] > 250 mM). Studies in the presence of the homopolymers [poly(dA-dT)]₂ and [poly(dG-dC)]₂ revealed negligible sequence specific recognition with both **70** and **71** showing slightly enhanced binding to stDNA over the homopolymers in the ground state. Further evidence for the PET process occurring with **70** and **71** in the presence of *guanine* was provided by the observation that its fluorescence was effectively quenched in the presence of [poly(dG-dC)]₂ and increased in the presence of [poly(dA-dT)]₂.

EtBr displacement assays showed that **70** and **71** are capable of displacing EtBr effectively when bound to stDNA in addition to T_m experiments that found that both complexes were capable of stabilising the helical structures of stDNA. The results from the CD studies revealed close association of both complexes with DNA and showed small ICD signals, pointing to a binding mode in which the metal centre is tightly associated with the helix. Finally, LD studies provided further evidence for the strong interaction of **70** and **71** with DNA and simultaneously confirmed that the π - π^* transition of the 1,8-naphthalimide chromophore of **70** is lying perpendicular to the DNA helix axis and is, thus, most likely intercalated between the base pairs.

The studies carried out above have identified that both complexes bind avidly to DNA concomitantly displaying varying spectroscopic behaviour in response to differing DNA polynucleotides. It is anticipated that both **70** and **71** in addition to binding to DNA may have photoreactivity with the DNA bases due to the π deficient character of their excited states. Equipped with both DNA binding ability and excited state reactivity it is anticipated that **70** and **71** will function as potential DNA photocleavage agents and thus may find application as diagnostic or phototherapeutic agents. The remainder of this Chapter will deal with biological studies including their DNA photocleavage ability as well as their cellular uptake.

4.5 DNA Photocleavage efficiency of **70** and **71**

The DNA photocleavage studies were carried out by treating pBR322 plasmid DNA (1 mg/ml) with each of the complexes **70** and **71** at varying ratios in 10 mM phosphate buffer solution in a similar manner as is described in Chapter 2. Shown in Figure 4.20 is the result of the DNA photocleavage studies. In order to provide a reference point and a comparison with the work carried out by Dr. Ryan,¹⁵⁴ the cleavage efficiency of **70** and **71** was compared to the known DNA photocleavage agent $[\text{Ru}(\text{bpy})_3]^{2+}$ and the $\text{Ru}(\text{bpy})_3$ -1,8-naphthalimide analogues **49** and **50**.

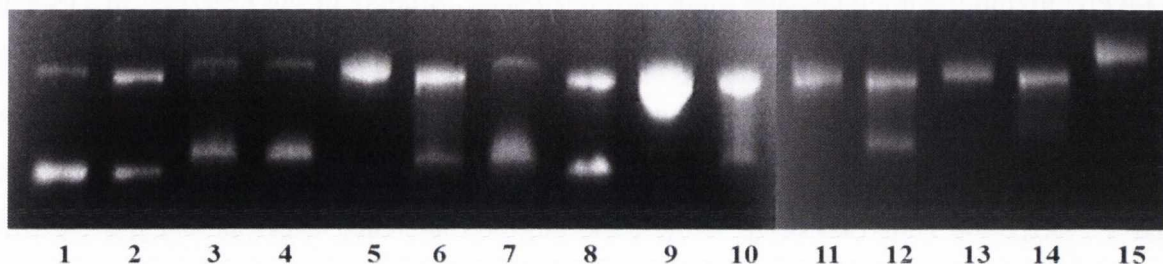


Figure 4.20: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after 30 min irradiation (2 J/cm^2) in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: $\text{Ru}(\text{bpy})_3^{2+}$ (P/D 20); Lanes 3 - 4: **70** and **71** in the dark (P/D 20); Lanes 5 - 6: **70** and **71** (P/D 20); Lanes 7 - 8: **49** and **50** (P/D 20); Lanes 9 - 10: **70** and **71** + NaN_3 (P/D 20); Lanes 11-12: **70** and **71** + D_2O (P/D 20); Lanes 13-14: **70** and **71** (P/D 10); Lane 15: **55** (P/D 20).

The relative intensity of the bands showed that the plasmid DNA was somewhat damaged to begin with, being comprised of 88% supercoiled and 12% open form DNA. The presence of $\text{Ru}(\text{bpy})_3^{2+}$ (P/D = 20) resulted in an increase to 40% open form after irradiation confirming its effectiveness as a DNA photocleaver.

When incubated in the dark neither **70** nor **71** showed any dark state DNA cleavage with 88% supercoiled form being detected in both cases. Moreover, as can be seen in Figure 4.20, lanes 5 and 6, irradiation and subsequent electrophoresis of pBR322 DNA in the presence **70** and **71** at a P/D ratio = 20 showed clear evidence of efficient DNA photocleavage, with **70** showing complete conversion of the plasmid to open form DNA and **71** exhibiting an increase in open form to 75%. Furthermore, when compared to the $\text{Ru}(\text{bpy})_3$ analogues **49** and **50**, which showed 15% and 48% cleavage respectively, both **70** and **71** were found to be more effective photocleavage agents. When irradiated in the presence of the known $^1\text{O}_2$ scavenger, NaN_3 (Lanes 9 and 10), it was observed that the photocleavage efficiencies of **70** and **71** were not reduced. Similarly, in D_2O , in which the lifetime of $^1\text{O}_2$ is significantly extended, the cleavage efficiency measured for **70** and **71** (Lanes 11 and 12) gave similar results to the damage observed for sample irradiated in phosphate buffer. Increasing the amount of **70** and **71** (P/D = 10) had little effect on the results obtained for **70** but slightly

increased the cleavage exhibited by **71**. Significantly, as seen in Lane 15, the cleavage efficiency of complex **55** discussed in Chapter 2 was seen to have a comparabale photocleavage efficiency to **70** suggesting its utility for phototherapeutic applications. The relative amounts of supercoiled vs. open form DNA are summarised in Table 4.7 and a representative graph is shown in Figure 4.21.

Table 4.7: Summary of percentages of supercoiled vs. open form pBR322

Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	12.5	87.5	9	100	0
2	39.3	60.7	10	89.1	10.9
3	12.1	87.9	11	100	0
4	11.3	88.7	12	73.4	26.6
5	100	0	13	100	0
6	74.8	25.2	14	91.90	8.1
7	14.9	85.1	15	100	0
8	47.5	52.5			

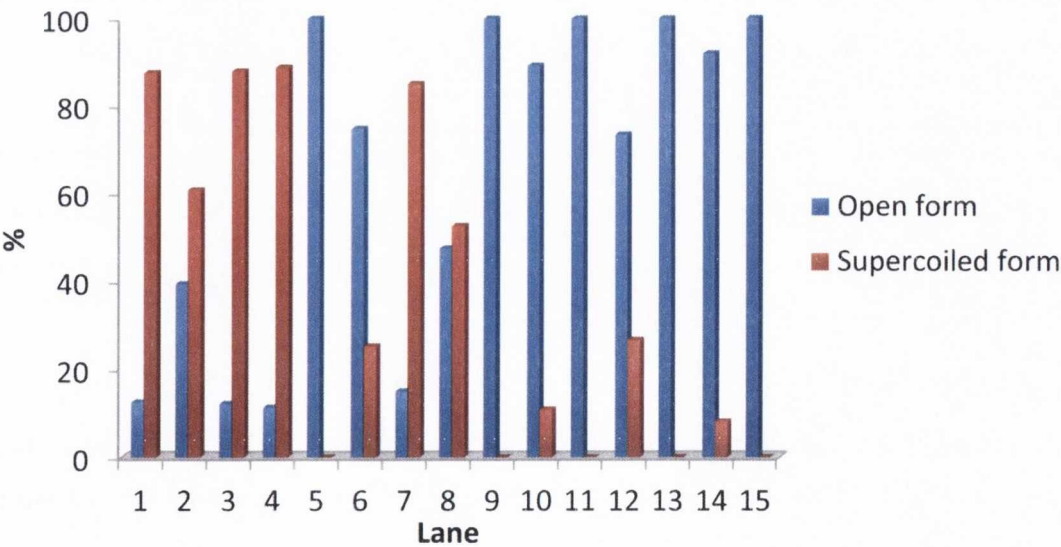


Figure 4.21: Relative percentages of supercoiled vs. open form pBR322 plasmid DNA from the photocleavage study of **70** and **71**

From the above results it is clear that the Ru(TAP)₂(bpy)-1,8-naphthalimide derivatives **70** and **71** display DNA photocleavage with efficiencies greater than those of [Ru(bpy)₃]²⁺ and the Ru(bpy)₃-1,8-naphthalimide derivatives **49** and **50** described by Dr. Ryan.¹⁵⁴ In addition, it appears that the nitro derivative, **70** is a more effective photocleaver than its amino substituted analogue **71**; most likely due to its increased Φ_F value as described in Section 4.3. Moreover, the photocleavage efficiencies of **70** and **71** were not affected to any great extent when irradiated in the presence of either NaN₃ or D₂O suggesting that ¹O₂ formation may not be the primary mechanism by which these complexes exert their activity.

Having established the efficient excited state reactivity and photocleavage ability of **70** and **71** the next step was to monitor the cellular uptake properties of these complexes within target cancer cell lines. The preceding section will attempt to reveal their cellular localisation.

4.6 Preliminary Cellular Uptake and Localisation Studies of **70** and **71**

The ability of **70** and **71** to be internalised in target cells is of paramount importance from the point of view of both diagnostics and therapeutics. Furthermore, due to the observed photocleavage ability of both complexes demonstrated in the previous section, access to cellular DNA would provide a novel and viable mechanism of action for new phototherapeutic candidates. As mentioned in Chapter 1, the work of Dr. Ryan and Dr. Frímannsson uncovered the ability of the Ru(bpy)₃ analogues of **70** and **71**, **49** and **50**, to selectively localise within the nucleus of cervical cancer HeLa cells *in vitro*.^{103,154} This was proposed to be one of the first examples of a Ru(II) complex located inside the nucleus of target cells as traditionally this property had inhibited the potential use of Ru(II) complexes as imaging agents and PDT candidates.^{214,215} The ability of **49** and **50** was tentatively ascribed to be due to the increased lipophilicity of these systems afforded by the inclusion of the 1,8-naphthalimide chromophore.¹⁰³ Taking into account the observed nuclear localisation of **49** and **50** a similar study of **70** and **71** was undertaken in collaboration with the Williams research group in the School of Biochemistry and Immunology at Trinity College Dublin.

Confocal microscopy was carried out in order to provide visual evidence of the localisation of **70** and **71** in cells. Populations of live HeLa cells (0.5×10^5) were incubated with **70** and **71** (100 μ M) at 37 °C for 4 hrs before being treated with the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope with a 60 $\times$ oil immersion lens. The results obtained are exemplified in Figure 4.22, which shows the fluorescence confocal laser scanning microscopy images, of live HeLa cells after incubation with **71** (the corresponding images for **70** are shown in Appendix 4). The observed images show the majority of cells exhibiting a large single intense fluorescent spot associated around

the nucleus with the appearance of ‘bean-shaped’ nuclei as seen in Chapter 2. Furthermore, both **70** and **71** appear to localise inside the nucleus, where fluorescence is observed from within the nuclear membrane, implied by the appearance of co-localisation with the nuclear stain DAPI. Interestingly, the fluorescence intensity measured within the nucleus is of much lower intensity in comparison to that observed surrounding the nuclear membrane. Such behaviour may imply the occurrence of photoredox processes between **70** and **71** the nuclear DNA causing the emission intensity of these complexes to be effectively quenched as was observed *in vitro* during the excited state DNA titrations.

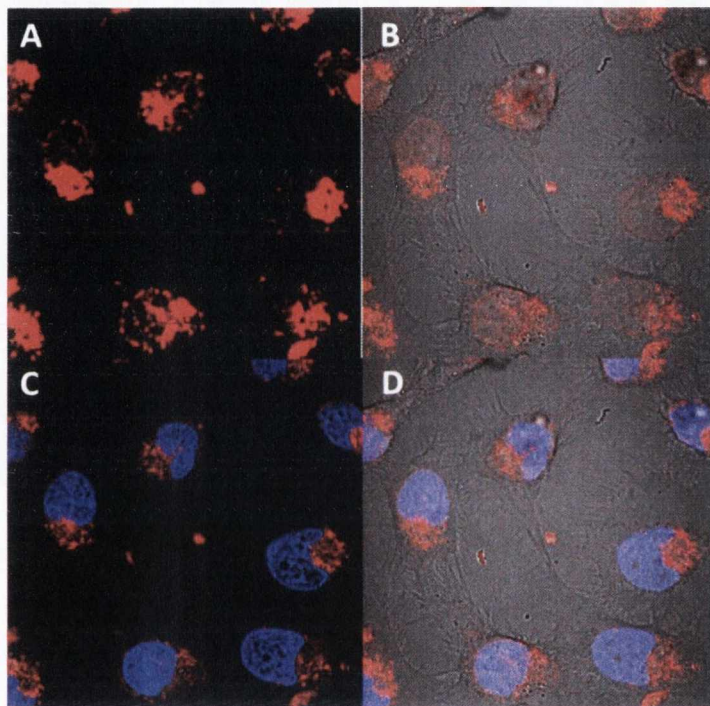


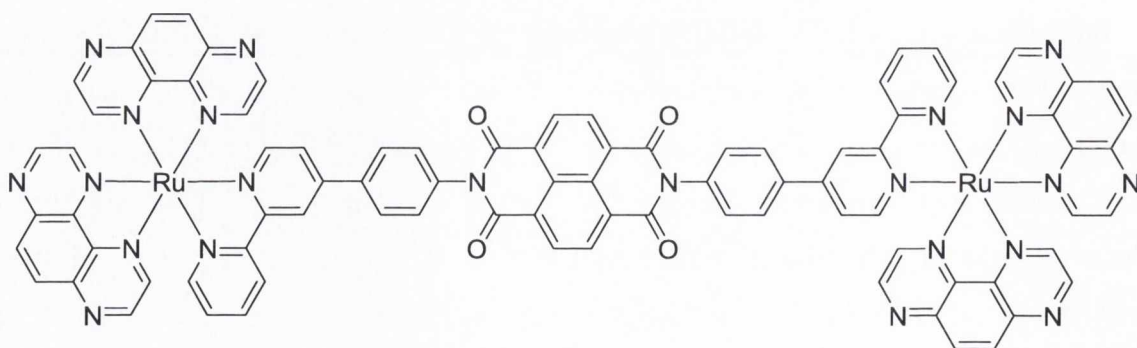
Figure 4.22: Confocal Laser Scanning Microscopy live cell images of **71** (50 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **71** (red), (B) overlay of **71** (red) and the bright field view, (C) overlay of **71** (red) and nuclear co-stain DAPI (blue) and (D) overlay of **71** (red), nuclear co-stain DAPI (blue) and the bright field view after 4 hrs of incubation.

With the observed DNA photoreactivity and nucleus localisation of **70** and **71**, their potential as PDT agents is clear. Such complexes could effectively damage DNA upon light irradiation causing apoptosis and leading to new unexplored families of phototherapeutic agents. The next step in this research will be to assess the dark and light cytotoxicity of complexes **70** and **71** in a similar manner to that outlined in Chapter 2, to establish their potential for use as PDT agents. These studies will be carried out the Williams research group and the results will be outlined in detail in the PhD Thesis of Ms. Erby. The following section will look to summarise the research and findings carried out in this Chapter.

4.7 Conclusions and Future Work

The results presented in this Chapter have further contributed to the library of bifunctional Ru(II)-1,8-naphthalimide conjugates developed within the Gunnlaugsson research group. We have demonstrated that **70** and **71** show water solubility and a distinctive set of photophysical properties that has allowed the nature of their interaction with DNA to be probed by various ground and excited state titrations. From these titrations DNA binding affinities of **70** and **71** with stDNA, [poly(dA-dT)]₂ and [poly(dG-dC)]₂ have been evaluated as being of the order of 10^6 M^{-1} . Furthermore, given the π deficient character of **70** and **71**, they have been shown to exhibit highly oxidising excited states which are effectively quenched by redox interactions with *guanine* sites on DNA. It has also been confirmed by EtBr displacement assays that **70** and **71** had a high affinity of binding for DNA, with binding constants in the range $10^6 - 10^7 \text{ M}^{-1}$. Similarly, the tight binding, typical of intercalative interaction was also apparent from thermal denaturation studies, where large shifts were observed in the T_m values of stDNA in the presence of both **70** and **71** at various binding ratios. From both circular and linear dichroism studies an overall binding mode for conjugates **70** and **71** with DNA was proposed, in which insertion of the 1,8-naphthalimide into the helix occurs with concomitant external association of the Ru(II) centre; a binding mode also proposed for **49** and **50** by Dr. Ryan.¹⁵⁴

Due to the excellent DNA binding ability exhibited by **70** and **71**, their ability to act as DNA photocleavers was also examined where both species were found to cleave supercoiled DNA with much increased efficiencies compared to the Ru(bpy)₃-1,8-naphthalimide analogues **49** and **50**. Furthermore, with the ultimate aim of applying them to phototherapeutic applications their cellular uptake ability was examined, with both species being found to accumulate in the cytoplasm and nucleus of HeLa cells, showing characteristic red MLCT emission.



79

4+

Future perspectives of this work will look to further evaluate the therapeutic potential of **70** and **71** where their ability to induce light activated cell death will be analysed using various biochemical techniques such as Alamar Blue cytotoxicity assays and FACS analysis, the results of which will be reported in the PhD Thesis of Ms. Erby. Moreover, development of bimetallic Ru(II) analogues such as **79** may give rise to complexes with increased photocleavage efficiency and, consequently, light activated cell cytotoxicity.

Chapter 5:

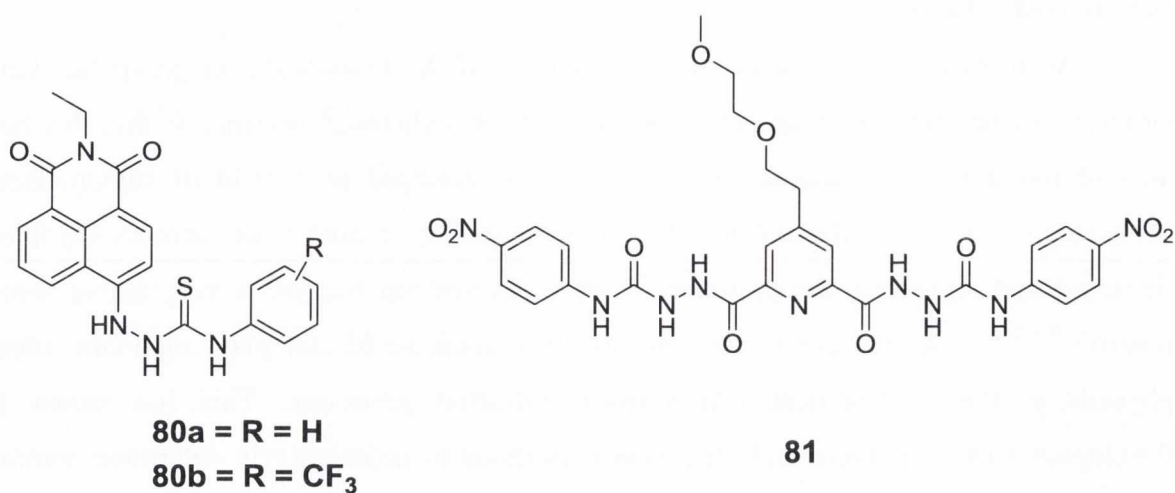
A Ru(II)-Mono-1,8-Naphthalimide Conjugate for Colorimetric and Luminescence Anion Sensing

5.1 Introduction:

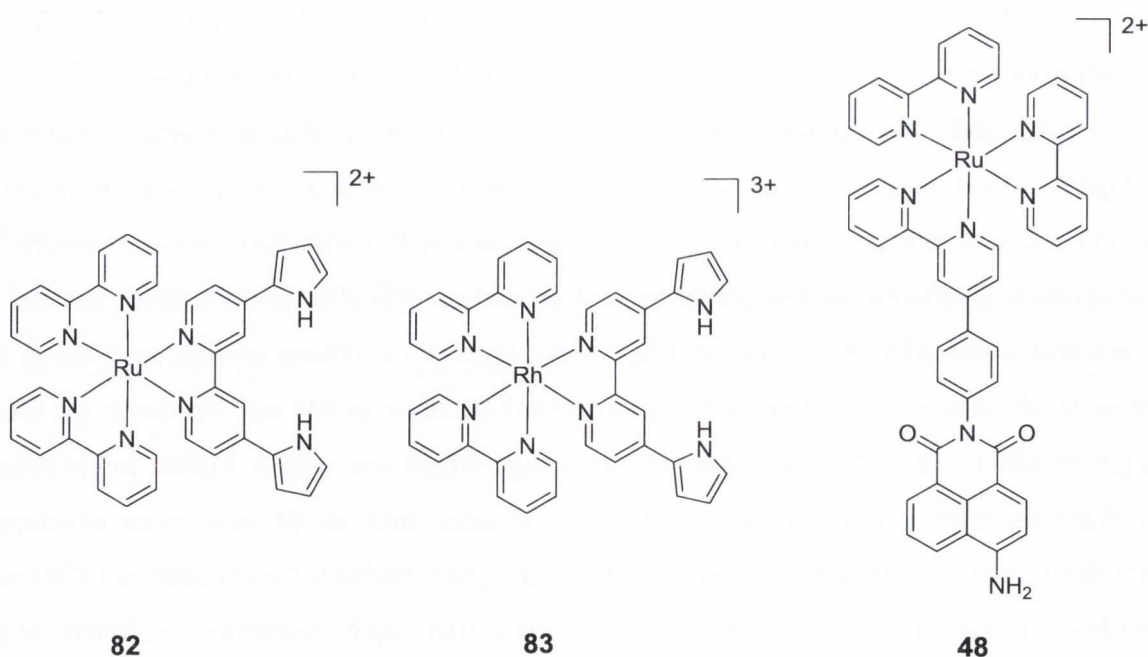
As highlighted in Chapter 1, the research of the Gunnlaugsson group has strong emphasis on the field of supramolecular chemistry and chemical sensing. Within this broad area of research anion sensing, in particular, has emerged as a field of supramolecular chemistry in its own right and the design of molecular receptors and sensors capable of detecting and signalling the presence of these species has become a very active area of research.^{96,216,217} Anions are everywhere in the natural world and play important roles in physiology, the environment and various industrial processes. This has meant that development of convenient and inexpensive methods to quantitatively determine anions in various environments is an important goal within many scientific disciplines such as industry, diagnostics, therapeutics and environmental/pollution control. For practical applications, these sensors also need to satisfy strict photophysical criteria, such as being able to absorb and emit light at long wavelengths to facilitate the use of naked eye detection or the use of inexpensive optics.⁹⁶

Due to its unique photophysical properties, the 1,8-naphthalimide structure has found application in many areas of chemistry such as the development of dyes, DNA sensing and anti-cancer agents.^{97,153,155} Its absorption and fluorescence emission spectra lie within the UV and visible regions, and the various photophysical properties can be easily fine-tuned through simple structural modifications. Consequently, the 1,8-naphthalimide structure has found extensive use within the field of anion recognition and sensing, and in the last number of years many examples of naphthalimide-based anion sensors have been published, clearly demonstrating the versatility of this structure within this fast growing field of research.⁹⁶

Compounds **80a** and **80b** were some of the first examples of such structures and were developed by Gunnlaugsson *et al.* where they were shown to be some of the first colorimetric and luminescence anion sensors that could function in highly competitive aqueous media.¹⁴⁶ These systems displayed defined photophysical properties with absorption maxima centred at 441 nm and a shoulder at 560 nm which, upon additions of various anions, resulted in an increase in absorbance at 560 nm and a concomitant decrease at 441 nm. Furthermore, these changes resulted in a yellow to purple colour change which was clearly visible to the naked eye. More recently, glycol based amidothiourea sensors such as **81** have been developed which show improved solubility in competitive hydrogen bonding solvents such as EtOH and EtOH/H₂O mixtures.²¹⁸ Moreover, it was shown that such receptors exhibited large spectroscopic modulations upon titration with biologically relevant anions such as adenosine triphosphate (ATP) and were also accompanied by colorimetric changes.



The use of transition metal ion-based polypyridyl complexes in anion sensing is another well-established field of research and a number of examples have been reported recently.^{197,201} Sessler and co-workers have used cationic anion receptors, based on the use of pyrrole-substituted bipyridine coordinated to Ru(II) and Rh(III) polypyridyl cores which have been functionalised to generate an anion binding site.¹⁹⁴ The ¹H NMR anion-binding studies in DMSO-*d*₆ revealed that **82** and **83** bind most simple anions with high affinity. In the case of chloride anions, structural studies, carried out by means of single-crystal X-ray diffraction analyses, were consistent with the solution-phase results and revealed that receptors **82** and **83** are both able to form stable complexes with this halide anion in the solid state.



As mentioned in Chapter 4, **48** developed by Ryan¹⁵⁴ in our own research group binds strongly to DNA in competitive media, where it exhibited concomitant changes in the ground

and the excited state properties of both the 1,8-naphthalimide and the Ru(II) metal to ligand charge transfer (MLCT) transitions.¹⁵⁵ Duke²¹⁸ and Ali²¹⁹ have shown that the interaction of some anions at the 4-amino position of the 1,8-naphthalimide chromophore results in significant modulations of its photophysical properties through both hydrogen bonding and deprotonation. Hence, we anticipated that **48** might also function as an anion sensor where, through modulations of the energy transfer mechanism from the naphthalimide moiety to the Ru(II) MLCT excited state, spectroscopic and colorimetric changes might occur. While the sensing of biologically relevant anions should ideally be achieved using water soluble agents/sensors, it is often carried out in organic media.^{206,207,220} In solvents such as DMSO, CHCl₃ and MeCN various recognition processes, including deprotonation, can take place while these processes cannot be studied in protic solvents.

This Chapter will detail the photophysical evaluation of **48** in MeCN and an investigation into the ability of complex **48** to bind and sense various anions using spectroscopic methods such as UV/Vis absorption and fluorescence spectroscopy. The results of these studies are presented in this Chapter. The synthesis and characterisation of **48** has been previously reported and thus will not be described herein.^{154,155}

5.2 Photophysical Evaluation of **48**

The titrations of **48** were carried out in MeCN and as such their photophysical behaviour was firstly evaluated in this solvent so that changes in its photophysical properties could be monitored upon titration with various anions.

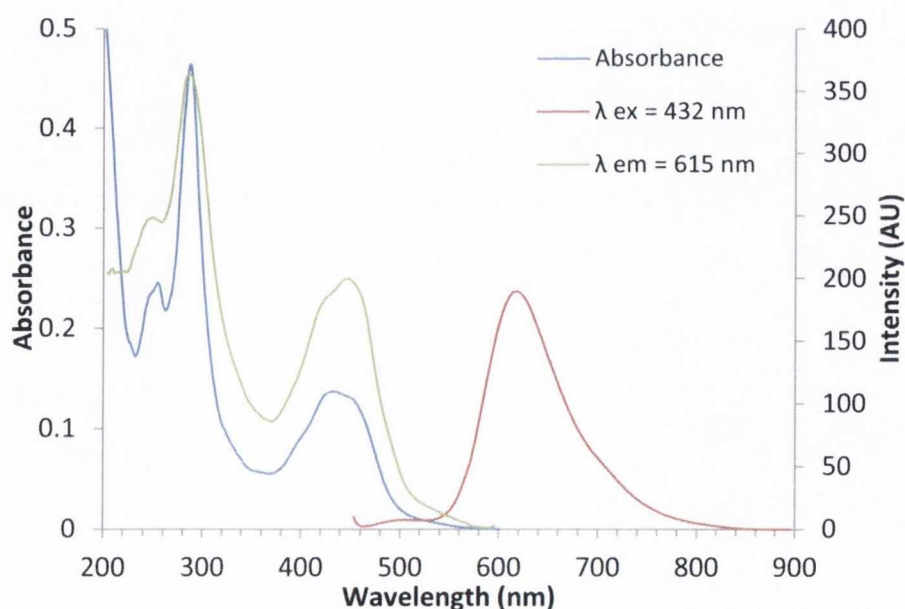


Figure 5.1: UV/Visible absorption, fluorescence excitation and emission spectra of **48** (10 μ M) in MeCN

The absorption, and the fluorescence excitation and emission spectra of **48**, recorded in MeCN are shown in Figure 5.1. The absorption spectrum of **48** gave rise to a broad band centred at 432 nm ($\epsilon = 11,700 \text{ M}^{-1} \text{ cm}^{-1}$), assigned to both the 4-amino-1,8-naphthalimide and the Ru(II) MLCT transitions and a narrower band at 290 nm ($\epsilon = 39,350 \text{ M}^{-1} \text{ cm}^{-1}$), attributed mainly to $\pi\text{-}\pi^*$ intra-ligand transitions. Excitation of **48** at both 290 nm and 432 nm resulted in essentially no emission being observed from the 1,8-naphthalimide portion, but a strong MLCT based emission band at 615 nm was observed. Considering excitation at 432 nm should address both excited states, the lack of any naphthalimide based emission suggests that an efficient energy transfer mechanism exists from the naphthalimide excited state to that of the MLCT state in MeCN. Further investigation of the excited states of **48** revealed an excitation spectrum identical to its absorption spectrum when the emission wavelength was fixed at 615 nm.

Having characterised the photophysical properties of **48** intense, long wavelength, MLCT based emission was seen to offer this system potential as a fluorescent anion sensor. Furthermore, it was anticipated that the brightly coloured solutions of **48** might offer colorimetric modulations upon interaction with various anions. The following section will discuss the ability of **48** to sense anions such as acetate (AcO^-), phosphate (H_2PO_4^-), chloride (Cl^-) and fluoride (F^-) some of which have the ability to function as effective bases in aprotic solution.

5.3 Spectroscopic Titrations of **48** with Anions

Spectroscopic anion titrations were conducted using both UV/Vis absorption and fluorescence emission titrations. Solutions of AcO^- , H_2PO_4^- , Cl^- and F^- as their tetrabutylammonium (TBA) salts in MeCN were titrated against a solution of **48** ($1 \times 10^{-5} \text{ M}^{-1}$) in MeCN. The changes observed were measured and quantified and all titrations were repeated to ensure reproducibility.

5.3.1 Preliminary UV/Vis Absorption and Emission Titration of **48** with Anions

In order to gain an immediate insight into the effect of various anions on the photophysical properties of **48**, an excess of each anion (500 eq.) was added and the changes in the ground and excited states were monitored.

Examination of the resulting spectra saw small changes being observed in the absorption spectrum of **48** in the presence of anions such as AcO^- and Cl^- , but with more significant changes being exhibited upon addition of F^- , where the long wavelength band was red shifted, and the appearance of a shoulder, centred at *ca.* 340 nm, was observed. This

shoulder was assigned to the naphthalimide $\pi-\pi^*$ transition and the bathochromic shift was deemed to be indicative of interaction of the anion with the amino 1,8-naphthalimide chromophore (Figure 5.2). The transitions were also clearly visible in the excitation spectrum where, again, the changes were greatest upon addition of F^- (see Appendix 5). Interestingly, the changes exhibited in this study were somewhat smaller than previously seen for such 1,8-naphthalimide-based sensors that possess acidic protons that can be deprotonated.²²¹

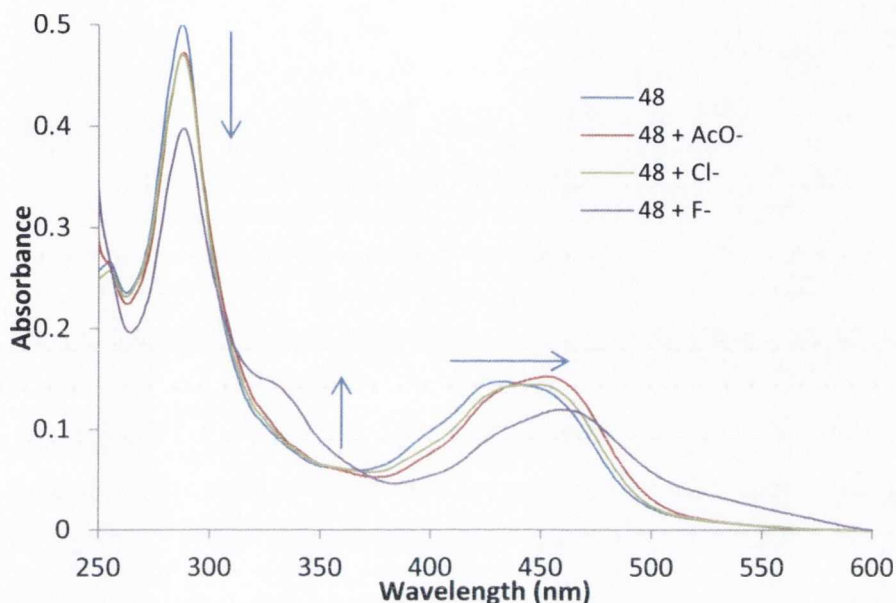


Figure 5.2: The changes in the absorption spectra of **48** in MeCN in the absence and presence of AcO^- , Cl^- and F^- as their TBA salts (500 eq.)

In contrast to the ground state results, more pronounced changes were observed in the emission spectrum of **48** in the presence of various anions (Figure 5.3). As stated above, excitation at 432 nm gave rise to the formation of a long wavelength emitting and broad emission band centered at 615 nm. Upon addition of the aforementioned anions, considerable changes were seen in this MLCT region, being most pronounced for F^- , where the emission was quenched by *ca.* 90%, while AcO^- and Cl^- gave rise to 73% and 39% emission quenching, respectively. Furthermore, no noticeable shifts were observed in the emission λ_{max} upon addition of these anions. In contrast, ions such as Br^- and I^- did not give rise to any changes in the absorption spectrum of **48**, and the fluorescence emission was only marginally quenched (less than 5%); excluding anion recognition by heavy atom effect quenching.²¹⁹ These spectroscopic studies were also carried out in DMSO and on all occasions, the same behaviour was observed as seen in MeCN mentioned above.

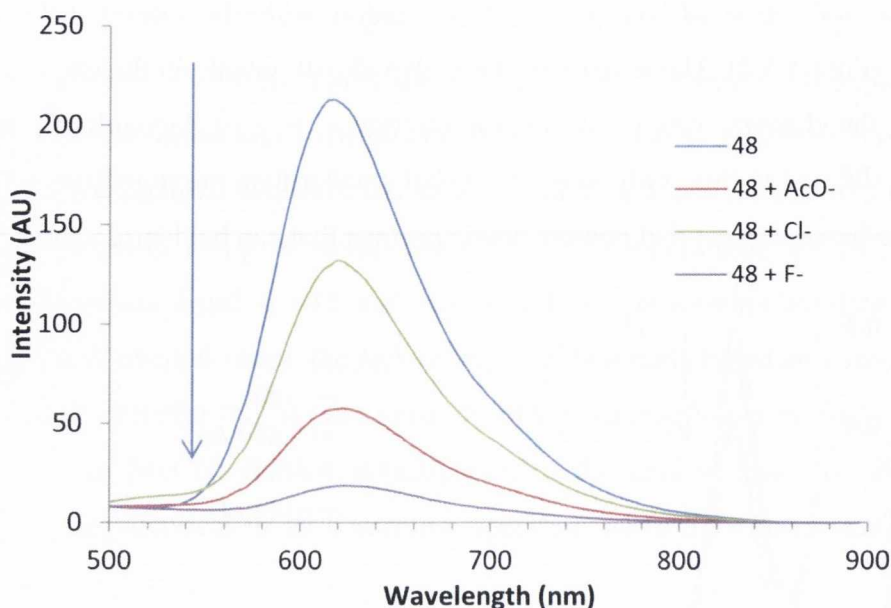


Figure 5.3: The changes in the fluorescence spectra of **48** (λ_{ex} 432 nm) in MeCN in the absence and presence of AcO^- , Cl^- and F^- as their TBA salts

Having established that these anions gave rise to slight changes in the UV/Vis absorption and to significant modulation of the MLCT-based emission spectrum of **48**, detailed anion titrations were carried out, observing the changes in both the ground and the excited state properties. The results of these detailed titrations and investigation into their host:guest stoichiometries are detailed in the following sections.

5.3.2 Absorption and Fluorescence Titrations of **48** with AcO^-

Detailed absorption titrations were firstly carried out with **48** in the presence of AcO^- . In agreement with the preliminary studies, the changes in the UV/Vis absorption spectrum were minor with just 20% hypochromism being observed at both 290 nm and 432 nm following the addition of 850 eq. of the anion (Appendix 5). Conversely, the fluorescence emission spectrum underwent more pronounced modulation, with addition of AcO^- giving rise to 70% emission quenching, as shown in Figure 5.4. No other significant changes were observed, such as changes in the shape or position of the λ_{max} of the spectra. The resulting emission quenching is typical of a photoinduced electron transfer (PET) to the excited MLCT state from the anion complexed naphthalimide, a phenomenon widely seen for naphthalimide based systems.⁹⁶

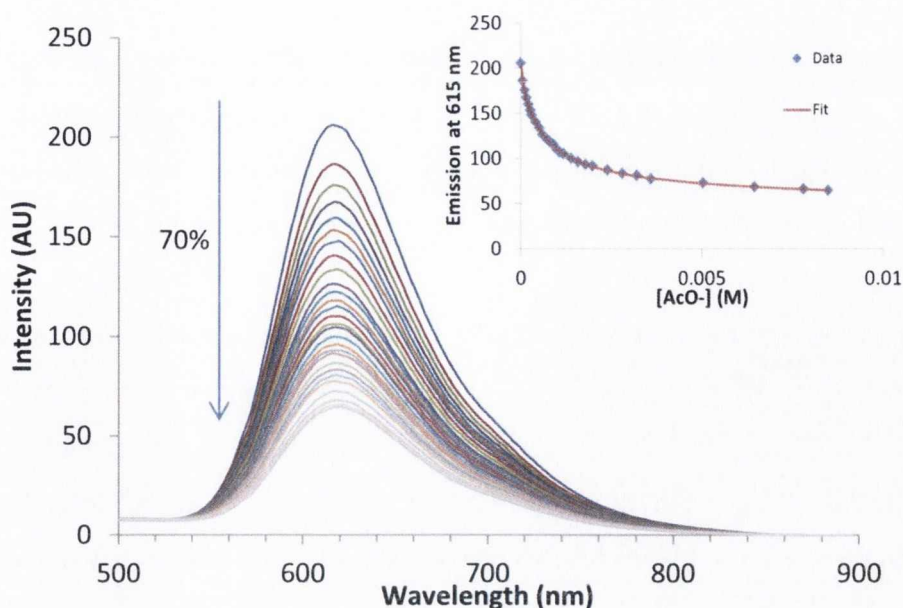


Figure 5.4: The changes in the emission spectrum of **48** (1.15×10^{-5} M) in MeCN upon titration with AcO⁻ (0→850 eq., [9 mM]). Inset: The binding isotherm with fit determined from the titration data at 615 nm, using SPECFITTM.

In order to gain information on the host:guest stoichiometries of **48** with AcO⁻ the changes in the fluorescence spectra were fitted using the non-linear regression analysis software program SPECFITTM. The data was best fitted to both 1:1 and 1:2 (Host:Guest) binding interactions, giving binding constants of $\log K_{1:1} = 4.25 (\pm 0.065)$ and $\log K_{1:2} = 3.11 (\pm 0.081)$. The speciation distribution diagram for this titration is shown in Figure 5.5, and clearly demonstrates an initial 65% formation of the 1:1 complex at low equivalents of AcO⁻ (0 → 30 eq.) which leads to 92% formation of the 2:1 complex after addition of very large equivalents of anion (850 eq.). In order to demonstrate the versatility of **48** as a fluorescent sensor detailed titrations of various other anions were also undertaken. The results of these

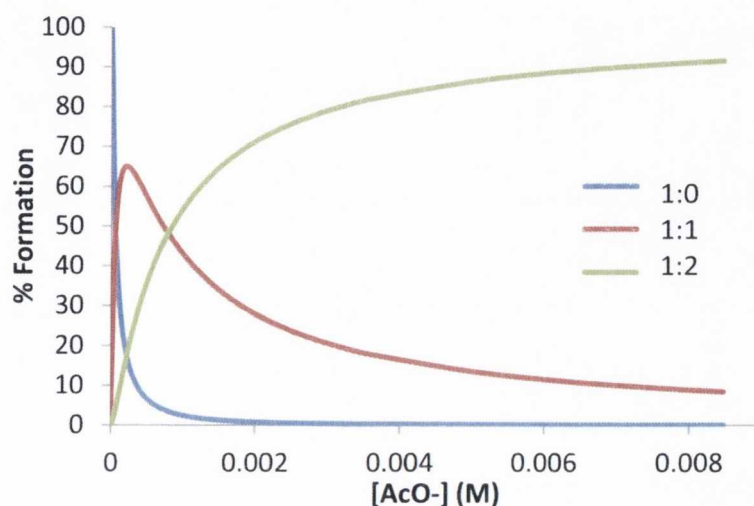


Figure 5.5: The speciation distribution diagram for the binding of **48** with AcO⁻

titrations will be outlined and discussed in the next sections.

5.3.3 Absorption and Fluorescence Titrations of **48** with H_2PO_4^-

In comparison to the minor changes observed in the UV/Vis absorption spectrum of **48** upon addition of AcO^- , more significant changes were exhibited in the presence of H_2PO_4^- . The long wavelength absorption band at 432 nm experienced a bathochromic shift of *ca.* 14 nm with a concomitant hypochromism of 28%. Similarly, the $\pi\text{-}\pi^*$ intraligand band centred at 290 nm also experienced both a red shift of *ca.* 5 nm and a hypochromism of 25% (Figure 5.6). This red shift at longer wavelength is indicative of interaction of the 4-amino naphthalimide moiety with H_2PO_4^- , most likely through hydrogen bonding interactions. Similarly, the fluorescence emission spectrum of **48** was also seen to be bathochromically shifted by *ca.* 20 nm from 615 nm to 635 nm upon addition of large equivalents of H_2PO_4^- (see Appendix 5). Furthermore, 61% quenching of the MLCT excited state was also observed, again, indicating the occurrence of PET processes.

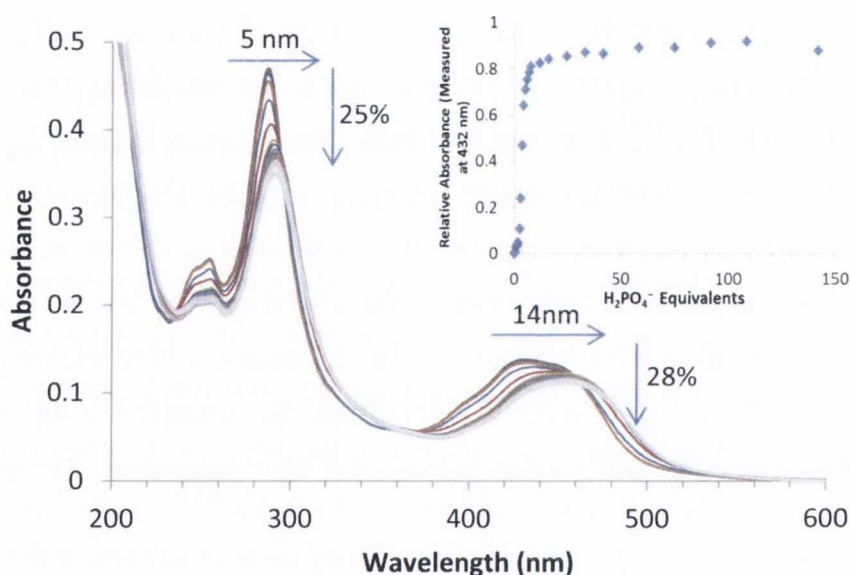


Figure 5.6: The changes in the absorption spectrum of **48** (1.18×10^{-5}) in MeCN upon titration with H_2PO_4^- (0 $\rightarrow$ 615 eq., [6.6 mM]). Inset: The relative absorption decrease at 432 nm as a function of H_2PO_4^- concentration (0 $\rightarrow$ 150 eq.).

The data from the fluorescence titrations was fitted using SPECFITTM in a similar manner to that of AcO^- above. The changes were best fitted using 1:1 binding stoichiometry, giving $\log K_{1:1} = 4.52 (\pm 0.084)$, the corresponding fit and speciation distribution diagram being shown in Figure 5.7. From this investigation it is clear that the 1:1 complex forms rapidly and exclusively with 95% formation occurring after the addition of just 15 eq. of H_2PO_4^- . The differing results to AcO^- , in both the ground and excited state titrations, support this rationalisation and suggest that different modes of interaction are occurring between **48**

and both AcO^- and H_2PO_4^- . This study was continued with the investigation of the ability of **48** to detect Cl^- . The changes observed are detailed and explained in the following section.

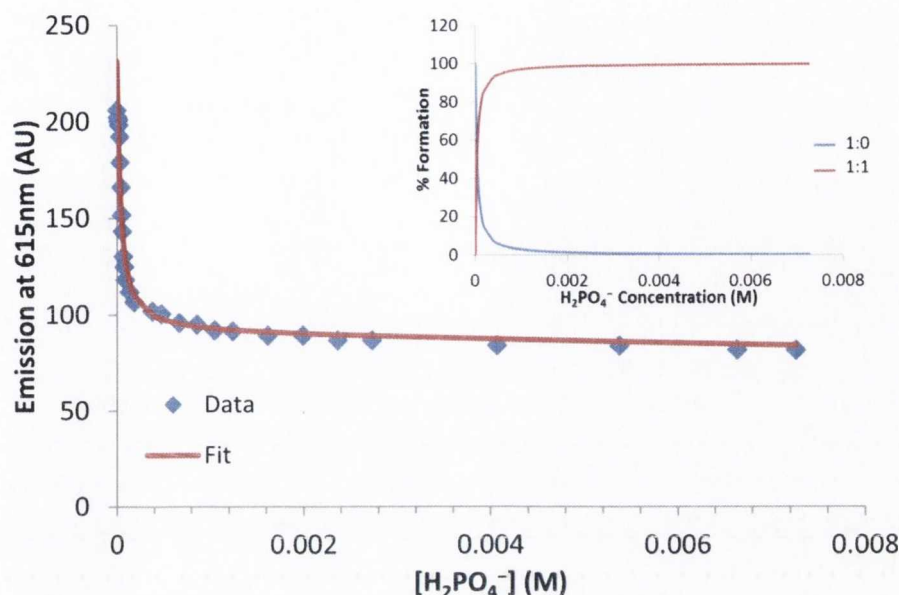


Figure 5.7: The best fit of the emission titration data measured at 615 nm. Inset: The corresponding speciation distribution diagram for the binding of **48** with H_2PO_4^-

5.3.4 Absorption and Fluorescence Titrations of **48** with Cl^-

The changes observed in the UV/Vis absorption spectrum of **48** upon addition of Cl^- were minor, resulting in a small hypochromism of just 12% at both 290 nm and 432 nm with no shifts in maximum absorbance (see Appendix 5). Such behaviour would imply very weak interaction between **48** and the Cl^- anions, possibly due to its large size and spherical shape. Moreover, changes in the fluorescence emission were also seen to be of smaller magnitude than those seen for both AcO^- and H_2PO_4^- (Appendix 5). A fluorescence quenching of 32% was exhibited, further suggesting that, although weak, some form of interaction is taking place between **48** and the Cl^- anions.

Despite their small magnitude, it was possible to fit these changes, again, using SPECFITTM and were best fitted to 1:1 stoichiometry, giving $\log K_{1:1} = 2.90 (\pm 0.056)$. Although no red shift was observed in either the ground or excited state titrations, a similar host:guest stoichiometry to H_2PO_4^- was seen for Cl^- (Figure 5.8). However, these changes occurred over a much larger range, further indicating the enhanced binding affinity of **48** for H_2PO_4^- over Cl^- .

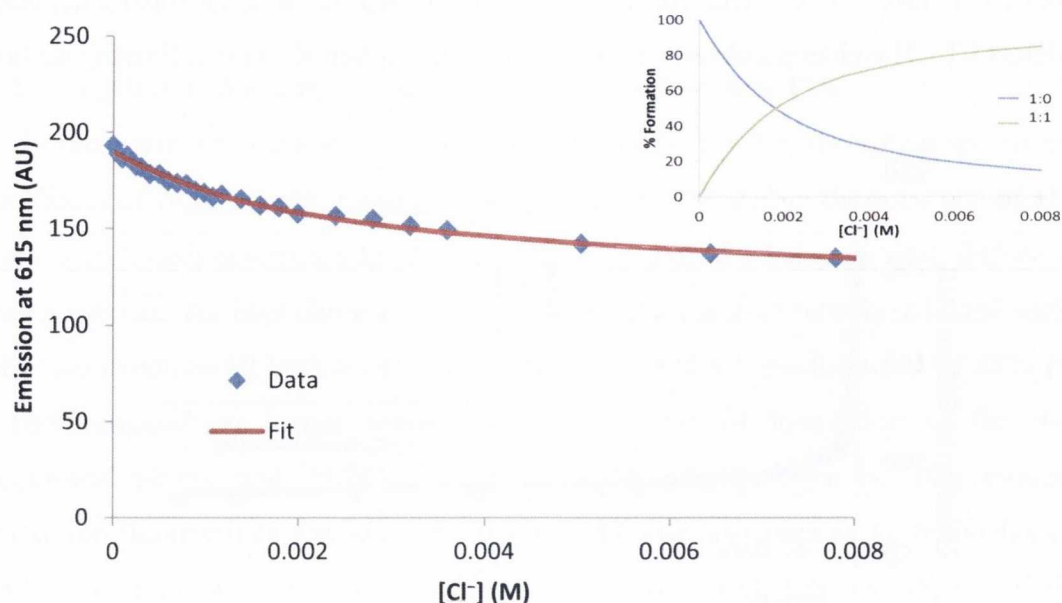


Figure 5.8: The best fit of the emission titration data measured at 615 nm. Inset: The corresponding speciation distribution diagram for the binding of **48** with Cl^-

Having observed large spectroscopic responses of naphthalimide based sensors to the F^- anion in the past,^{150,222} due to deprotonation of the amino moiety, and having seen large changes in the preliminary titrations with **48**, it was next decided to quantify the interaction of F^- with **48** by carrying out detailed titration studies. The results of this investigation are outlined in the next section.

5.3.5 Absorption and Fluorescence Titrations of **48** with F^-

In comparison to the results obtained with **48** and the various anions investigated above, more significant changes were seen in both the UV/Vis absorption and the fluorescence emission spectrum of **48** upon interaction with F^- . The MLCT absorption of **48** underwent a more significant change in the absorbance maximum from 432 nm to 460 nm, and a concomitant hypochromism of 45% at 432 nm upon addition of F^- . Furthermore, at lower wavelengths a hypochromism of 33% was exhibited in the the $\pi-\pi^*$ intraligand band at 290 nm and, as had been seen in the preliminary anion studies, there was an emergence of a shoulder centred at *ca.* 340 nm which was assigned to the naphthalimide $\pi-\pi^*$ transition. Moreover, upon addition of F^- , a colour change was also exhibited from orange to red further demonstrating the strong interaction of F^- with **48** and was clearly visible to the naked eye (Figure 5.9). The changes observed in the absorption spectrum may be due to hydrogen bonding, as has been postulated for the various anions tested thus far, however, due to the basic nature of F^- , deprotonation of the amino moiety in solution is also likely and has been previously demonstrated.²¹⁸ This hypothesis is particularly likely upon addition of excess F^-

as removal of hydrogen bonded F^- leads to the formation of bifluoride (HF_2^-). This species has a unique stability and is the driving force behind such deprotonation events.²²³

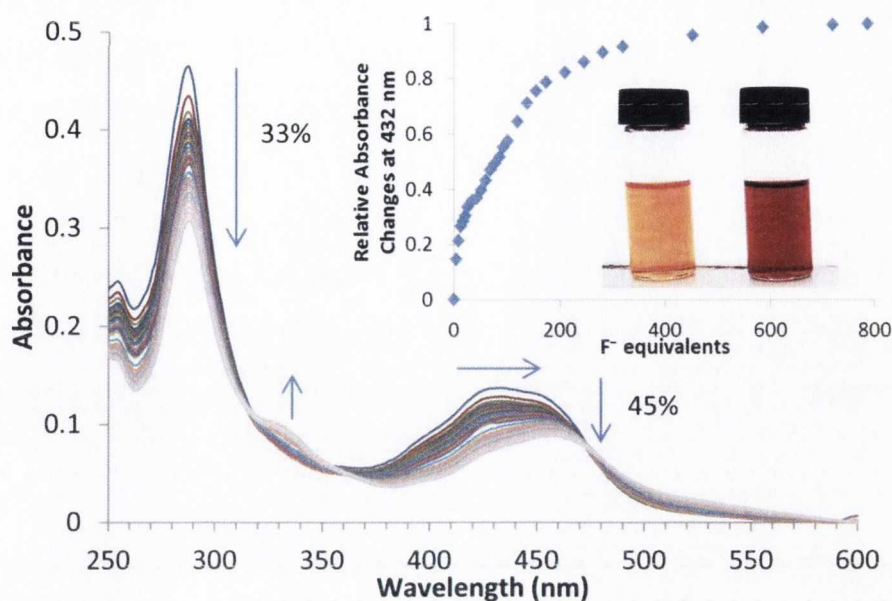


Figure 5.9: The changes in the absorption spectrum of **48** ($1.17 \times 10^{-5} M$) in MeCN upon titration with F^- (0 $\rightarrow$ 800 eq., $[8.4 mM]$). Inset: The relative absorbance decrease at 432 nm as a function of F^- concentration and the resultant colour change from orange to red

Concomitant with these large changes in the ground state, the fluorescence emission spectrum of **48** was also affected showing a dramatic 90% emission decrease over the entire titration range (0 $\rightarrow$ 800 eq.) (Figure 5.10).

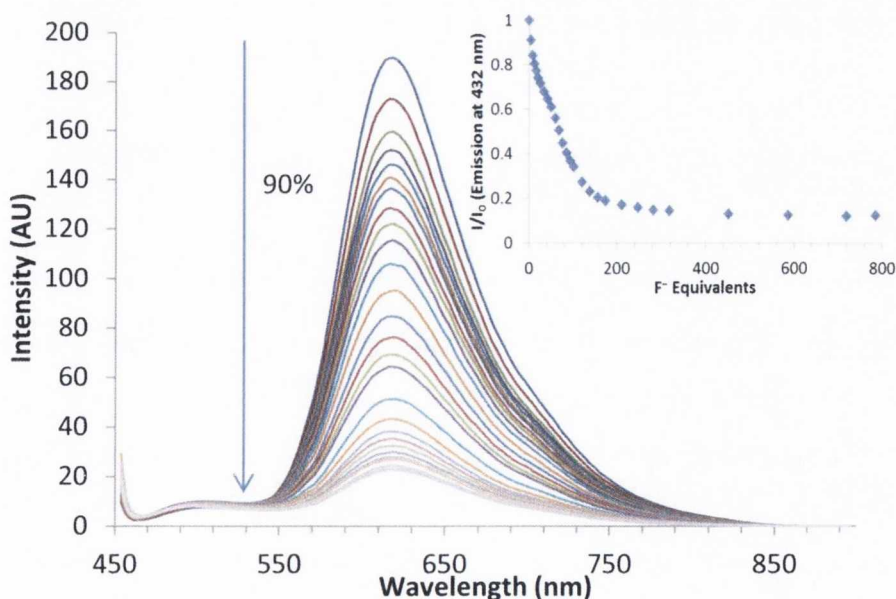


Figure 5.10: The changes in the fluorescence emission spectrum of **48** ($1.17 \times 10^{-5} M$) in MeCN upon titration with F^- (0 $\rightarrow$ 800 eq., $[8.4 mM]$). Inset: The integrated fluorescence decrease measured at 615 nm

The changes observed were significantly more pronounced than those exhibited with **48** in the presence of the other anions tested, and demonstrate that the PET process previously described is more effective upon interaction of the amino naphthalimide moiety with F^- . This efficiency is likely to be brought about by both hydrogen bonding of F^- and deprotonation, causing the naphthalimide moiety to become highly electron rich, resulting in an enhancement of the PET quenching of **48**. Such behaviour correlates well with previous examples of naphthalimide based anion sensors, where F^- has been shown to exhibit an enhanced quenching of the fluorophore over various other anions.²²⁴ The ‘on-off’ fluorescence quenching exhibited by **48** was also shown to be fully reversible, as upon addition of protic solvent, the emission was restored. Similarly, the addition of protic solvent also resulted in a restoration of the original orange colour of solutions of **48**, deeming this system to be a selectively reversible colorimetric and fluorescent sensor for F^- .

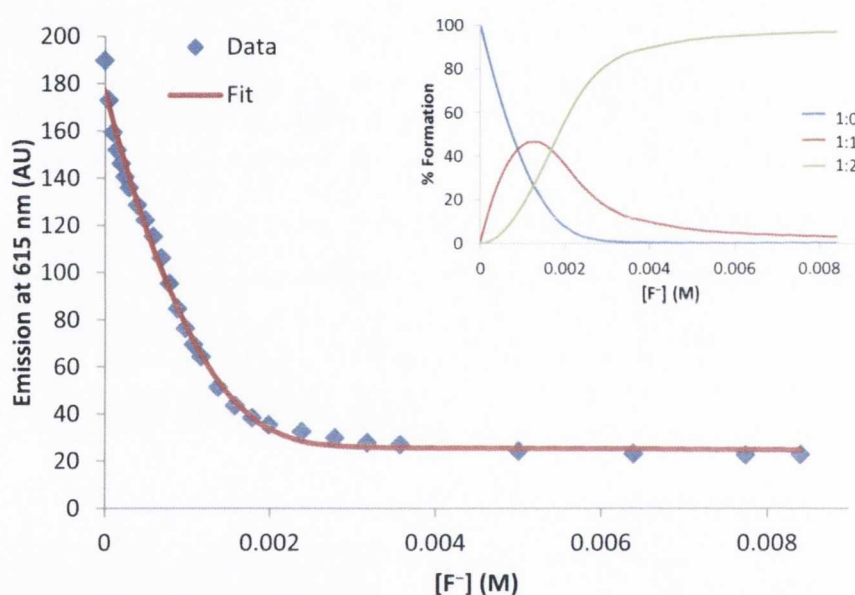
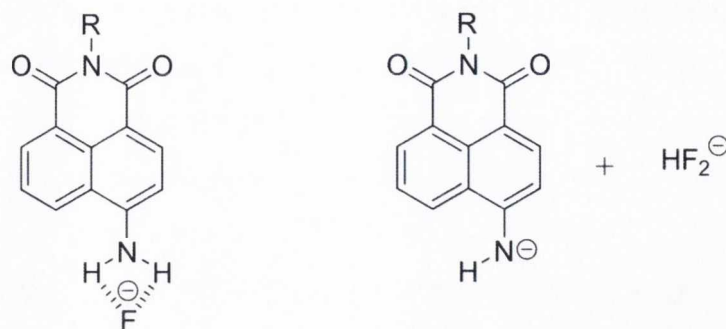


Figure 5.11: The best fit of the emission titration data measured at 615 nm. Inset: The corresponding speciation distribution diagram for the binding of **48** with F^-

The changes in both the absorbance and fluorescence spectra were fitted using SPECFITTM with the results being complimentary to each other. Both 1:1 and 1:2 (host:guest) binding stoichiometries for F^- were observed in both cases. The UV/Vis absorption titrations yielded binding constants of $\log K_{1:1} = 4.24 (\pm 0.01)$ and $\log K_{1:2} = 2.6 (\pm 0.13)$, and similarly fitting of the fluorescence changes were best fitted to two-step binding interactions, which gave $\log K_{1:1} = 4.20 (\pm 0.25)$ and $\log K_{1:2} = 3.7 (\pm 0.37)$. From these results it was possible to derive a speciation distribution diagram that is shown in Figure 5.11, which demonstrates that there were three significant species in solution. One of these species corresponds to sensor **48**, while the other two are most likely to be the F^- hydrogen bonded species **48**: F^- and, as the

equivalents increase, the third species that emerges is likely to be the deprotonated naphthalimide receptor with concomitant formation of HF_2^- , as shown in Scheme 5.2.



Scheme 5.1: Possible 1:1 and 1:2 Host:Guest binding interaction between **48** and F^-

Sensor **48** has shown itself to be a highly effective anion sensor and, moreover, has proven to be selective for F^- . The final section under this heading will attempt to summarise the results observed in this investigation.

5.3.6 Discussion of Spectroscopic Titrations of **48** with Anions

In this Chapter, the binding affinities and host:guest stoichiometries of **48** towards various structurally simple anions have been studied using both UV/Vis absorption and fluorescence emission spectroscopy.

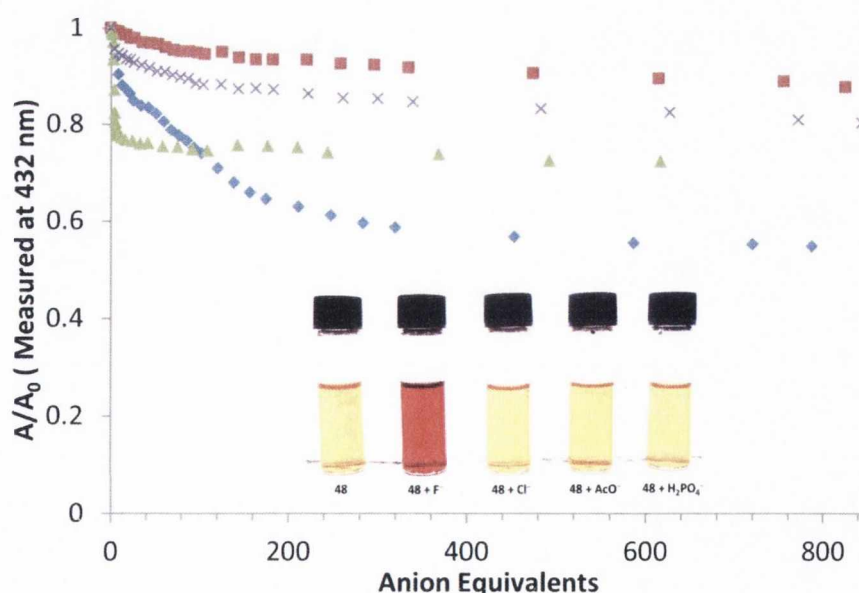


Figure 5.12: Changes in the MLCT absorption of **48** ($12\ \mu\text{M}$) in MeCN upon addition of Cl^- ($\blacksquare$), AcO^- ($\times$), H_2PO_4^- ($\blacktriangle$) and F^- ($\blacklozenge$). Inset: The corresponding solutions of **48** observed upon the addition of excess of each anion

The comparison curve of the changes in the absorption of **48** upon titration with F^- , Cl^- , AcO^- and H_2PO_4^- are shown in Figure 5.12. This graph illustrates the varying response of **48** resulting from the addition of each anion and clearly indicates the preference shown for F^- over the other anions studied, where the largest hypochromism is exhibited. Similarly, each comparison curve exhibits a different binding profile, reflecting the different modes of interaction of the various anions. In addition, as shown as an insert in Figure 5.12, colorimetric changes have been observed for **48** upon addition of F^- while such changes have not been observed for any of the other anions studied, making sensor **48** a selective colorimetric ‘naked eye’ sensor for F^- anions.¹⁵⁰

In a manner similar to the absorption studies, fluorescence emission titrations of **48** also revealed varying effects in response to the different anions. As shown in Figure 5.13, F^- again showed the largest degree of modulation, causing the fluorescence to be quenched by 90%. The remaining anions, AcO^- , H_2PO_4^- and Cl^- also showed similar quenching effects; thought to be as a result of PET quenching from the anion complexed naphthalimide to the Ru(II) centre. In the case of H_2PO_4^- , more complex behaviour was observed where the initial fluorescence decrease took place over a smaller range of equivalents of anion (0 – 30 eq.), in comparison to the other anions studied. Moreover, the emission maximum of **48** was seen to be bathochromically shifted by 20 nm from 615 nm to 635 nm, suggesting major perturbation of the excited state of **48** upon binding of H_2PO_4^- . Again, the varying behaviour of each of the anions towards **48** indicates the different affinity of each anion for this system and also gives an insight into the varying host:guest stoichiometries involved.

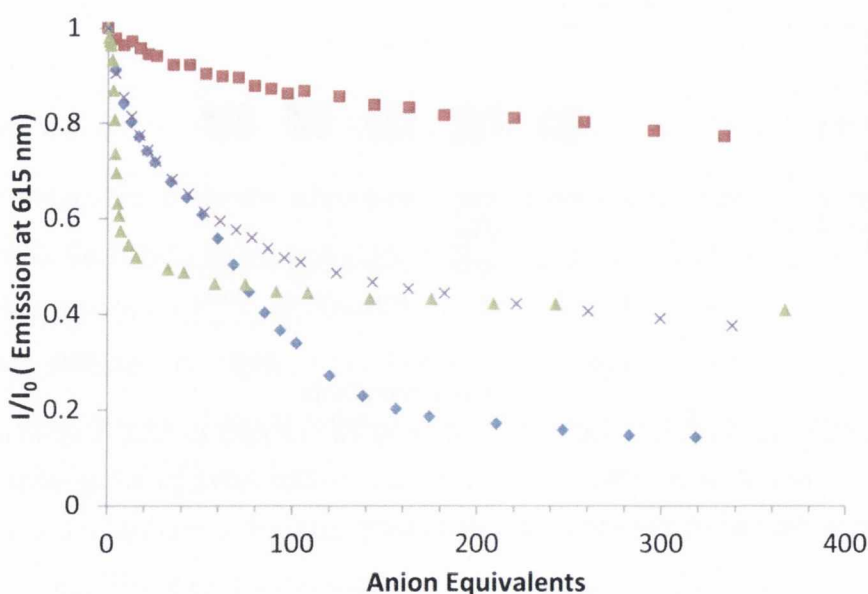


Figure 5.13: Changes in the fluorescence emission of **48** (12 μM) ($\lambda_{\text{ex}} = 432 \text{ nm}$) in MeCN upon addition of Cl^- (■), AcO^- (×), H_2PO_4^- (▲) and F^- (◆).

From the observed changes in both the UV/Vis and fluorescence emission studies, it was expected binding affinities for each anion could be calculated from the titration data by using SPECFITTM. The values calculated are summarised in Table 5.1. As can be seen, Cl⁻ was seen to exclusively form a 1:1 species in solution and was also shown to have the lowest binding affinity for **48**, as expected from the small magnitude of the changes observed in the titration data. H₂PO₄⁻ was also seen to exclusively form 1:1 species in solution but, conversely, was seen to be bound most strongly; possibly due to the binding of the bulky tetrahedral H₂PO₄⁻ anion by the 4-amino group. In the case of F⁻ and AcO⁻, both anions exhibited 1:1 and 2:1 binding stoichiometries in solution with **48**, where the species formed upon interaction with F⁻ were found to be more stable. Furthermore, all of the changes observed in both the ground and excited state titrations were found to be reversible upon addition of more competitive solvents such as MeOH and EtOH.

Table 5.1: Binding constants and host:guest stoichiometries obtained from fitting the changes in the fluorescence emission spectra of **48** using SPECFITTM

Anion	Host:Guest	LogK	Standard Deviation
F ⁻	1:1	4.20	± 0.25
	1:2	3.7	± 0.37
Cl ⁻	1:1	2.9	± 0.056
AcO ⁻	1:1	4.25	± 0.065
	1:2	3.11	± 0.081
H ₂ PO ₄ ⁻	1:1	4.52	± 0.084

Having observed and quantified the affinity of anions for **48** using UV/Vis absorption and fluorescence emission titrations, ¹H NMR studies were also undertaken in order to gain a better insight into the processes involved in the anion recognition event. ¹H NMR spectroscopy has been used to monitor host:guest interactions and can provide an insight into the mechanisms behind such host:guest interactions.²²⁴ Moreover, in aprotic solvents such as DMSO-*d*₆, the relative shifts of exchangeable NH protons can be easily monitored; an important factor in this investigation as **48** was expected to interact with anions *via* its NH₂ moiety. The results of those studies will be presented and discussed in the following section.

5.4 ^1H NMR Studies of **48** with Anions

In order to shed some light on the possible sensing mechanisms involved in the changes seen in the spectroscopic studies, ^1H NMR studies were also undertaken for **48** in the presence of each of the anions studied in the previous sections; AcO^- , H_2PO_4^- , Cl^- and F^- . All spectra were obtained at 20°C in $\text{DMSO}-d_6$ upon addition of excess of each anion (200 eq.) to a solution of **48** (1×10^{-2} M).

In all cases, these anions gave rise to significant changes in the ^1H NMR spectrum of **48**, where several resonances were seen to be shifted downfield upon addition of the anions. The ^1H NMR spectra of **48** alone and in the presence of Cl^- (200 eq.) are shown in Figure 5.14. These substantial changes would indicate interaction of the anions with the sensor *via* possible hydrogen bonding between the anion and the 4-amino moiety of the naphthalimide fluorophore as discussed in the previous section. This observation is further supported by the apparent shifts in the H_3 and H_5 naphthalimide aromatic protons adjacent to the amino moiety, found at 7 ppm and 8.5 ppm, respectively. These signals are seen to be shifted significantly to 7.3 ppm and 9.8 ppm, respectively indicating a dramatic change in the electron density distribution, possibly caused by anion recognition. Similar shifts were exhibited by all signals associated with the naphthalimide moiety, however, due to the highly aromatic character of **48**, many of these signals are overlapping and, as such clear observation of each signal was not possible. A further observation from these studies was the apparent broadening and disappearance of the NH_2 signal at 6 ppm, which is seen to disappear after addition of the anion. Similar behaviour has been observed with urea- and thiourea-based anion receptors studied in the Gunnlaugsson lab and has been attributed to the formation of a hydrogen bonded complex between the amino moiety and the anion or due to deprotonation of the amino NH_2 moiety.^{218 219}

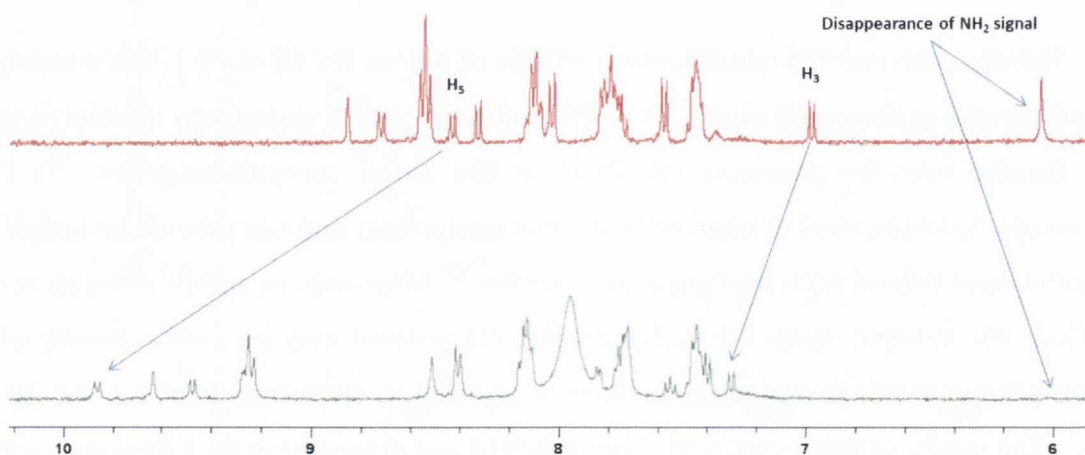


Figure 5.14: Stack plot of the ^1H NMR spectrum (400 MHz) of **48** (1×10^{-2} M) after addition of Cl^- (200 eq.) in $\text{DMSO}-d_6$

More definitive evidence for deprotonation of the amine moiety came from the addition of F^- to **48**. As has been seen for various naphthalimide based systems, significant broadening occurred in the aromatic region of the 1H NMR spectrum concomitant with the strong formation of a triplet peak at 16 ppm ($J = 123$ Hz) (Figure 5.15). The observed broadening and appearance of the triplet at 16 ppm is likely to be due to deprotonation of the 4-amino moiety occurring with the concomitant formation of HF_2^- , a species with unique stability believed to be the driving force behind such deprotonation events.²²³ This hypothesis is further supported by the disappearance of the NH_2 signal of **48** at 6 ppm indicating that this is the most likely site of deprotonation.

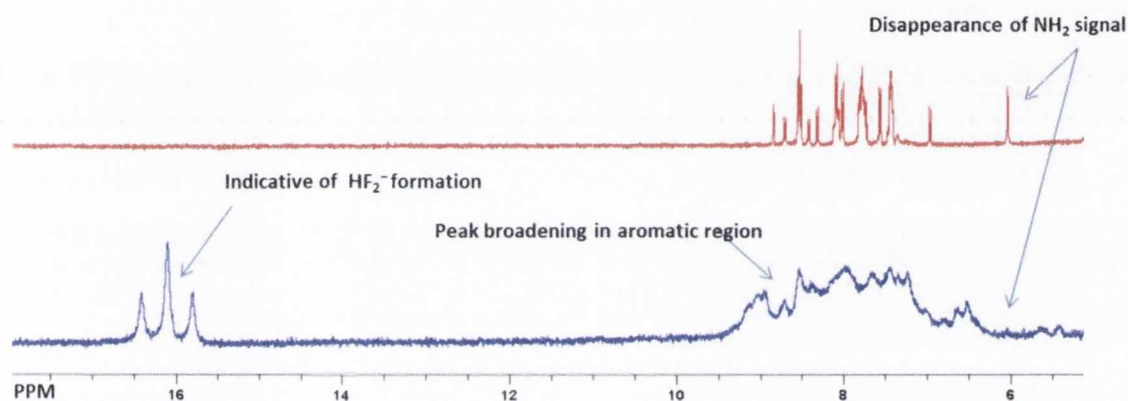


Figure 5.15: Stack plot of the 1H NMR spectrum (400 MHz) of **48** (1×10^{-2} M) after addition of F^- (200 eq.) in $DMSO-d_6$

5.5 Conclusions and Future Perspectives

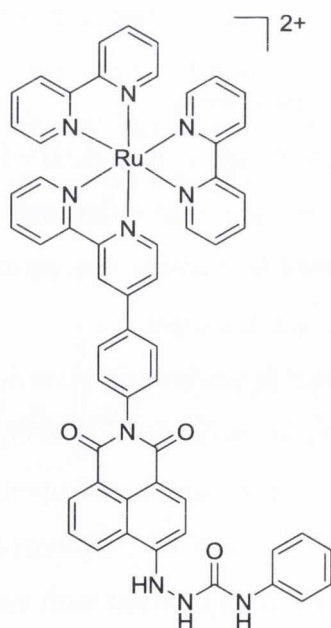
The work carried out in this Chapter has detailed the ability of a Ru(II)-1,8-naphthalimide conjugate, **48** as a fluorescent and colorimetric sensor for anions. Its anion recognition ability has been investigated by various spectroscopic methods such as UV/Vis absorption, fluorescence and 1H NMR spectroscopy.

It has been observed that hypochromic behaviour was exhibited by **48** in its UV/Vis absorption spectrum upon titration with AcO^- , $H_2PO_4^-$, Cl^- and F^- . Furthermore, **48** exhibited a selective colour change from orange to red upon addition of F^- ; behaviour not observed in the cases of AcO^- , $H_2PO_4^-$ and Cl^- . Moreover, MLCT based fluorescence quenching of **48** has also been observed upon titration with each of the anions, with F^- again showing the largest degree of modulation. This behaviour has been ascribed to PET quenching of the Ru(II) excited state due to the anion interaction with the amino-naphthalimide moiety. In addition, from fitting of the titration data using SPECFITTM, the binding affinity and speciation distribution of each anion was evaluated. Cl^- was seen to exclusively form 1:1

species in solution and was also shown have the lowest binding affinity for **48**, as expected from the small magnitude of the changes observed. H_2PO_4^- was also seen to exclusively form 1:1 species in solution but, conversely, was seen to be bound most strongly. In the case of F^- and AcO^- , both anions exhibited 1:1 and 2:1 binding stoichiometries in solution with **48**, where the species formed upon interaction with F^- were found to be more stable.

In order to provide additional evidence for the interaction of anions with **48**, ^1H NMR studies were also carried out upon addition of excess AcO^- , H_2PO_4^- , F^- and Cl^- . The changes observed correlated well with the UV/Vis absorption and fluorescence titrations where significant changes were exhibited in the chemical shifts of all signals associated with the naphthalimide moiety. Furthermore, in the case of F^- a signal at 16 ppm evolved after addition to **48**, providing strong evidence for the formation of H_2F^- , leading to the conclusion that deprotonation is the most likely process by which **48** senses this anion.

The system detailed in this Chapter, **48** is an example of a transition metal based anion sensor that exhibits modulations in its MLCT fluorescence character upon interaction with anions. The system has many advantages over others reported as it is a selective colorimetric sensor of F^- in addition to causing fluorescence modulations occurring at long wavelengths. Future work in this field may include the development of Ru(II) conjugates containing various recognition motifs. For example complex **84** would provide a system that may recognise anions more selectively, and may also have the ability to sense anions in competitive media

**84**

Chapter 6:

Synthesis, Photophysical and DNA Binding Studies of Ru(II) 4-Amino- 1,8-Naphthalimide Derived Tröger's Bases

6.1 Introduction

The rigid V-shaped Tröger's base framework and its inherent chirality have promoted the preparation of a diverse range of receptor systems since its discovery in 1887.²²⁵ Originally prepared from *p*-toluidine, Tröger's base, **85**, was not structurally characterised until half a century later when Spielman²²⁶ established the correct structure of **85** which was later confirmed when Wilcox²²⁷ reported its X-ray crystal structure in 1985. As a result, in the late 1980s, interest in functionalised analogues of Tröger's base emerged, due to the central bicyclic framework which results in the two aromatic rings being fused nearly perpendicular to each other, creating the rigid, V-shaped molecular scaffold. Hence, Tröger's base was seen to offer itself as a unique, nanometer-sized building block for molecular designs and has since been heavily utilised in molecular recognition and supramolecular chemistry.²²⁸⁻²³⁰ Furthermore, due to the blocked conformation of the two nitrogens of the methanodiazocine bridge, the molecule is chiral with a C₂ axis of symmetry, giving it the potential for enantiospecific recognition of molecules.²³¹

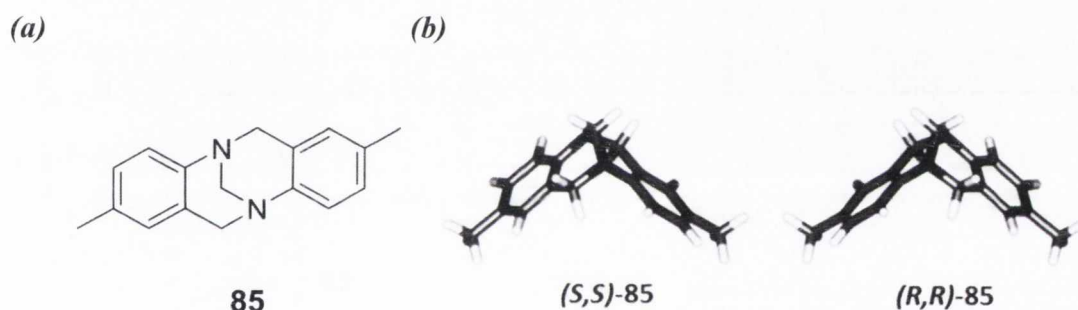
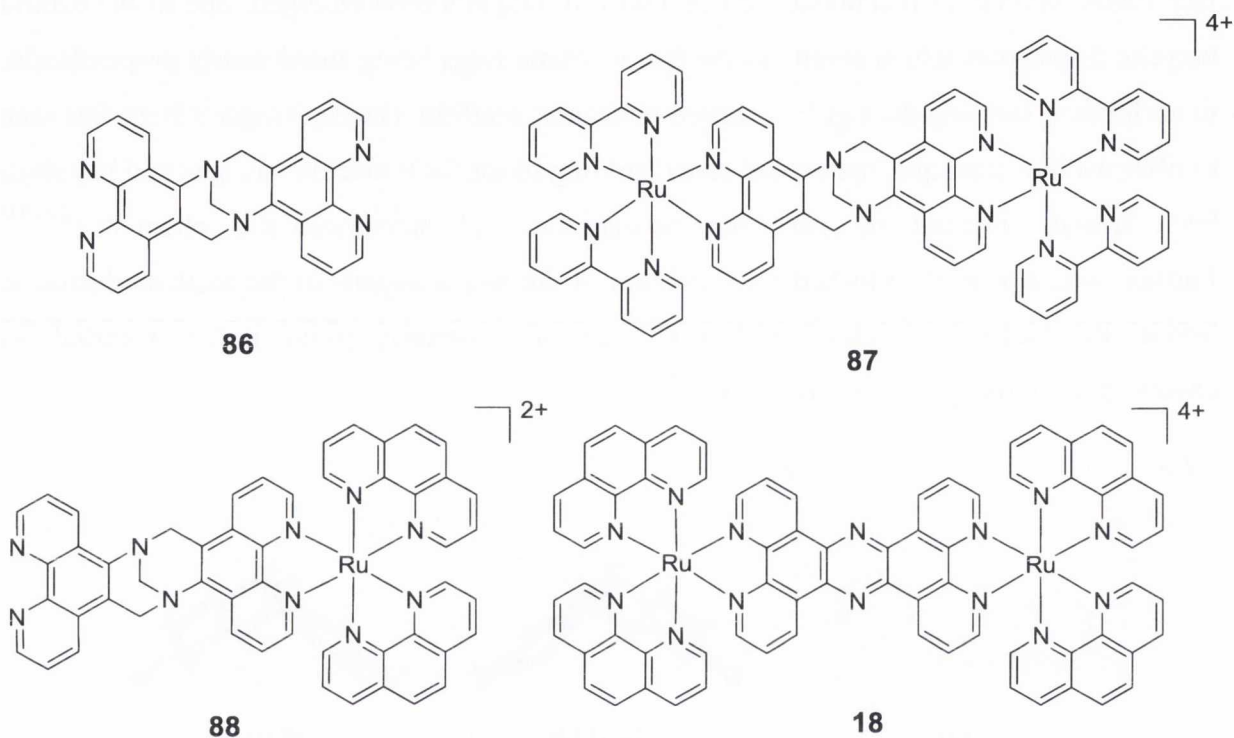


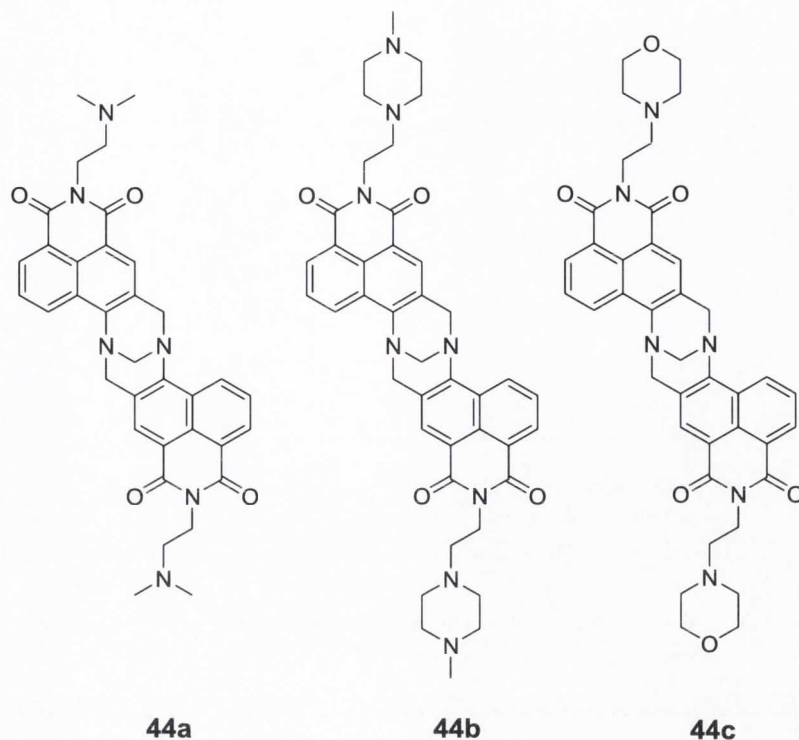
Figure 6.1: (a) The chemical structure of Tröger's base, **85** and (b) PM3-optimised geometry of the two enantiomers of **85**.

More recently, nucleic acids, which are inherently chiral, have been targeted by the use of Tröger's base analogues as DNA-binding motifs. The chirality of the Tröger's base framework plays an important role in their interaction with DNA and it is expected that such species may give rise to enantiospecific binders of DNA. One of the earliest systems developed for DNA recognition came from Yashima *et al.*²³² who reported the initial synthesis of a racemic bis-(1,10-phenanthroline) containing Tröger's base, **86**, and showed that a racemic mixture of **86** could interact with DNA from CD spectroscopy results. Furthermore, it was demonstrated that **86** could cleave DNA when complexed with Cu(I) by conversion of closed circular pUC18 plasmid to open circular DNA.²³² Kirsch De Mesmaeker *et al.* have exploited the available bipyridyl ligand binding sites of **86** by the synthesis of both mono- and bimetallic Ru(II) species.^{233,234} Although interaction of the bimetallic species, **87**, with DNA has not yet been reported, three stereoisomers of the monometallic species, **88** have been

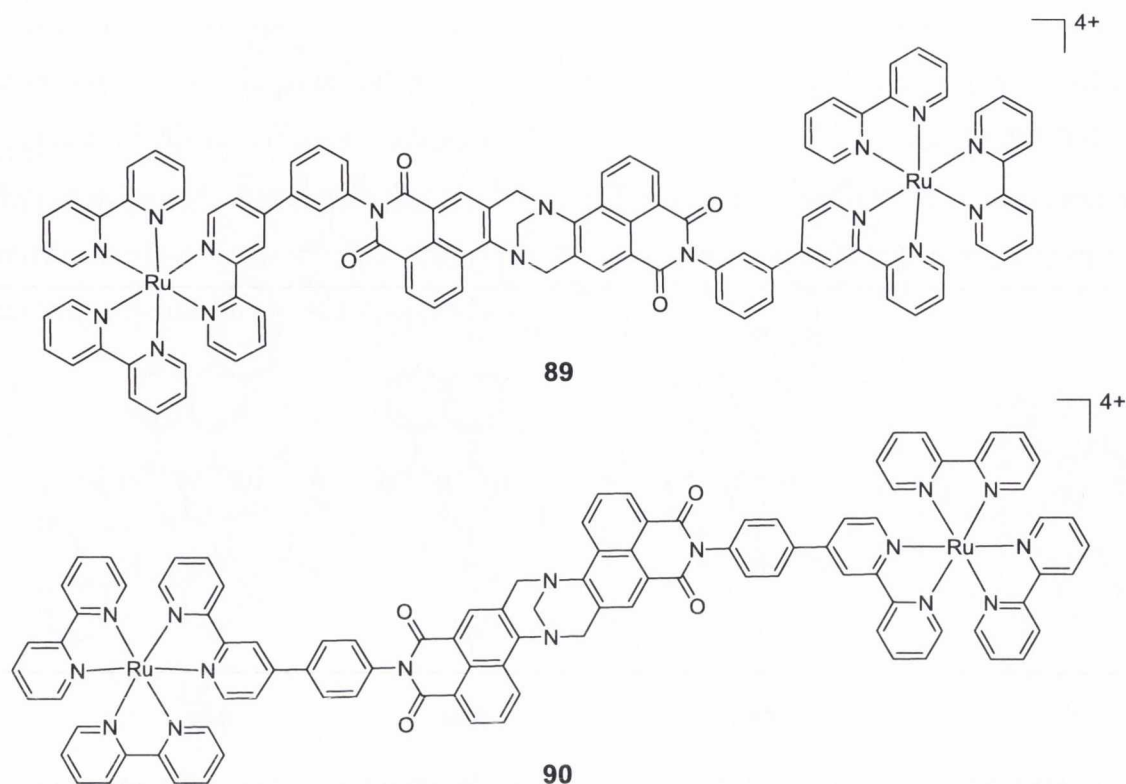
isolated through crystallisation and their interaction with DNA explored.^{234,235} Steady-state emission measurements in the presence of calf thymus DNA showed that the DNA binding of **88** depends on the stereoisomer, where the affinity constant for the stereoisomer Δ -**88** is two orders of magnitude higher than that for Λ -**88**. Moreover, the binding affinity was seen to be mainly controlled by the absolute configuration of the metal centre of the complex as opposed to the chirality induced by the Tröger's base framework.²³⁵



Bimetallic Ru(II) complexes assembled by polypyridyl bridging ligands, have also been shown to bind avidly to DNA and have found numerous applications, most recently in cellular imaging.⁵³ As described in Chapter 1, the work of Thomas *et al.* has led to the development of **18**, which has shown potential as an *in cellulo* nuclear DNA stain, well tolerated by live cells.⁵³ In a related study, also detailed in Chapter 1, the Tröger's base structure has been exploited by Veale *et al.* for use as dual imaging–therapeutic agents where the organic derivatives **44 a - c** were also shown to selectively localise with the nucleus of K562 Leukaemia cells.^{152,153} These bis-1,8-naphthalimide containing Tröger's base species, **44 a - c** also exhibited a number of other advantageous properties, such as the ability to bind strongly to DNA, showing concomitant changes in their ground and excited state properties and, furthermore, these species were shown to induce apoptosis within the K562 Leukaemia cells with IC₅₀ values as low as 5.53 μ M making them potential candidates as anti-cancer therapeutic agents.



Taking into consideration the examples from the literature above and continuing in the search for new Ru(II) based DNA binding motifs, it was decided to modify the design of **44 a - c** to incorporate a bipyridyl portion that would allow the formation of bimetallic Ru(II)-1,8-naphthalimide containing Tröger's bases, **89** and **90**. This would enable the preparation of these new bimetallic species that were expected to be capable of binding DNA with further possible applications in cellular imaging and therapeutics. It was envisioned that the Tröger's base framework may orientate the two functional units; the metallic Ru(II) centres and the organic bis-1,8-naphthalimide containing bipyridyl ligand, in a rigid architecture that may enable tight DNA binding as seen for **44 a - c**. The bimetallic Ru(II) character may enable cellular localisation through a similar manner as seen for the bimetallic complex **18** and, in addition, the highly positive charge of both **89** and **90** would be likely to further increase the DNA binding ability of such systems through electrostatic interactions. Furthermore, in the synthesis of these bimetallic species, the connectivity around the metal centre was seen as a pivotal design feature, where the Ru(II) centres and 1,8-naphthalimide units would have either a *meta* (**89**) or *para* (**90**) arrangement with respect to each other. These arrangements do not allow for changes in the overall distance between the two chromophores but do allow for changes in their orientation with respect to each other. It was expected that such arrangements would have significant effects on the DNA binding ability of each of these systems as has been previously observed for the Ru(II) conjugated 4-amino-1,8-naphthalimide analogues, **48** and **50** described by Ryan¹⁵⁴.



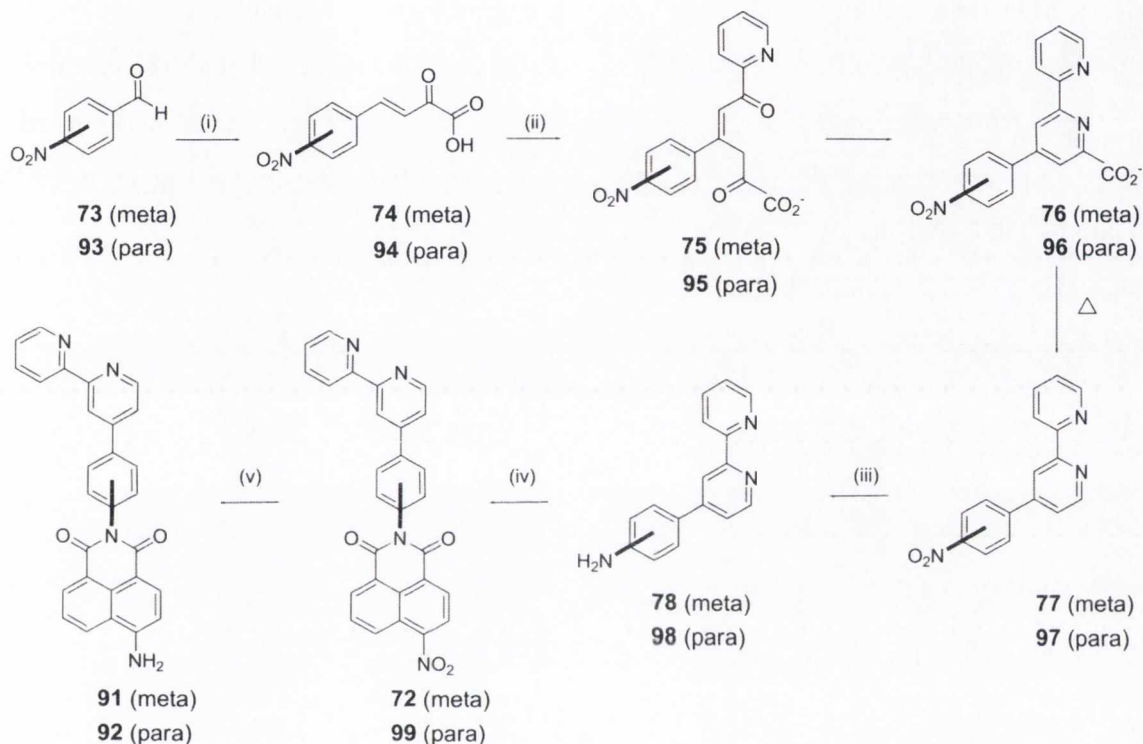
The work described in this Chapter aims to develop and study systems **89** and **90** as potential DNA binding units. This Chapter will focus on a description of the synthesis and characterisation of both complexes. Furthermore, a discussion of the photophysical properties of each complex in aqueous solution will be described followed by a preliminary investigation into the DNA binding ability of each system as assessed by DNA denaturation. With the presence of numerous stereoisomers (up to 6) due to the combination of two Ru(II) centres (Δ or Λ) with the chiral Tröger's base framework (R or S) the stereochemical characteristics of these compounds will not be discussed here and have been isolated as a mixture of enantiomers.

6.2 Synthesis of **89** and **90**

The target 1,8-naphthalimide containing Tröger's base derivatives **89** and **90** were prepared as racemates from the commercially available 4-nitro-1,8-naphthalic anhydride. The *N*-substituted 4-amino-1,8-naphthalimide precursors were firstly synthesised using the procedures developed within the Gunnlaugsson lab as discussed previously in Chapter 4. The following section will deal with a brief description of the methods utilised for the synthesis of the *meta*- and *para*-substituted bipyridine functionalised 4-amino-1,8-naphthalimide precursors.

6.2.1 Synthesis of bipyridine functionalised 4-amino-1,8-naphthalimide precursors

Both bipyridine functionalised amino-1,8-naphthalimide precursors, **91** and **92** were synthesised following a similar procedure. As formation of the nitro-1,8-naphthalimide *meta* derivative has already been discussed in Chapter 4, the route to **92**, in which the bipyridine and 1,8-naphthalimide moieties are *para* to each other will be briefly discussed as an example.

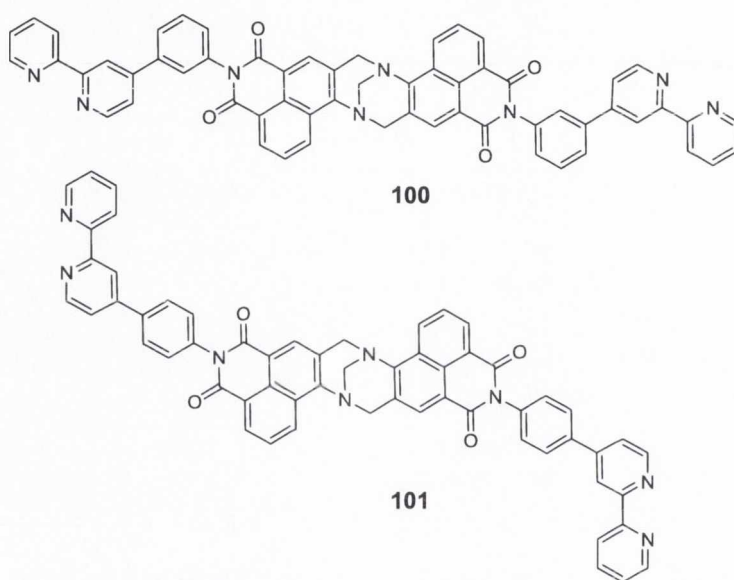


Scheme 6.1: (i) EtOH, sodium pyruvate, NaOH; (ii) ammonium acetate, H₂O; (iii) N₂H₂, 10% Pd, EtOH; (iv) EtOH, 4-nitro-1,8-naphthalic anhydride; (v) N₂H₂, 10% Pd/C, DMF.

Initially, the condensation of 4-nitrobenzaldehyde, **93**, with sodium pyruvate was achieved under basic conditions at 0 °C followed by acidification of the reaction mixture with 0.1 M HCl which gave rise to chalcone **94** as a yellow solid in 66% yield. Pyridacyl pyridinium iodide was refluxed in water in the presence of **94** to give intermediate **95**, incorporating the first pyridine moiety. NH₄OAc, also present in the reaction mixture, subsequently reacted at the two carbonyl groups to initiate the second ring closure and installing the second pyridine ring. This resulted in the carboxyl intermediate **96** which was heated strongly under high vacuum to cause decarboxylation and afforded the desired nitro intermediate **97** in 50% yield. Subsequent reduction of the nitro group using hydrazine in the presence of 10% Pd/C catalyst gave the aniline functionalised bipyridine **98** as an off white solid in 98% yield. Following condensation of **98** with 4-nitro-1,8-naphthalic anhydride in

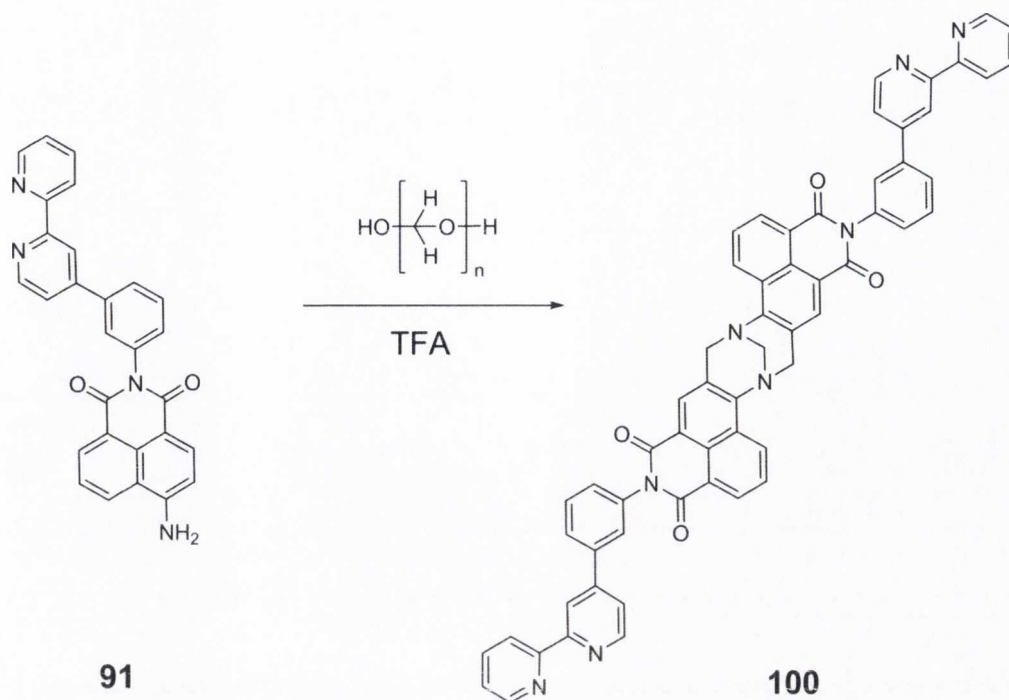
anhydrous ethanol, under an argon atmosphere for 24 hours gave the desired nitro species **99** as a beige solid in 84% yield. Finally, both bipyridine functionalised amino-1,8-naphthalimide precursors, **91** and **92** were synthesised by reduction of the nitro groups in DMF using hydrazine in the presence of 10% Pd/C to give meta substituted **91** in 96% yield and para substituted **92** in 94% yield. Both precursors were fully characterised using ^1H NMR, ^{13}C NMR, IR and HRMS analysis. ^1H NMR peaks were assigned using CH and HH COSY analysis. Full experimental details and characterisation are given in Chapter 8.

Having successfully synthesised and characterised precursors **91** and **92**, syntheses of the Tröger's base analogues were next undertaken. The following section will detail the synthesis and characterisation of the resulting bis-1,8-naphthalimide containing Tröger's base derivatives **100** and **101**.



6.2.2 Synthesis of bipyridine functionalised 4-amino-1,8-naphthalimide containing Tröger's bases

The synthesis of **100** and **101** was undertaken according to a procedure developed in the Gunnlaugsson lab for the formation of the Tröger's base structure in high yields.²³⁶ This involved treating the 4-amino-1,8-naphthalimide precursors, **91** and **92** with 1.5 equivalents of formaldehyde in neat TFA over 12 hours at room temperature. Upon completion, the reaction mixture was initially neutralised and further basified to pH 10 by the slow drop wise addition of an aqueous NaOH solution (6M) resulting in the precipitation of a bright yellow solid which was isolated by suction filtration. Dissolution in DMSO, followed by recrystallisation by addition of MeOH, gave the pure Tröger's base systems **100** and **101** in 68% and 70% yields respectively. The synthesis of **100** is depicted in Scheme 6.2.



Scheme 6.2: The synthesis of **100** by the reaction of **91** with paraformaldehyde in the presence of TFA.

As before, both **100** and **101** were fully characterised using ^1H NMR, ^{13}C NMR, IR and HRMS analysis. The ^1H NMR resonances were assigned using C-H and H-H COSY analysis. The C_2 plane of symmetry of these molecules is reflected in their ^1H NMR spectra where the aromatic region shows sharp, well defined signals with integrations corresponding to the desired species. Similarly, ^{13}C NMR spectra confirm the C_2 symmetric nature of **100** and **101** showing signals that are consistent with their assigned structures. Formation of the Tröger's base framework was confirmed by the presence of two well-separated doublets between 5.3 and 4.7 ppm, pertaining to the methylene protons of the diazocine ring, and a singlet at 4.8 ppm, integrated for two protons, which is characteristic of the diazocine bridge. C-H COSY analysis confirmed these results, where clear coupling was observed between the methylene protons and their corresponding ^{13}C resonances giving a clear indication of the formation of the Tröger's base framework. The ^1H NMR and C-H COSY spectra of **101** (600 MHz, $\text{DMSO}-d_6$) are shown in Figure 6.2(a) and 6.2(b), respectively while those of **100** are shown in Appendix 6. Successful formation of both compounds was also confirmed from accurate mass spectrometry, where **100** displayed a peak at 921.2965, corresponding to the $[\text{M}+\text{H}]$ ion and **101** displayed a peak at 921.2941 also corresponding to the $[\text{M}+\text{H}]$ ion.

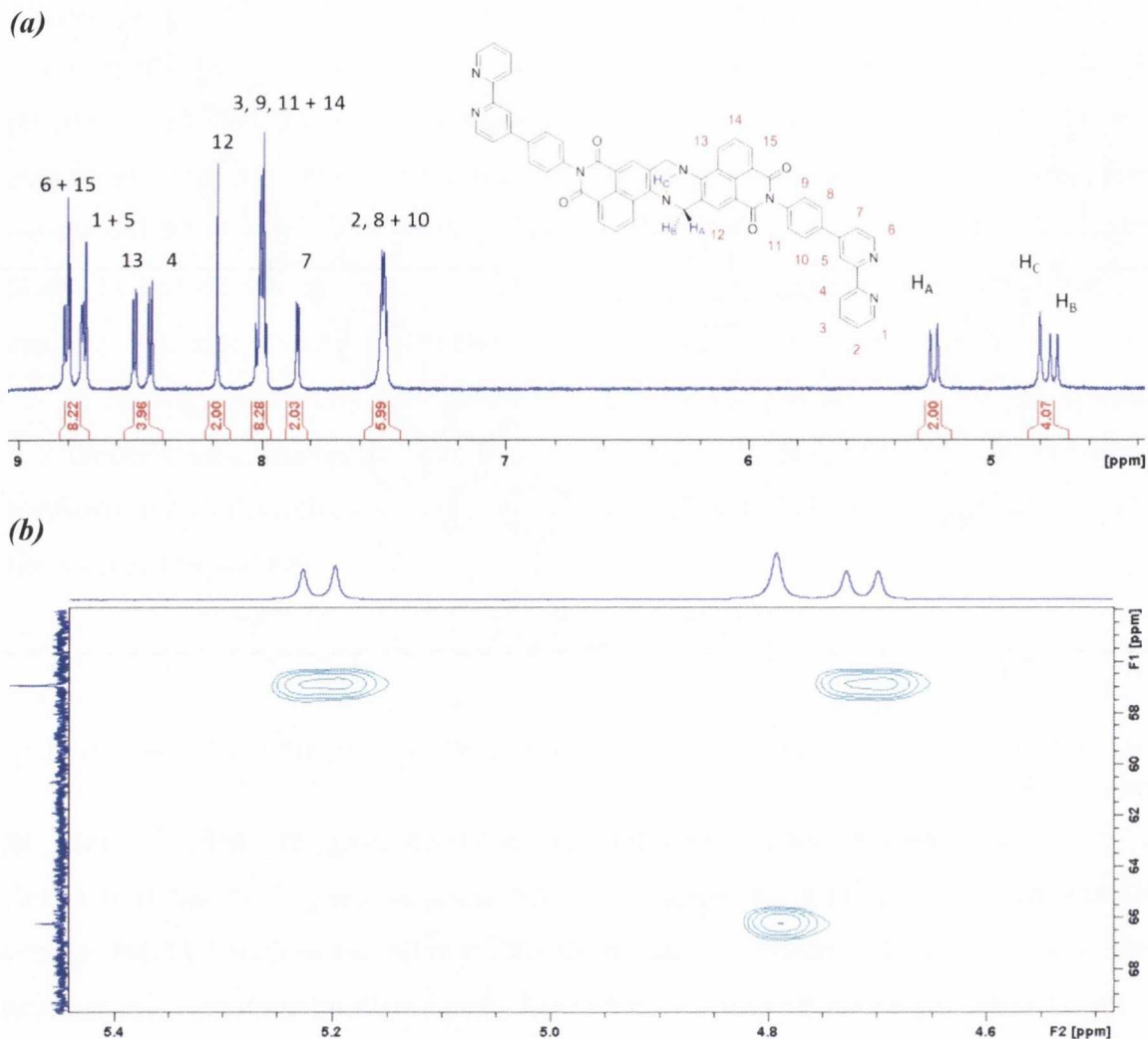
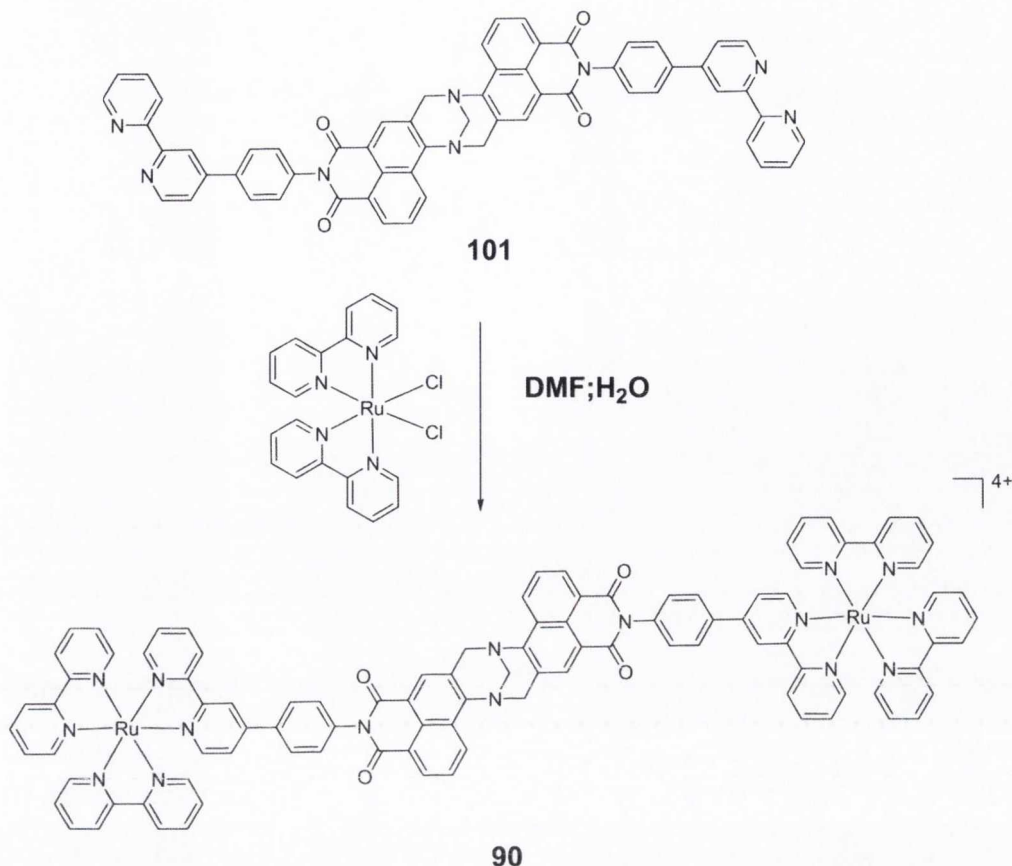


Figure 6.2: (a) ^1H NMR spectrum of **101** (600 MHz, $\text{DMSO}-d_6$). (b): The C-H COSY spectrum of **101** (600 MHz, $\text{DMSO}-d_6$) showing the interaction of the methylene protons of the diazocene bridge with their corresponding ^{13}C resonances

6.2.3 Complexation of **100** and **101** with $\text{Ru}(\text{bpy})_2\text{Cl}_2$

In a manner similar to that described in Chapters 2 and 4, microwave-assisted synthesis was utilised for the complexation of each of the ligands **100** and **101** with $\text{Ru}(\text{bpy})_2\text{Cl}_2$, shown for complex **90** in Scheme 6.3. Reaction of **100** and **101** at $140\text{ }^\circ\text{C}$, in the presence of $\text{Ru}(\text{bpy})_2\text{Cl}_2$, in a DMF/ H_2O mixture, gave the characteristic dark purple to orange colour change, signifying the formation of a tris-Ru(II) complex in solution. For each reaction, the solvent was removed under reduced pressure, before redissolution in H_2O and filtration followed by precipitation of the crude complexes as their PF_6 salts by addition of a concentrated ethanolic solution of NH_4PF_6 . The crude complexes **89** and **90** were purified by slow diffusion of Et_2O into a concentrated CH_3CN solution by placement in an Et_2O -saturated closed vessel. The resulting deep red solids were collected by centrifugation in 68% and 63% yield for **89** and **90** respectively.



Scheme 6.3: The complexation of $\text{Ru}(\text{bpy})_2\text{Cl}_2$ to ligand **101** using microwave assisted synthesis to form complex **90**.

These novel structures were characterised using the full range of techniques described in each of the previous Chapters (see Chapter 8 for full experimental details). The C_2 plane of symmetry of these novel complexes was, again, reflected in their ^1H NMR spectra where the signals observed in aromatic region were well defined signals with integrations corresponding to the desired species. In addition, through the use of H-H COSY, C-H COSY and selective TOCSY analysis an assignment of all resonances was possible. The presence of the Tröger's base framework was also confirmed by the appearance of the two familiar well-separated methylene proton resonances between 5.3 ppm and 4.7 ppm and the singlet at 4.8 ppm, characteristic of the diazocine bridge. In a manner similar to that described for **100** and **101**, C-H COSY analysis confirmed the coupling between the methylene protons and their corresponding ^{13}C resonances giving a clear indication of the retention of the Tröger's base structure. The ^1H NMR spectrum of **90** (600 MHz, CD_3CN) is shown in Figure 6.3 with that of **89** shown in Appendix 6 with the corresponding 2-D spectra.

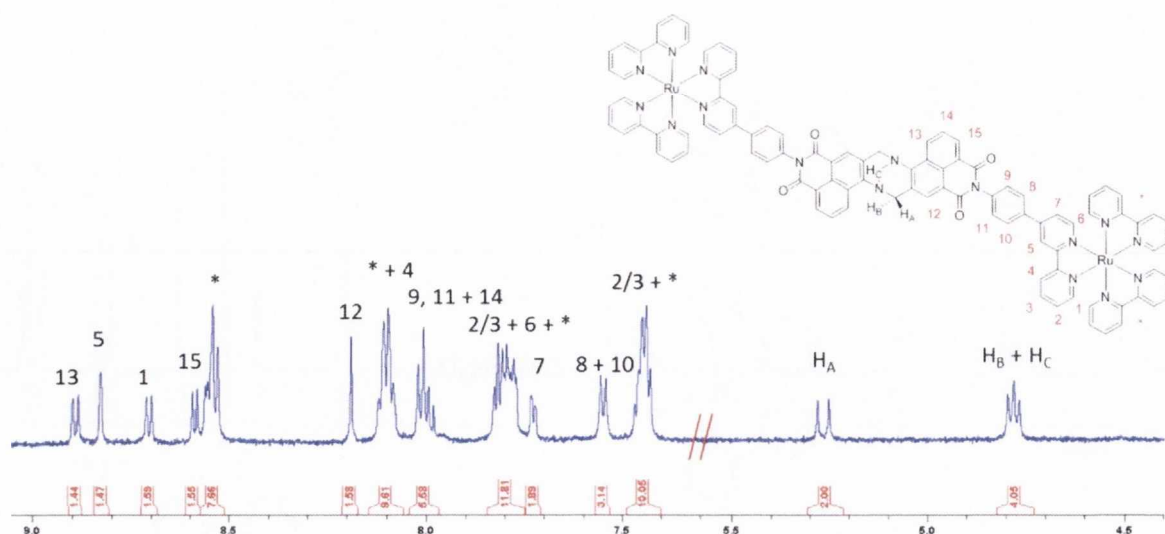


Figure 6.3: The ^1H NMR spectrum of **90** (600 MHz, CD_3CN). Assignment of the various resonances was achieved by various 2-D NMR experiments.

The ^{13}C NMR spectra (recorded in CD_3CN) were also obtained, in which all the expected resonances were observed, with signature resonances of the naphthalimide carbonyl groups being observed at 165.3 ppm and 164.7 ppm for **89** and at 164.2 ppm and 163.6 ppm for **90**. The successful formation of the complexes was also clearly evident from accurate MALDI-TOF mass spectrometry analysis, where **89** displayed a peak at 2183.2554, corresponding to its M^{4+} ion + 3PF_6 counterions. Similarly, **90** displayed a peak at 2183.2517, also pertaining to its M^{2+} ion + 3PF_6 counterions. A comparison between the calculated and obtained isotopic distribution pattern is shown for **89** in Figure 6.4 with that for **90** shown in Appendix 6. Obtained as their chloride salts, both complexes were water-soluble and their photophysical properties were thus studied in aqueous solution.

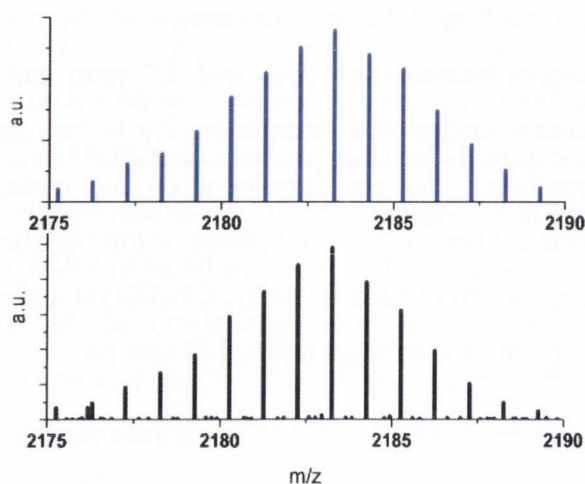


Figure 6.4: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for **89** from high resolution mass spectrometry analysis.

In summary, the desired bimetallic Ru(II)-1,8-naphthlaimide containing Tröger's bases, **89** and **90** have been successfully synthesised in high purity. Moreover, both of their structures have been fully characterised using standard spectroscopic techniques. The following section will deal with a further characterisation of these compounds by evaluating their photophysical properties in solution.

6.3 Photophysical Characterisation of **89** and **90**

In order to further characterise the solution properties of the two bimetallic Ru(II)-1,8-naphthalimide containing Tröger's bases, **89** and **90**, the following sections will deal with an investigation of the photophysics of both the organic precursors **100** and **101**, and their corresponding complexes **89** and **90**. While the latter were carried out in phosphate buffer solution at pH 7.4, the analyses of **100** and **101** were carried out in organic solution due to a lack of solubility in H₂O.

6.3.1 Photophysical properties of ligands **100** and **101**

The UV/Vis absorption, fluorescence emission and excitation spectra of the 1,8-naphthlaimide containing Tröger's base, **101** in CH₂Cl₂ are shown in Figure 6.5 with those of **100** shown in Appendix 6.

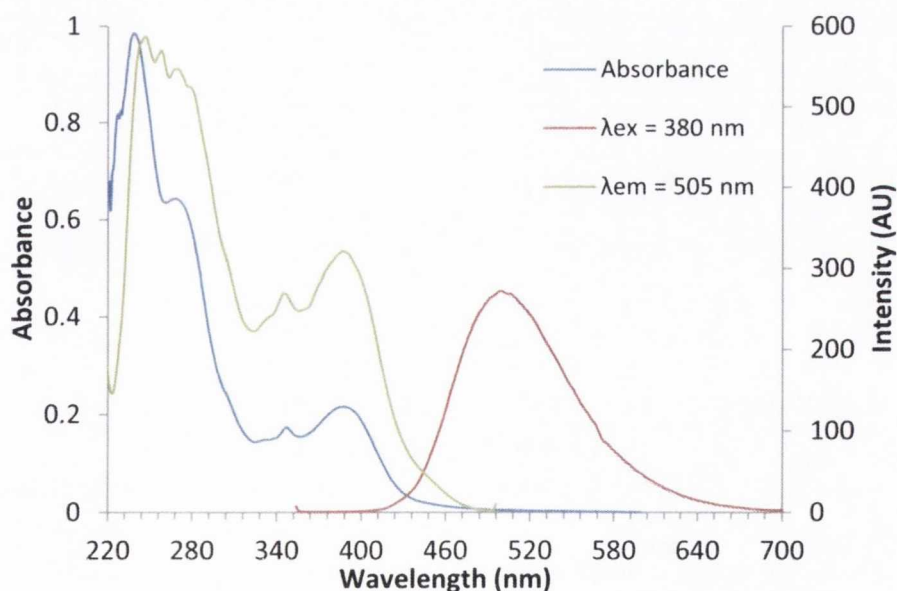


Figure 6.5: UV/Visible, excitation and emission spectra of **101** (1×10^{-5} M) in CH₂Cl₂.

Four different absorption maxima are shown in the UV/Vis absorption spectrum of both **100** and **101** with no major differences seen between both species. Maxima are observed at 240 nm, 270 nm, 350 nm and 395 nm, respectively. The intense band at 240 nm was attributed to π - π^* intraligand transitions within the bipyridine portion of these species. The

less intense shoulder at 270 nm was assigned to the of $\pi\text{-}\pi^*$ transitions within the naphthalimide part of the ligand while the second main absorption region between 350 nm and 400 nm was assigned to the internal charge transfer (ICT) character of the naphthalimide fluorophore, arising due to the push–pull nature of the electron-donating amine and the electron-withdrawing diimide.²³⁷ Excitation of both **100** and **101** into all of these absorbance maxima resulted in an ICT based emission band occurring between 400 nm – 600 nm with λ_{max} at 505 nm. Furthermore, the excitation spectrum ($\lambda_{\text{em}} = 505$ nm) of each compound structurally matches those of the absorption spectra reflecting the relative contributions of each absorption band.

6.3.2 Photophysical Characterisation of Complexes **89** and **90**

Complexation of **100** and **101** with two Ru(II) polypyridyl metal centres was expected to have a significant effect on the photophysical properties of these systems. The UV/Vis absorption, excitation and emission spectra of **90**, were recorded in 10 mM phosphate buffer solution at pH 7.4 and these are shown in Figure 6.6.

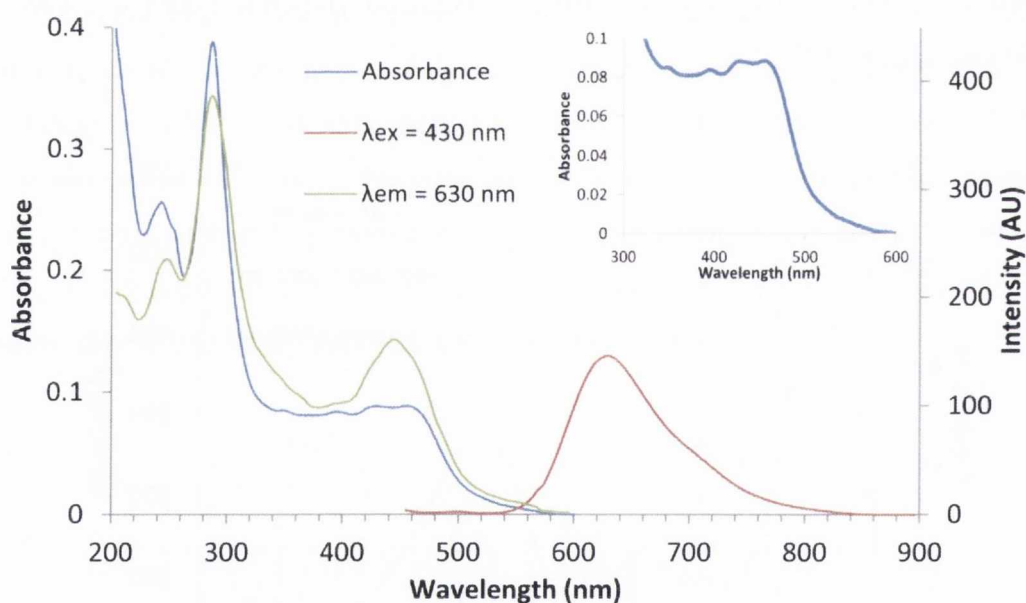


Figure 6.6: UV/Visible, excitation and emission spectra of **90** in 10 mM phosphate buffer at pH 7.4. Inset: UV/Visible absorption region between 300 nm and 600 nm showing the four absorbance maxima.

The absorption spectrum of both **89** and **90** were found to be identical and showing two main absorbance regions, consisting of two bands at 240 nm and 290 nm, and a broad absorption band composed of maxima at 350 nm, 400 nm, 430 nm and 460 nm. The former were characteristic of $\pi\text{-}\pi^*$ intra-ligand transitions within the bipyridine ligands, while the less intense bands between 350 nm and 500 nm were assigned to the 1,8-naphthalimide ICT transitions (350 nm and 400 nm), and the MLCT transitions associated with the Ru(II) centres

at 430 nm and 460 nm, respectively. A summary of the absorption properties of **89** and **90** as well as their respective molar absorptivities in 10 mM phosphate buffer are given in Table 6.1.

Table 6.1: Absorption properties of **89** and **90** in 10 mM phosphate buffer, at pH 7.4

Complex	$\lambda_{\max}$ (nm) [$\epsilon(\text{M}^{-1} \text{cm}^{-1})$] in phosphate buffer ($\pm 10\%$)				
	$\pi\text{-}\pi^*$ IL	$\pi\text{-}\pi^*$ Naph		MLCT	
89	285 [129500]	350 [27500]	400 [29200]	430 [32100]	460 [30300]
90	285 [138300]	350 [30600]	400 [30100]	430 [32900]	460 [32900]

Excitation into all of the aforementioned absorption bands of **89** and **90** resulted in the appearance of the MLCT based emission centred at *ca.* 615 nm. As previously discussed, the absence of any 1,8-naphthalimide associated emission band suggests that sensitisation of the MLCT excited state occurs through some energy transfer process between the naphthalimide moiety and the Ru(II) centres, as the ICT emission centred at 505 nm overlaps with the MLCT absorbance of the Ru(II) centre. Such behaviour has been previously observed for the Ru(II) conjugated 4-amino-1,8-naphthalimide analogues described by Ryan *et al.* and was rationalised in a similar manner.¹⁵⁵ The observed $\lambda_{\max}$ of emission is similar but slightly blue shifted in comparison to the parent complex $[\text{Ru}(\text{bpy})_3]^{2+}$ which has a $\lambda_{\max}$ of 622 nm in aqueous solution.²³³ The quantum yields measured **89** and **90** were, again, found to be identical with $\Phi_{\text{MLCT}} = 0.015 (\pm 10\%)$, which is significantly lower than that of $[\text{Ru}(\text{bpy})_3]^{2+}$ (0.042 in H_2O)¹⁷⁶ but of similar magnitude to that reported for the amino substituted Ru(II)-1,8-naphthalimide conjugate, **50** described by Ryan which was reported as 0.018.¹⁵⁵ Lower quantum yields are again attributed to an interaction between the 1,8-naphthalimide and the MLCT triplet state of the Ru(II) centres. Excited state lifetime measurements (τ_{em}) were also obtained in H_2O with $\tau_{\text{em}} = 187$ ns and 174 ns for **89** and **90** respectively; again indicating the similar photophysical behaviour of **89** and **90** despite the structural differences between the two analogues; these results are summarised in Table 6.2.

Table 6.2: Emission properties of **89** and **90** in phosphate buffer solution at pH 7.4 at 298K ($\lambda_{\text{ex}} = 430$ nm)

Complex	$\lambda_{\text{emission}}$ (nm)	Φ_{f} ($\pm 10\%$)	τ_{em} (ns) (air)
89	615	0.015	187
90	615	0.015	174

From the above photophysical results it is clear that the inclusion of two Ru(II) centres has significantly affected the photophysical properties of **100** and **101**. The strategy employed in the design of **89** and **90** was successful in yielding systems with varying structural attributes, but with very similar photophysical behaviour. We anticipate that the variation in structure may have important consequences on the nature of the interaction of these complexes with DNA and the following sections will deal with a preliminary evaluation of the interaction of **89** and **90** with DNA using various spectroscopic techniques.

6.4 DNA Binding Interactions of **89** and **90**

As has been observed in the previous Chapters, the photophysical properties of species in solution are often modulated upon interaction with DNA. This was also the case in the study reported by Veale *et al.* where significant modulations of the photophysical characteristics of the Tröger's base analogues, **44** – **46** were observed upon interaction with DNA.¹⁵³ Furthermore, all three systems exhibited greater affinity for DNA over their 4-amino-1,8-naphthalimide precursors which are known binders of DNA.⁹⁷ In addition, as discussed in the introduction, Ru(II) polypyridyl complexes are also known binders of DNA and, significantly, bimetallic complexes have been observed to exhibit increased DNA affinity over their monometallic counterparts.⁴⁷ As a result, preliminary studies on **89** and **90** were undertaken in order to get an insight into the possible binding ability of these systems with DNA. These studies will be detailed in the preceding sections.

6.4.1 Spectroscopic Titrations of **89** and **90** with DNA

The UV/Vis absorption spectroscopy titrations with stDNA were initially carried out to probe the interaction of **89** and **90** with DNA. The titrations were undertaken as described before, by addition of small aliquots of stDNA to a 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached.

As can be seen in Figure 6.7 for **89**, although the shape of the spectra are essentially unchanged, addition of a sufficient excess of DNA ($P/D = 50$) caused a minor red shift of the absorption maxima coupled to an 8% hypochromism at 400 nm and a 6% hypochromism at 460 nm. Similar behaviour was seen for **90** whereby a slightly larger magnitude of change was exhibited with a 13% hypochromism at 400 nm and a 10% hypochromism at 460 nm (see Appendix 6). Examination of the titration curves for **89** and **90** reveals a biphasic interaction of these complexes with DNA. The hypochromic effect as a function of P/D is illustrated as an inset in Figure 6.7. This is consistent with the existence of more than one site of binding as has been proposed for other bimetallic Ru(II) species.⁴⁸ Closer inspection of the hypochromic effect reveals that the bimetallic complexes **89** and **90** exhibit a P/D ratio dependent two-stage

process. An initial marked decrease in absorbance is followed, at higher P/D ratios, by a gradual increase in the absorbance leading to a plateau when essentially 100% of the complexes were bound to the DNA. Similar absorption results were reported for the organic systems **44** – **46** and the biphasic-binding seen was attributed to a “template directed effect” where the initial decrease in absorbance is thought to be as a result of DNA-induced aggregation of **89** and **90**, causing them to stack on the DNA backbone, and is followed by a subsequent increase in absorbance due to a destacking event, allowing a redistribution of the species over the length of the DNA polyanion.¹⁵³ It is also important to point out, however, that these changes are minor in comparison to those reported by Ryan *et al.* for the Ru(II) conjugated 4-amino-1,8-naphthalimide analogues and may suggest weak interaction of these systems with DNA.¹⁵⁵

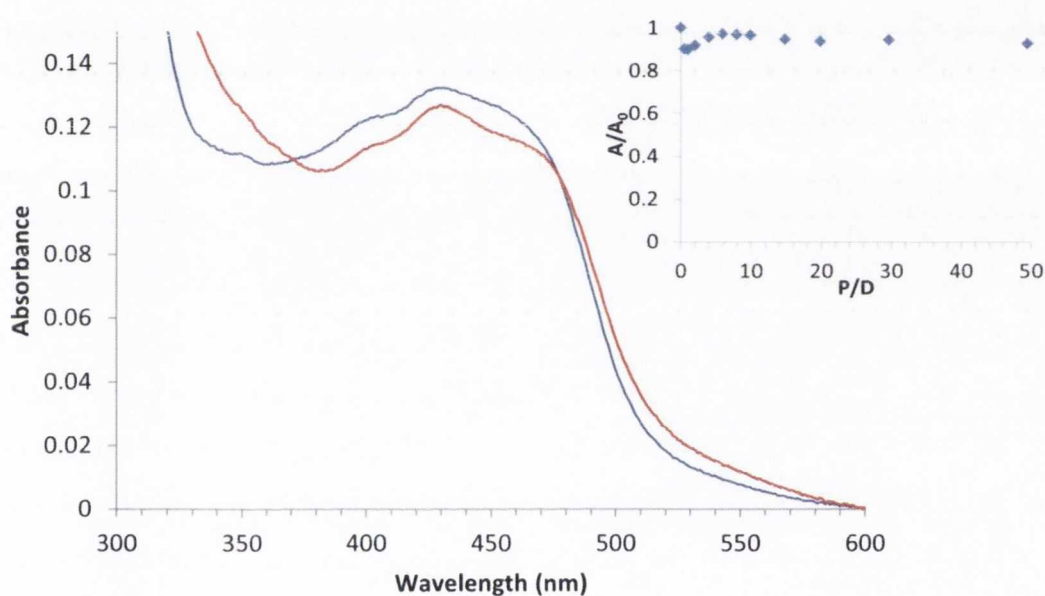


Figure 6.7: Change in the UV/Visible absorption spectrum of **89** (----)(4 μ M) upon addition of stDNA (----) (0-187 μ M). Inset: Plots of the change in integrated MLCT absorption as a function of P/D ratio.

Similarly, the effect on the luminescence intensity upon the addition of stDNA to solutions of **89** and **90** over the same range (P/D 0 $\rightarrow$ 50) were minor. Upon binding of **89** to stDNA a 3% intensity enhancement was observed, coupled with a slight red-shift of the λ_{max} of emission by *ca.* 2 nm. An enhancement of 5% was also observed for **90** with a red-shift of the λ_{max} of the MLCT emission by *ca.* 2 nm (Figure 6.8). Again, examination of the titration curves for **89** and **90**, as a function of P/D ratio, reveals the same biphasic interaction seen in the absorbance titrations, however, due to such small changes this data is difficult to interpret.

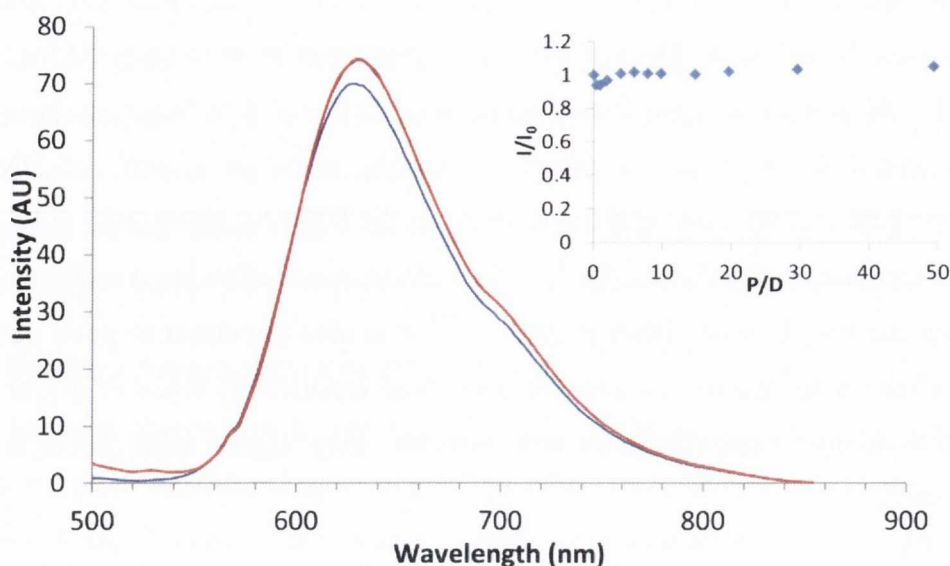


Figure 6.8: Change in the fluorescence emission spectrum of **90** (----) (λ_{ex} 460 nm) ($4\mu\text{M}$) upon addition of stDNA (----) (0 – $187\mu\text{M}$). Inset: Plots of the change in integrated MLCT emission as a function of P/D ratio.

An inset in Figure 6.8 illustrates the change in intensity of **89** and **90** as a function of P/D ratio where the emission is initially seen to be slightly quenched (P/D $0 \rightarrow 2$) followed by a small increase leading to a plateau (P/D $2 \rightarrow 50$) which corresponds to a situation where **89** and **90** are fully bound to DNA. In a similar manner to the behaviour described for the absorbance titration, the observed emission behaviour is most likely explained by a biphasic interaction. Such interactions occur where the complexes experience an extra enhancement of luminescence quenching resulting from DNA induced stacking of the complexes at low P/D ratios. As more binding sites become available the complexes are redistributed along the DNA helix resulting in a slight increase in the emission intensity due to sheltering afforded by the DNA helix microenvironment.²¹³

Due to the small magnitude of the changes and the observed biphasic behaviour of both **89** and **90** a binding constant could not be determined accurately from the absorption or emission data. However, in order to further evaluate the binding affinity of these systems the melting behaviour of DNA was evaluated in the presence of various concentrations of **89** and **90**. The results of these studies will be discussed in the following section.

6.4.2 Thermal Denaturation Studies of **89** and **90**

As the cationic complexes **89** and **90** were expected to interact with DNA, thermal denaturation studies were carried out in order to quantify this interaction as has been described in previous Chapters. In the absence of metal complex, the T_m value for stDNA was

determined to be 68 °C. As seen for the other Ru(II) based complexes described in this Thesis, the melting transition for DNA in the presence of both **89** and **90** showed significant changes at the two P/D ratios evaluated. Figure 6.9 demonstrates the changes exhibited by both **89** and **90** at a P/D ratio of 10 with the corresponding changes at a P/D ratio of 25 shown in Appendix 6. The observed changes reflect the stabilisation of the helix structure by both of these species, where the melting transition had not gone to completion at 90 °C for **89** and was shifted significantly to 74.1 °C for **90**. Similarly, at P/D = 25 the melting transition for **89** had not gone to completion at 90 °C and was shifted to 71.4 °C for **90**. Interestingly, **89** was observed to exhibit a much increased stabilisation over that of **90** at both P/D = 10 and at P/D = 25, indicating a higher binding affinity for DNA in comparison to its structural analogue **90**. This is most likely due to the orientation of the two Ru(II) centres with respect to the Tröger's base framework, where the configuration, in space, of **89** seems to be more favourable for a strong interaction with DNA. This result is complementary to those of Ryan¹⁵⁴ where he also showed that a meta arrangement between the Ru(II) centre and the 1,8-naphthalimide precursor system **50** was shown to result in greater affinity for DNA, as demonstrated by T_m measurements and changes in the spectroscopic properties, due to greater complementarity in the shape of the complex to that of the DNA. Further analysis in to the particular binding mode of these species with DNA may enable more definitive answers, however, the experiments described here have demonstrated significant stabilisation of the helix associated with binding of these systems and further emphasises the high affinity that they both possess for DNA.

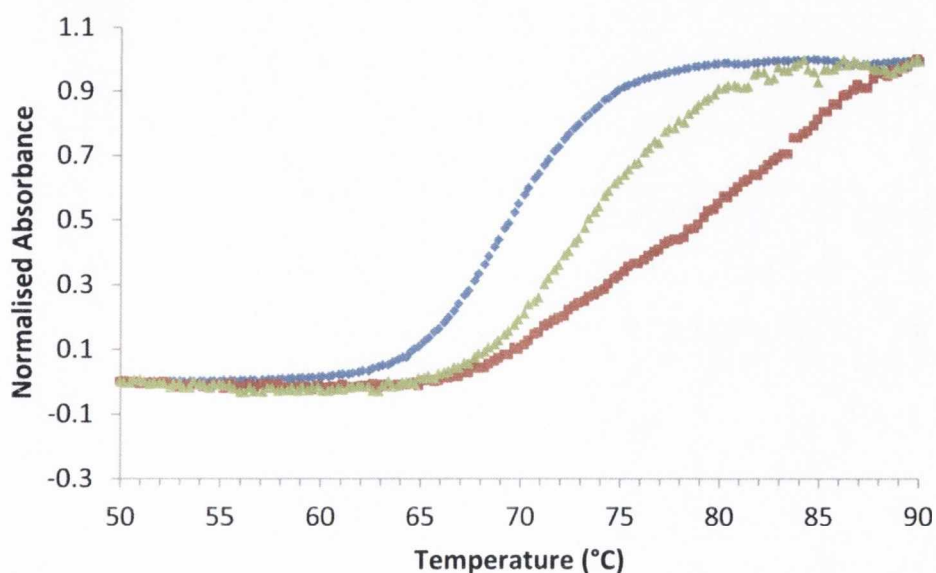


Figure 6.9: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacklozenge$) and presence of **89** ($\blacksquare$) and **90** ($\blacktriangle$) at P/D = 10.

In order to probe this interaction more thoroughly and to evaluate the role of electrostatic interactions in the binding of **89** and **90** to DNA, T_m studies were also undertaken at varying concentrations of NaCl (25 mM and 50 mM). As both **89** and **90** are highly cationic, electrostatic binding to DNA was expected to play a significant role in the binding process and it was expected that these measurements would reveal the relative contribution of electrostatic vs. non-electrostatic modes of interaction. As demonstrated in Figure 6.10 in the absence of **89** and **90**, the T_m for stDNA was determined as 76 °C in the presence of 25 mM NaCl concentration; but this value was increased significantly in the presence of **89** and **90**, where the melting transition had not fully completed at 90 °C for **89** and was again, shifted significantly to 79 °C for **90**. Similarly, at 50 mM NaCl concentration, the melting transition for DNA was seen to be increased to 80 °C in the absence of metal complex but upon addition of **89** the melting transition had still not completed at 90 °C and was shifted to 82 °C for **90**. On both occasions, **89** was observed to have an increased effect on the stabilisation of stDNA over that of **90** further emphasising the higher binding affinity for DNA it has in comparison to **90**. It is clear from these results that the interaction of **89** and **90** with DNA is dependent on the ionic strength of the medium, as DNA stabilisation in 10 mM phosphate buffer is increased in comparison to those at high ionic strength. Nevertheless, as both complexes continue to show DNA stabilisation even at 50 mM NaCl concentration it would suggest that, although electrostatic interactions have a role to play in the binding process, it appears that there are other factors that affecting the association of these complexes with DNA.

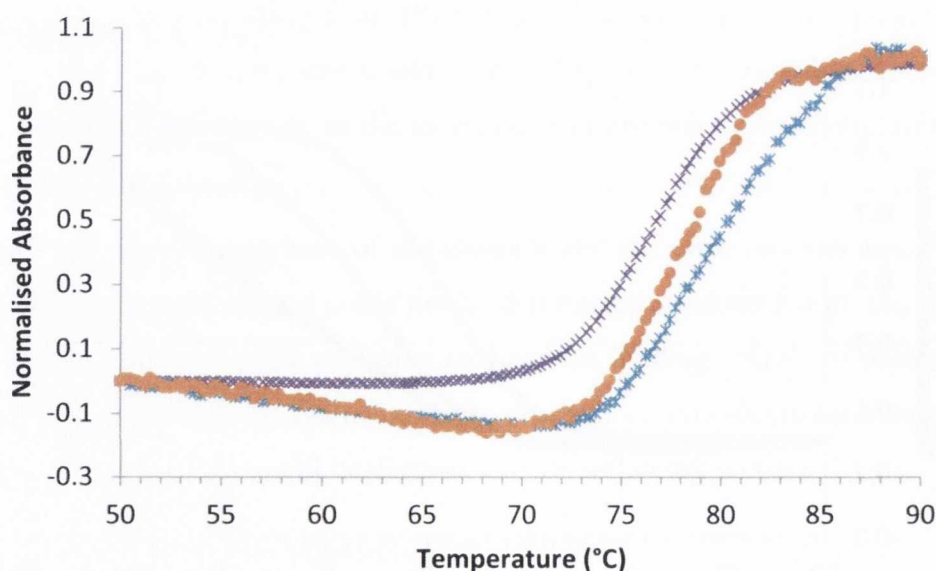


Figure 6.10: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer + 25 mM NaCl, pH 7.4, in the absence ($\times$) and presence of **89** ($*$) and **90** ($\bullet$) at P/D = 10

Further studies will be necessary to give a more thorough insight into the particular binding mode of both **89** and **90** with DNA; however, for the purposes of this Chapter, the following section will deal confocal microscopy studies to determine the applicability of **89** and **90** as fluorescent cellular probes or as cytotoxic agents.

6.5 Preliminary Cellular Uptake and Localisation Studies of **89** and **90**

The potential of **89** and **90** to act as fluorescent cellular probes relies on their ability to be internalised in target cells, preferably in an organelle specific manner, where their fluorescent properties can be exploited for both diagnostic and therapeutic applications. As mentioned previously, the work of Veale^{152,153} explored the ability of the Tröger's base analogues **44a - c** to localise in K562 Leukaemia cells and through confocal microscopy it was discovered that these species were capable of selectively localising within the nucleus. Similarly, as mentioned in Chapter 1, the dinuclear monointercalating Ru(II) complex studied by Thomas *et.al.*,⁵³ **18** has been used as an *in cellulo* nuclear DNA stain, well tolerated by live cells, for use with both luminescence and transition electron microscopy. Taking these factors into account an investigation into the cellular uptake ability of the bimetallic Ru(II)-1,8-naphthlaimide containing Tröger's bases **89** and **90** was undertaken in collaboration with the Williams Lab in the School of Biochemistry and Immunology at Trinity College Dublin.

Confocal microscopy was carried out in order to provide visual evidence of the localisation of **89** and **90** in cells. Populations of live HeLa cells (0.5×10^5) were incubated with **89** and **90** (25 μ M) at 37 °C for 4 hrs before being treated with the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope with a 60 $\times$ oil immersion lens. Figure 6.11 shows the fluorescence confocal microscopy picture of the live HeLa cells after incubation with **89** and their comparison with a population of healthy, untreated control cells. The image clearly demonstrates the localisation of **89** in all cells, with apparent localisation in the cytoplasm and around the nucleus. Similar results were found for **90**, the results of which are shown in Appendix 6. Moreover, the addition of **89** and **90** to HeLa cells seems to have adverse effects on the cell integrity. Visualisation of cells treated with **89** compared to the untreated control cells clearly demonstrates significant differences in the cell morphologies, where the extracellular membrane of cells treated with the complexes seem to undergoing membrane 'blebbing'; an irregular bulge in the plasma membrane of a cell caused by localised damage of the cytoskeleton commonly associated with apoptosis.²³⁸

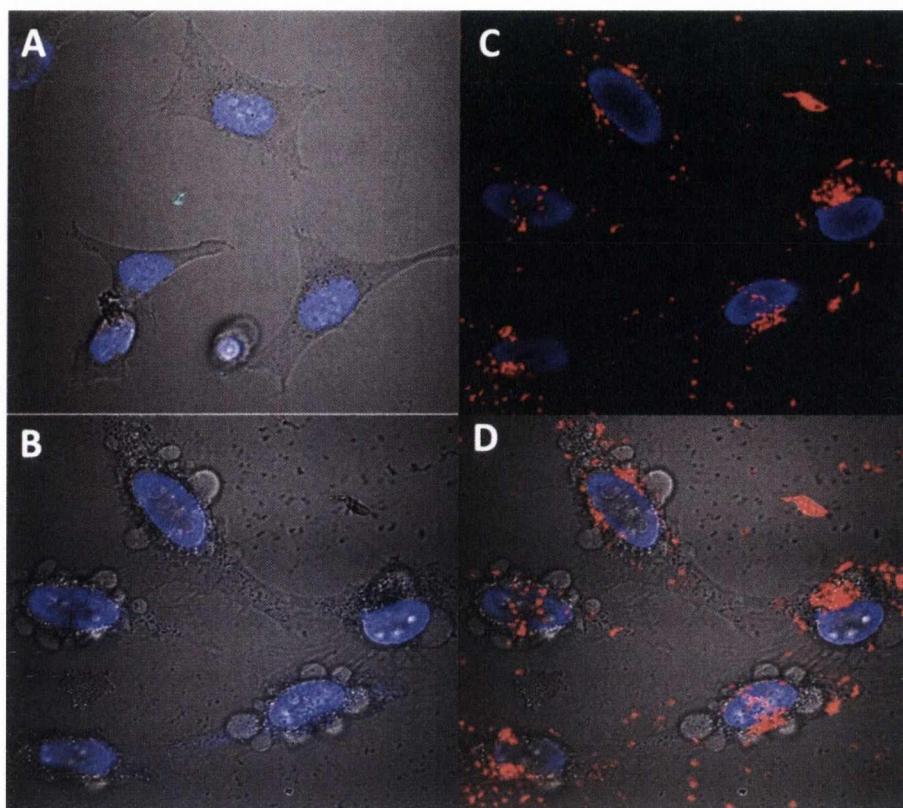


Figure 6.11: Confocal Laser Scanning Microscopy live cell images of **89** (25 μ M) with HeLa cells. Shown is the image obtained with (A) control cells and (B) the bright field view of cells treated with **89** after 4 hrs incubation, stained with DAPI (blue), (C) overlay of **89** (red) and nuclear co-stain DAPI (blue) and (D) overlay of **89** (red), nuclear co-stain DAPI (blue) and the bright field view.

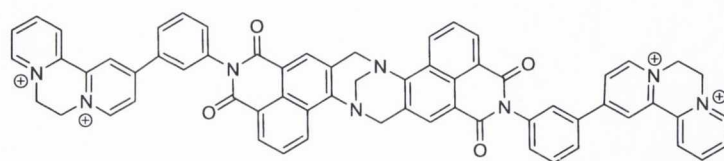
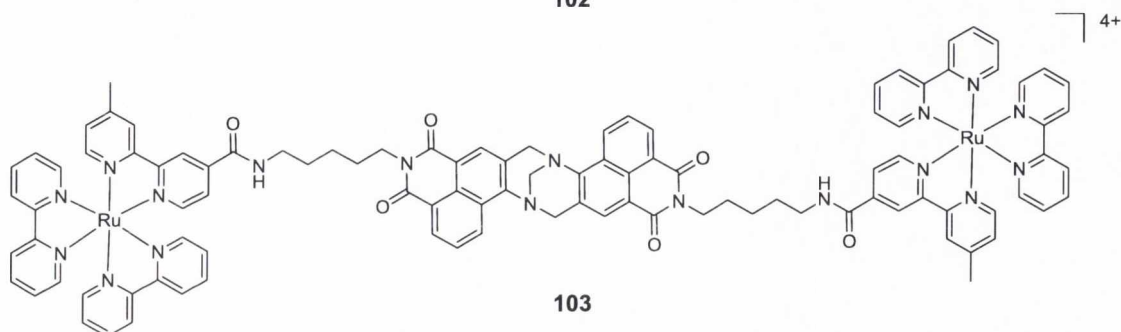
At such low doses and such short incubation times, the observed cellular damage indicates that **89** and **90** may find application as cytotoxic agents. Indeed, when treated with **89** and **90** at a higher concentration and incubation time (100 μ M over a 24 hr period; as carried out with the Ru(II) complexes described in Chapter 2) no remaining cells could be visualised suggesting that treatment with **89** or **90** has effectively irradiated the cell population. Further studies are underway to further ascertain the exact effects of **89** and **90** within these cell lines and these results will be reported in the thesis of Ms. Erby in the School of Biochemistry and Immunology at Trinity College Dublin.

6.6 Conclusions and Future Perspectives

This Chapter has detailed the design, synthesis and characterisation of two bimetallic Ru(II)-1,8-naphthlaimide containing Tröger's bases, **89** and **90**. A description of their photophysical properties in aqueous solution at pH 7.4 has been described where both species were shown to exhibit behaviour characteristic of Ru(II) polypyridyl complexes showing MLCT based absorption at 460 nm and the corresponding MLCT based fluorescence emission

at 615 nm. Absorption bands at 400 nm were also observed for both species due to the 1,8-naphthalimide chromophore. However, the ICT based emission at ≈ 500 nm associated with such absorption was not clearly seen, suggesting an efficient energy transfer from the 1,8-naphthalimide portion to the Ru(II) centre. The DNA binding affinities of **89** and **90** in aqueous solution have also been explored through UV/Vis absorption titrations, fluorescence titrations and T_m experiments. Although the changes in both the UV/Vis absorption and fluorescence titrations were of small magnitude, T_m experiments revealed large stabilisation of the DNA helix upon interaction with both **89** and **90**, where **89** was seen to be bound more strongly. Furthermore, studies in the presence of varying ionic strength revealed the stabilisation effect of **89** and **90** was still seen at 50 mM NaCl concentration, suggesting that, although electrostatic interactions play a role in their binding to DNA, there are other factors contributing to this interaction. Finally, the ability of **89** and **90** to localise in cancer cells was evaluated through the use of confocal microscopy, where both species were seen to localise within the cytoplasm of HeLa cells, where they were also observed to cause significant damage to the cell populations at relatively low doses and short incubation times.

Future work in this project will focus on more detailed investigations into the specific mode of interaction of **89** and **90** with DNA and the source of their observed cytotoxic effect. Moreover, synthesis of an organic analogue such as **102** will look to exploit the use of an ethylene bridge in a manner similar to that of **67** described in Chapter 3. Such a system may give rise to a water soluble species that lacks the bulky Ru(II) centres enabling more close association with DNA. Concomitantly such a species should display advantageous photophysical properties allowing the binding process to be monitored. Alternatively, a Ru(II)-1,8-naphthalimide containing Tröger's base such as **103** which makes use of a flexible linker from the Ru(II) centre to the Tröger's base framework may be an approach that could yield species with increased binding affinity and photophysical response.

**102****103**

Chapter 7:

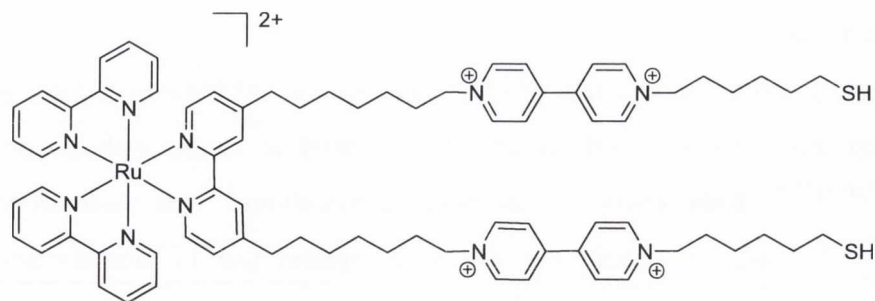
Luminescent Ru(II) Polypyridyl Functionalised Gold Nanoparticles

7.1 Introduction

Recently, gold nanoparticle (AuNP) conjugates and their properties have led to new and exciting developments with enormous potential in areas such as optoelectronics,²³⁹ sensors,^{136,156,157,240} light energy conversion applications²⁴¹ as well as in biology and medicine.¹¹⁷⁻¹¹⁹ With the sensitivity and wide spread use of fluorescence spectroscopy, fluorophore-bound AuNPs, in particular, have been proposed as useful probes for biomolecular imaging^{125,242} with such hybrid probes offering the possibility of using complementary sensing *via* fluorescence confocal microscopy and electron microscopy. The binding of chromophores to the metal surface, however, often results in quenching of the excited state due, for the most part, to energy or electron transfer deactivation pathways.²⁴³

As has been demonstrated throughout this Thesis, Ru(II) polypyridyl complexes have been intensively studied due to their useful photophysical properties and selective photochemistry, especially in the presence of DNA. In addition, characteristic photostability and water solubility all combine to make Ru(II) polypyridyl complexes excellent candidates for potential therapeutics as well as spectroscopic probes and photoreagents.³¹ Many applications have been explored for such species, for instance in luminescent recognition and sensing,^{33,164,244} and as structure-specific DNA probes.^{53,178} Moreover, luminescent Ru(II) complexes have often been proposed as useful fluorophores for cellular imaging,^{245,246} but until very recently their actual applications have remained scarce.^{246,247}

In a bid to combine the advantageous properties of both Ru(II) polypyridyl complexes and gold nanostructures, there have recently been a small number of reports which have looked to functionalise gold surfaces with Ru(II) polypyridyl complexes.^{141,142} One such example is the Ru(II)-viologen linked thiol complex **104** which has been fabricated on a gold electrode in the form of a self-assembled monolayer (SAM) with a view to exploiting the properties of $[\text{Ru}(\text{bpy})_3]^{2+}$ for solar energy conversion applications.²⁴⁸ Similar species have also been utilised for functionalisation of AuNPs, where significant quenching of the excited state fluorescence was proposed to occur by both energy transfer and electron transfer mechanisms.^{243,249} Many of these studies were undertaken with a view to elucidating the excited state behaviour of the Ru(II) polypyridyl units upon conjugation to AuNPs, however, to the best of our knowledge systems such as these have not yet been utilised for biological applications and we expect that such luminescent AuNP systems might offer attractive photophysical properties, ideal for application in cellular imaging.

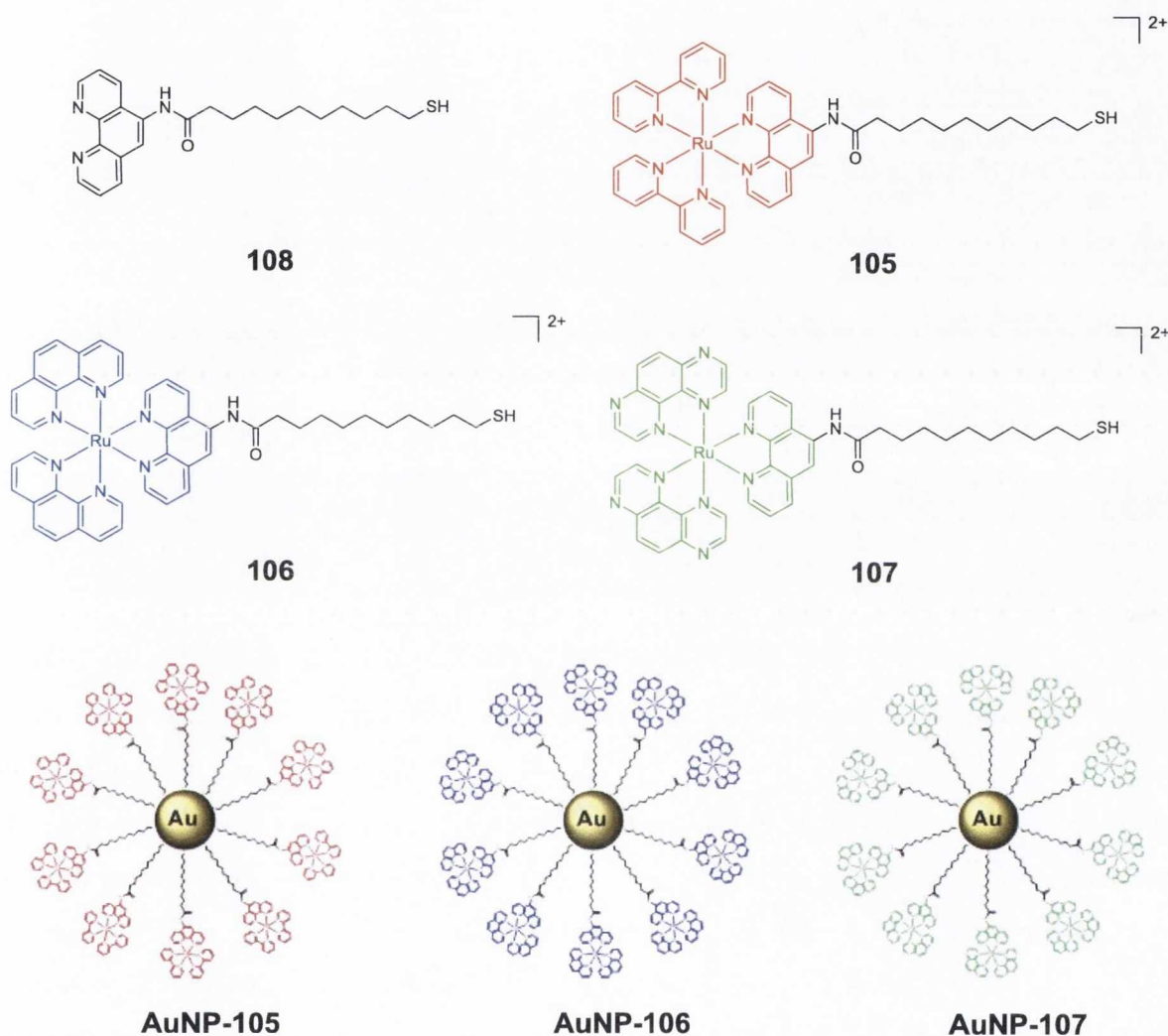


104

As work within the Gunnlaugsson group has already focused on the development of luminescent cellular targeting (therapeutic/imaging) agents^{152,153} and surface modified AuNPs,^{156,157} we envisaged that the combination of Ru(II) polypyridyl complexes, spacially separated from the surface of AuNPs by a sufficiently long covalent spacer, could be employed as luminescent probes/imaging agents for various biological applications. This Chapter will deal with the synthesis of the Ru(II) complexes **105** - **107**, all of which possess a terminal alkyl thiol group which will facilitate their adsorption onto AuNPs; leading to the formation of three water soluble AuNP systems **AuNP-105**, **AuNP-106** and **AuNP-107**. In a manner similar to that described in Chapter 2, variation of the ancilliary ligands around the Ru(II) centre, utilising bipyridine (bpy), phenanthroline (phen) and tetraazaphenanthrene (TAP), was expected to provide potential for recognition and sensing of DNA, owing to each of their characteristic luminescent properties. In addition, the use of the π -deficient TAP ligands should confer a strongly oxidising excited state on such a complex,^{60,164} potentially resulting in a photoinduced-electron transfer (PET) between *guanine* bases of DNA and the Ru(II) functionalised AuNPs. This process often results in efficient DNA cleavage, as was observed and discussed in Chapter 2 for **55**. Thus, Ru(II) AuNP conjugates containing at least two TAP ligands are expected to yield highly photoreactive species capable of causing selective damage to DNA upon light irradiation; a highly desirable characteristic from the point of view of PDT treatments.

The aim of the work presented in this Chapter will be to synthesise the alkyl thiol functionalised phenanthroline ligand **108**, the successful synthesis of which, will enable the synthesis, characterisation and photophysical evaluation of the alkyl thiol containing Ru(II) complexes, **105**, **106** and **107**. Their spectroscopic response in solution and in the presence of DNA will also be evaluated using various techniques before their adsorption onto the surface of AuNPs is undertaken. This will be followed by a characterisation of the resulting conjugates **AuNP-105**, **AuNP-106** and **AuNP-107** using traditional methods such as UV/Vis absorption spectroscopy, fluorescence spectroscopy, dynamic light scattering (DLS) and

transmission electron microscopy (TEM) analysis and a preliminary observation of their DNA binding response. Finally, preliminary biological studies will be described detailing the DNA photocleavage ability of **AuNP-105**, **AuNP-106** and **AuNP-107** as well as their cellular uptake and localisation ability. The following section will firstly deal with the synthesis and characterisation of the alkyl thiol functionalised phenanthroline ligand **108** and the resulting free complexes **105 - 107**.

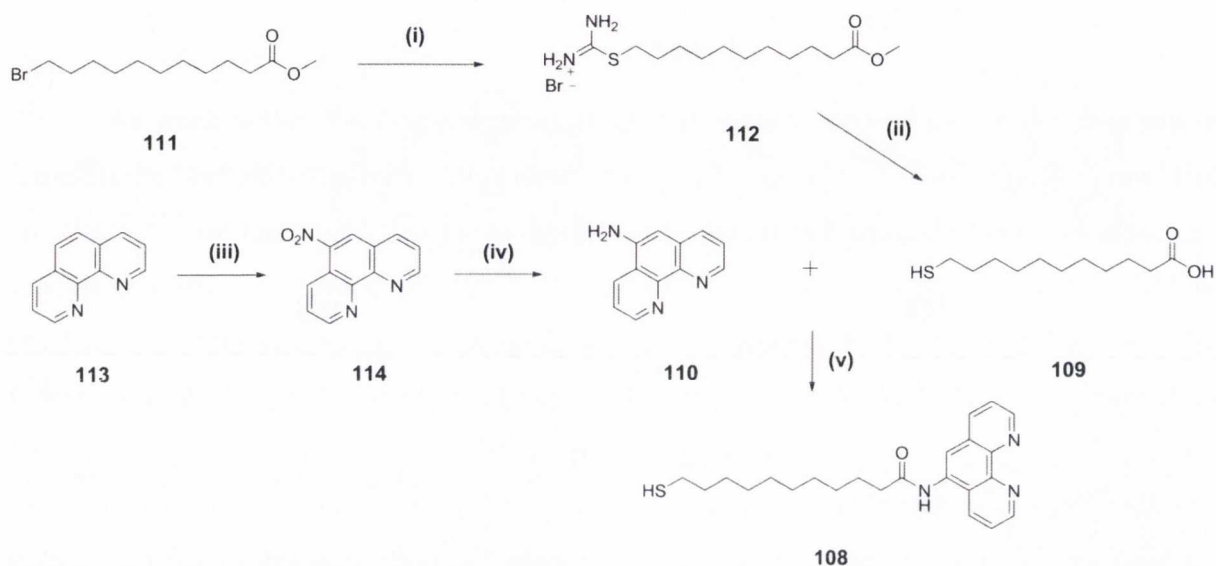


7.2 Synthesis of Complexes 105 - 107

The target compounds **105 - 107** were prepared from commercially available starting materials. The same procedure was applied to the synthesis of all analogues and full experimental details are provided in Chapter 8. The following section will deal with a description of the methods used for the synthesis and characterisation of **105 - 108**.

7.2.1 Synthesis of alkyl thiol functionalised phenanthroline **108**

The alkyl thiol functionalised system **108** was synthesised as outlined in Scheme 7.1. The synthetic scheme firstly involved the synthesis of two intermediates, 11-mercaptoundecanoic acid, **109** and 5-amino-1,10-phenanthroline, **110** before coupling of the two fragments to afford **108**.



Scheme 7.1: (i) EtOH:H₂O, thiourea; (ii)(a) aq. NaOH; (b) 6N H₂SO₄; (iii) H₂SO₄, HNO₃, H₂O; (iv) N₂H₄, 10% Pd/C, EtOH; (v) DCM, EDCI, DMAP.

11-Mercaptoundecanoic acid, **109** was synthesised from the commercially available methyl 11-bromoundecanoate, **111** according to a procedure by Zhu *et al.*²⁵⁰ by the nucleophilic displacement of bromide with an excess of thiourea to yield the isothiuronium bromide salt intermediate **112**. Subsequent base hydrolysis of **112** in the presence of aqueous NaOH followed by acidification with 6 N H₂SO₄ resulted in the desired intermediate 11-mercaptoundecanoic acid, **109**. Purification by recrystallisation from hot EtOH yielded the pure compound **109** as a white powder in 72% yield. Simultaneously, 5-amino-1,10-phenanthroline, **110** was synthesised by aromatic nitration of commercially available 1,10-phenanthroline, **113** using a mixture of concentrated H₂SO₄ and HNO₃ to yield 5-nitro-1,10-phenanthroline **114**, in 65% yield. Reduction of the nitro group of **114** using hydrazine in the presence of 10% Pd/C catalyst gave the amino functionalised phenanthroline derivative **110** as a yellow solid in 98% yield. The final step of the synthesis of **108** involved the peptidic coupling of **109** with **110** in DCM using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) in the presence of 4-dimethylaminopyridine (DMAP) to giving **108** as a grey solid in 78% yield after precipitation with H₂O.

The novel ligand **108** was characterised by ^1H NMR, ^{13}C NMR, melting point analysis and IR spectroscopy. The ^1H NMR spectrum (600 MHz, $\text{DMSO}-d_6$) is shown in Figure 7.1. Assignment of the peaks was achieved by H-H COSY and C-H COSY analysis.

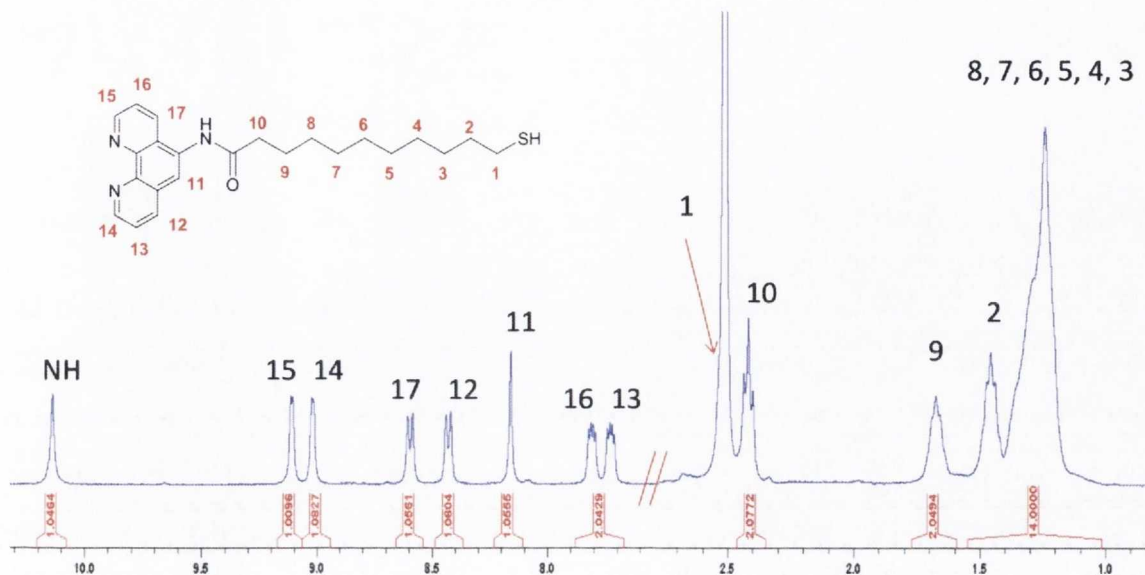


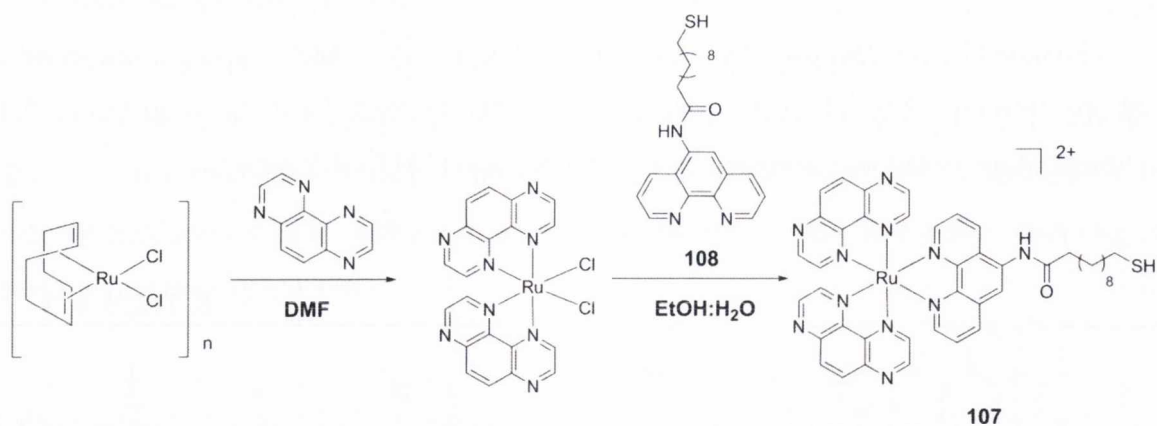
Figure 7.1: The ^1H NMR spectrum of **108** (600 MHz, $\text{DMSO}-d_6$)

The successful formation of this species was immediately apparent due to the the characteristic NH signal at 10.1 ppm. The remainder of the spectrum was also easily assigned owing to the clear distinction observed between the aromatic and aliphatic regions of the ^1H NMR spectrum. Seven clear signals in the aromatic region between 7.5 ppm and 9.5 ppm all integrated to one proton and were assigned as shown in Figure 7.1 by H-H and C-H COSY analysis. Similarly, the aliphatic resonances were all characterised using the same analysis but with the signal α to the thiol group being overlapped by that of the residual H_2O peak, selective TOCSY analysis was necessary. Full experimental details and characterisation are given in Chapter 8.

Having successfully synthesised and characterised the precursor ligand **108**, syntheses of the Ru(II) alkyl thiol complexes were next attempted. The following section will detail the synthesis and characterisation of the resulting complexes **105** - **107**.

7.2.2 Synthesis of alkyl thiol functionalised Ru(II) complexes **105** - **107**

In a manner similar to that described in Chapters 2, 4 and 6, microwave-assisted synthesis was exploited for the complexation of ligand **108** with $\text{Ru}(\text{bpy})_2\text{Cl}_2$, $\text{Ru}(\text{phen})_2\text{Cl}_2$ and $\text{Ru}(\text{TAP})_2\text{Cl}_2$ to yield complexes **105**, **106** and **107**, respectively. The general synthetic pathway is exemplified in Scheme 7.2 showing the synthesis of complex **107**.



Scheme 7.2: Complexation of $\text{Ru}(\text{TAP})_2\text{Cl}_2$ to ligand **108** using a microwave activated reaction to yield complex **107**.

The microwave-assisted syntheses of each of the $\text{Ru}(\text{L-L})_2\text{Cl}_2$ derivatives in DMF, where L-L is either bpy, phen or TAP, was achieved by reaction of the respective ancilliary ligands with $\text{Ru}(\text{cod})\text{Cl}_2$. Ligand **108** was then complexed with the $\text{Ru}(\text{II})$ precursors using a $\text{H}_2\text{O}/\text{EtOH}$ mixture. After 30 minutes irradiation in the microwave at 140°C , precipitation of the resultant complexes as their PF_6 salts was achieved by addition of a concentrated ethanolic solution of NH_4PF_6 . Isolation by suction filtration afforded the products **105**, **106** and **107** as red/brown solids in 75%, 65% and 54% yield respectively after purification by column chromatography [flash silica; 40:4:1 $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{NaNO}_3$ (sat)]. The chloride salts of the complexes were re-formed by dissolution in MeOH followed by stirring with Amberlite ion exchange resin (Cl^- form) for 1 hour.

The three complexes were characterised by ^1H NMR, ^{13}C NMR, elemental analysis, HRMS, melting point analysis and IR spectroscopy. The ^1H NMR spectrum (600 MHz, CD_3CN) of **105** is shown in Figure 7.2 with those of **106** and **107** given in Appendix 7. Again, assignment of the various resonances in the ^1H NMR spectra was achieved by H-H and C-H COSY analysis and is indicated in Figure 7.2. Full experimental details and characterisation are given in Chapter 8.

As for ligand **108**, the ^1H NMR spectra for each complex were easily separated between the aromatic and aliphatic regions with each of the peaks being assigned to their respective protons as shown in Figure 7.2 for **105** and in Appendix 7 for **106** and **107**. The aromatic region of all three systems gained added complexity due to the appearance of signals corresponding to the ancilliary bpy, phen and TAP ligands for **105**, **106** and **107**, respectively. The aliphatic regions of the spectra were seen to be similar to those observed for the ligand **108** with the CH_2 signal α to the thiol group being shifted furthest downfield, with the CH_2 signal α to the amide being shifted slightly less downfield. As these spectra were measured in MeOD, the NH signal could not be observed, however, when measured in CD_3CN the NH signal for each of these complexes could be clearly observed at *ca.* 10 ppm.

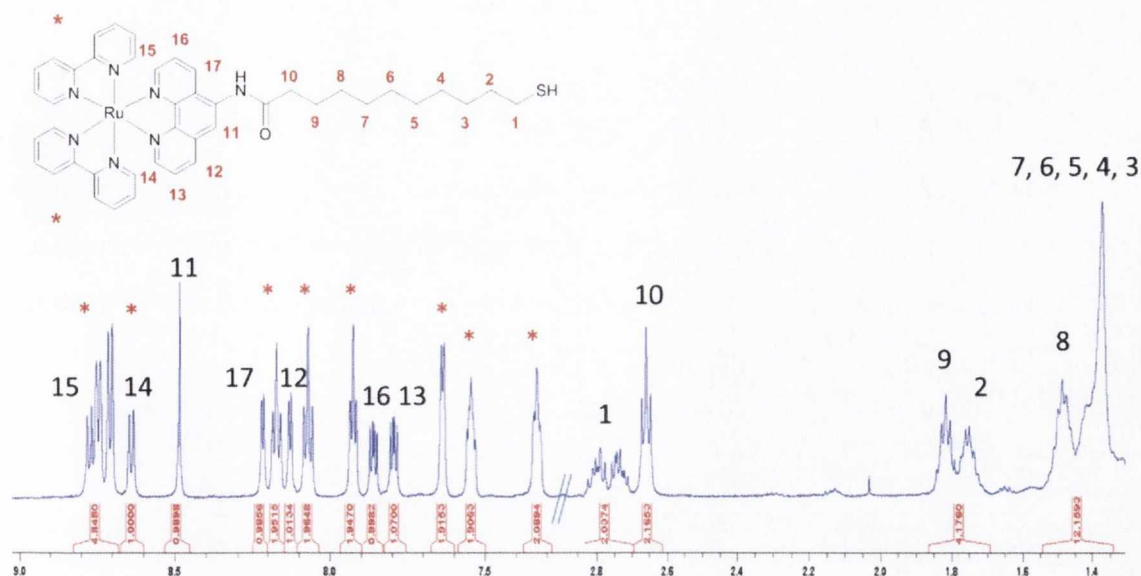


Figure 7.2: The ^1H NMR spectrum of **105** (600 MHz, MeOD)

Successful formation of the complexes was also evident from MALDI high resolution mass spectrometry analysis, where **105**, **106** and **107** displayed $m/z = 2051.3718$, 2147.3765 and 2155.3379 , respectively corresponding to their $2\text{M}^{2+} - 2\text{H}$ ions plus three PF_6 counter ions. This observation was seen for each of the complexes due to oxidation of the thiol groups during the ionisation process leading to bimetallic disulfide species being observed. A comparison between the calculated and observed isotopic distribution pattern is shown for **106** in Figure 7.3 with those for **105** and **107** shown in Appendix 7. Synthesised as their chloride salts, all complexes were water-soluble, and their photophysical properties were investigated in 10 mM phosphate-buffered aqueous solutions at pH 7.4.

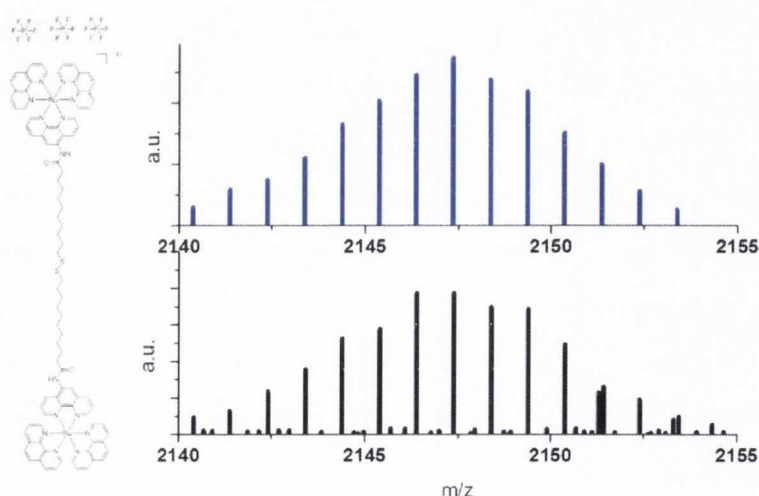


Figure 7.3: Comparison between the calculated (**blue**) and observed (**black**) isotopic distribution pattern for **105** from high resolution mass spectrometry analysis

7.3 Photophysical Characterisation of **105** - **107**

An investigation into the absorption and emission properties of **105** - **107** was necessary to evaluate their suitability for application towards biological imaging agents. In addition, it was important to be able to compare the photophysics of the free complexes with those of the AuNP conjugates to determine any interaction between the Ru(II) centres and the surface of the AuNPs. As such this section details the photophysical behaviour of each of the complexes **105** - **107** at pH 7.4 in phosphate buffer solution.

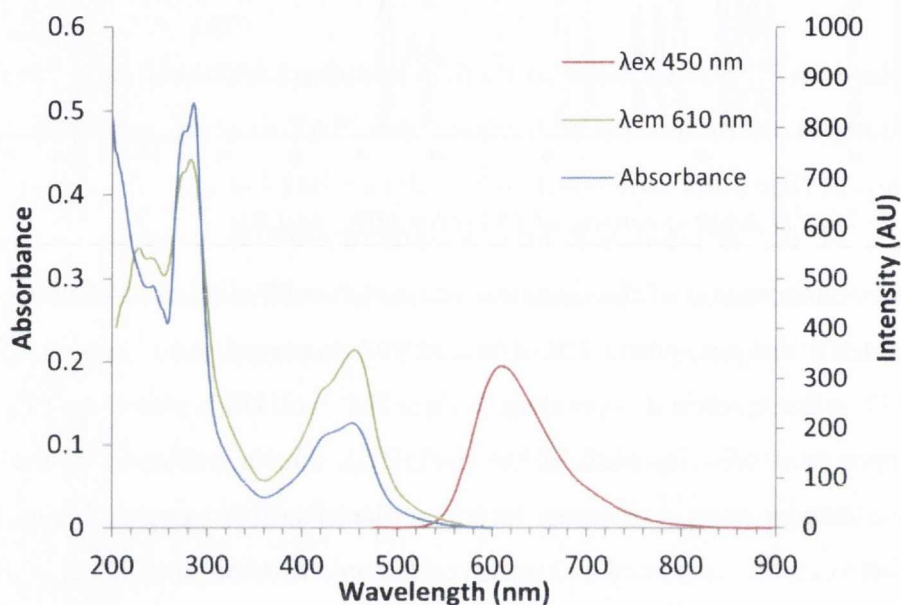


Figure 7.4: The UV/Visible, excitation and emission spectra of **105** (10 μ M) in 10 mM phosphate buffer at pH 7.4

The absorption, excitation and emission spectra of **105**, recorded in 10 mM phosphate buffer at pH 7.4, are shown in Figure 7.4. The spectra of **106** and **107** are shown in Appendix 7. The absorption spectrum of **105** showed absorbance maxima at *ca.* 285 nm and 450 nm. The band at 285 nm was characteristic of π - π^* intra-ligand transitions of the ancilliary bpy ligands while the broad, longer wavelength band with a maximum at 450 nm can be attributed to the MLCT transitions of the Ru(II) centre. Similarly, the absorption spectrum of **106** and **107** showed similar absorbance maxima with the short wavelength transitions being attributed to π - π^* intra-ligand transitions of the ancilliary ligands while the broad band centred at *ca.* 450 nm was characteristic of the MLCT transitions. A summary of the absorption properties of **105** - **107** as well as their respective molar absorptivities is given in Table 7.1.

Table 7.1: Absorption properties of **105**, **106** and **107** in 10 mM phosphate buffer, at pH 7.4.

Complex	$\lambda_{\max}$ (nm) [$\epsilon(\text{M}^{-1} \text{cm}^{-1})$] in phosphate buffer ($\pm 10\%$)	
	$\pi\text{-}\pi^*$ IL	MLCT
105	285 [49500]	450 [12400]
106	260 [72700]	450 [14300]
107	270 [50900]	415 [10700]

Excitation into all absorption bands of each complex resulted in characteristic MLCT based emission with $\lambda_{\max}$ at 610 nm, 605 nm and 635 nm for **105**, **106** and **107**, respectively. Similarly, excitation spectra of **105**, **106** and **107** were shown to be structurally identical to their absorption spectra. The fluorescence quantum yields (Φ_F) (aerated) for **105** - **107** were found to be 0.054, 0.056 and 0.028, respectively. The values for **105** and **106** were somewhat higher than the value reported for $[\text{Ru}(\text{bpy})_3]^{2+}$ (0.042 in H_2O)¹⁷⁶. Furthermore, the value of 0.028 obtained for **107** is double the value recorded for $[\text{Ru}(\text{TAP})_3]^{2+}$ in H_2O (0.014).⁷⁵

Excited state lifetime measurements (τ_{em}) (aerated) were recorded at pH 7.4 in 10 mM phosphate buffer solution in order to further characterise the excited state properties of **105** - **107** by using time correlated single photon counting (TCSPC). All data was best fitted to a monoexponential decay function confirming the presence of just one fluorescent species in solution. Complexes **105** and **106** showed τ_{em} values of 610 ns and 648 ns respectively, that were slightly longer than analogous complexes $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ which have been shown to have τ_{em} values of 450 ns and 530 ns in aerated H_2O .²⁵¹ Complex **107** exhibited a τ_{em} value of 563 ns which was slightly shorter than its parent complex $[\text{Ru}(\text{TAP})_2(\text{phen})]^{2+}$ which has a τ_{em} value of 730 ns in aerated H_2O , respectively. A summary of the excited state properties of **105** - **107** in 10 mM phosphate buffer is given in Table 7.2.

Table 7.2: Emission properties of **105**, **106** and **107** in phosphate buffer solution at pH 7.4 at 298K

Complex	$\lambda_{\text{emission}}$ (nm)	Φ_f ($\pm 10\%$)	τ_{em} (ns) (air)
105	610	0.054	610
106	605	0.056	648
107	635	0.028	563

During this photophysical study it has been observed that **105** - **107** displayed characteristic Ru(II) polypyridyl complex ground and excited state behaviour in aqueous solution, leading to the conclusion that the presence of the long chain alkyl thiol did not have a profound effect on their solution properties. In addition, each complex displays multiple absorption bands and intense fluorescence emission which is seen as an advantage in terms of potential imaging applications. As has been observed in previous Chapters of this Thesis with other Ru(II) complexes, the photophysical properties of **105** - **107** are expected to be sensitive to their local environment and, as a result, may be modulated upon interaction with DNA or other biomolecules. Moreover, as complex **107** contains two π deficient TAP ligands, photo-induced electron transfer behaviour between the DNA nucleobases and the π deficient Ru(II) complex may occur, leading to a direct and profound effect on the fluorescence emission spectrum of **107**. In order to explore the nature of the interaction between **105**, **106** and **107** with DNA, the following sections will discuss a photophysical study in which changes upon interaction with stDNA will be monitored. In addition to providing information on the DNA binding ability of the free complexes **105**, **106** and **107**, it will also enable a direct comparison to be made with the DNA binding ability of **AuNP-105**, **AuNP-106** and **AuNP-107** at a later stage in this Chapter.

7.4 DNA Binding Interactions of **105**, **106** and **107**

With their cationic nature **105**, **106** and **107** were all expected to have a strong affinity for the negatively charged DNA backbone. Furthermore, the π deficient nature of **107** makes it a possible DNA photoreactive agent and it was anticipated that any modulations in its photophysical behaviour would provide valuable information on the processes occurring during the binding event. The following sections will detail spectroscopic titrations carried out to investigate the nature and binding affinity of **105**, **106** and **107** with DNA.

7.4.1 Spectroscopic Titrations of **105** - **107** with DNA

7.4.1.1 UV/Vis Absorption Titrations of **105** - **107** with DNA

The changes in the ground state of **105** are shown in Figure 7.5 with those of **106** and **107** shown in Appendix 7. The interaction of **105** - **107** with DNA was examined using UV/Vis absorption spectroscopy in a similar fashion to that described in previous Chapters by addition of small aliquots of stDNA to a 10 mM phosphate buffer solution of each complex at pH 7.4, until a plateau in absorbance was reached. All titrations were repeated a number of times to ensure reproducibility.

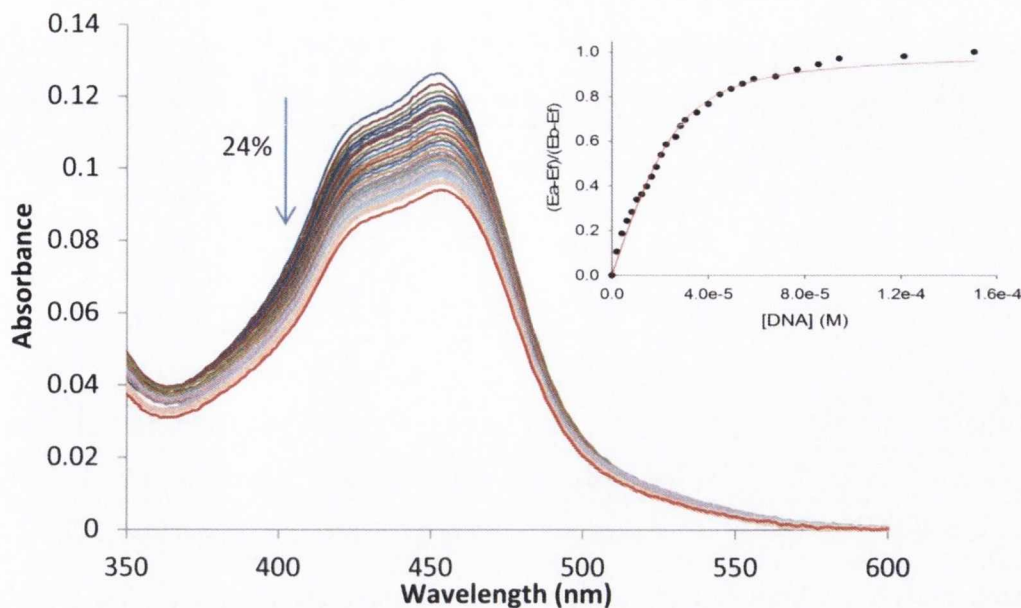


Figure 7.5: Changes in the UV/Visible spectrum of **105** (10 μM) upon additions of stDNA (0-290 μM) in 10mM phosphate buffer, (pH 7.4). Inset: Plot of $(\epsilon_a - \epsilon_f) / (\epsilon_b - \epsilon_f)$ vs. $[DNA] (M)$, P using data with a P/D between 0-15 and the best fit of the data (---) using the Bard Eqn.

The titration of **105** - **107** with stDNA resulted in significant modulations to their absorption spectra. Complex **105** exhibited a 24% decrease in absorbance of its MLCT band at 450 nm with **106** experiencing an initial red shift of 7 nm followed by a 30% hypochromism at 450 nm. The MLCT band of **107** exhibited a less obvious red shift of 4 nm and this coincided with a hypochromism of 26% at 415 nm. These results are similar to those obtained previously for the addition of DNA to other Ru(II) complexes; the observed changes in the MLCT band having been attributed to electrostatic interactions between the complex and the phosphate backbone of DNA.²⁷ Interestingly, in the case of **106**, the initial additions of DNA seemed to induce a slight red shift in the absorbance maximum which was followed by the observed hypochromic effect. Such behaviour may be due to a different mode of interaction at low DNA ratios, in which the ancilliary phenanthroline ligands are partially intercalated.²⁵² Further additions of DNA might give rise to a redistribution of the complexes as more binding sites become available. The spectroscopic changes observed, *i.e.* the significant hypochromism and red shift of the absorption maximum, are consistent with strong association of each of these complexes with DNA and, as such, the intrinsic binding constant K_b , and binding site size n were determined using the model of Bard *et al.* using these changes.¹⁸¹ A representative plot of $(\epsilon_a - \epsilon_f) / (\epsilon_b - \epsilon_f)$ vs. $[DNA]$ and the corresponding best fit of the data to the Bard eqn. for **105** are shown as an inset in Figure 7.5 and a summary of the results obtained are detailed in Table 7.3.

Table 7.3: DNA binding parameters from fits to absorbance data

Complex	$\lambda(\text{MLCT})$ Hypochromism	Binding constant K_b (M^{-1})	Binding site size n (base pairs)	R^2
105	24%	$4.7 \times 10^5 (\pm 0.7)$	1.3 (± 0.08)	0.99
106	30%	---	---	---
107	26%	$6.4 \times 10^5 (\pm 1.7)$	1.9 (± 0.1)	0.99

The above results confirmed the affinity of the complexes **105** - **107** for DNA where **105** showed binding with $K_b = 4.7 \times 10^5 (\pm 0.7)$ and $n = 1.3 (\pm 0.08)$. A fit could not be obtained for **106** due to the biphasic nature of the interaction mentioned above, however, **107** was shown to have a slightly stronger affinity for DNA compared to **105** with $K_b = 6.4 \times 10^5 (\pm 1.7)$ and $n = 1.9 (\pm 0.1)$. The small magnitude of changes and the observed red shift had an effect on the data for **107** and it should be noted that the error calculated from this curve is quite high as a result. In any case the changes observed gave rise to binding constants of the order of 10^5 M^{-1} for both **105** and **107**, which are higher than those previously determined for parent complexes such as $[\text{Ru}(\text{phen})_3]^{2+}$ which has been calculated as being $3 - 5 \times 10^4 \text{ M}^{-1}$ (5 mM Tris/HCl, 50 mM NaCl, pH 7.1) depending on its enantiomeric specificity.³⁷ The higher binding affinity of **105** and **107** is likely to be due to the presence of the long chain alkyl thiol group which may increase affinity through hydrophobic interactions. Moreover, it is also likely that the binding of **106** to DNA is of similar magnitude, if not even higher, than both **105** and **107** due to the ability of its ancilliary phen ligands to partially intercalate between the base pairs of DNA.³⁷ This observation is supported by examination of the titration curves for **105** - **107** (Figure 7.6) which reveals that the largest changes are observed for **106**.

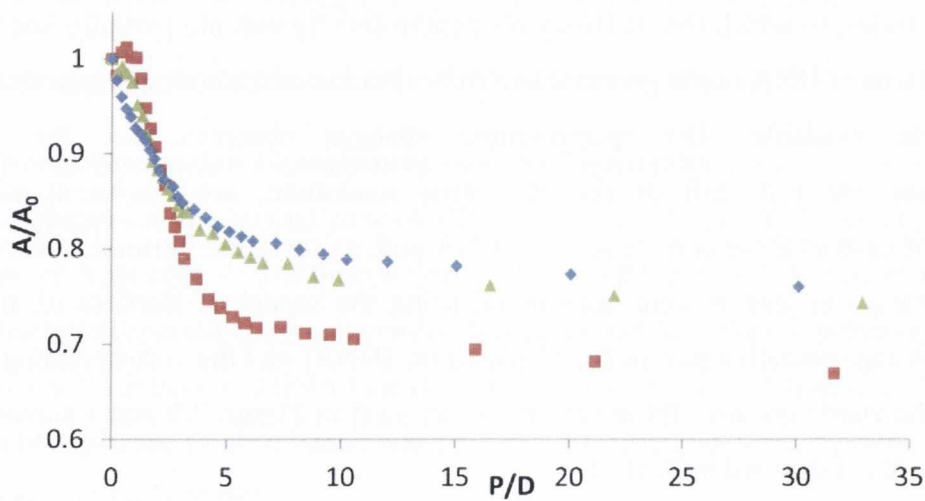


Figure 7.6: Plots of the change in integrated MLCT absorption of **105** (♦), **106** (■) and **107** (▲) upon addition of stDNA in 10 mM phosphate buffer, at pH 7.4

Having gained information on the binding processes occurring between **105** - **107** and DNA through UV/Vis absorption titrations, changes were also monitored in the excited state properties of **106** - **107**. These results will be presented in the following section.

7.4.1.2 Fluorescence Emission Titrations of **105** - **107** with DNA

Given the excited state character of **105** - **107** it was anticipated that addition of DNA would lead to significant modulations of the emission response, similar to those observed in previous Chapters. Indeed, the incorporation of two TAP ligands in the structure of **107** make it especially likely and it was anticipated that perturbations of its emission behaviour would provide evidence for any photo-electron transfer processes occurring with DNA.

The changes in the excited character of **107** are shown in Figure 7.7 with those of **105** and **106** shown in Appendix 7. Upon addition of stDNA to complex **105** the MLCT emission band centred at 610 nm was seen to undergo a biphasic interaction where, after the first addition, the emission was seen to decrease before increasing to a plateau 13% above the initial emission value. Complex **106** showed a slightly different biphasic behaviour in which the emission centred at 605 nm was seen to be quenched initially by 36% (P/D 0 $\rightarrow$ 2.5) before a subsequent slight increase to a plateau (P/D = 2.5 $\rightarrow$ 30). Interestingly, this resulted in an overall fluorescence emission decrease of 31% for **106** when fully bound to the DNA helix in solution. Complex **107** (Figure 7.7) showed more efficient quenching as a result of interaction with DNA where the emission centred at 635 nm was efficiently quenched by 66% over the entire range of the titration (P/D 0 $\rightarrow$ 30).

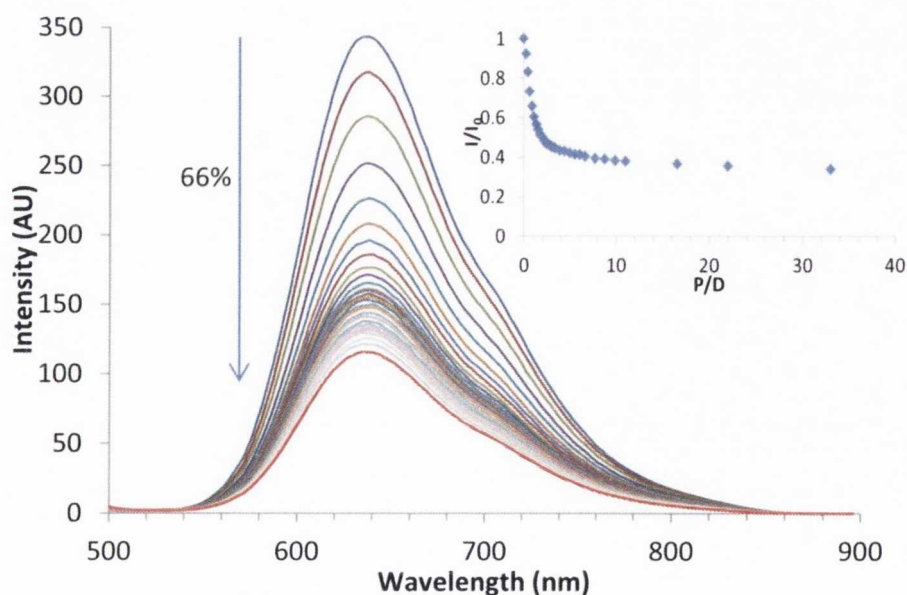


Figure 7.7: Changes in the emission spectrum of **107** (10 μM) (λ_{ex} 415 nm) with increasing concentration of stDNA (0-285 μM). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.

The fluorescence data for both **105** and **106** suggests a biphasic interaction of these complexes with different distributions of the complex on the polynucleotide at different stages of the titration. The luminescence at the plateau value is likely to correspond to that of the isolated complex bound to DNA. In the case of **105**, the slight enhancement of luminescence might originate from closely bound metal complexes which are sheltered from the bulk solution by the DNA helix environment, resulting in an overall fluorescence increase. Conversely, in the case of **106**, the initial decrease was followed by a slight increase, however, the overall changes resulted in a 30% decrease in the observed fluorescence, perhaps suggesting the occurrence of redox processes between **106** and DNA. The emission behaviour of **107** follows the trend observed with other π deficient TAP complexes, due to quenching of the excited state of **107** by photo-electron transfer between *guanine* residues and the MLCT state of **107**.^{32,79,158,182}

For comparison, the emission profiles for each of the systems, **105** - **107** are shown in Figure 7.8. Examination of the overlaid changes demonstrates the different excited state responses observed for each complex. The fact that such minor structural differences between these systems can exhibit such a large variation in their photophysical response serves to further emphasise the sensitivity of Ru(II) complexes to their local environment. Furthermore, the changes exhibited by **105** - **107** reflect interactions with DNA that may also be monitored using various other techniques. The following sections will attempt to further quantify the binding affinities of complexes **105** - **107** for DNA by utilising various other spectroscopic and biophysical techniques.

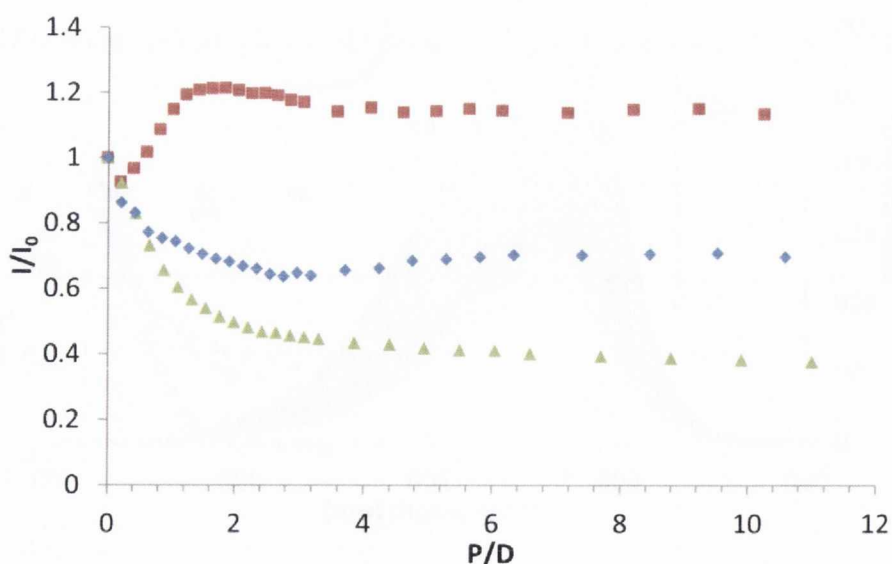


Figure 7.8: Relative changes in the MLCT emission intensity of **105** (■), **106** (◆) and **107** (▲) upon addition of stDNA in at pH 7.4 in 10 mM phosphate buffer solution

7.4.2 Ethidium Bromide Displacement Assays

The binding efficiency of **105** - **107** was also monitored by displacement of DNA bound EtBr as described in previous Chapters. The addition of **105** - **107** to a mixture of EtBr and DNA was found to displace 50% EtBr at molar ratios less than one indicating a strong interaction of these complexes with DNA, confirming the results obtained from the UV/Vis absorption and emission titrations discussed above. As can be seen in Figure 7.9, the addition of **105** - **107** caused the emission intensity of intercalated EtBr to decrease, thus displacing the known intercalator from the DNA helix.

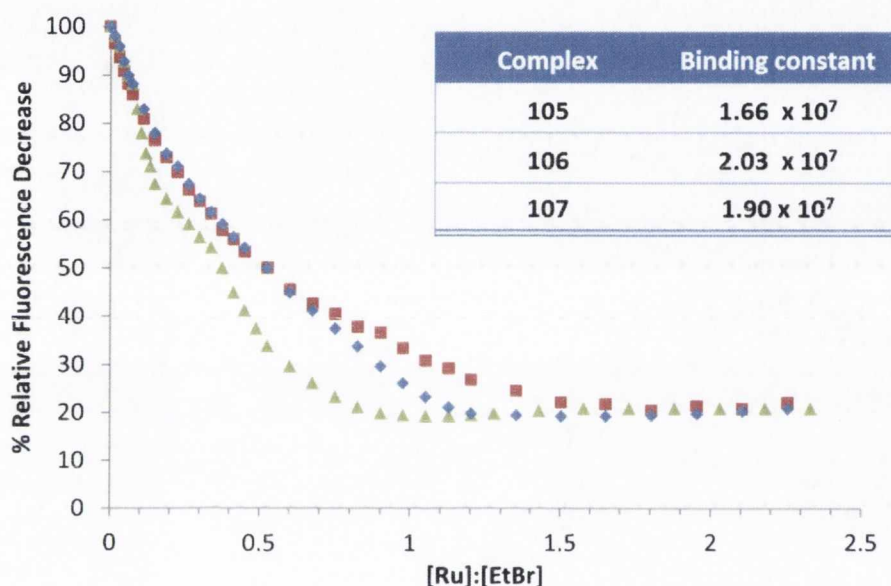


Figure 7.9: Decrease in fluorescence of DNA bound EtBr upon addition of **105** (■), **106** (▲) and **107** (◆) in 10 mM phosphate buffer, pH 7.4. Inset: Table of binding affinities of **105**, **106** and **107** calculated from EtBr displacement assays

The apparent binding constants K_{app} were again calculated according to the competitive binding model described by Boger *et al.*¹⁸⁵ as demonstrated previously and a summary of binding constants derived is displayed as an inset in Figure 7.9. The displacement capabilities correlate well with the behaviour observed in the UV/Vis and fluorescence titrations where the displacement capability is in the order of **106** > **107** > **105** and this is reflected in the values obtained for the binding constants (Figure 7.9). As seen previously for EtBr displacement assays, the values obtained are considerably higher than those determined by UV/Vis and fluorescence data, being nearly two orders of magnitude higher. This is thought to be due to binding of **105** - **107** at high P/D ratios, where many different processes may be occurring, including perturbation of the DNA helical microstructure. Such behaviour is likely to have a significant effect on the binding ability of an intercalator, such as EtBr, and may effect displacement of the DNA bound EtBr molecule more readily resulting in a higher

value of K_{app} being determined. Aside from these observations, it is clear from these results that **105** - **107** display significant interaction with DNA and confirm the previous results obtained from the ground and excited state spectroscopic investigations. The following section will look to further emphasise this binding ability by observing the effect of **105** - **107** on the DNA helix stability using T_m measurements.

7.4.3 Thermal Denaturation Studies of **105** - **107**

Thermal denaturation studies were carried on **105** - **107** in the presence of stDNA, as in the previous Chapters. In the absence of metal complex the T_m value for DNA was determined to be 69 °C. Shown in Figure 7.10 are the melting profiles of DNA alone and in the presence of **105**, **106** and **107**.

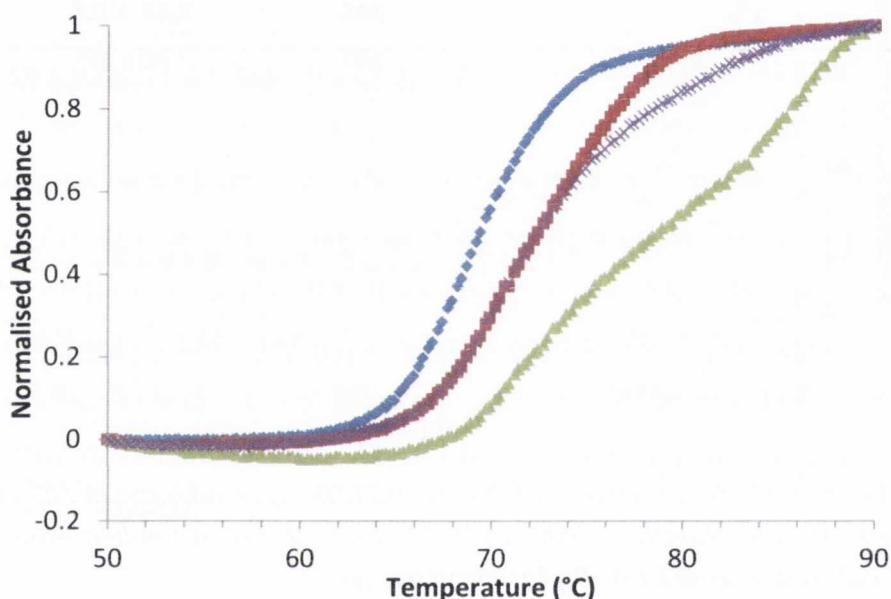


Figure 7.10: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence (♦) and presence of **105** (■), **106** (▲) and **107** (×) at P/D = 10. Inset: The T_m values from first derivative plots of the melting curve

The changes observed reflect the stabilisation of the DNA helix structure upon interaction with each of the complexes at a P/D ratio of 10. Both **105** and **107** showed modest stabilisation of the helix being slightly shifted to 73 °C with a perturbed profile being observed for **107**. For complex **106** the melting transition had not gone to completion at 90 °C and, consequently, it was not possible to fit the data to a sigmoidal function. Although an exact value could not be reported it may be regarded as being greater than 75 °C. It is also noteworthy that the different extent of the stabilisation observed for each complex again correlates well with both the spectroscopic titrations and the EtBr displacement assays where stDNA was seen to exhibit maximum stabilisation upon interaction with **106**.

Overall this study has demonstrated significant stabilisation of the DNA helix associated with interaction with **105** - **107** and further emphasises the affinity that they possess for DNA. The following section will look to further monitor the affinity of these complexes for DNA and attempt to shed light on the particular binding mode of this family of complexes to DNA using circular dichroism.

7.4.4 Circular Dichroism Studies of **105** - **107** in the Presence of DNA

In an attempt to further probe the interaction of **105** - **107** with DNA, circular dichroism titrations were carried out in the same manner as described in previous sections, in which the concentration of DNA was kept constant and that of the complexes was varied to give a range of P/D ratios. The spectra for **106** are shown in Figure 7.11 with those of **105** and **107** shown in Appendix 7.

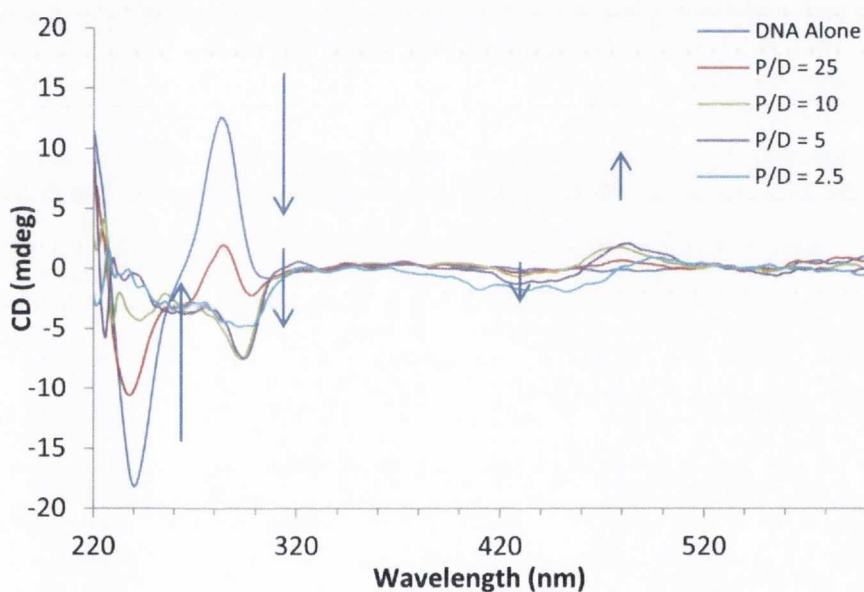


Figure 7.11: Circular dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **106** at varying P/D

The data obtained for all three complexes, **105** - **107** in the presence of DNA show CD spectra with significant modulations. Most obviously, large decreases in the DNA region of the CD spectra between 220 nm and 300 nm were observed for all three complexes. Simultaneously, evolution of a negative band in this region was also seen in each case at *ca.* 290 nm and most likely corresponds to π - π^* intra-ligand transitions of the ancilliary ligands. Behaviour such as this has been observed in Chapter 2 for complexes **53** - **55** and implies strong association of the metal centre of **105** - **107** with the DNA helix. At high loading (P/D = 2.5) there was also the evolution of small ICD signals in the charge transfer region, from 350 nm – 500 nm. For **105** this culminates in a small negative signal centred at 460 nm, while

for **106** the appearance of both positive (at 420 nm) and negative (at 470 nm) CD bands are apparent. For complex **107** a similar ICD signal to **105** was observed, however, the small negative signal was centred at 420 nm corresponding to the MLCT maximum absorption of **107** at 415 nm.

From the above study it is clear that in all cases the CD spectrum of DNA was substantially affected, with the characteristic DNA CD signal practically disappearing. This UV region is sensitive to changes in conformation resulting from complex binding, indeed binding of $[\text{Ru}(\text{phen})_3]^{2+}$ by insertion of two phen ligands into the minor groove of a $[\text{d}(\text{CGCGATCGCG})_2]$ oligonucleotide has been shown to cause distortions of the DNA helical structure.²⁵² However, **53** - **55** all exhibit transitions in the UV region, which overlap with the DNA absorption bands, thus it is difficult to discern if these bands result from an ICD, or from structural changes in the DNA. Taking these observations into consideration, in addition to the ICD signal observed in the charge transfer region, overall results point to a binding mode in which the metal centre is tightly associated with the DNA helix, thus experiencing its chirality.

Considered with the other spectroscopic studies presented so far, it can be concluded that **105** - **107** all bind to DNA with specific interactions with the helical microenvironment ensuring tight association of the metal centre. Such interactions are likely to occur through external binding or partial insertion into the grooves of the ancilliary ligands as has been postulated for parent systems such as $[\text{Ru}(\text{phen})_3]^{2+}$.²⁵² This important evidence also confirms that the presence of the long chain alkyl thiol on these complexes does not have a negative effect on DNA binding. The proceeding section will summarise the results from the DNA binding studies undertaken.

7.4.5 Summary of DNA Binding Studies of **105** - **107**

The work described in this Chapter has looked to evaluate the DNA binding capability of **105** - **107** in order to compare the free complexes with those attached to AuNPs. The investigations have looked at the photophysical modulations exerted on **105** - **107** upon addition of DNA using UV/Vis and fluorescence spectroscopy and these results have been further confirmed by the use of EtBr displacement assays, T_m experiments and CD studies.

The photophysical properties of **105** - **107** were first evaluated in phosphate buffer solution before changes in the ground and the excited state interactions in the presence of stDNA were observed and quantified. Analysis of the ground state data allowed the calculation of binding constants of the order of 10^5 M^{-1} for both **105** and **107**, however, due to a biphasic interaction of **106** with DNA reliable binding data could not be obtained for this

complex. Concomitantly, all three complexes exhibited excited state modulations upon interaction with DNA where **105** was seen to have its emission increased while **106** and **107** exhibited emission quenching.

Using EtBr displacement assays it was shown that **105** - **107** were capable of displacing EtBr effectively when bound to stDNA, while T_m experiments found that the helical structure of stDNA was stabilised in the presence of all three complexes. The general trend of stabilisation and EtBr displacement ability both correlated well with the DNA binding affinities observed from the UV/Vis titrations where **106** > **107** > **105** were the observed preferences from all three studies. The results from the CD studies revealed close association of **105** - **107** with DNA and showed small ICD signals in the charge transfer region, pointing to a binding mode in which the metal centre is tightly associated with the helix.

The studies carried out thus far have identified that **105** - **107** bind to DNA; each displaying varied spectroscopic behaviour in response to stDNA. The next section of this Chapter will deal with the synthesis of AuNPs and the adsorption of the alkyl thiol based complexes **105** - **107** on to the gold surface to create **AuNP-105**, **AuNP-106** and **AuNP-107**. Once functionalised, it was anticipated that such AuNP conjugates may also have binding affinity for DNA that may be exploited for use in luminescent biological imaging applications. The preceding sections of this Chapter will deal with the synthesis of **AuNP-105**, **AuNP-106** and **AuNP-107** and their physical and photophysical characterisation. This will be followed by an investigation into their DNA binding ability and will be followed by preliminary biological studies including their DNA photocleavage ability as well as their cellular uptake and localisation.

7.5 Synthesis of Ru(II) Functionalised AuNP-105, AuNP-106 and AuNP-107

Functionalisation of AuNPs with self-assembled monolayers provides a method for introducing diverse functionality to the nanoparticle surface and, as a result, many preparative methods for the synthesis of AuNPs have been developed to date.²⁵³⁻²⁵⁶ Depending on the preparative conditions utilised, many AuNPs have a tendency to slowly flocculate, causing precipitation from solution.²⁵⁴ One of the most widely used and versatile synthetic methods for the formation of stable AuNPs is the Brust-Schiffrin method which involves a two-phase approach resulting in the formation of AuNPs with a diameter of *ca.* 5 nm.²⁵⁴ Given the water soluble nature of **105** - **107** such a phase transfer synthesis is ideally suited to the formation of **AuNP-105**, **AuNP-106** and **AuNP-107** and is the basis for the method described.

The syntheses of **AuNP-105**, **AuNP-106** and **AuNP-107** were achieved using a modified two-phase Brust method where HAuCl_4 was first solubilised in H_2O and was then transferred to toluene using tetraoctylammonium bromide (TOAB) as the phase-transfer agent. Subsequent reduction using NaBH_4 gave TOAB-stabilised gold nanoparticles (TOAB-AuNP), where the organic phase was seen to change colour from orange to deep purple within a few seconds indicating the formation of the colloidal species. Exchange of TOAB on the surface of the AuNPs with the Ru(II) alkyl thiol functionalised complexes **105**, **106** and **107** was achieved by vigorously stirring an aqueous solution of each complex with TOAB-AuNP in toluene over a 12 hour period at room temperature. The initial orange aqueous solution turned a dark brown colour confirming the phase transfer of the AuNPs from the toluene layer to the aqueous layer. Separation of the aqueous layer and subsequent addition of a saturated aqueous solution of NH_4PF_6 to the aqueous solution of AuNPs resulted in the formation of a flocculate which was isolated by centrifugation and repeatedly washed with a mixed $\text{H}_2\text{O}/\text{MeOH}$ solution and dried under vacuum. Any unbound complex was then removed by a second anion exchange step where, after dissolution in MeCN, an acetone solution of tetrabutylammonium chloride (TBACl) was added resulting in the immediate formation of a flocculate which was isolated by centrifugation, repeatedly washed with acetone to remove excess salts (TBA^+ or NH_4^+) and dried with Et_2O . The resultant solid was readily soluble in H_2O and formed a stable red coloured colloidal solution. Figure 7.12 outlines the synthetic approach undertaken. The resulting water-soluble gold nanoparticles **AuNP-105**, **AuNP-106** and **AuNP-107** were found to be stable for many months when kept at room temperature in aqueous buffered solution at pH 7.4 and were characterised using dynamic light scattering (DLS), TEM, and UV/Visible studies. Details of their characterisation are described in the following section.

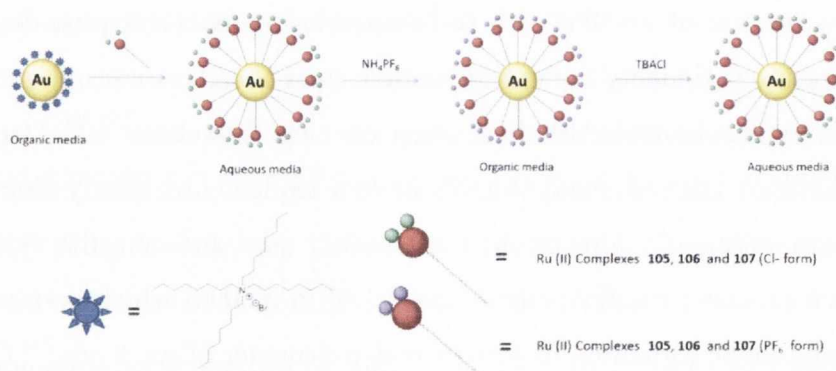


Figure 7.12: Representation of exchange of TOAB on the surface of AuNPs with the Ru(II) alkyl thiol functionalised complexes **105**, **106** and **107** and aqueous/organic medium transfers by anionic exchange around the Ru(II) stabilised AuNPs

7.6 Characterisation of Ru(II) Functionalised AuNP-105, AuNP-106 and AuNP-107

7.6.1 Dynamic Light Scattering Studies of AuNP-105, AuNP-106 and AuNP-107

In order to estimate the size of the AuNP conjugate species, **AuNP-105**, **AuNP-106** and **AuNP-107** dynamic light scattering (DLS) measurements were undertaken using a Malvern Zetasizer Instrument. DLS is a non-invasive technique used for measuring the size of molecules and particles typically in the submicron region.²⁵⁷ Particles in suspension undergo Brownian motion induced by bombardment by solvent molecules that themselves are moving due to their thermal energy. If the particles are illuminated with a laser, the intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles and analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size.²⁵⁷ The diameter that is measured by DLS is called the hydrodynamic diameter and refers to how a particle diffuses within a fluid. The diameter obtained by this technique is that of a sphere and will depend not only on the size of the particle “core”, but also on any bound surface structure.²⁵⁷

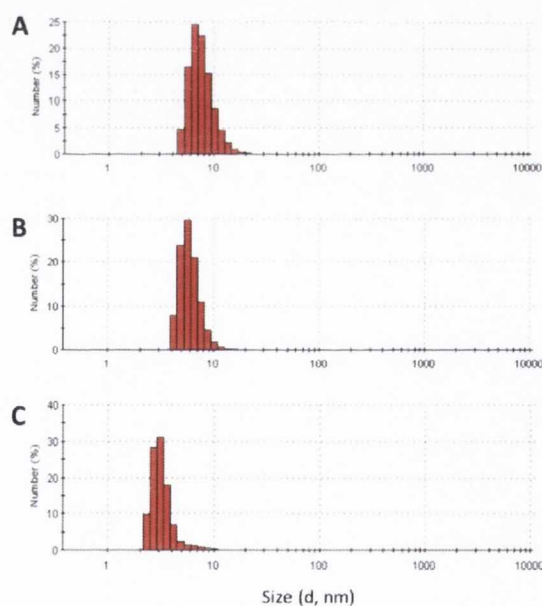


Figure 7.13: Particle size distribution plots for (A) **AuNP-105**, (B) **AuNP-106** and (C) **AuNP-107** as determined from DLS analysis

DLS measurements were carried out on filtered samples of **AuNP-105**, **AuNP-106** and **AuNP-107** and gave hydrodynamic diameter readings of 7.73 nm, 6.03 nm and 3.29 nm respectively, the size distribution graphs being shown in Figure 7.13.

This hydrodynamic diameter range is consistent with previously reported AuNPs synthesised using similar methods and suggests successful formation of **AuNP-105**, **AuNP-106** and **AuNP-107**.^{141,156,254} The DLS data also revealed a very narrow size distribution of AuNPs indicating that no agglomeration of the AuNPs has occurred. In order to further characterise **AuNP-105**, **AuNP-106** and **AuNP-107**, TEM measurements were subsequently carried out, the results of which will be discussed in the next section.

7.6.2 Transmission Electron Microscopy Studies of **AuNP-105**, **AuNP-106** and **AuNP-107**

TEM studies were undertaken to provide information on the shape, size and size distribution of the AuNPs. An example of TEM images obtained for **AuNP-105** is shown in Figures 7.14 (A) and 7.14 (B) and the corresponding size distribution graph in Figure 7.14 (C). Equivalent images and spectra for **AuNP-106** and **AuNP-107** are contained in Appendix 7. Formation of the desired species, **AuNP-105**, **AuNP-106** and **AuNP-107** was confirmed, where clear formation of monodisperse particles was observed. From examination of the TEM images of each of the AuNP conjugates, the particle size distribution was calculated by the measurement of approximately 100 particles in a field of view for each sample. Mainly spherical nanoparticles were observed which were measured as having average diameters of

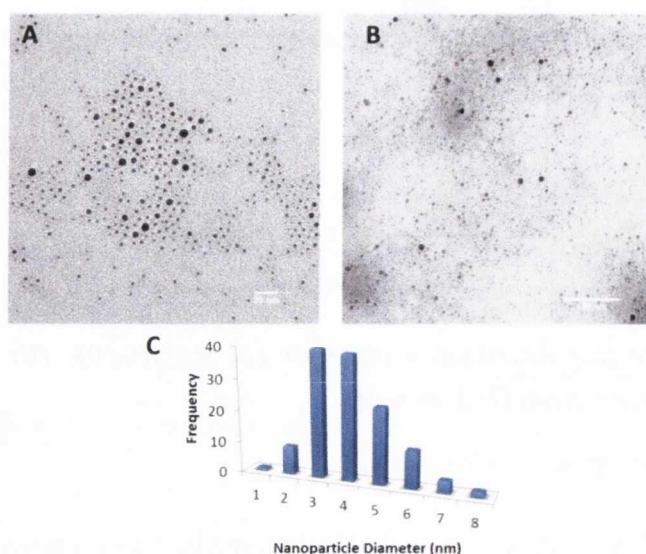


Figure 7.14: TEM images of **AuNP-105** after deposition on copper grids showing: (A) close up image and (B) a larger field view. (C) The corresponding particle size distribution calculated from the TEM data

4.0 nm $\pm$ 1.3 nm, 4.3 nm $\pm$ 1.3 nm and 3.2 nm $\pm$ 1.1 nm for **AuNP-105**, **AuNP-106** and **AuNP-107** respectively, with no evidence of aggregation.

The results from the TEM studies correlate well with the results obtained from the DLS measurements discussed in the previous section where **AuNP-105** and **AuNP-106** have been shown to have similar particle size distributions to each other while **AuNP-107** has been shown on both occasions to have a smaller average particle size. However, the hydrodynamic diameters of **AuNP-105** and **AuNP-106** were measured to be 7.73 nm and 6.03 nm compared to their TEM images which revealed smaller average diameters of 4.0 nm and 4.3 nm, respectively. Such an observation may be accounted for by noting that TEM analysis will only depend on the size of the particle “core”, while any bound surface structure will not be revealed. Conversely, the diameter obtained by DLS is that of the particle “core” in addition to the bound alkyl thiol functionalised Ru(II) complexes. Curiously, such behaviour is not observed for **AuNP-107** where the measurements obtained from both DLS and TEM analysis are almost identical with diameters measured as 3.29 nm and 3.2 nm.

Further information on the sizes of **AuNP-105**, **AuNP-106** and **AuNP-107** can be obtained by UV/Vis spectroscopy which has been widely used to characterise the relationship between the particle size and its optical properties due to NP size playing a critical role in its optical response.^{258,259} Metallic NPs with diameters larger than 3 nm typically show a broad surface plasmon resonance (SPR) band at *ca.* 520 nm due to oscillation of the conduction band electrons induced by the interacting electromagnetic field.²⁵⁸ Taking this into account an investigation into the photophysical properties of each system, **AuNP-105**, **AuNP-106** and **AuNP-107** was undertaken, the results of which will be discussed in the following section.

7.7 Photophysical Studies of Ru(II) Functionalised AuNP-105, AuNP-106 and AuNP-107

In addition to providing information on particle size, the photophysical behaviour of **AuNP-105**, **AuNP-106** and **AuNP-107** can provide evidence for surface functionalisation and information on excited state interactions between the Ru(II) centres and the AuNP surface by comparing them with the absorption and emission properties of complexes **105** - **107**. The following section will detail the ground and excited state properties of each of the AuNP conjugates **AuNP-105**, **AuNP-106** and **AuNP-107** in phosphate buffer solution at pH 7.4 and a comparison will be made with the results discussed in Section 7.3 for the free complexes **105**, **106** and **107**.

The absorption, excitation and emission spectra of **AuNP-105**, recorded in 10 mM phosphate buffer at pH 7.4, are shown in Figure 7.15. The spectra of **AuNP-106** and **AuNP-107** are shown in Appendix 7. The absorption spectrum of **AuNP-105** shows absorbance maxima at *ca.* 290 nm and 450 nm with a broad shoulder extending to *ca.* 700 nm. As was the case for the free complex **105**, the band at 290 nm is characteristic of the π - π^* intra-ligand transitions of the ancilliary bpy ligands while the broad band centred at 450 nm can be attributed to the MLCT transitions of the Ru(II) centre. The shoulder seen extending to 700 nm can be characterised as the SPR absorption band, associated with the AuNPs, and confirms the adsorption of **105** on the surface. Similarly, the absorption spectrum of **AuNP-106** showed absorbance bands centred at 265 nm and 450 nm with the shoulder extending to *ca.* 700 nm. The absorption spectrum of **AuNP-107** followed the same behaviour where a π - π^* intra-ligand band at 270 nm was observed with an MLCT maximum at 415 nm and an SPR shoulder extending to *ca.* 700 nm.

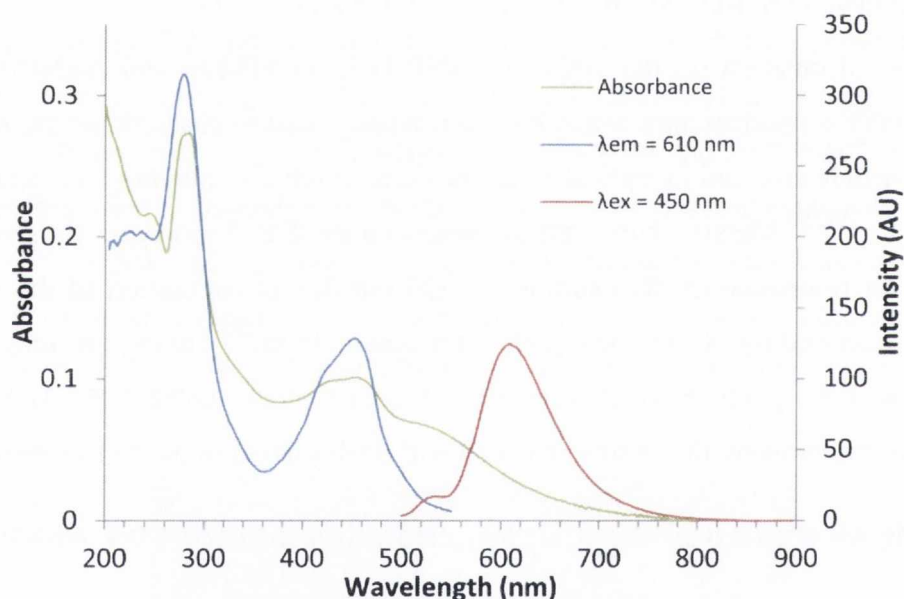


Figure 7.15: The UV/Visible, excitation and emission spectra of **AuNP-105** in 10 mM phosphate buffer solution at pH 7.4

Interestingly, when compared to the free complexes **105**, **106** and **107**, no major shifts were observed in the spectral band positions of **AuNP-105**, **AuNP-106** and **AuNP-107**. However, the relationship of absorbance between the π - π^* intra-ligand band and the MLCT band was significantly affected with the π - π^* intra-ligand absorption decreasing for all of the AuNP conjugates. As shown in Figure 7.16 for **AuNP-105**, the π - π^* intra-ligand absorption underwent a 35% hypochromism upon attachment to AuNPs in relation to its MLCT absorption. Similarly, **AuNP-106** and **AuNP-107** experienced similar hypochroic behaviours of their π - π^* intra-ligand absorption bands, with *ca.* 44% and 36% decreases observed,

respectively (Appendix 7). It is likely that this is due to the overlap of the MLCT absorption band with the SPR band. Because of these spectral perturbations, accurate Ru(II) concentrations are difficult to determine accurately using the extinction coefficients found for the free complexes **105**, **106** and **107** (Table 7.1).

As was the case for the complexes **105**, **106** and **107**, excitation into both the $\pi\text{-}\pi^*$

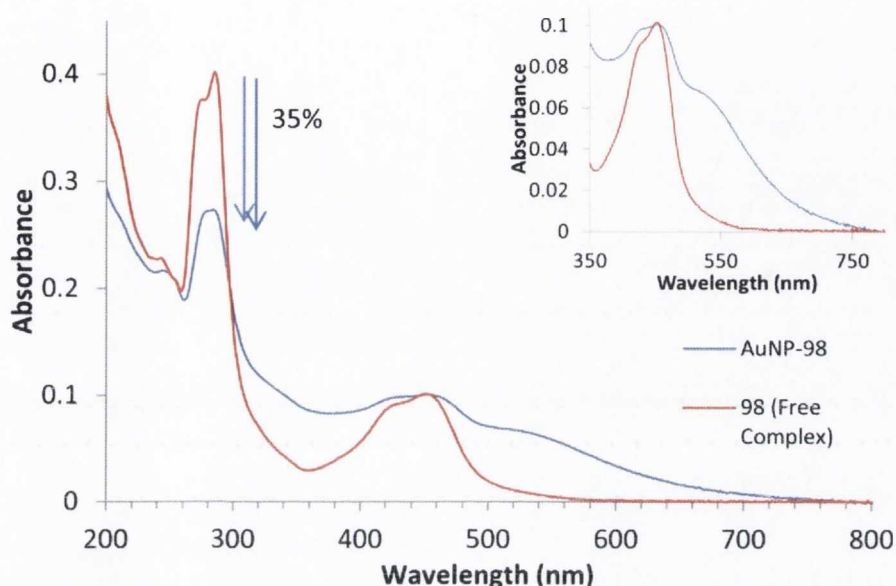


Figure 7.16: UV/Visible spectra showing the comparison between the free complex **105** and the gold conjugates **AuNP-105**. Inset: Close up of the MLCT/SPR region

intra-ligand bands and the MLCT bands resulted in characteristic MLCT based emission with λ_{max} at 610 nm, 605 nm and 635 nm for **AuNP-105**, **AuNP-106** and **AuNP-107**, respectively, however, excitation in to the SPR band did not yield a measurable fluorescence signal. Similarly, excitation spectra recorded for **AuNP-105**, **AuNP-106** and **AuNP-107** were shown to be structurally identical to the absorption spectra of the free complexes **105**, **106** and **107** and showed no contribution to the observed fluorescence from the AuNP SPR band. The fluorescence quantum yields (Φ_F) for **AuNP-105**, **AuNP-106** and **AuNP-107** were measured as being significantly reduced in comparison to those determined for **105**, **106** and **107** with values measured as 0.002 for all of the AuNP conjugates. Such behaviour suggests that the excited states of **105**, **106** and **107** undergo rapid deactivation when bound to AuNPs. Such behaviour has previously been reported, being attributed to energy-transfer quenching of the Ru(II) luminescence by interaction with the AuNPs surface.¹⁴³ More recently, however, the quenching process has been observed to be due to the close proximity of the complexes on periphery of the AuNP core which can lead to an electron transfer reaction between neighbouring complexes.²⁴³

In order to further evaluate the excited state properties of **AuNP-105**, **AuNP-106** and **AuNP-107** excited state lifetime measurements (τ_{em}) were recorded in 10 mM phosphate buffer solution at pH 7.4 and compared to the excited state properties of **105 - 107** as shown for **105** and **AuNP-105** in Figure 7.17. In contrast to **105 - 107**, all data for **AuNP-105**, **AuNP-106** and **AuNP-107** was best fit to a double exponential decay function with contributions from long and short lived components.

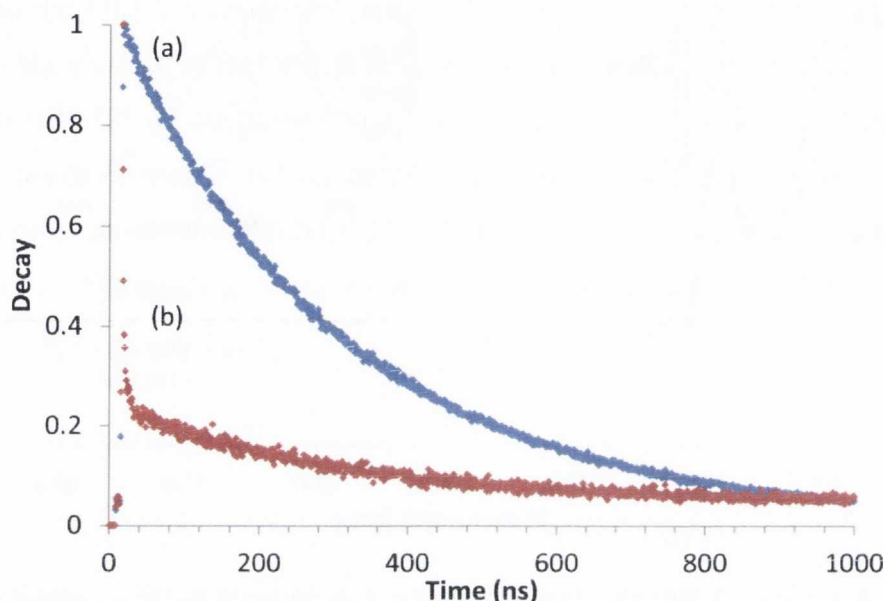


Figure 7.17: Comparative luminescence lifetime profiles of (a) **105** and (b) **AuNP-105**

The lifetime of the long-lived components were similar to that of unbound **105**, **106** and **107** with values of 533 ns, 594 ns and 583 ns for **AuNP-105**, **AuNP-106** and **AuNP-107** respectively, and can be attributed to the unquenched complexes bound to the surface of the AuNPs. Similarly, the short-lived components of the complexes were found to be 4.8 ns, 5.2 ns and 6.1 ns and are likely to be due to the quenched excited state of the complexes, either through an energy or electron-transfer process.^{243,249} A summary of the excited state properties of **AuNP-105**, **AuNP-106** and **AuNP-107** in 10 mM phosphate buffer solution is given in Table 7.4.

Table 7.4: Emission properties of **AuNP-105**, **AuNP-106** and **AuNP-107** in phosphate buffer solution at pH 7.4 at 298K

Complex	$\lambda_{\text{emission}}$ (nm)	Φ_f ($\pm 10\%$)	$\tau_{em \text{ long}}$ (ns)	$\tau_{em \text{ short}}$ (ns)
AuNP-105	610	0.002	533	4.8
AuNP-106	605	0.002	594	5.2
AuNP-107	635	0.002	583	6.1

In summary, having examined the photophysical properties of **AuNP-105**, **AuNP-106** and **AuNP-107** it is clear that, like their free complex analogues **105**, **106** and **107**, the AuNP conjugates display characteristic and useful photophysical properties in aqueous solution which may be amenable for use in biological imaging or therapeutic applications. In addition to showing characteristic fluorescent behaviour, it is expected that the photophysical properties of **AuNP-105**, **AuNP-106** and **AuNP-107** will be significantly modulated upon binding to DNA and, consequently, a photophysical study documenting changes in the photophysical response of **AuNP-105**, **AuNP-106** and **AuNP-107** upon interaction with DNA was undertaken. As well as providing information on the DNA binding ability of **AuNP-105**, **AuNP-106** and **AuNP-107** this study also allowed a direct comparison to be made with the DNA binding ability of the free complexes **105**, **106** and **107**.

7.8 DNA Binding Interactions of AuNP-105, AuNP-106 and AuNP-107

As seen in previous section of this Thesis, binding of Ru(II) complexes to DNA typically results in changes in their electronic spectra and can give valuable information about the binding processes involved. As such, these Ru(II) functionalised AuNPs were expected to interact with DNA through a combination of electrostatic interactions and DNA photoreactions. To determine the nature of their affinity for DNA a number of spectroscopic titrations were carried out and these results are presented in the following sections.

7.8.1 Spectroscopic Titrations of AuNP-105, AuNP-106 and AuNP-107 with DNA

7.8.1.1 UV/Vis Absorption Titrations of AuNP-105, AuNP-106 and AuNP-107 with DNA

The interaction of **AuNP-105**, **AuNP-106** and **AuNP-107** with DNA was firstly examined using UV/Vis absorption spectroscopy in a similar fashion to that for the free complexes above. Changes in the Ru(II) complex MLCT absorption and the SPR absorption were monitored for each AuNP conjugate with the MLCT absorption being kept at an optical density identical to that used during the DNA titrations of the free complexes **105**, **106** and **107**. This allowed for direct comparison between the interaction of the free complexes and the AuNP conjugates **AuNP-105**, **AuNP-106** and **AuNP-107** and corresponded to Au concentrations of $1.98 \times 10^{-8} \text{ M}^{-1}$, $1.79 \times 10^{-8} \text{ M}^{-1}$ and $2.54 \times 10^{-8} \text{ M}^{-1}$ for the conjugates **AuNP-105**, **AuNP-106** and **AuNP-107**, respectively, calculated from the molar absorptivity of the SPR band.²⁵⁹ All titrations were repeated a number of times to ensure reproducibility. The changes in the ground state of **AuNP-105** are shown in Figure 7.19 with those of **AuNP-106** and **AuNP-107** shown in Appendix 7.

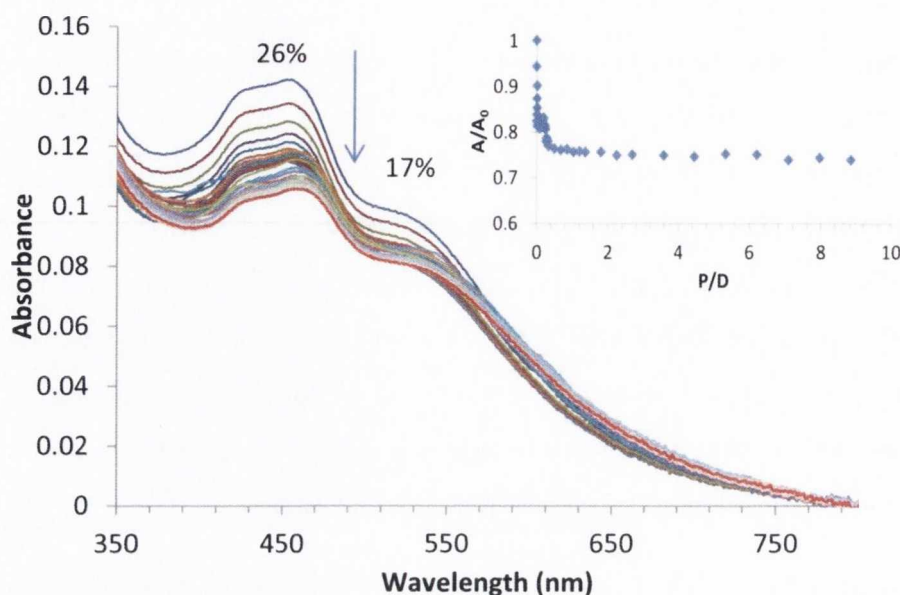


Figure 7.19: Changes in the UV/Visible absorption spectrum of **AuNP-105** ($1.98 \times 10^{-8} M$) upon titration with stDNA (0-290 μM) in 10 mM phosphate buffer, (pH 7.4). Inset: Plot of the change in integrated MLCT absorption as a function of P/D ratio

The addition of DNA to **AuNP-105**, **AuNP-106** and **AuNP-107** resulted in significant changes in their absorption spectra. In the case of **AuNP-105** (Figure 7.19), an initial red shift followed by a 26% hypochromism was observed for the MLCT band with a concomitant 17% decrease of the SPR band. **AuNP-106** also exhibited a slight red shift followed by a hypochromism of 29% in the MLCT band and an SPR decrease of 21% while **AuNP-107** showed a decrease of 31% coupled with the initial slight red shift and an SPR decrease of 25%. The initial red shift observed for each of the AuNP species may indicate DNA induced aggregation at very low P/D ratio ($P/D \leq 1$) with their redistribution as more DNA become available (P/D 1-8). In addition, defined hypochromicities coupled with the magnitude of the changes would indicate close association of the nanostructures with the DNA duplex in solution. Moreover, when compared to the changes observed for the free complexes **105**, **106** and **107**, the UV/Vis absorption properties of **AuNP-105**, **AuNP-106** and **AuNP-107** were seen to be similar in the MLCT region showing hypochromicities of *ca.* 25 - 30% for free complexes and AuNP conjugates alike. However, the observed absorbance decreases were seen to occur at a much lower P/D ratio for the AuNP conjugates giving very different binding profiles. A comparison between the DNA binding profile of **105** and **AuNP-105** exemplifies this and is shown in Figure 7.20. In agreement with the ground state studies, significant changes were also expected in the emission spectra of **AuNP-105**, **AuNP-106** and **AuNP-107** upon binding to DNA and the results of this study are presented in the following section.

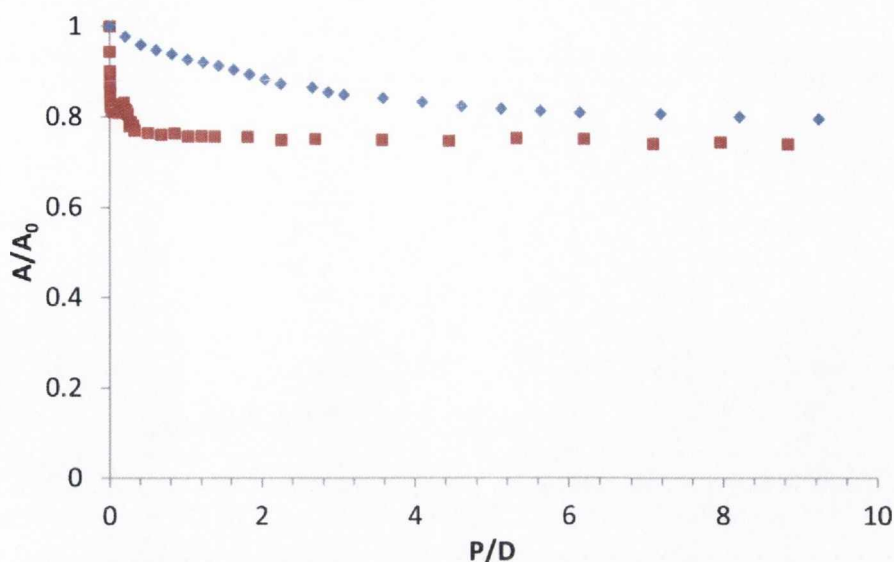


Figure 7.20: Comparison plots of the change in integrated MLCT absorption of **105** (♦) and **AuNP-105** (■) upon addition of stDNA at pH 7.4 in 10 mM phosphate buffer solution

7.8.1.2 Fluorescence Emission Titrations of AuNP-105, AuNP-106 and AuNP-107 with DNA

Given the excited state characters of **AuNP-105**, **AuNP-106** and **AuNP-107** and their similarity to the free complexes **105**, **106** and **107** it was anticipated that addition of DNA would also lead to modulations of their emission responses. Thus, luminescent titrations were carried out in parallel with the UV/Vis absorption titrations as described above.

The changes in the excited character of **AuNP-107** are shown in Figure 7.21 with those of **AuNP-105** and **AuNP-106** shown in Appendix 7. In the case of **AuNP-105**, an initial sharp decrease was observed in the MLCT based emission at 610 nm ($P/D \leq 1$). This was followed by a subsequent slight increase with an effective change in fluorescence over the course of the titration resulting in a 14% decrease when bound at a P/D ratio of 8. Similarly **AuNP-106** saw an initial sharp intensity decrease followed an effective 47% overall quenching when bound at a P/D ratio of 8. As the excited state reduction potentials of Ru(II) complexes of this type are expected to be less than the oxidation potentials of the nucleobases *adenine* and *guanine* quenching of the emission by redox interaction is not likely. However, this behavior may be ascribed to the close proximity of the AuNPs along the DNA backbone resulting in an increase in the effect described above whereby luminescence may suffer from energy/electron-transfer quenching by interaction with neighbouring AuNPs.^{243,249}

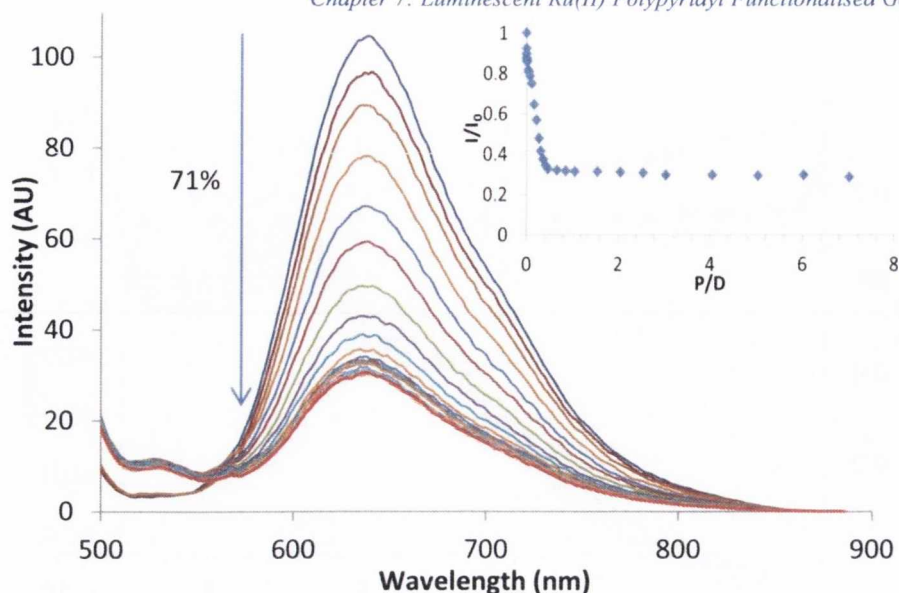


Figure 7.21: Changes in the fluorescence emission spectrum of **AuNP-107** ($2.54 \times 10^{-8} \text{ M}^1$) upon titration with *stDNA* ($0\text{--}77 \mu\text{M}$) in 10mM phosphate buffer, ($\text{pH } 7.4$). Inset: Plot of the change in integrated MLCT emission ($\lambda = 635 \text{ nm}$) as a function of P/D ratio.

Conversely, in the case of **AuNP-107** a steady, sharp fluorescence decrease of 71% was observed (Figure 7.21). This is in agreement with the behaviour of other π -deficient Ru(II) complexes,^{77,260} as those complexes containing two TAP ligands have been shown to photo-oxidise *guanine* containing nucleotides through a proton coupled photoinduced electron transfer (PCET) process as described in previous Chapters.^{60,71} In a similar manner to that described for the ground state titrations, the observed luminescent changes for **AuNP-105**, **AuNP-106** and **AuNP-107** mirrored those of the free complexes **105**, **106** and **107**. However, the observed changes were again seen to occur over a much shorter P/D ratio, and as such gave rise to very different binding profiles for each *e.g.* as shown in Figure 7.22 for **AuNP-107** and its corresponding free complex **107**.

In conclusion, from the spectroscopic titrations of **AuNP-105**, **AuNP-106** and **AuNP-107**, large changes have been observed in both the ground and excited state DNA studies. In the case of the former, such large and well defined hypochromicities are indicative of a strong association with the DNA helix and have been seen in previous Chapters for other Ru(II) species. Similarly, the large fluorescence changes observed for each of the AuNP conjugates upon titration with DNA supports interaction of the Ru(II) portion of these AuNP species with the DNA helix. These changes have been most clearly demonstrated in the case of **AuNP-107** where the observed fluorescence quenching has been attributed to redox processes occurring between the excited state of AuNP bound **107** and *guanine* nucleobases; a phenomenon observed for structurally related π -deficient Ru(II) complexes. In order to further confirm the DNA binding ability of **AuNP-105**, **AuNP-106** and **AuNP-107** various other techniques have

been utilised and the following sections will deal with a description of each of the techniques involved.

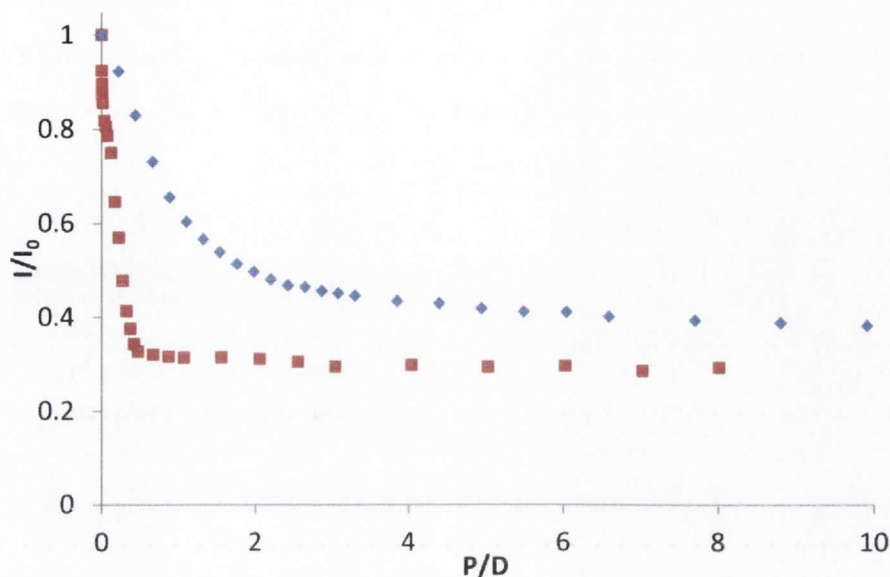


Figure 7.22: Comparison plots of the change in integrated MLCT emission of **107** (♦) and **AuNP-107** (■) upon addition of stDNA in 10 mM phosphate buffer, at pH 7.4

7.8.2 Ethidium Bromide Displacement Assays

The binding efficiencies of **AuNP-105**, **AuNP-106** and **AuNP-107** were also monitored by displacement of DNA bound EtBr as described previously for **105 - 107**. The addition of **AuNP-105**, **AuNP-106** and **AuNP-107** to a mixture of EtBr and DNA was also found to displace 50% EtBr at molar ratios less than or equal to one, further indicating a strong interaction of these AuNP conjugates with DNA. As can be seen in Figure 7.23, the addition of **AuNP-105**, **AuNP-106** and **AuNP-107** caused the emission intensity of intercalated EtBr to decrease, thus displacing the known intercalator from the DNA helix.

The data from the UV/Vis DNA titrations did not allow for accurate calculation of the DNA binding affinities of **AuNP-105**, **AuNP-106** and **AuNP-107**, however, the apparent binding constants K_{app} were again calculated according to the competitive binding model described by Boger *et al.*¹⁸⁵ and a summary of binding constants derived is displayed as an inset in Figure 7.23. The binding constants obtained further confirm a strong interaction of **AuNP-105**, **106** and **107** with DNA and each of the systems show comparable displacement capabilities but **AuNP-107** is seen to have a slightly reduced affinity in comparison to **AuNP-105** and **AuNP-106**. Interestingly, all of the binding constants obtained were reduced in comparison to the free complexes **105**, **106** and **107** and this may indicate that their

attachment to the AuNP surface substantially affects the binding affinity of **105**, **106** and **107** for DNA. Although EtBr displacement assays are not an indicator of any particular mode of binding, the observed behaviour for **AuNP-105**, **AuNP-106** and **AuNP-107** in these assays shows significant interaction with the DNA helix, perhaps through electrostatic interaction or through perturbation of the helix microstructure. The following section will look to further emphasise this binding ability by observing the effect of **AuNP-105**, **AuNP-106** and **AuNP-107** on the DNA helix stability using T_m measurements.

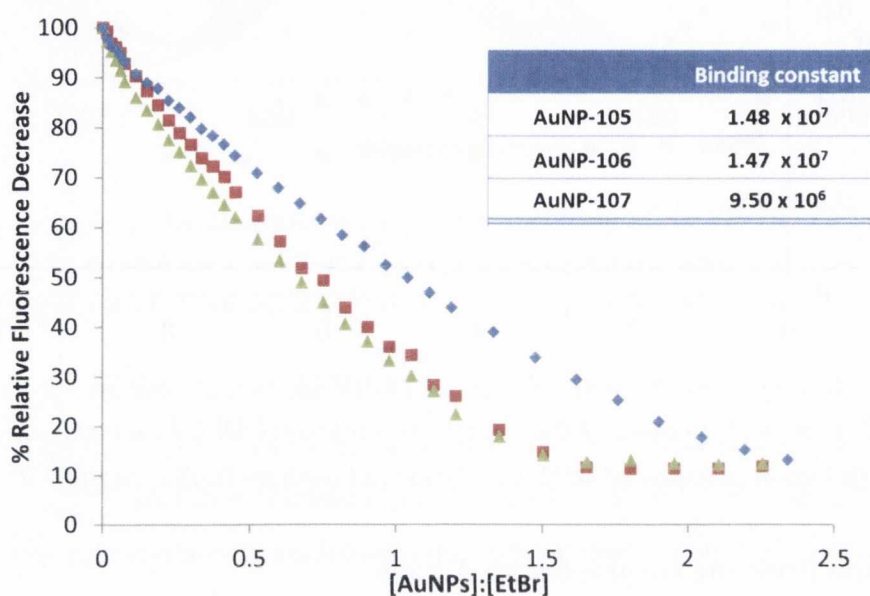


Figure 7.23: Decrease in fluorescence of DNA bound EtBr upon addition of **AuNP-105** (■), **AuNP-106** (▲) and **AuNP-107** (◆) in 10 mM phosphate buffer, pH 7.4. Inset: Table of binding affinities (K_{app}) of **AuNP-105**, **AuNP-106** and **AuNP-107** calculated from EtBr displacement assays

7.8.3 Thermal Denaturation Studies of **AuNP-105**, **AuNP-106** and **AuNP-107**

Thermal denaturation studies were carried on **AuNP-105**, **AuNP-106** and **AuNP-107** in the presence of stDNA, as in Section 7.4.3. In the absence of metal complex the T_m value for DNA was determined to be 68 °C.

Shown in Figure 7.24 are the melting profiles of DNA alone and in the presence of **AuNP-105**, **AuNP-106** and **AuNP-107**. Curiously, no changes are observed in the DNA helix stability of stDNA upon interaction with **AuNP-105**, **AuNP-106** or **AuNP-107** at a P/D ratio of 10, results which are contradictory to those observed in Sections 7.8.1 and 7.8.2. Similarly, no stabilisation of the helix structure upon interaction with each of the AuNP conjugates at a higher loading of P/D = 5 was observed (Appendix 7). Such behaviour is unexpected as it was envisaged that the highly positive nature of **AuNP-105**, **AuNP-106** and **AuNP-107** would lead to, at least, modest stabilisation of the helix, as was the case with the free complexes **105**,

106 and **107**. Thus, the results of this study have proven to be contradictory to results that have previously been obtained using UV/Vis and fluorescence techniques and indicate that no interaction is occurring between **AuNP-105**, **AuNP-106** and **AuNP-107** and DNA. The following section will look to further evaluate the behaviour of **AuNP-105**, **106** and **107** upon interaction with DNA by observing changes in the circular dichroism behaviour of DNA upon addition of the AuNP conjugates.

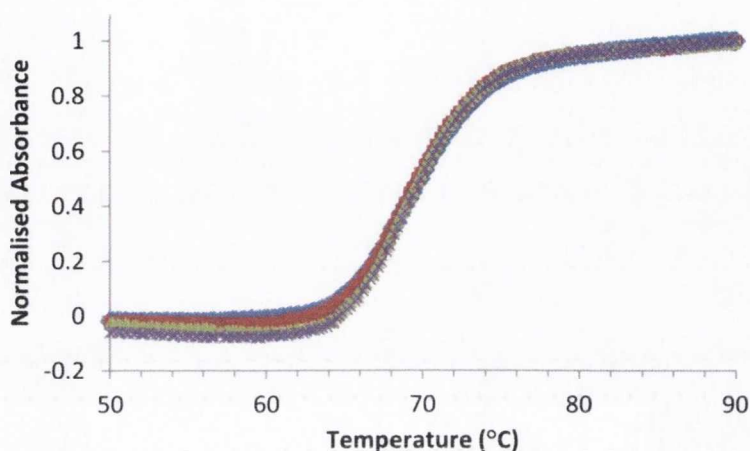


Figure 7.24: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacklozenge$) and presence of **AuNP-105** ($\blacksquare$), **AuNP-106** ($\blacktriangle$) and **AuNP-107** ($\times$) at $P/D = 10$.

7.8.4 Circular Dichroism Studies of **AuNP-105**, **AuNP-106** and **AuNP-107** in the Presence of DNA

To further probe the DNA interaction of **AuNP-105**, **AuNP-106** and **AuNP-107**, circular dichroism titrations were carried out as described in Section 7.4.4, the resulting spectra for **AuNP-106** are shown in Figure 7.25 with those of **AuNP-105** and **AuNP-107** shown in Appendix 7.

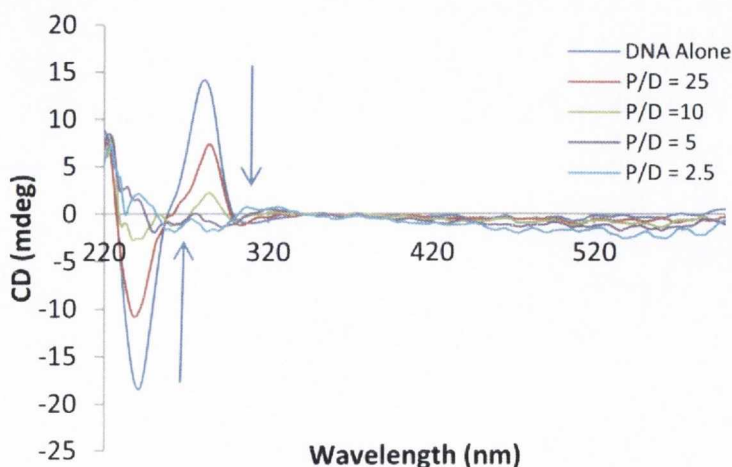


Figure 7.25: Circular dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **AuNP-106** at varying ratios

The data obtained for all three species, **AuNP-105**, **AuNP-106** and **AuNP-107** in the presence of DNA show CD spectra with significant modulations. As was the case with the free complexes, **105**, **106** and **107**, the most obvious and large changes occurred in the DNA region of the CD spectra between 220 nm and 300 nm. Little or no changes were seen outside of this region and no ICD signals were apparent in the MLCT region, from 350 nm – 550 nm.

In a similar manner to the free complexes, **105**, **106** and **107** the CD spectrum of DNA is substantially affected, with the characteristic DNA signal practically disappearing upon addition of **AuNP-105**, **AuNP-106** and **AuNP-107**. As stated in Section 7.4.4, this UV region is sensitive to changes in the DNA helix conformation resulting from external binding of foreign species,²⁵² however, like the free complexes **105**, **106** and **107** all three of the AuNP conjugates exhibit transitions in the UV region, which overlap with the DNA absorption bands, thus it is difficult to discern if these bands result from an ICD, or from structural changes in the DNA. Considered with the other spectroscopic studies presented so far, it is still unclear as to whether **AuNP-105**, **AuNP-106** or **AuNP-107** bind to DNA. However, during the course of carrying out these experiments it was observed that, at a sufficiently high concentration, upon addition of DNA, **AuNP-105**, **AuNP-106** and **AuNP-107** were seen to aggregate and precipitate from solution. In order to examine the observed aggregates confocal microscopy studies were carried out to measure the characteristic MLCT emission from the precipitates formed. The preceding section will describe the results obtained from these experiments.

7.8.5 DNA Induced Aggregation of AuNP-105, AuNP-106 and AuNP-107 Observed by Confocal Microscopy

Aggregation of AuNPs is a known phenomenon and generally results from a screening effect which minimises electrostatic repulsion between the AuNPs. The dampening effect of this electrostatic repulsion thus allows the AuNPs to come into close proximity with each other forming AuNP aggregates that grow and eventually precipitate from solution.²⁶¹ In the case of **AuNP-105**, **AuNP-106** and **AuNP-107**, it was observed that addition of DNA at sufficiently high concentrations (1×10^{-3} M) at low P/D ratios ($P/D = 1$) brought about aggregation of the AuNP conjugates leading to the formation of a dark precipitate. Shown in Figure 7.26 are confocal microscopy images of the aforementioned precipitate showing the bright field images obtained and the observed red MLCT based emission from the DNA induced **AuNP-105** aggregates. Interestingly, in some of the samples analysed the aggregates formed seemed to reveal a fibrous network of fluorescence barely visible in the bright field

image (Figure 7.26A). Alternatively, numerous aggregated ‘clumps’ were formed that were found to be highly fluorescent with no obvious ordering (Figure 7.26B).

The observed MLCT based fluorescence emanating from the AuNP aggregates confirms that the surface of the AuNPs remains functionalised with their MLCT Ru(II) complexes. In addition, the observed fibrous pattern exhibited by the aggregates would indicate a certain degree of ordered interaction with the DNA in solution, perhaps binding of the AuNP conjugates along the DNA backbone as shown by Sleiman *et al.* for a Ru(II) polypyridyl complex bound to streptavidin conjugated AuNPs.¹³⁹ Further analysis, such as high resolution TEM analysis is necessary to confirm this explanation, and is the aim of current work being carried out within the Gunnlaugsson research group. In any case, the work presented here confirms that these Ru(II) conjugated AuNPs do interact with DNA in solution and bring about aggregation and precipitation of the nanostructures. The aggregation effect is most likely brought about by electrostatic interactions between the AuNP species and DNA which at high AuNP loading leads to a high proportion of AuNPs being in close proximity to each other along the DNA helix, leading to aggregation and eventually precipitation. The proceeding section will attempt to summarise the results from the various DNA binding studies undertaken thus far.

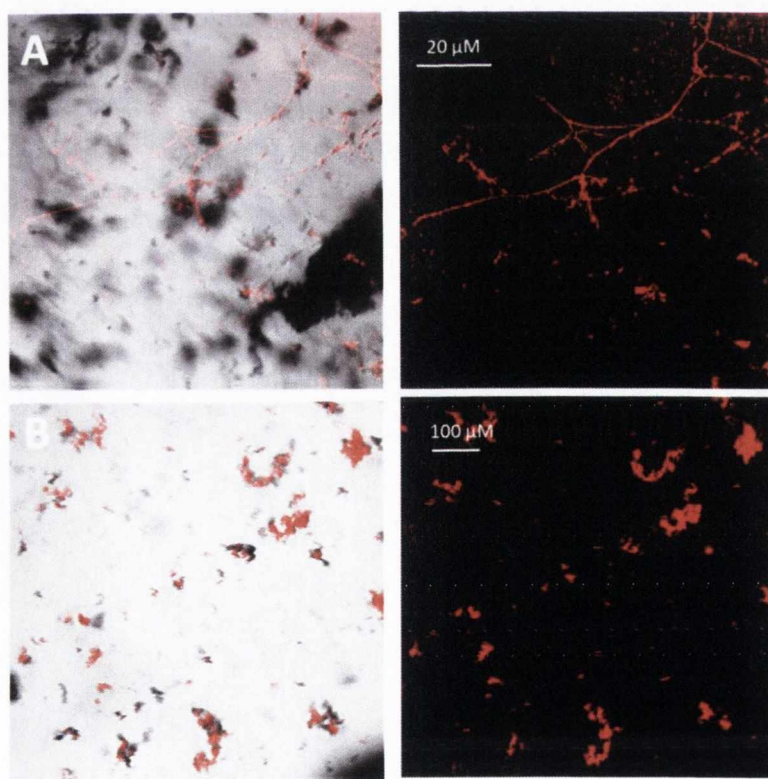


Figure 7.26: Confocal fluorescence images of **AuNP-105**, showing the Ru(II) MLCT emission, arising from the aggregated AuNPs after addition of DNA

7.8.6 Summary of DNA Binding Studies of AuNP-105, AuNP-106 and AuNP-107

The work described above has attempted to evaluate the DNA binding capability of **AuNP-105**, **106** and **107** and compare the results with those of the free complexes **105**, **106** and **107**. The photophysical properties of **AuNP-105**, **AuNP-106** and **AuNP-107** were initially investigated in aqueous solution and showed characteristic MLCT absorption and fluorescence spectra confirming the adsorption of the Ru(II) complexes on the AuNP surface. Subsequently, changes in their ground and the excited state properties in the presence of stDNA were monitored and revealed significant modulations, showing absorbance decreases in the MLCT absorption region and, concomitantly, each of the AuNP conjugates **AuNP-105**, **AuNP-106** and **AuNP-107** exhibited excited state quenching. The observed quenching was most apparent for the π -deficient species, **AuNP-107** which, upon interaction with DNA, was seen to have its emission efficiently quenched by 71%, indicating the occurrence of PET processes between the AuNP species and the nucleobases of DNA.

EtBr displacement assays confirmed the results observed from the spectroscopic titrations and showed that **AuNP-105**, **AuNP-106** and **AuNP-107** were all capable of displacing EtBr effectively when bound to stDNA. Peculiarly, T_m experiments found that the helical structure of stDNA was not stabilised in the presence of any of the nanoconjugates, even at a high loading of P/D = 5. The results from the CD studies were found to be largely inconclusive and only revealed changes in the UV region, where it was impossible to differentiate whether such changes were brought about by perturbations in the DNA helix conformation or by absorption overlap in the UV region between the DNA base absorption and Ru(II) π - π^* intraligand absorption bands. Subsequently, confocal microscopy analysis of DNA induced AuNP aggregates revealed the formation of ordered fibrous networks in the presence of **AuNP-105** and suggested that indeed interaction between the AuNP species and DNA was occurring. Although far from conclusive the majority of the evidence presented thus far has pointed to some form of interaction between **AuNP-105**, **AuNP-106** and **AuNP-107** and DNA. Further studies are on-going in order to confirm this interaction and the redesign of the Ru(II) complex portion should allow for more precise control over the DNA binding ability of such structures.

Nevertheless, the main aims of this Chapter set out from the beginning were the evaluation of **AuNP-105**, **AuNP-106** and **AuNP-107** for potential application in biological imaging and therapeutic applications. Consequently, the remainder of this Chapter will deal with the DNA photocleavage ability, cellular uptake and cellular localisation of **AuNP-105**, **AuNP-106** and **AuNP-107**.

7.9 DNA Photocleavage Efficiency of AuNP-105, AuNP-106 and AuNP-107

The ability **AuNP-105**, **AuNP-106** and **AuNP-107** to initiate light sensitised DNA cleavage was investigated in order to determine the excited state reactivity of the AuNP conjugates and determine their applicability to light activated therapeutics. Due to the known DNA photocleavage properties of Ru(II) complexes, it was anticipated that the incorporation of **105**, **106** and **107** on their surface would also furnish **AuNP-105**, **AuNP-106** and **AuNP-107** with DNA photocleavage capability. The cleavage efficiencies of **AuNP-105**, **AuNP-106** and **AuNP-107** were compared to the known DNA photocleavage agent $[\text{Ru}(\text{bpy})_3]^{2+}$ in order to provide a reference standard as well as the free complexes **105**, **106** and **107** to determine the effect of their adsorption on to the AuNP surface. The photocleavage studies were carried out as described in previous Chapters by treating pBR322 plasmid DNA (1 mg/mL) with **AuNP-105**, **AuNP-106** and **AuNP-107** at varying P/D ratios, in 10 mM phosphate buffer solution. The samples were then subjected to 2 J/cm^2 using a Hg-Xe lamp equipped with a $> 390 \text{ nm}$ cut off filter before being separated using horizontal agarose gel electrophoresis in TBE buffer.

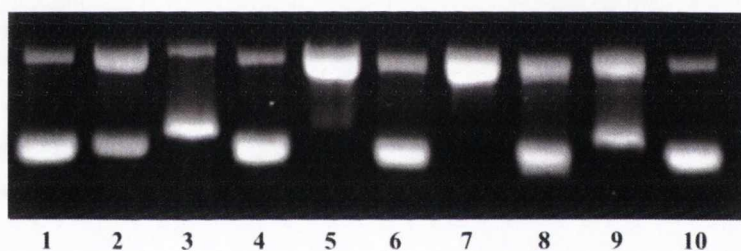


Figure 7.27: Agarose gel electrophoresis of pBR322 DNA (1mg/mL) after 30 min irradiation (2 J/cm^2) in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: $[\text{Ru}(\text{bpy})_3]^{2+}$ (P/D 20); Lane 3: **107** in the dark (P/D 10); Lane 4 : **AuNP-107** in the dark (P/D 10); Lanes 5-6: **107** and **AuNP-107** (P/D 20); Lanes 7-8: **107** and **AuNP-107** (P/D 10); Lanes 9-10: **107** and **AuNP-107** + 10mM NaN_3 (P/D 10).

The results obtained for **AuNP-107** and **107** are shown in Figure 7.27, the complete Figures and corresponding tables for **AuNP-105**, **AuNP-106**, **105** and **106** are contained in Appendix 7. The relative intensity of the bands for each study showed that the plasmid DNA was somewhat damaged to begin with, being comprised of *ca.* 80% supercoiled and *ca.* 20% open form DNA. The presence of $[\text{Ru}(\text{bpy})_3]^{2+}$ (P/D = 20) in each case resulted in an increase to *ca.* 55% open form after irradiation confirming its effectiveness as a DNA photocleaver. When incubated in the dark neither the AuNP conjugates **AuNP-105**, **AuNP-106** and **AuNP-107** nor the free complexes **105**, and **107** showed any DNA cleavage activity. Conversely, when subjected to light irradiation, the free complexes **105** - **107** were all shown to effectively convert supercoiled plasmid DNA to open form DNA with **107** showing the most effective

cleavage ability giving rise 98% conversion to open form at a P/D = 20. Similarly, when an increased proportion of **105**, **106** and **107** were present (P/D = 10) an increase in photocleavage efficiency was observed exhibiting 43%, 54% and 100% conversion to open form DNA for **105**, **106** and **107** (Figure 7.27; Lanes 5 and 7) respectively. Importantly, when irradiated in the presence of the known singlet oxygen scavenger, NaN₃, it was observed that the photocleavage efficiency of all three complexes **105** - **107** was significantly reduced, leading to 23%, 38% and 56% conversion to open form DNA for **105**, **106** and **107** (Figure 7.27; Lane 9) respectively. In contrast to the results observed for the free complexes, **AuNP-105**, **AuNP-106** and **AuNP-107** were shown to cause little or no photocleavage, with no observed photocleavage effect being recorded for either **AuNP-105** or **AuNP-106** at either P/D = 20 or P/D = 10. **AuNP-107** showed moderate cleavage ability with 30% and 38% conversion to open form at P/D ratios of 20 and 10 respectively. However, this process occurred with efficiencies much reduced in comparison to Ru(bpy)₃²⁺ (Figure 7.27; Lanes 6 and 8) and, furthermore, this small degree of photocleavage capability was extinguished in the presence of NaN₃ (Figure 7.27; Lane 10). The relative amounts of supercoiled vs. open form DNA are summarised in Table 7.5 and a representative graph is shown in Figure 7.28.

Table 7.5: Summary of percentages of supercoiled vs. open form pBR322

Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	17.2	82.8	6	29.5	70.5
2	55.7	44.3	7	100	0
3	19.2	80.8	8	37.9	62.1
4	16.8	83.2	9	56.1	43.9
5	98.1	1.9	10	17.7	82.3

From this study it is clear that complexes **105** and **106** showed similar efficiencies to Ru(bpy)₃²⁺ while **107** was seen to be a much more efficient photocleavage agent with almost 100% conversion to open form DNA being observed at P/D = 10 and 20. This is most likely due to the ability of these complexes to generate ¹O₂ thus causing single strand breaks. The appearance of 'dragging' on the gel also further indicates the binding ability of these positively charged complexes **105**, **106** and **107** to DNA as their accumulation on the DNA molecules increases the overall positive charge of the species resulting in the mobility of the

DNA through the gel being inhibited. It is expected that the increased DNA photocleavage efficiency exhibited by **107** is due to its ability to photoreact with *guanine* nucleobases in addition to its $^1\text{O}_2$ production capability. Moreover, the damage to DNA caused by **105**, **106** and **107** was seen to be inhibited in the presence of NaN_3 further indicating that the involvement of $^1\text{O}_2$ formation is likely to be a contributing factor to the observed DNA damage. The inability of **AuNP-105** and **AuNP-106** to initiate light sensitised DNA cleavage was obvious from this study and is most likely due to the decreased Φ_F value of the AuNP conjugates in comparison to their free complex analogues. Such a reduction in Φ_F leads to a reduction in $^1\text{O}_2$ generation capability thus leading to less efficient photocleavage ability. **AuNP-107** showed a slight increase compared to **AuNP-105** and **AuNP-106**, however, as stated above the efficiencies measured were reduced in comparison to $\text{Ru}(\text{bpy})_3^{2+}$ and, furthermore, the presence of NaN_3 caused a further reduction in the photocleavage ability.

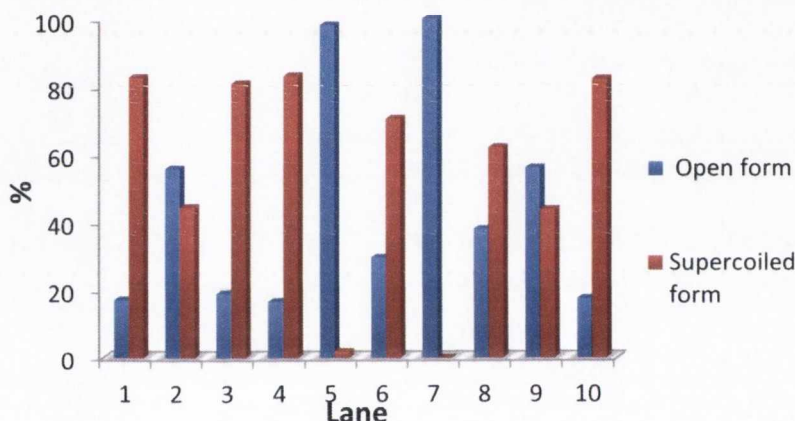


Figure 7.28: Relative percentages of supercoiled vs. open form pBR322 plasmid DNA from the photocleavage study of **AuNP-107**

This section has attempted to monitor the ability **AuNP-105**, **AuNP-106** and **AuNP-107** to initiate light sensitised DNA cleavage and to determine the excited state reactivity of the AuNP conjugates in comparison to their free complex analogues. It is clear from the results discussed herein that **AuNP-105**, **AuNP-106** and **AuNP-107** do not show a general applicability to light activated therapeutics, however, due to their observed unphotoreactive nature and fluorescent properties, their potential use as biological imaging agents is well founded. In addition, synthetic design may yield similar systems which will give rise to similar AuNP conjugates with real therapeutic applications. As such, one of the most important factors to consider for **AuNP-105**, **AuNP-106** and **AuNP-107** is their ability to enter cellular environments. The preceding section will attempt to reveal the cellular localisation behaviour of **AuNP-105**, **AuNP-106** and **AuNP-107** using both confocal microscopy and TEM analysis.

7.10 Cellular Uptake and Localisation Studies of AuNP-105, AuNP-106 and AuNP-107

With the aforementioned DNA binding ability and fluorescent character of **AuNP-105**, **AuNP-106** and **AuNP-107** it was expected that these systems may have applicability to biological imaging and diagnostics. In this study the aim was to evaluate the ability of these compounds to enter cells, *in vitro* and determine their specific sub-cellular localisation. Sub-cellular uptake and localisation studies of **AuNP-105**, **AuNP-106** and **AuNP-107** in HeLa (cervical cancer cell line) cells were undertaken using both confocal microscopy and transmission electron microscopy experiments. The results discussed here are the result of a collaboration between the Gunnlaugsson research group and the Williams research group in the School of Biochemistry and Immunology at Trinity College Dublin. The work discussed here has been undertaken in collaboration with Dr. Suzanne M. Cloonan and Ms. Kim Orange and the results of more detailed studies undertaken hereafter will be reported in the PhD Thesis of Ms. Orange.

7.10.1 Confocal Microscopy Cellular Localisation Study of AuNP-105, AuNP-106 and AuNP-107

Confocal microscopy analysis of **AuNP-105**, **AuNP-106** and **AuNP-107** was carried out on populations of live HeLa cells (0.5×10^5) after having been incubated with **AuNP-105**, **AuNP-106** and **AuNP-107** at concentrations of 100 μM and, subsequently 20 μM at 37 °C for 4 hrs before being treated with the fluorescent nuclear stain DAPI and viewed using an Olympus FV1000 point scanning microscope with a 60 $\times$ oil immersion lens.

Figure 7.29, shows the fluorescence confocal laser scanning microscopy images, of live HeLa cells upon excitation at 450 nm after incubation with **AuNP-105** at (A) 100 μM concentration and (B) 20 μM concentration prior to imaging. Figure 7.29 (C) shows a close up image of the same population of cells at 20 μM . The corresponding images for **AuNP-106** and **AuNP-107** are shown in the Appendix. At 100 μM concentration a clear demonstration of the uptake of **AuNP-105** was observed with bright red fluorescence being observed from all cells, however, it was also clear that at such high concentrations very little information could be extracted about the sub-cellular localisation of **AuNP-105**, **AuNP-106** and **AuNP-107**. Subsequently, a five-fold reduction in the amount of AuNPs incubated with the cells led to images which showed apparent localisation in the cytoplasm and nucleus of the HeLa within defined areas of fluorescence, as demonstrated in Figure 7.29(C). Furthermore, localisation is also seen in the bright field image by the appearance of the AuNPs as dense ‘dark spots’. These dense areas are seen to overlap perfectly with the red fluorescence emanating from

within the cell interior and suggest the aggregation of the AuNP species in specific areas of the cells.

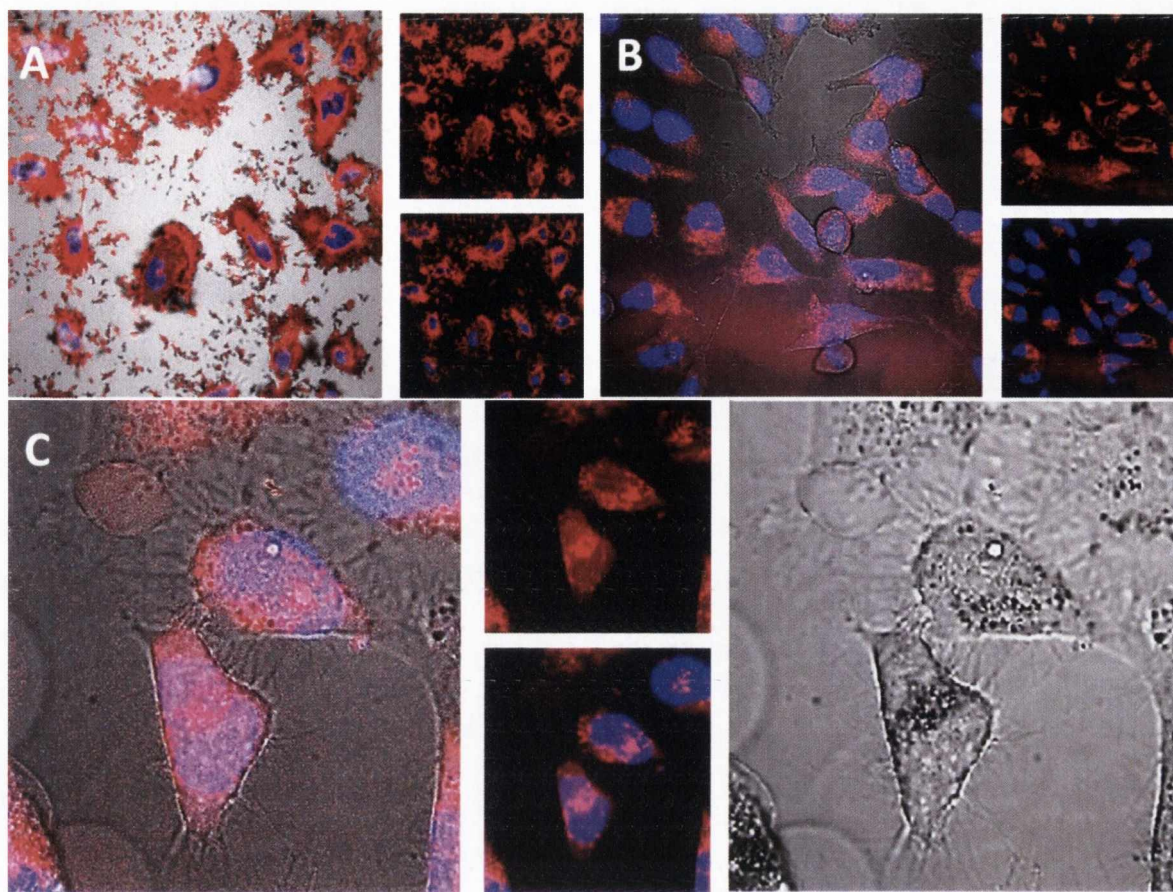


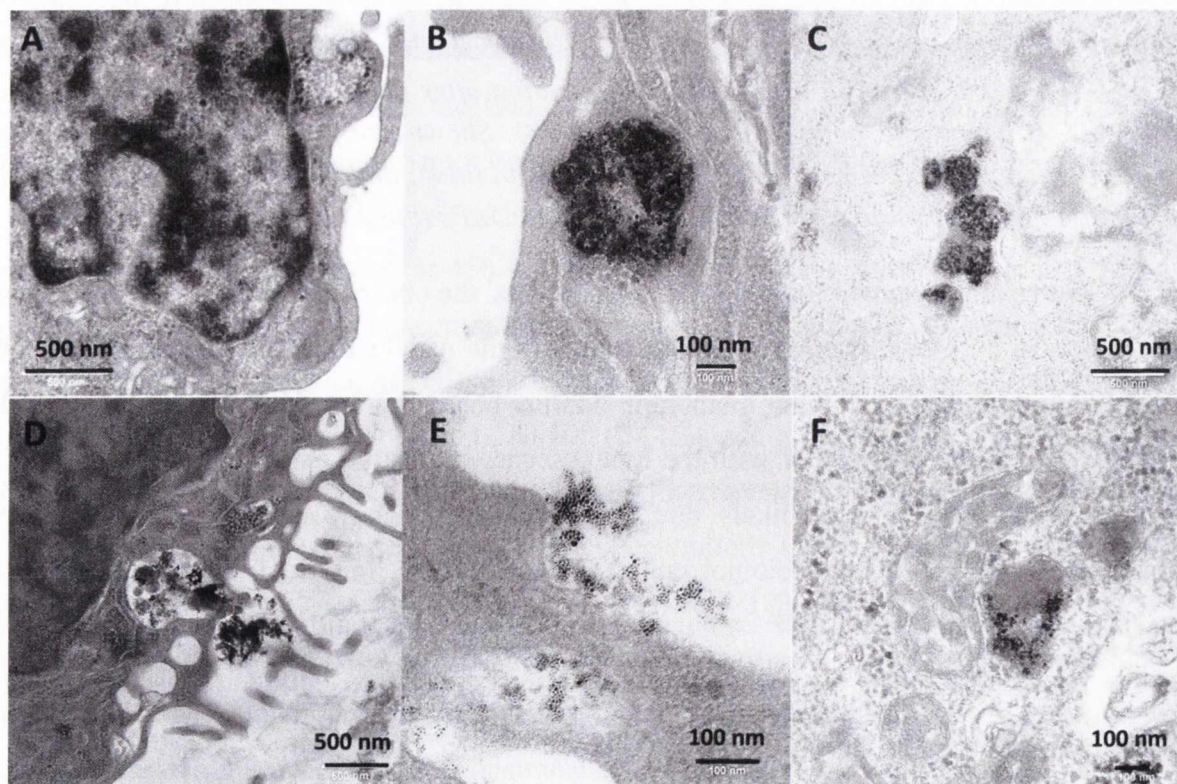
Figure 7.29: Confocal microscopy study of **AuNP-105** in HeLa cells co stained with DAPI (A) at 100 μM and (B) at 20 μM AuNP concentration after 4hrs. (C) represents a close up image of the same cell population depicted in (B). Shown are the images obtained with **AuNP-105** (red), **AuNP-105** + DAPI nuclear co-stain (blue) and an enlarged optical image of the cells (grey) overlaid with **AuNP-105**(red) and DAPI (blue).

Moreover, as shown from Z-stack experiments, the observed luminescence was shown to be spherical in three dimensions, corresponding to the AuNPs being located inside the cells and not bound to the extracellular membrane. Similar behaviour was also observed for **AuNP-105**, **AuNP-106** and **AuNP-107** with the luminescence intensity being slightly diminished in the case of **AuNP-107** most likely due fluorescence quenching caused by photoelectron transfer processes with various biomolecules (Appendix 7).^{60,82} In order to confirm the cellular uptake capability of these AuNP conjugates a biological TEM study was also undertaken to observe **AuNP-105** inside the cell membranes. The results of this study are discussed in the following section.

7.10.2 TEM Cellular Localisation Study of AuNP-105, AuNP-106 and AuNP-107

Due to the ability of AuNP species to be visualised by TEM microscopy, it was envisaged that analysis of biological samples of cells incubated with **AuNP-105** would support the confocal microscopy cellular uptake studies described in the previous section. Moreover, it was expected that such a study would provide valuable information on the specific cellular location of this species by its ability to observe cellular environments on the nanoscale. In a manner similar to that described in Chapter 2, cells were fixed, dehydrated, embedded in epoxy resin, ultra-sliced, mounted on a copper grid and stained with uranyl acetate and lead citrate before examination on a JEM-2100 transmission electron microscope. Images were taken with an X1500-3000 objective.

Figure 7.30 shows the TEM images of **AuNP-105** inside HeLa cells where the AuNP species seem to be aggregated and ‘trapped’ inside specific cellular vesicles in the cytoplasm, behaviour previously seen for AuNP species.¹²⁰ For a single cell, multiple vesicles containing **AuNP-105** were observed where, within the vesicles, the AuNPs appeared to be clumped together with a large number of AuNPs located in each vesicle. In addition, the nuclei of the cells examined also showed extremely dense staining which may suggest nuclear localisation, however, definitive evidence for this observation is difficult as vesicles cannot form inside the nucleus and, hence individual AuNPs are difficult to observe at this resolution.



: Transmission electron microscopy images of HeLa cells treated with **AuNP-105** showing (A) densely stained nuclear material and (B)-(F) AuNP aggregates inside the cell cytoplasm.

The above localisation studies together have attempted to evaluate the cellular localisation behaviour of **AuNP-105**, **AuNP-106** and **AuNP-107**. It is clear from the above results that each of the AuNP species readily enter cells and it is likely that with the overall positive surface charge of these AuNPs (due to the Ru(II) complexes on the surface) that their entry into cells is *via* receptor-mediated endocytosis as has been proposed for other AuNP species¹²⁰ however, more detailed studies are necessary to confirm this. Nevertheless, with their observed cellular uptake ability **AuNP-105**, **AuNP-106** and **AuNP-107** show vast potential for use as biological imaging agents. Moreover, these results provide a fundamental platform on which future studies can build in order to tune the delivery of such species to various sub cellular locations enabling the use of such metal stabilized AuNPs for diagnostic and therapeutic applications.

7.11 Overall Conclusions and Future Perspectives

The study undertaken in this Chapter has attempted the Ru(II) functionalisation of the surface of AuNPs with a view to evaluating their potential as biological imaging and therapeutic agents. Within this research, the synthesis of three alkyl thiol functionalised Ru(II) polypyridyl complexes, **105**, **106** and **107** have been described in addition to their characterisation and photophysical evaluation. Their DNA-binding affinities in aqueous solution have also been explored through a variety of techniques such as UV/Vis absorption titrations, fluorescence titrations, EtBr displacement assays, T_m experiments and CD studies to enable a better understanding of the nature of their DNA-binding interactions. Analysis of the ground state data allowed the calculation of DNA binding constants of the order of 10^5 M^{-1} for each of the complexes with fluorescence studies showing significant emission modulation upon interaction with stDNA. EtBr displacement assays and T_m experiments in addition to CD studies confirmed that **105 - 107** are capable of binding to DNA where it was observed that **106** brings about the most profound stabilisation of the DNA helical structure.

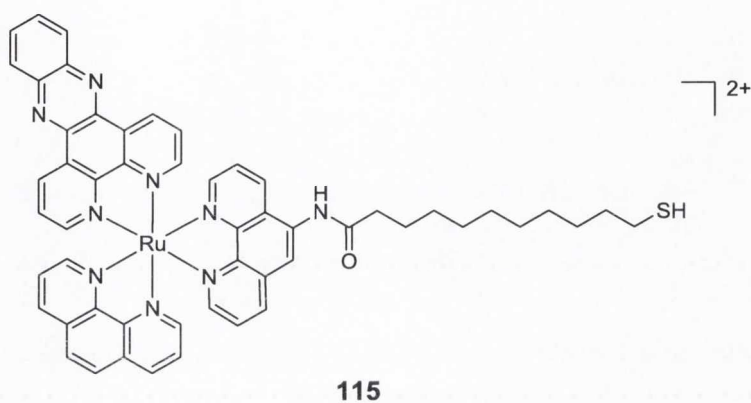
Once the properties of **105 - 107** had been fully explored, a subsequent description of the synthesis and functionalisation of AuNPs was given, yielding the Ru(II)-AuNP conjugates **AuNP-105**, **AuNP-106** and **AuNP-107**. These species were characterised using DLS and TEM analysis before being photophysically evaluated by UV/Vis and fluorescence techniques. Each of the Ru(II)-AuNP conjugates showed absorption and fluorescent behaviour characteristic of both the AuNP SPR absorption and the Ru(II) chromophore MLCT absorption and emission, which subsequently allowed a comparison between the observed changes upon interaction of **105**, **106** and **107** with DNA with their AuNP counterparts **AuNP-105**, **AuNP-106** and **AuNP-107**. As seen for the free complexes, the photophysical

properties of the AuNP conjugates were significantly perturbed in the presence of DNA, indicating interaction between the biomolecule and **AuNP-105**, **AuNP-106** and **AuNP-107** in solution. Furthermore, in the case of **AuNP-107**, which contains a π deficient Ru(II) centre, steady state fluorescence quenching was observed which is most likely as a result of photoinduced electron transfer reactions between the Ru(II)-AuNP conjugate and the *guanine* nucleobases of DNA. Curiously, T_m measurements contradicted this evidence where none of the AuNP species **AuNP-105**, **AuNP-106** and **AuNP-107** were shown to effectively stabilise the DNA structure. Nevertheless, DNA induced aggregation of the AuNP conjugates, as observed by confocal microscopy, supported the photophysical studies where an ordered fibrous pattern exhibited upon aggregation was observed indicating a certain degree of interaction of the AuNP species with the DNA in solution.

In addition to their DNA binding parameters, **AuNP-105**, **AuNP-106** and **AuNP-107** and their free complex analogues, **105**, **106** and **107** were evaluated for their ability to function as efficient photocleavage agents for DNA. It was observed that the photocleavage efficiencies of **AuNP-105**, **AuNP-106** and **AuNP-107** were greatly reduced in comparison to their free complex analogues; an observation attributed to the reduction in fluorescence quantum yield caused by their adsorption onto the AuNP surface. Regardless of their lack of observed photoreactivity, with a view to evaluating their potential as biological imaging agents, confocal microscopy studies revealed the rapid cellular localisation of **AuNP-105**, **AuNP-106** and **AuNP-107** within HeLa cells where intense emission was observed in the cytoplasm and nucleus. Furthermore, TEM analysis of HeLa cells supported their cellular uptake, where aggregated clumps of AuNPs were found to exist inside vesicles within the cell cytoplasm. Moreover, intense staining of the nuclear material also suggested a certain degree of nuclear localisation, supporting the results of the confocal microscopy study.

The work presented here, thus, represents a new strategy in developing Ru(II) stabilised AuNP conjugates as potential biological imaging agents. Although, their observed photoreactivity was diminished in comparison to the free complexes, excitation of these species with a higher power light source, may give rise to the photothermal activation of the AuNP species. With simultaneous generation of the Ru(II) MLCT excited state such species may provide a two pronged attack in cancer therapies, utilising both photothermal and photodynamic effects in what may yield a new generation in nanomaterials and their exploitation in light activated therapies.

Future investigations will look to increase the DNA binding ability of the AuNP conjugates by functionalisation of the AuNP surface with a stronger DNA binding moiety such as **115**. Similarly, a systematic study on the role played by AuNP size in cellular uptake ability and their effect on the fluorescence of the attached Ru(II) system may help to yield species more suitable for specific cellular targeting and therapeutic applications.



Chapter 8:

Experimental Section

8.1 General Experimental Techniques

All NMR spectra were recorded using either a 400 MHz Bruker Spectrospin DPX-400 or AV-600 spectrometer, operating at 400.1/600.1 MHz for ^1H NMR and 100.2/150.2 MHz for ^{13}C NMR respectively. Shifts are referenced relative to the internal solvent signals. Electrospray mass spectra were recorded on a Micromass LCT spectrometer or a MALDI QToF Premier, running Mass Lynx NT V 3.4 on a Waters 600 controller connected to a 996 photodiode array detector with HPLC-grade methanol or acetonitrile. High resolution mass spectra were determined by a peak matching method, using leucine enkephaline, (Tyr-Gly-Gly-Phe-Leu), as the standard reference ($m/z = 556.2771$). All accurate mass were reported within ± 5 ppm. Melting points were determined using an IA9000 digital melting point apparatus. Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer fitted with a Universal ATR Sampling Accessory. Elemental analysis was conducted at the Microanalytical Laboratory, School of Chemistry and Chemical Biology, University College Dublin.

UV-visible absorption spectra were recorded on a Varian CARY 50 spectrophotometer with a wavelength range of 200-800 nm and a scan rate of 600 nm min^{-1} . Baseline correction measurements were used for all spectra. Fluorescence measurements were made with a Varian Carey Eclipse Fluorimeter in either 1 cm or 3 cm quartz cuvettes. The luminescence quantum yields were calculated relative to the reference value of $[\text{Ru}(\text{bpy})_3]^{2+}$ (0.028 in H_2O). The quantum yields were determined from the integration of fluorescence spectra obtained in the reference solvent. The spectra were obtained under the same conditions using a solution of the same absorbance and excited at the same wavelength. Fluorescence lifetime experiments were carried out on a Horiba Scientific FluoroLog - Modular Spectrofluorometer equipped with Time Correlated Single Photon Counting (TCSPC) capability. Linear and Circular Dichroism (CD) spectra were recorded at a concentration corresponding to an optical density of approximately 1.0, in buffered solutions, on a J-815 Circular Dichroism Spectropolarimeter equipped with a Linear Dichroism Accessory (LD) or a Jasco J-810-150S CD spectropolarimeter (CD). EtBr displacement assays were performed in 10 mM phosphate buffer solution (pH 7.4) according to the procedure of Boger *et al.*¹⁸⁵ The fluorescence of EtBr was initially measured ($\lambda_{\text{max}} = 595 \text{ nm}$) and normalized to 0% relative fluorescence. An appropriate amount of stDNA was then added at a ratio of EtBr to DNA (base pairs) of 2:1. At this ratio it could be estimated that all intercalation sites were occupied and the fluorescence was measured again and normalised to 100% relative fluorescence. The changes in the emission spectra of EtBr bound to DNA were then monitored upon the successive additions of

the appropriate DNA binding molecule. The titrations were continued until the decrease in emission reached saturation and remained constant. Thermal denaturation experiments were performed on a thermoelectrically coupled Perkin Elmer LAMBDA 25 UV/Vis Spectrophotometer. The temperature in the cell was ramped from 30 to 90 °C, at a rate of 1 °C min⁻¹ and the absorbance at 260 nm was measured every 0.2 °C. All other reagents and solvents were purchased commercially and used without further purification. Solutions of DNA in 10mM phosphate buffer (pH 7.4) gave a ratio of UV absorbance at 260 nm and 280 nm of 1.86:1, indicating that the DNA was sufficiently free of protein. Its concentration was determined spectrophotometrically using the molar absorptivity of 6600 M⁻¹ cm⁻¹ (260 nm). The concentration of homopolymers, [poly(dAdT)]₂ and [poly(dGdC)]₂ was also determined spectrophotometrically using the molar absorptivities of 6600 M⁻¹ cm⁻¹ (262nm) and 8400 M⁻¹ cm⁻¹ (254nm), respectively. Phosphate buffer was made from two 1M stock solutions of NaH₂PO₄ and Na₂HPO₄ (using 10 mL volumetric flasks) in millipore water. Portions of each solution were diluted together to achieve 10 mM phosphate buffer and the pH was adjusted to pH 7.4 by addition of NaOH.

Titrations were carried out on samples of the dyes at $1 (\pm 0.5) \times 10^{-5}$ M at ambient temperature by monitoring changes in the absorption and emission spectra, at pH 7.4 in 10 mM phosphate buffer upon successive additions of aliquots of stDNA. The results are quoted using the concentration of stDNA expressed as a nucleotide phosphate to dye ratio (P/D ratio).

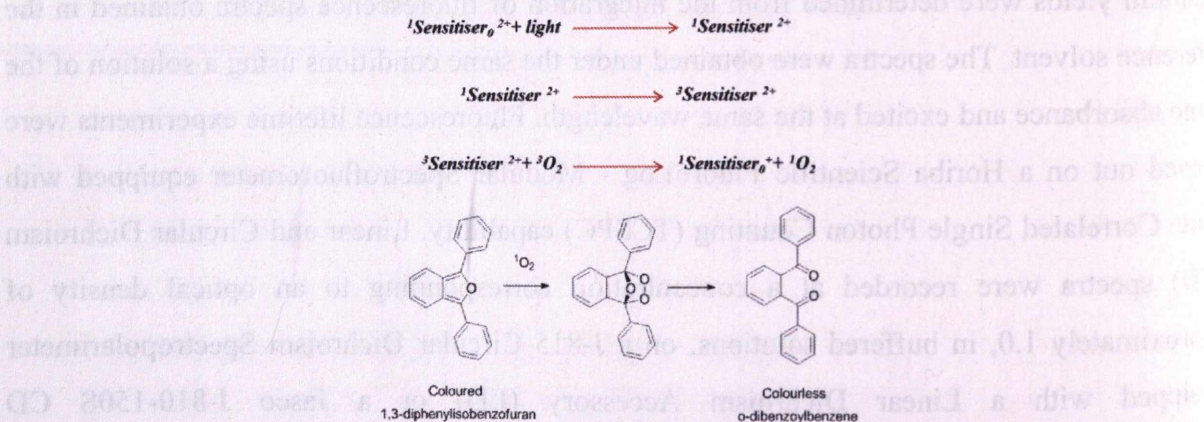


Figure 8.1: Representation of the mechanism by which light induced degradation of DPBF occurs

Photo-oxidation studies were undertaken by monitoring the light induced degradation of 1,3-diphenylisobenzofuran (DPBF), with ¹O₂ generation achieved following a modified procedure of Frimansson *et al.*, by irradiating solutions containing the sensitizer (1×10^{-8} M) and DPBF (initial concentration 5×10^{-5} M) and following the disappearance of its absorption band at 415 nm over a time period of 180 min.¹⁹³ Filtered light of wavelength 470 nm was

chosen for excitation of the sensitiser as well as $[\text{Ru}(\text{bpy})_3]^{2+}$ in order to irradiate the complexes selectively over the DPBF. $[\text{Ru}(\text{bpy})_3]^{2+}$ (1×10^{-8} M) was chosen as a comparative reference from which relative rates of reactive oxygen species (ROS) production could be determined because of its known $^1\text{O}_2$ quantum yield (Φ_Δ) (0.86 in oxygen-saturated MeOH at 1 atm).¹⁹⁴ The mechanism by which the light induced degradation of DPBF occurs is outlined in Figure 8.1.¹⁹²

8.1.1 Calculation of binding constants from UV/Vis Titration Data

The intrinsic binding constant K_b , and binding site size n were determined using the model of Bard *et al.*,¹⁸¹ equation (1) from a plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}]$. ϵ_a , ϵ_f , ϵ_b correspond to the apparent extinction coefficient, the extinction coefficient for the free dye, and the extinction coefficient for the dye in the fully bound form. C_t is the total dye concentration, $[\text{DNA}]$ is the DNA concentration expressed in nucleotide phosphate. The data was fit to this model using non-linear regression with Sigmaplot 11.0.

$$\frac{(\epsilon_a - \epsilon_f)}{(\epsilon_b - \epsilon_f)} = \frac{[b - (b^2 - 2K_b C_t [\text{DNA}]/n)^{1/2}]}{(2K_b C_t)} \quad (1)$$

$$b = 1 + K_b C_t + K_b [\text{DNA}]/2 \quad (2)$$

8.1.2 Calculation of binding constants from Fluorescence Titration Data

K_b was determined from both the absorbance and luminescence data according to the McGhee and Von Hippel model.²⁰⁵ The concentration of the bound compound C_b , and free compound C_f , at a given concentration were firstly determined from equations (3) and (4):

$$C_b = \frac{I_f - I}{I_f - I_b} \cdot C \quad (3)$$

$$C_f = C - C_b \quad (4)$$

I_f and I_b are the fluorescence intensities of the free and bound compound, I is the fluorescence response and C is the dye concentration. The absorbance data was treated in a similar manner. Data analysis was then carried out as described in equation (5), where, $r = C_b/[DNA]$, ($[DNA]$ is the concentration of DNA expressed in base pairs). Analysis was achieved using the plot of r/C_f versus r and the curve was fit to equation (5) using Sigmaplot 11.0.

$$\frac{r}{C_f} = K_b(1 - nr) \left(\frac{1 - nr}{1 - (n-1)r} \right)^{n-1} \quad (5)$$

8.1.3 Calculation of binding constant from ethidium bromide displacement assays

The apparent binding constant K_{app} was calculated according to the competitive binding model described by Tse and Boger using equation (6).¹⁸⁵

$$K_{app} = K_{EB} [EB]/[agent] \quad (6)$$

where K_{EB} is the binding constant for ethidium bromide, $[EB]$ is the concentration of ethidium bromide and $[agent]$ is the dye concentration at 50% of the fluorescence.

8.2 General Biological Procedures

The DNA photocleavage studies were carried out by treating pBR322 plasmid DNA (1 mg/mL) with each of the complexes at varying ratios. The samples were then subjected to 2 J/cm² (30 mins irradiation) using an Hamamatsu L2570 200 Watt HgXe Arc Lamp equipped with a NaNO₂ filter before being separated using horizontal agarose gel electrophoresis in a TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. A 0.8% (w/v) agarose solution was prepared by dissolving 0.8 g of agarose in 100 mL of TBE buffer. The agarose was melted by boiling, and the gel was poured while warm and left to cool. Electrophoresis was carried out at *ca.* 5 V/cm (40 mA, 90 V) to separate covalently closed circular (**Form I**), open circular (**Form II**), and linear (**Form III**) forms of the plasmid DNA. A loading dye solution composed of sucrose (40%), xylene-cyanol (0.25%), and bromophenol (0.25%) in TBE was added to the samples to help them sink in the wells of the gel. DNA migrated less rapidly than the xylene-cyanol dye (green), with bromophenol blue (violet) moving much more rapidly. Visualisation of the DNA after electrophoresis was achieved by staining the gel for 90 mins with an aqueous solution of ethidium bromide, which fluoresces strongly when bound to DNA. The dye within the gel was illuminated with a transilluminator (Bioblock 254 UV illuminator) and the gel photographed to provide a record of the distances migrated by the

various DNA fragments. The ratio of the different forms was estimated using ImageJ Gel analysis software.

HeLa cells were grown in a cell culture flask using low-glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂. Real-time confocal microscopy experiments were carried out using HeLa cells, seeded at a density of *ca.* 1×10^5 cells/2mL. Cells were left for the appropriate incubation time before treatment with the required compound for varying incubation times. Cells were washed twice with fresh media to remove excess compound before detection of fluorescence between 580 and 680 nm using an Olympus FV1000 point scanning microscope fitted with a 60x oil immersion lens with an NA (numerical aperture) of 1.42. The software used to collect images was FluoView Version 7.1 software. TEM of images of HeLa cells were obtained after the cells were fixed, dehydrated, embedded in epoxy resin, ultra-sliced, mounted on a copper grid and stained with uranyl acetate and lead citrate before examination on a JEM-2100 transmission electron microscope. Images were taken with an X1500-3000 objective.

Cell cytotoxicity tests were undertaken in the cervical cancer cell line, HeLa, using an alamar blue cytotoxicity assay in which the non-fluorescent resazurin dye becomes reduced to its pink coloured and highly red fluorescent resorufin dye which indicates oxidation-reduction processes that can be related to cell viability.

8.4 Materials

All chemicals were obtained from Sigma-Aldrich, TCI or Alfa Aesar and unless specified, were used without further purification. Deuterated solvents for NMR use were purchased from Apollo Ltd. Dry solvents were prepared using standard procedures, according to Vogel's Textbook of Practical Organic Chemistry (5th Edition), with distillation prior to each use. Chromatographic columns were run using Silica gel 60 (230-400 mesh ASTM). Solvents for synthetic purposes were used at GPR grade unless otherwise stated. Analytical TLC was performed using Merck Kieselgel 60 F₂₅₄ silica gel plates. *cis*-[Ru(bpy)₂Cl₂],²⁶² *cis*-[Ru(TAP)₂Cl₂],¹⁷¹ [RuCl₂(η⁴-COD)],²⁶³ 1,10-phenanthroline-5,6-dione,¹⁶⁸ 4,4'-dicarboxy-2,2'-bipyridine,²⁶⁴ 5-nitro-1,10-phenanthroline and 5-amino-1,10-phenanthroline²⁶⁵ were prepared by the literature routes. The stDNA, and homopolymers, [poly(dA-dT)]₂ and [poly(dG-dC)]₂, were obtained from Sigma Aldrich as their sodium salts and were stored at -20 °C.

8.5 General Synthetic Procedures

Procedure 1: Complexation of pdppz ligand, 52 with Ru(L-L)₂Cl₂

Pyrazino[2,3-*h*]dipyrido[3,2-*a*:2',3'-*c*]phenazine (1 eq.) and the appropriate Ru(L-L)₂Cl₂ complex (1 eq.) were suspended in an EtOH:H₂O mixture (1:1, 7 mL) before the suspension was degassed by bubbling with argon for 15 mins. The mixture was heated at 140 °C for 40 mins using microwave irradiation and filtered before the PF₆ salt of the complex was precipitated from solution by addition of a conc. ethanolic solution of NH₄PF₆. The resultant precipitate was collected *via* centrifugation before being washed with H₂O (5 mL), EtOH (5 mL) and Et₂O (5 mL). Purification was achieved through silica gel flash column chromatography eluting with CH₃CN/H₂O/Aq. NaNO₃(sat) (40:4:1). The product was dried under vacuum before the chloride form of the complex was reformed by stirring a solution of the PF₆ complex in MeOH (15 mL) in Amberlite ion exchange resin (chloride form) for 1 hr.

Procedure 2: Chalcone formation

The appropriate nitro-benzaldehyde (1 eq.) was added to EtOH and the mixture heated to 70 °C until the solid dissolved. Sodium pyruvate (1.1 eq.) dissolved in H₂O was added, and the mixture cooled to 0 °C in an ice bath. NaOH solution (2M, 30 mL) was added dropwise and the mixture stirred at 0 °C for 3 hrs. The mixture was subsequently neutralised with 2M HCl, filtered, the solid washed with EtOH (2 × 10 mL) and dried.

Procedure 3: 4-(nitrophenyl)-2,2'-bipyridine formation

The appropriate chalcone (1 eq.), 2-pyridacyl pyridinium iodide (1 eq.) and ammonium acetate (8 eq.) were added to H₂O and the mixture heated at reflux for 5 hrs. The solid was filtered, and washed with H₂O (2 × 10 mL) and acetone (2 × 5 mL). The ammonium salt was heated under high vacuum with a heat gun until evolution of CO₂ ceased. The resulting black solid was dissolved in EtOAc (150 mL), activated charcoal added and the mixture refluxed for 15 mins. The mixture was filtered through a pad of celite and the solvent removed under reduced pressure.

Procedure 4: 4-(nitrophenyl)-2,2'-bipyridine reduction

The appropriate 4-(nitrophenyl)-2,2'-bipyridine derivative (1 eq.) and 10% Pd/C (0.2 g) were added to EtOH (30 mL) and the mixture heated at reflux for 1 hr. Hydrazine monohydrate (98%, 20 eq.) was added and the mixture heated at reflux for 1 hr. The mixture was filtered through a pad of celite and the solid washed with CH₂Cl₂ (40 mL). The filtrate was washed with water, dried over MgSO₄, and the solvent removed under reduced pressure.

Procedure 5: 4-[N-phenyl-4-nitro-1,8-naphthalimide]-2,2'-bipyridine formation

The relevant 4-(aminophenyl)-2,2'-bipyridine (1 eq.) and 4-nitro-1,8-naphthalic anhydride (1 eq.) were suspended in HPLC grade EtOH (30 mL) and the mixture refluxed in a pressure tube for 48 hrs. The reaction mixture was cooled to room temperature before the product was collected by suction filtration and washed with EtOH (30 mL).

Procedure 6: 4-[N-phenyl-4-nitro-1,8-naphthalimide]-2,2'-bipyridine reduction

The relevant 4-(aminophenyl)-2,2'-bipyridine (1 eq.) was dissolved in anhydrous DMF (40 mL) and heated to 100 °C before 10% Pd/C (0.2 g) was added and the mixture stirred for 1 hr. Hydrazine monohydrate (98%, 10 eq.) was added and the mixture was again heated at reflux for 4 hrs. The mixture was filtered hot through a pad of celite and the solid washed with DMF (20 mL). The filtrate was evaporated simultaneously with toluene (4 × 30 mL). The resulting solid was suspended in toluene before being isolated by suction filtration and dried under high vacuum.

Procedure 7: Formation of Bis-1,8-naphthalimide containing Tröger's base derivatives

A mixture of the relevant 4-amino-1,8-naphthalimide-bipyridine ligand (1 eq.) and paraformaldehyde (1.5 eq.) in neat TFA (3.5 mL) was stirred at room temperature for 18 hrs under an argon atmosphere. The reaction was subsequently brought to pH 9 by slow addition of aq. NaOH (6 M). The resulting precipitate was isolated by suction filtration and washed with EtOH (3 × 10 mL) and Et₂O (3 × 10 mL). The crude product was purified by precipitation from DMSO using MeOH and dried under high vacuum.

Procedure 8: Complexation of Bis-1,8-naphthalimide containing Tröger's base derivatives with Ru(bpy)₂Cl₂

The appropriate Bis-1,8-naphthalimide containing Tröger's base ligand (1 eq.) and Ru(bpy)₂Cl₂ (1 eq.) were suspended in EtOH:H₂O (1:1) and the suspension was degassed by bubbling with argon for 15 mins. The reaction mixture was heated at 150 °C for 40 mins using microwave irradiation before being allowed to cool and filtered. The PF₆ salt of the complex was formed by addition of a conc. ethanolic solution of NH₄PF₆ with the resulting precipitate being collected by centrifugation. The dried solid was redissolved in MeCN before purification by slow diffusion of Et₂O into MeCN. The crystalline solid was converted to the chloride form of the complex by stirring a solution of the PF₆ salt in MeOH with Amberlite anion exchange resin (Cl⁻ form) for 1 hr.

Procedure 9: Complexation of alkyl thiol functionalised 1,10-phenanthroline ligand with $\text{Ru(L-L)}_2\text{Cl}_2$

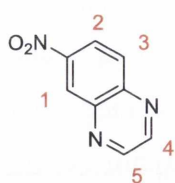
Ligand 11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide (1 eq.) and the appropriate bis polypyridyl Ru dichloride (1 eq.) were suspended in ethylene glycol:H₂O (8:2) and the suspension was degassed by bubbling with argon for 15 mins. The reaction mixture was heated at 150 °C for 40 mins using microwave irradiation before being filtered. The PF₆ salt was formed by addition of a conc. ethanolic solution of NH₄PF₆ with the resulting precipitate being collected by centrifugation. The dried solid was redissolved in MeCN before purification by silica flash column chromatography eluting with MeCN:H₂O:Aq. NaNO₃(sat) (40:4:1). The chloride form of the complex was reformed by stirring a solution of the PF₆ salt in MeOH with Amberlite anion exchange resin (Cl⁻ form) for 1 hr.

Procedure 10: Synthesis of Ru(II) stabilised AuNPs

HAuCl₄.3H₂O (1 eq.) was dissolved in H₂O (1 mL) before TOAB (1.5 eq.) in toluene (4 mL) was added. The resulting mixture was stirred vigorously before NaBH₄ (1.2 eq.) was added and the mixture stirred for a further 1 hr. The appropriate Ru(II) complex (1 eq.) in H₂O (20 mL) was added and the resulting mixture was stirred for 1 hr. The aqueous layer was subsequently separated before addition of a conc. aqueous solution of NH₄PF₆ (0.5 mL) afforded flocculation of a dark solid which was collected by centrifugation and washed with H₂O (3 × 10 mL). The solid was redissolved in MeCN (10 mL) before addition of a conc. solution of TBACl resulted in the formation of a flocculate which was collected by centrifugation and washed with MeCN (3 × 10 mL) before being dried under high vacuum.

8.6 Synthesis and characterisation of compounds described in Chapter 2

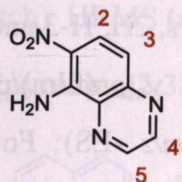
6-nitroquinoxaline (57)¹⁶⁷



4-nitrobenzene-1,2-diamine (4 g, 27.95 mmol, 1 eq.) was suspended in EtOH (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 DCM (4 × 50 mL). The solvent was again removed under reduced pressure after being dried over MgSO₄. The resulting orange solid was recrystallised twice from isopropanol (2 × 150 mL) to yield golden crystalline needles (4.07 g, 83%). m.p. 183–184 °C. δ_{H} (400 MHz, [D₆] DMSO): 9.17 (s, 2H, H-4 and H-5), 8.91 (d, 1H, $J = 2.52$, H-1), 8.57 (dd, 1H, $J = 2.52$, $J = 9.04$, H-2), 8.36 (d, 1H, $J = 9.04$, H-3). δ_{C} (100 MHz, [D₆] DMSO): 148.87, 148.13, 147.60, 144.66, 140.99, 131.26, 125.25, 123.56. ν_{max} (film)/cm⁻¹:

1520 (C-NO₂), 1344 (C-NO₂). HRMS (*m/z* -ES): Found: 176.0474 (M+H. C₈H₆N₃O₂ Requires: 176.0460).

6-nitroquinoxalin-5-amine (58)¹⁶⁶



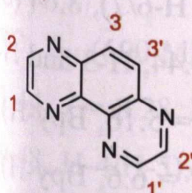
Sodium metal (2.3 g, 100 mmol, 3.3 eq.) was added to distilled MeOH (125 mL). A solution of hydroxylamine (3.13 g, 45 mmol, 1.5 eq.) in distilled MeOH (50 mL) was added after cooling to 0 °C on ice. The resulting NaCl precipitate was allowed to settle before the supernatant solution was added to a well stirred suspension of 6-nitroquinoxaline in boiling MeOH (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, 43%). m.p. 239-240 °C (Literature: 240-241 °C)¹⁶⁶ δ_H (400 MHz, [D₆] DMSO): 9.10 (d, 1H, *J* = 2.00, H-4/5), 8.94 (d, 1H, *J* = 2.04, H-4/5), 8.53 (s, 2H, NH₂), 8.30 (d, 1H, *J* = 9.52, H-2), 7.19 (d, 1H, *J* = 9.52, H-3). δ_C (100 MHz, [D₆] DMSO): 148.85, 145.84, 145.09, 143.22, 134.16, 126.11, 114.34. ν_{max} (film)/cm⁻¹: 3297 (N-H Stretch), 1615 (N-H bend), 1520 (C-NO₂), 1344 (C-NO₂). HRMS (*m/z* -ES): Found: 191.0568 (M+H. C₈H₇N₄O₂ Requires: 191.0569).

Quinoxaline-5,6-diamine (59)¹⁶⁷



6-nitroquinoxalin-5-amine (1.5 g, 7.86 mmol, 1 eq.) and 10% Pd/C (0.2 g) were added to EtOH (80 mL) and the mixture heated at reflux for 1 hr. Hydrazine monohydrate (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 (400 MHz, [D₆] DMSO): 8.58 (d, 1H, *J* = 2.00, H-4/5), 8.51 (d, 1H, *J* = 2.00, H-4/5), 7.26 (d, 1H, *J* = 9.04, H-2/3), 7.20 (d, 1H, *J* = 8.52, H-2/3), 5.28 (s, 2H, NH₂), 5.16 (s, 2H, NH₂). δ_C (100 MHz, [D₆] DMSO): 141.90, 140.12, 136.92, 133.03, 132.41, 126.04, 121.80, 116.33. ν_{max} (film)/cm⁻¹: 3306 (N-H Stretch), 1610 (N-H bend). HRMS (*m/z* -ES): Found: 161.0816 (M+H. C₈H₉N₄ Requires: 161.0827).

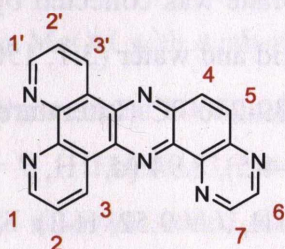
1,4,5,8-tetraazaphenanthrene (60)¹⁶⁷



Quinoxaline-5,6-diamine (1 g, 6.24 mmol, 1 eq.) was suspended in EtOH (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

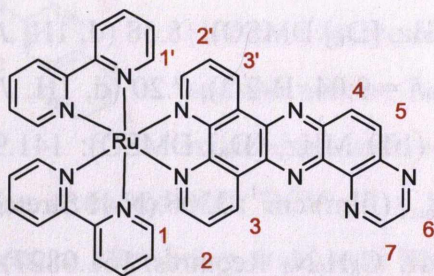
under reduced pressure before being dissolved in water (100 mL) and the aqueous layer extracted with DCM (5×40 mL). The solvent was again removed under reduced pressure after being dried over MgSO_4 . The resulting orange solid was recrystallised from isopropanol (50 mL) to yield yellow crystalline needles (0.83 g, 72%). δ_{H} (400 MHz, $[\text{D}_6]$ DMSO): 9.24 (d, 2H, $J = 2.04$, H-1 and H-1'), 9.21 (d, 2H, $J = 2.04$, H-2 and H-2'), 8.38 (s, 2H, H-3 and H-3'). δ_{C} (400 MHz, $[\text{D}_6]$ DMSO): 146.90, 145.68, 143.47, 140.20, 131.53. ν_{max} (film)/ cm^{-1} : 3016 (Aromatic C-H Stretch), 1493 (Aromatic C=C stretch). HRMS (m/z -ES): Found: 183.0685 (M + H. $\text{C}_{10}\text{H}_7\text{N}_4$ Requires: 183.0671).

Pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (52)



Quinoxaline-5,6-diamine (0.1 g, 0.476 mmol, 1 eq.) and 1,10-phenanthroline-5,6-dione (0.084 g, 0.524 mmol, 1.1 eq.) were suspended in a mixture of EtOH and water (1:1, 15 mL) and heated at 140 °C in a pressure tube overnight. The resulting brown precipitate was collected by suction filtration, washed with H_2O (10 mL), EtOH (10 mL) and Et_2O (15 mL) before being dried under high vacuum. The product was obtained as a grey solid (0.151 g, 95%). m.p. > 250 °C. Calculated for $\text{C}_{20}\text{H}_{10}\text{N}_6 \cdot 0.5\text{H}_2\text{O}$: C, 69.96; H, 3.23; N, 24.48. Found: C, 69.25; H, 3.24; N, 24.2. δ_{H} (600 MHz, $[\text{D}_6]$ DMSO): 9.82 (d, 1H, $J = 7.62$, H-1/1'), 9.70 (d, 1H, $J = 9.36$, H-1/1'), 9.38 (d, 1H, $J = 1.74$, H-3/3'), 9.32 (m, 2H, H-7 and H-6'), 9.30 (d, 1H, H-3/H-3'), 8.63 (d, 1H, $J = 9.42$, H-4/5), 8.52 (d, 1H, $J = 9.12$, H-4/5), 8.10 (m, 2H, H-2 and H-2'). ν_{max} (film)/ cm^{-1} : 3258 (Aromatic C-H stretch), 1378 (C-N stretch). HRMS (m/z -ES): Found: 335.1048 (M+H. $\text{C}_{20}\text{H}_{11}\text{N}_6$ Requires: 335.1045).

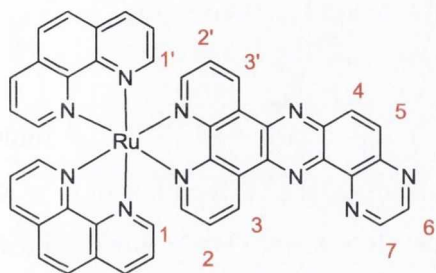
$\text{Ru}(\text{pyrazino}[2,3\text{-h}]\text{dipyrido}[3,2\text{-a:2',3'-c}]\text{phenazine})(\text{bipyridine})_2\text{Cl}_2$ (53)



Complex **53** was synthesised according to Procedure 1 using pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (0.05 g, 0.15 mmol, 1 eq.) and $\text{Ru}(\text{bpy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.072 g, 0.15 mmol, 1 eq.), giving the product as an orange/brown solid (0.012 g, 77%). Calculated for $\text{C}_{40}\text{H}_{26}\text{F}_{12}\text{N}_{10}\text{P}_2\text{Ru} \cdot 1\text{H}_2\text{O}$: C, 45.51; H, 2.67; N, 13.27. Found: C, 45.68; H, 2.45; N, 13.24. m.p. > 250 °C. δ_{H} (600 MHz, CD_3CN): 9.76 (d, 1H, $J = 8.10$, H-1/1'), 9.76 (d, 1H, $J = 8.22$, H-1/1'), 9.29 (s, 1H, H-6/7), 9.25 (s, 1H, H-6/7), 8.64 (d, 1H, $J = 9.06$, Bpy-H), 8.58 (m, 5H, H-4, H-5 and Bpy-H), 8.27 (d, 2H, $J = 4.44$, H-3 and H-3'), 8.16 (t, 2H, $J = 7.86$, Bpy-H), 8.06 (m, 2H, H-2 and H-2'), 7.90 (d, 2H, $J = 5.16$, Bpy-H), 7.79 (d, 1H, $J = 5.58$, Bpy-H), 7.76 (d, 1H, $J = 5.52$, Bpy-H), 7.51 (t, 2H, $J = 6.6$, Bpy-H),

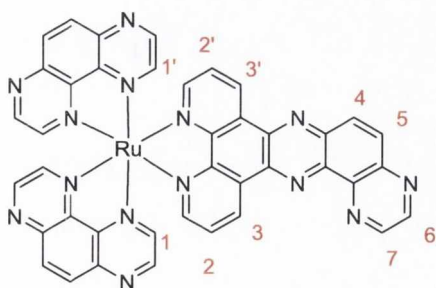
7.29 (m, 2H, Bpy-H). δ_{C} (150 MHz, CD_3CN): 154.02, 153.82, 152.26, 152.09, 151.89, 150.12, 149.97, 147.34, 145.52, 145.19, 140.94, 140.51, 140.04, 138.88, 137.92, 137.90, 137.83, 134.19, 133.15, 132.95, 131.22, 130.26, 129.86, 127.56, 127.50, 127.40, 127.28, 127.18, 124.29, 124.24, 124.18. ν_{max} (film)/ cm^{-1} : 3353 (Aromatic C-H stretch), 1359 (C-N stretch). HRMS (m/z -ES): Found: 748.1370 (M^{2+} . $\text{C}_{40}\text{H}_{26}\text{N}_{10}\text{Ru}$, Requires: 748.1385).

Ru(pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine)(phenanthroline) $_2$ Cl $_2$ (54)



Complex **54** was synthesised according to **Procedure 1** using pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (0.1 g, 0.3 mmol, 1 eq.) and $\text{Ru}(\text{phen})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.160 g, 0.3 mmol, 1 eq.), giving the product as an orange/red solid (0.224 g, 69%). Calculated for $\text{C}_{44}\text{H}_{26}\text{F}_{12}\text{N}_{10}\text{P}_2\text{Ru} \cdot \text{NaNO}_3 \cdot 2.5\text{NH}_4\text{PF}_6$: C, 38.54; H, 2.34; N, 11.24. Found: C, 38.02; H, 2.25; N, 11.15., m.p. > 250 °C. δ_{H} (600 MHz, CD_3CN): 9.84 (dd, 1H, $J = 1.20$, $J = 8.16$, H-1/H-1'), 9.74 (dd, 1H, $J = 1.26$, $J = 8.22$, H-1/H-1'), 9.28 (d, 1H, $J = 1.98$, H-6/H-7), 9.24 (d, 1H, $J = 1.92$, H-6/H-7), 8.65 (m, 5H, H-4/H-5 and Phen-H), 8.57 (d, 1H, $J = 9.42$, H-4/H-5), 8.30 (m, 4H, Phen-H), 8.27 (dd, 1H, $J = 1.08$, $J = 5.28$, Phen-H), 8.25 (dd, 1H, $J = 1.08$, $J = 5.34$, Phen-H), 8.20 (m, 2H, H-3 and H-3'), 8.07 (m, 2H, Phen-H), 7.86 (m, 2H, H-2 and H-2'), 7.68 (m, 4H, Phen-H). δ_{C} (150 MHz, CD_3CN): 155.60, 155.42, 154.26, 154.22, 153.96, 151.81, 151.67, 148.86, 148.79, 148.51, 146.73, 146.47, 145.43, 142.44, 141.96, 141.65, 140.52, 137.94, 137.90, 135.36, 134.59, 132.44, 132.05, 132.03, 131.67, 131.23, 129.04, 128.27, 128.20, 126.90. ν_{max} (film)/ cm^{-1} : 3400 (Aromatic C-H stretch), 1331 (C-N stretch). HRMS (m/z -ES): Found: 796.1362 (M^{2+} . $\text{C}_{44}\text{H}_{26}\text{N}_{10}\text{Ru}$, Requires: 796.1385).

Ru(pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine)(tetraazaphenanthrene) $_2$ Cl $_2$ (55)



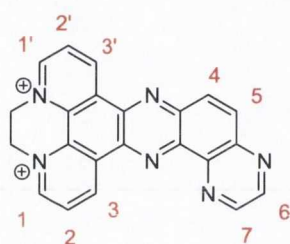
Complex **55** was synthesised according to **Procedure 1** using pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (0.05 g, 0.15 mmol, 1 eq.) and $\text{Ru}(\text{TAP})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (0.08 g, 0.15 mmol, 1 eq.), giving the product as a brown solid (0.085 g, 52%). Calculated for $\text{C}_{40}\text{H}_{22}\text{F}_{12}\text{N}_{14}\text{P}_2\text{Ru} \cdot 1\text{CH}_3\text{OH}$: C, 43.90; H, 2.34; N, 17.48. Found: C, 44.24; H, 2.62; N, 17.10. m.p. > 250

°C. δ_{H} (600 MHz, CD_3CN): 10.41 (dd, 1H, $J = 1.08$, $J = 8.28$, H-1/H-1'), 9.98 (dd, 1H, $J = 1.44$, $J = 8.28$, H-1/H-1'), 9.35 (s, 2H, H-6 and H-7), 9.08 (m, 4H, TAP-H), 8.75 (d, 1H, $J = 9.48$, H-4/H-5), 8.73 (s, 4H, TAP-H), 8.64 (d, 1H, $J = 9.42$, H-4/H-5), 8.49 (d, 2H, $J = 3.6$,

TAP-H), 8.46 (d, 2H, $J = 2.8$, TAP-H), 8.41 (m, 2H, H-3 and H-3'), 8.05 (m, 2H, H-2 and H-2'). δ_{C} (150 MHz, CD_3CN): 156.56, 156.37, 150.93, 150.78, 150.51, 150.35, 150.26, 149.90, 149.87, 148.61, 146.76, 146.55, 146.50, 145.58, 143.35, 143.24, 142.62, 141.93, 141.32, 140.18, 136.12, 136.08, 135.53, 133.84, 133.77, 132.46, 131.90, 131.46, 128.64. ν_{max} (film)/ cm^{-1} : 3411 (Aromatic C-H stretch), 1330 (C-N stretch). HRMS (m/z -ES): Found 800.1224 (M^{2+} . $\text{C}_{40}\text{H}_{22}\text{N}_{14}\text{Ru}$ Requires: 800.1195).

8.7 Synthesis and characterisation of compounds described in Chapter 3

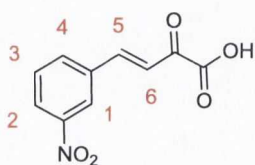
Pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (67)



Pyrazino[2,3-h]dipyrido[3,2-a:2',3'-c]phenazine (0.17 g, 0.5 mmol, 1 eq.) was suspended in dibromoethane (25 mL) before the suspension was heated at 135 °C for 24 hrs. The resulting brown precipitate was isolated by suction filtration before the chloride form of the complex was formed by stirring a solution of the bromide complex in MeOH (35 mL) with Amberlite ion exchange resin (chloride form) for 1 hr. The solution was filtered and reduced in volume before being precipitated from solution by slow addition of diethyl ether. The resulting precipitate was isolated by suction filtration and washed with diethyl ether (2 x 10 mL) yielding a brown solid (0.17 g, 78%). m.p. > 250 °C. Calculated for $\text{C}_{22}\text{H}_{14}\text{N}_6\text{Cl}_2 \cdot 5\text{H}_2\text{O}$: C, 50.48; H, 4.62; N, 16.06. Found: C, 50.46; H, 4.17; N, 16.04. δ_{H} (600 MHz, MeOD): 11.21 (dd, 1H, $J = 1.24$, $J = 8.48$, H-1/H-1'), 10.76 (dd, 1H, $J = 1.12$, $J = 8.44$, H-1/H-1'), 9.89 (dt, 2H, $J = 5.04$, $J = 5.92$, H-3 and H-3'), 9.41 (s, 2H, H-6 and H-7), 8.95 (m, 2H, H-2 and H-2'), 8.85 (AB sys, 2H, $J_{\text{AB}} = 9.48$, H-4 and H-5), 5.85 (s, 4H, H-8). δ_{C} (100 MHz, MeOD): 150.19, 149.96, 148.89, 146.53, 146.08, 146.07, 145.18, 144.46, 142.90, 140.56, 139.00, 137.78, 136.02, 132.87, 132.72, 132.63, 132.18, 131.84, 129.65, 129.64, 53.08, 53.06. ν_{max} (film)/ cm^{-1} : 3258 (Aromatic C-H stretch), 1378 (C-N stretch). HRMS (m/z -ES): Found: 393.1446 ($\text{M}^{2+}\text{MeO}^-$. $\text{C}_{23}\text{H}_{17}\text{N}_6\text{O}_2$ Requires: 393.1453).

8.8 Synthesis and characterisation of compounds described in Chapter 4

(*E*)-4-(3-nitrophenyl)-2-oxo-3-butenic acid (74)¹⁵⁴



Compound **74** was synthesised according to **Procedure 2** using 3-nitrobenzaldehyde (7.7 g, 51.1 mmol, 1 eq.) and sodium pyruvate (6.2 g, 56.2 mmol, 1.1 eq.), giving the product as a bright yellow solid (6.19 g, 54%). δ_{H} (400 MHz, $[\text{D}_6]$ DMSO): 8.45 (s, 1H, H-1), 8.22 (dd, 1H, $J = 2.0$, $J = 8.0$, H-2), 8.15 (d, 1H, $J = 8.0$, H-4), 7.70 (t, 1H, $J = 8.0$, H-3), 7.54 (d, 1H, $J = 16.6$, H-5), 7.05 (d, 1H, $J = 16.6$, H-6). δ_{C} (100 MHz, $[\text{D}_6]$ DMSO): 196.81, 169.16, 148.80, 141.32, 137.08, 134.52, 130.94, 127.86, 124.82, 123.01. ν_{max} (film)/ cm^{-1} : 3502 (-O-

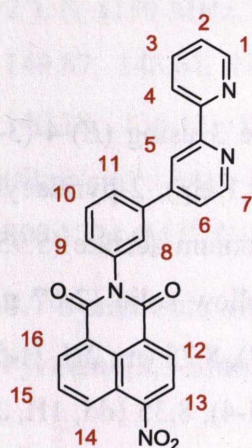
H), 1688 (C=O), 1535 (C-NO₂), 1346 (C-NO₂). HRMS (*m/z* -ES): Found: 220.0252 (M-H. C₁₀H₆NO₅ Requires: 220.0246).

4-(3-nitrophenyl)-2,2'-bipyridine (77)¹⁵⁴

Compound **77** was synthesised according to **Procedure 3** using (*E*)-4-(3-nitrophenyl)-2-oxo-3-butenic acid (2.13 g, 9.63 mmol, 1 eq.), 2-pyridacetyl pyridinium iodide (3.14 g, 9.63 mmol, 1 eq.) and ammonium acetate (5.95 g, 77.21 mmol, 8 eq.) giving the product as a pale yellow solid (2.67 g, 51%). δ_{H} (400 MHz, CDCl₃): 8.83 (d, 1H, *J* = 5.04, H-7), 8.75 (m, 2H, H-5 and H-1), 8.65 (app d, 1H, H-8), 8.50 (d, 1H, *J* = 8.0, H-4), 8.35 (dd, 1H, *J* = 2.0, *J* = 8.0, H-9), 8.13 (d, 1H, *J* = 7.52, H-11), 7.89 (dt, 1H, *J* = 7.52, *J* = 2.0, H-3), 7.72 (t, 1H, *J* = 7.52, H-10), 7.60 (dd, 1H, *J* = 2.0, *J* = 5.0, H-6), 7.41 (dt, 1H, *J* = 1.52, *J* = 6.04, H-2). δ_{C} (100 MHz, CDCl₃): 156.71, 155.12, 149.62, 148.79, 148.39, 146.39, 139.70, 136.67, 132.69, 129.69, 123.72, 123.30, 121.66, 121.01, 120.89, 118.50. ν_{max} (film)/cm⁻¹: 1529 (C-NO₂), 1349 (C-NO₂). HRMS (*m/z* -ES): Found: 278.0923 (M+H. C₁₆H₁₂N₃O₂ Requires: 278.0930).

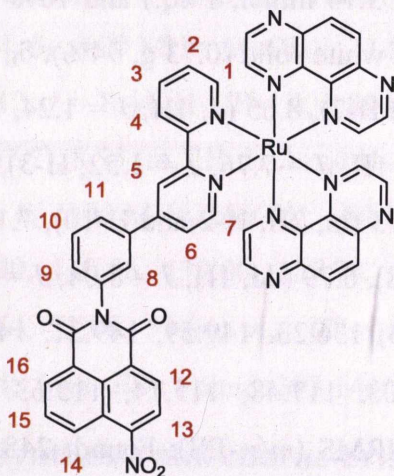
4-(3-aminophenyl)-2,2'-bipyridine (78)¹⁵⁴

Compound **78** was synthesised according to **Procedure 4** using 4-(3-nitrophenyl)-2,2'-bipyridine (0.87 g, 3.14 mmol, 1 eq.) and 10% Pd/C (0.2 g) yielding the product as an off white solid (0.73 g, 94%). δ_{H} (400 MHz, CDCl₃): 8.75 (m, 2H, H-1 and H-7), 8.65 (d, 1H, *J* = 1.24, H-5), 8.47 (d, 1H, *J* = 8.0, H-4), 7.86 (dt, 1H, *J* = 7.76, *J* = 1.52, H-3), 7.53 (dd, 1H, *J* = 5.0, *J* = 1.76, H-6), 7.33 (m, 2H, H-2 and H-10), 7.17 (d, 1H, *J* = 7.76, H-11), 7.11 (s, 1H, H-8), 6.79 (dd, 1H, *J* = 8.04, *J* = 1.76, H-9), 3.85 (s, 1H, NH₂). δ_{C} (100 MHz, CDCl₃): 156.53, 156.23, 149.59, 149.21, 147.04, 139.44, 136.99, 129.99, 123.80, 121.67, 121.30, 119.03, 117.48, 115.74, 113.63. ν_{max} (film)/cm⁻¹: 3429 (NH₂ Stretch), 1632 (Aromatic C-N). HRMS (*m/z* -ES): Found: 248.1198 (M+H. C₁₆H₁₄N₃ Requires: 248.1188).

4-[*N*-(*m*-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine (72)¹⁵⁴

Compound **72** was synthesised according to **Procedure 5** using 4-(3-aminophenyl)-2,2'-bipyridine (0.54 g, 2.17 mmol, 1 eq.) and 4-nitro-1,8-naphthalic anhydride (0.53 g, 2.17 mmol, 1 eq.) giving the product as a yellow/brown solid (0.86 mg, 84%). δ_{H} (400 MHz, CDCl_3): 8.94 (d, 1H, H-14), 8.83 (d, 1H, $J = 7.52$, H-16), 8.78 (m, 2H, H-7 and H-13), 8.73 (s, 1H, H-5), 8.71 (d, 1H, $J = 5.0$, H-1), 8.48 (m, 2H, H-4 and H-12), 8.07 (t, 1H, $J = 7.52$, H-15), 7.95 (d, 1H, $J = 8.04$, H-9), 7.86 (t, 1H, $J = 8.04$, H-3), 7.75 (m, 2H, H-8 and H-10), 7.61 (d, 1H, $J = 5.0$, H-6), 7.45 (d, 1H, $J = 7.52$, H-11), 7.35 (t, 1H, $J = 6.0$, H-2). δ_{C}

(100 MHz, CDCl_3): 162.17, 156.34, 155.47, 149.49, 149.31, 148.72, 147.68, 139.49, 136.54, 134.98, 132.51, 129.90, 129.82, 129.65, 129.38, 129.01, 128.62, 127.44, 126.94, 126.54, 123.55, 123.45, 123.41, 122.58, 121.22, 120.85, 118.56. ν_{max} (film)/ cm^{-1} : 1717 (–CO–N–CO–), 1524 (C–NO₂), 1350 (C–NO₂). HRMS (m/z –ES): Found: 473.1233 (M+H. C₂₈H₁₇N₄O₄ Requires: 473.1250).

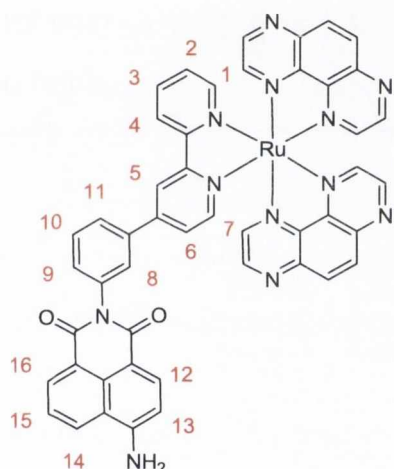
Ru(4-[*N*-(*m*-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine)(1,4,5,8-tetraazaphenanthrene)₂Cl₂ (70)

4-[*N*-(*m*-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine (0.104 g, 0.22 mmol, 1 eq.) and Ru(TAP)₂Cl₂ (0.118 g, 0.22 mmol, 1 eq.) were suspended in DMF:H₂O (1:1) and the suspension was degassed by bubbling with argon for 15 mins. The reaction mixture was heated at 150 °C for 40 mins using microwave irradiation before being allowed to cool and filtered. The solvent from the resulting solution was removed under reduced pressure before redissolution in H₂O (5 mL). The PF₆ salt of the complex was formed by addition of a conc. ethanolic solution of NH₄PF₆ with

the resulting precipitate being collected by centrifugation. The dried solid was redissolved in MeCN before purification by slow diffusion of Et₂O in to MeCN giving the product as a red/brown solid (0.140 g, 52%). The crystalline solid was converted to the chloride form of the complex by stirring in MeOH with Amberlite anion exchange resin (Cl[–] form) for 1 hr. m.p. > 250 °C. δ_{H} (600 MHz, CD₃CN): 9.15 (m, 2H, TAP-H), 8.93 (m, 2H, TAP-H), 8.88 (m, 2H, H-7 and H-16), 8.74 (m, 2H, H-1 and H-5), 8.69 (d, 1H, $J = 7.92$, H-13), 8.66 (m, 4H, TAP-H), 8.51 (d, 1H, $J = 7.92$, H-12), 8.41 (d, 1H, $J = 2.70$, TAP-H), 8.33 (d, 1H, $J = 2.46$, TAP-H), 8.15

(m, 4H, H-3, H-6 and 2TAP-H), 8.07 (d, 1H, $J = 7.98$, H-9), 7.94 (s, 1H, H-8), 7.84 (t, 1H, $J = 7.84$, H-10), 7.80 (d, 2H, $J = 5.94$, H-4 and H-14), 7.64 (m, 2H, H-11 and H-15), 7.41 (t, 1H, $J = 6.12$, H-2). δ_c (150 MHz, CD_3CN): 164.66, 163.88, 158.06, 157.58, 154.21 (C-H, 4/14), 154.09 (C-H, 4/14), 151.06, 150.91, 150.52 (C-H, TAP), 150.50 (C-H, TAP), 150.47 (C-H, TAP), 149.76 (C-H, TAP), 149.72 (C-H, TAP), 149.37 (C-H, TAP), 146.62, 146.59, 146.49, 143.34, 143.31, 142.98, 142.95, 140.22 (C-H, 3), 138.02, 137.56, 133.87 (C-H, TAP), 133.80 (C-H, TAP), 133.79 (C-H, TAP), 133.12 (C-H, 5), 132.19 (C-H, 11), 131.55 (C-H, 10), 131.05 (C-H, 6), 130.88 (C-H, 13), 130.55 (C-H, 7), 130.29, 128.98 (C-H, 8), 128.93 (C-H, 2), 128.80 (C-H, 9), 128.17, 125.96 (C-H, 15), 125.70 (C-H, 1), 125.22 (C-H, 12), 124.59, 124.31, 123.16 (C-H, 16). ν_{max} (film)/ cm^{-1} : 1711 (C=O), 1665 (C=O), 1535 (NO₂ stretch), 1355 (NO₂ stretch). HRMS (m/z -ES): Found: 1083.1088 ($M+PF_6^+$; $C_{48}H_{28}F_6N_{12}O_4PRu$, Requires: 1083. 1042).

Ru(4-[N-(m-phenyl)-4-amino-1,8-naphthalimide]-2,2'-bipyridine)(1,4,5,8-tetraazaphenanthrene)₂Cl₂ (71)



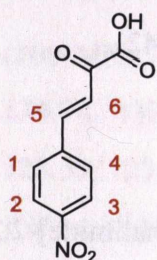
Ru(4-[N-(m-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine)(1,4,5,8-tetraazaphenanthrene)₂Cl₂ (0.1 g, 0.081 mmol, 1 eq.) was dissolved in HPLC grade MeOH (20 mL) and 10% Pd/C added. The reaction mixture was subjected to 3 atm H₂ for 24 hrs. The reaction mixture was filtered through a celite plug and the solvent removed under reduced pressure to give the product as a red/brown solid (0.092 g, 95%). The crystalline solid was converted to the chloride form of the complex by stirring a solution of the PF₆ salt in MeOH with Amberlite anion exchange resin (Cl⁻ form) for 1

hr. Calculated for $C_{48}H_{30}F_{12}N_{12}O_2P_2Ru \cdot 2H_2O$: C, 46.72; H, 2.78; N, 13.62. Found: C, 46.65; H, 2.30; N, 13.15. m.p. > 250 °C. δ_H (600 MHz, CD_3CN): 9.15 (d, 1H, $J = 2.76$, TAP-H), 9.14 (d, 1H, $J = 2.70$, TAP-H), 8.93 (d, 1H, $J = 2.94$, TAP-H), 8.92 (d, 1H, $J = 2.76$, TAP-H), 8.86 (d, 1H, $J = 1.74$, H-5), 8.74 (d, 1H, $J = 8.16$, H-1), 8.64 (m, 4H, TAP-H), 8.55 (d, 1H, $J = 6.90$, H-16), 8.43 (d, 1H, $J = 8.88$, H-14), 8.40 (d, 1H, $J = 2.76$, TAP-H), 8.34 (d, 1H, $J = 2.70$, TAP-H), 8.32 (d, 1H, $J = 8.32$, H-12), 8.15 (m, 3H, H-2 and 2TAP-H), 8.00 (d, 1H, $J = 8.70$, H-9), 7.94 (s, 1H, H-8), 7.76 (m, 4H, H-4, H-7, H-10 and H-15), 7.66 (dd, 1H, $J = 1.98$, $J = 6.12$, H-6), 7.56 (d, 1H, $J = 7.92$, H-11), 7.40 (dt, 1H, $J = 1.08$, $J = 5.70$, H-3), 6.97 (d, 1H, $J = 8.28$, H-13), 6.06 (s, 2H, NH₂). δ_c (150 MHz, CD_3CN): 165.62, 164.84, 157.98, 157.58, 154.12 (C-H, 7), 154.02 (C-H, 4), 152.92, 150.99, 150.49, 150.48 (C-H, TAP), 150.46 (C-H, TAP), 149.74 (C-H, TAP), 149.69 (C-H, TAP), 149.36 (C-H, TAP), 149.34 (C-H, TAP), 146.57, 146.55, 146.46,

146.44, 143.31, 143.30, 142.96, 142.94, 140.17 (C-H, 2), 139.33, 137.10, 134.87 (C-H, 12), 133.84 (C-H, TAP), 133.78 (C-H, TAP), 133.75, 132.64 (C-H, 11), 131.37, 131.19 (C-H, 10), 129.73 (C-H, 14), 129.34 (C-H, 8), 128.87 (C-H, 3), 128.01 (C-H, 9), 125.82 (C-H, 6), 125.70 (C-H, 1), 125.61 (C-H, 15), 123.82, 123.03 (C-H, 5), 120.89, 110.84, 109.72 (C-H, 13). $\nu_{\max}$ (film)/ cm^{-1} : 3356 (Aromatic C-H stretch), 1675 (C=O), 1628 (C=O), 1584 (NH bend), 1371 (C-N stretch). HRMS (m/z -ES): Found: 1053. 1266 ($M+PF_6^+$; $C_{48}H_{30}F_6N_{12}O_2PRu$, Requires: 1053. 1300).

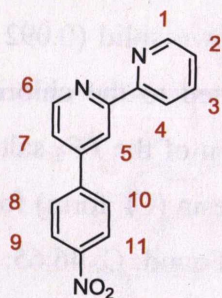
8.9 Synthesis and characterisation of compounds described in Chapter 6

(*E*)-4-(4-nitrophenyl)-2-oxo-3-butenic acid (**94**)²⁰⁸

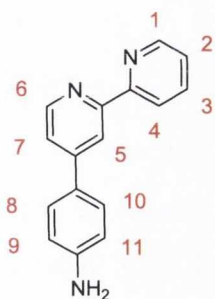


Compound **94** was synthesised according to **Procedure 2** using 4-nitrobenzaldehyde (7.7 g, 51.1 mmol, 1 eq.) and sodium pyruvate (6.2 g, 56.2 mmol, 1.1 eq.) giving a bright yellow solid (7.486 g, 66%). δ_H (400 MHz, $[D_6]$ DMSO): 8.24 (d, 2H, $J = 8.84$, H-2 and H-3), 7.96 (d, 2H, $J = 8.76$, H-1 and H-4), 7.55 (d, 1H, $J = 16.4$, H-5), 7.05 (d, 1H, $J = 16.4$, H-6). δ_C (100 MHz, $[D_6]$ DMSO): 196.97, 168.89, 148.29, 141.88, 140.85, 129.65, 129.30, 124.46. $\nu_{\max}$ (film)/ cm^{-1} : 3461 (-O-H), 1677 (C=O), 1514 (C-NO₂), 1346 (C-NO₂). HRMS (m/z -ES): Found: 220.0255 (M-H. $C_{10}H_6NO_5$ Requires: 220.0246).

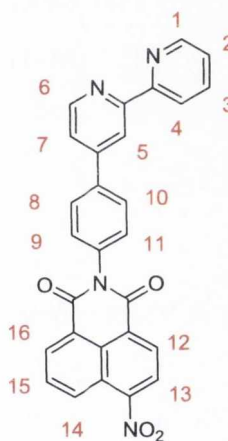
4-(4-nitrophenyl)-2,2'-bipyridine (**97**)²⁰⁸



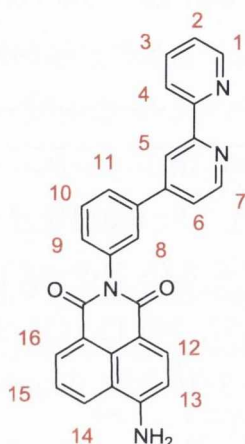
Compound **97** was synthesised according to **Procedure 3** using (*E*)-4-(4-nitrophenyl)-2-oxo-3-butenic acid (2.13 g, 9.63 mmol, 1 eq.), 2-pyridacyl pyridinium iodide (3.14 g, 9.63 mmol, 1 eq) and ammonium acetate (5.95 g, 77.21 mmol, 8 eq.) to yield a pale yellow solid (1.34 g, 50%). δ_H (400 MHz, $CDCl_3$): 8.82 (d, 1H, $J = 5.04$, H-6), 8.74 (m, 2H, H-1 and H5), 8.50 (d, 1H, $J = 7.96$, H-4), 8.39 (d, 2H, $J = 8.84$, H-9 and H-11), 7.94 (d, 2H, $J = 8.88$, H-8 and H-10), 7.89 (dt, 1H, $J = 7.68$, $J = 1.64$, H-2/3), 7.58 (dd, 1H, $J = 5.12$, $J = 1.8$, H-7), 7.39 (m, 1H, H-2/3). δ_C (100 MHz, $CDCl_3$): 154.88, 149.57, 148.58, 147.76, 146.57, 144.19, 136.95, 127.73, 123.87, 123.82, 121.26, 121.05, 118.81. $\nu_{\max}$ (film)/ cm^{-1} : 1516 (C-NO₂), 1360 (C-NO₂). HRMS (m/z -ES): Found: 278.0919 (M+H. $C_{16}H_{12}N_3O_2$ Requires: 278.0930).

4-(4-aminophenyl)-2,2'-bipyridine (98)²⁰⁸

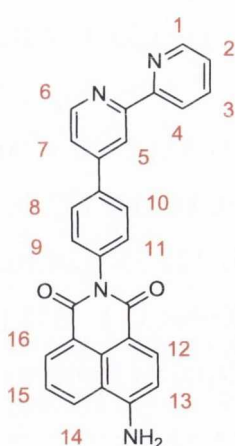
Compound **98** was synthesised according to **Procedure 4** using 4-(4-nitrophenyl)-2,2'-bipyridine (1.00 g, 3.61 mmol, 1 eq.) and 10% Pd/C (0.2 g) yielding the product as an off white solid (0.745 g, 83%). δ_{H} (400 MHz, CDCl_3): 8.73 (d, 1H, $J = 4.24$, H-1), 8.67 (d, 1H, H-6), 8.63 (d, 1H, $J = 1.6$, H-5), 8.45 (d, 1H, $J = 8.0$, H-4), 7.85 (dt, 1H, $J = 7.76$, $J = 1.76$, H-2/3), 7.66 (d, 2H, $J = 8.52$, H-8 and H-10), 7.51 (dd, 1H, $J = 5.2$, $J = 1.8$, H-7), 7.34 (dt, 1H, $J = 7.44$, $J = 0.84$, H-2/3), 6.81 (d, 2H, $J = 8.56$, H-9 and H-11), 3.90 (s, 2H, $-\text{NH}_2$). δ_{C} (100 MHz, CDCl_3): 155.97, 149.09, 148.57, 147.12, 136.46, 127.74, 127.48, 123.21, 120.79, 120.12, 117.44, 114.81. ν_{max} (film)/ cm^{-1} : 3319 (NH_2 Stretch), 1582 (Aromatic C-N). HRMS (m/z -ES): Found: 248.1175 ($\text{M}+\text{H}$). $\text{C}_{16}\text{H}_{14}\text{N}_3$ Requires: 248.1188).

4-[N-(p-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine (99)¹⁵⁴

Compound **99** was synthesised according to **Procedure 5** using 4-(4-aminophenyl)-2,2'-bipyridine (0.54 g, 2.17 mmol, 1 eq.) and 4-nitro-1,8-naphthalic anhydride (0.53 g, 2.17 mmol, 1 eq.) to give a pale yellow/brown solid (0.86 g, 84%). δ_{H} (400 MHz, $[\text{D}_6]$ DMSO): 8.78 (m, 4H, H-1, H-5, H-6 and H-16), 8.65 (m, 3H, H-12, H-13 and H-14), 8.47 (d, 1H, $J = 7.9$, H-4), 8.16 (t, 1H, $J = 7.4$, H-15), 8.07 (d, 2H, $J = 8.52$, H-8 and H-9 or H-10 and H-11), 8.01 (dt, 1H, $J = 7.68$, $J = 1.72$, H-3/H-2), 7.90 (dd, 1H, $J = 5.12$, $J = 1.84$, H-7), 7.64 (d, 2H, $J = 8.48$, H-8 and H-9 or H-10 and H-11), 7.52 (dt, 1H, $J = 5.8$, $J = 1.04$, H-3/H-2). δ_{C} (100 MHz, $[\text{D}_6]$ DMSO): 163.74, 162.93, 156.54, 155.56, 150.63, 149.83, 149.80, 148.10, 137.94, 137.90, 137.12, 132.22, 130.63, 130.43, 130.12, 129.41, 129.32, 128.05, 127.75, 124.90, 124.75, 123.86, 123.37, 122.29, 121.18, 118.24. ν_{max} (film)/ cm^{-1} : 1662 ($-\text{CO}-\text{N}-\text{CO}-$), 1532 ($\text{C}-\text{NO}_2$), 1239 ($\text{C}-\text{NO}_2$). HRMS (m/z -ES): Found: 495.1066 ($\text{M}+\text{Na}$; $\text{C}_{28}\text{H}_{16}\text{N}_4\text{O}_4\text{Na}$ Requires: 495.1069).

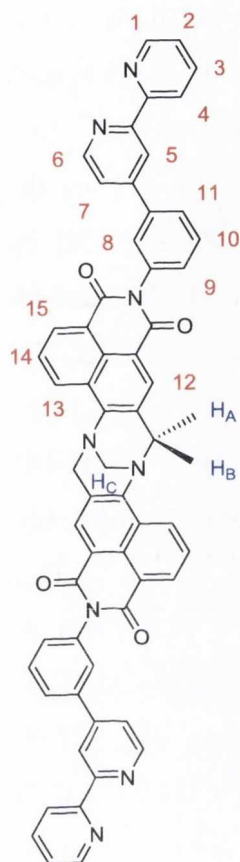
4-[*N*-(*m*-phenyl)-4-amino-1,8-naphthalimide]-2,2'-bipyridine (**91**)

Compound **91** was synthesised according to **Procedure 6** using 4-[*N*-(*m*-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine (0.450 g, 0.95 mmol, 1 eq.), 10% Pd/C (0.2 g) and hydrazine monohydrate (98%, 0.49 mL, 9.5 mmol, 10 eq.) to afford a brown solid. (0.40 g, 96%). δ_{H} (400 MHz, $[\text{D}_6]$ DMSO): 8.78 (d, 1H, $J = 5.12$, H-7), 8.70 (m, 3H, H-1, H-5 and H-16), 8.46 (m, 2H, H-16 and H-14), 8.23 (d, 1H, $J = 8.36$, H-12), 7.98 (m, 3H, H-2, H-8 and H-9), 7.89 (dd, 1H, $J = 4.9$, $J = 2.56$, H-6), 7.70 (m, 2H, $J = 7.88$, H-15 and H-10), 7.50 (m, 4H, H-3 H-8, H-11 and NH_2), 6.90 (d, 1H, $J = 8.36$, H-13). δ_{C} (100 MHz, $[\text{D}_6]$ DMSO): 164.60, 163.71, 156.50, 155.52, 153.37, 150.61, 149.73, 147.93, 138.37, 138.18, 137.87, 134.51, 131.61, 130.83, 130.77, 130.21, 130.00, 128.24, 126.76, 124.86, 124.49, 122.80, 122.01, 121.12, 120.00, 118.04, 108.68, 108.38. ν_{max} (film)/ cm^{-1} : 1686 (-CO-N-CO-), 3335 (N-H stretch), 1649 (N-H bend). HRMS (m/z -ES): Found: 443.1499 ($\text{M}+\text{H}$. $\text{C}_{28}\text{H}_{19}\text{N}_4\text{O}_2$ Requires: 443.1508).

4-[*N*-(*p*-phenyl)-4-amino-1,8-naphthalimide]-2,2'-bipyridine (**92**)

Compound **92** was synthesised according to **Procedure 6** using 4-[*N*-(*p*-phenyl)-4-nitro-1,8-naphthalimide]-2,2'-bipyridine (0.450 g, 0.95 mmol, 1 eq.), 10% Pd/C (0.2 g) and hydrazine monohydrate (98%, 0.49 mL, 9.5 mmol, 10 eq) to give the product as a brown solid. (0.39 mg, 94%). δ_{H} (400 MHz, $[\text{D}_6]$ DMSO): 8.82 (d, 1H, $J = 5.04$, H-7), 8.76 (m, 2H, H-1 and H-5), 8.69 (d, 1H, $J = 8.3$, H-16), 8.47 (m, 2H, H-4 and H-14), 8.23 (d, 1H, $J = 8.36$, H-12), 8.00 (m, 3H, $J = 7.68$, H-2 and either H-8 and H-9 or H-10 and H-11), 7.89 (dd, 1H, $J = 5.0$, $J = 1.4$, H-6), 7.71 (t, 1H, $J = 7.88$, H-15), 7.53 (m, 5H, H-3, NH_2 and either H-8 and H-9 or H-10 and H-11), 6.90 (d, 1H, $J = 8.36$, H-13). δ_{C} (100 MHz, CDCl_3): 164.51, 163.61, 159.46, 156.52, 155.59, 153.43, 150.61, 149.83, 148.21, 138.26, 137.88, 137.31, 134.56, 131.67, 130.76, 130.67, 127.79, 124.87, 124.51, 122.69, 122.23, 121.17, 120.01, 118.17, 108.71, 108.21. ν_{max} (film)/ cm^{-1} : 1637 (-CO-N-CO-), 3341 (N-H stretch), 1574 (N-H bend). HRMS (m/z -ES): Found: 907.2765 ($2\text{M}+\text{Na}$. $\text{C}_{56}\text{H}_{36}\text{N}_8\text{O}_4\text{Na}$ Requires: 907.2757)

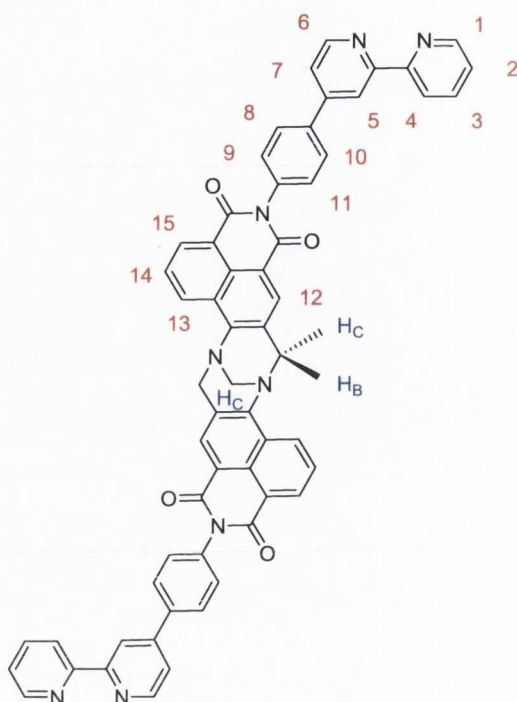
Bis- $\{N$ -[3-(2,2'-dipyridin-4-yl)phenyl]-9,18,-methano-1,8-naphthalimido[b,f][1,5]diazocene (100)



Compound **100** was synthesised according to **Procedure 7** using a mixture of 3-[N -(m -phenyl)-4-amino-1,8-naphthalimide]-2,2'-bipyridine (0.3 g, 0.68 mmol, 1 eq.) and paraformaldehyde (0.03 g, 1.02 mmol, 1.5 eq.) in neat TFA (3.5 mL) to give the product as a bright yellow solid (0.21 g, 68 %). δ_{H} (600 MHz, $[\text{D}_6]$ DMSO): 8.78 (d, 2H, $J = 5.64$, H-15), 8.75 (d, 2H, $J = 3.04$, H-6), 8.65 (m, 4H, H-5 and H-1), 8.50 (d, 2H, $J = 4.84$, H-13), 8.41 (d, 2H, $J = 5.04$, H-4), 8.15 (s, 2H, H-12), 7.97 (m, 8H, H-3, H-8, H-11 and H-14), 7.79 (br s, 2H, H-7), 7.67 (t, 2H, $J = 5.16$, H-9), 7.44 (br m, 4H, H-2 and H-10), 5.20 (d, 2H, $J = 11.68$, H_{A}), 4.79 (s, 2H, H_{C}), 4.70 (d, 2H, $J = 11.68$, H_{B}). δ_{C} (150 MHz, $[\text{D}_6]$ DMSO): 163.73 (Nap C=O), 163.15 (Nap C=O), 155.98, 154.93, 150.08 (C-6), 149.13 (C-1), 147.27, 137.94, 137.30 (C-3), 137.01, 130.55 (C-12), 130.20 (C-15), 130.07 (C-10), 129.78 (C-9), 129.18 (C-13), 127.97, 127.52 (C-11), 127.12 (C-14), 126.76, 126.54 (C-8), 126.11, 124.27 (C-2), 123.04, 121.41 (C-7), 120.58 (C-4), 118.25, 117.43 (C-5), 66.16 ($\text{CH}_2\text{-C}$), 56.87 ($\text{CH}_2\text{-A/B}$). ν_{max} (film)/ cm^{-1} : 1353 (C-N stretch), 1664 (-

CO-N-CO-), 3414 (Aromatic C-H stretch). HRMS (m/z -ES): Found: 921.2965 ($\text{M}+\text{H}$. $\text{C}_{59}\text{H}_{36}\text{N}_8\text{O}_4$ Requires: 921.2938)

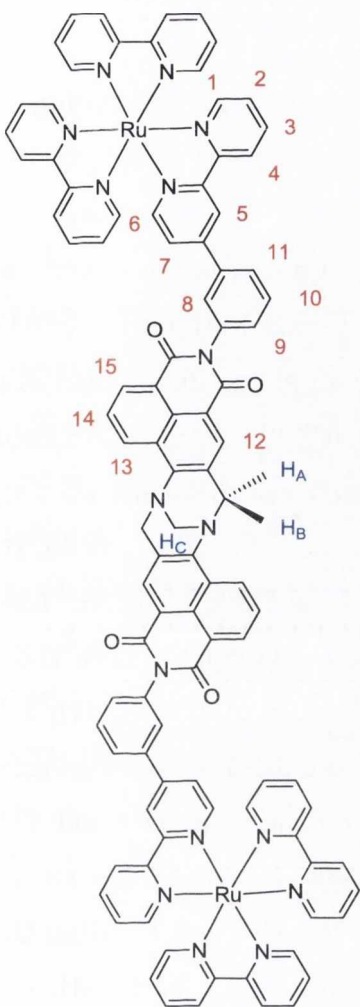
Bis- $\{N$ -[4-(2,2'-dipyridin-4-yl)phenyl]}-9,18,-methano-1,8-naphthalimido[b,f][1,5]-diazocene (101)



Compound **101** was synthesised according to **Procedure 7** using a mixture of 4- $[N$ -(p -phenyl)-4-amino-1,8-naphthalimide]-2,2'-bipyridine (0.3 g, 0.68 mmol, 1 eq.) and paraformaldehyde (0.03 g, 1.02 mmol, 1.5 eq.) in neat TFA (3.5 mL) yielding the product as a bright yellow solid (0.21 g, 70 %). δ_H (600 MHz, $[D_6]$ DMSO): 8.80 (m, 4H, H-6 and H-15), 8.73 (m, 4H, H-1 and H-5), 8.52 (d, 2H, $J = 4.76$, H-13), 8.45 (d, 2H, $J = 5.32$, H-4), 8.18 (s, 2H, H-12), 7.99 (m, 8H, H-3, H-9, H-11 and H-14), 7.85 (dd, 2H, $J = 1.2$, $J = 3.44$, H-7), 7.50 (m, 6H, H-2, H-8 and H-10), 5.23 (d, 2H, $J = 11.72$, H_A), 4.80 (s, 1H, H_B), 4.73 (d, 1H, $J = 11.68$, H_C). δ_C

(150 MHz, $[D_6]$ DMSO): 156.02, 155.06, 150.08 (C-6), 149.29 (C-1), 147.62, 137.35 (C-3), 137.13, 137.04, 130.66 (C-12), 130.26 (C-13), 129.99 (C-8, C-10), 129.28 (C-15), 127.99, 127.40 (C-9, C-11), 127.12 (C-14), 126.82, 126.19, 124.34 (C-2), 122.98, 121.70 (C-7), 120.66 (C-4), 118.17, 117.68 (C-5), 66.17 (CH₂), 64.64, 56.88 (CH₂). $\nu_{\max}$ (film)/cm⁻¹: 1356 (C-N stretch), 1657 (-CO-N-CO-), 3387 (Aromatic C-H stretch), HRMS (m/z -ES): Found: 921.2941 (M+H. C₅₉H₃₇N₈O₄ Requires: 921.2938).

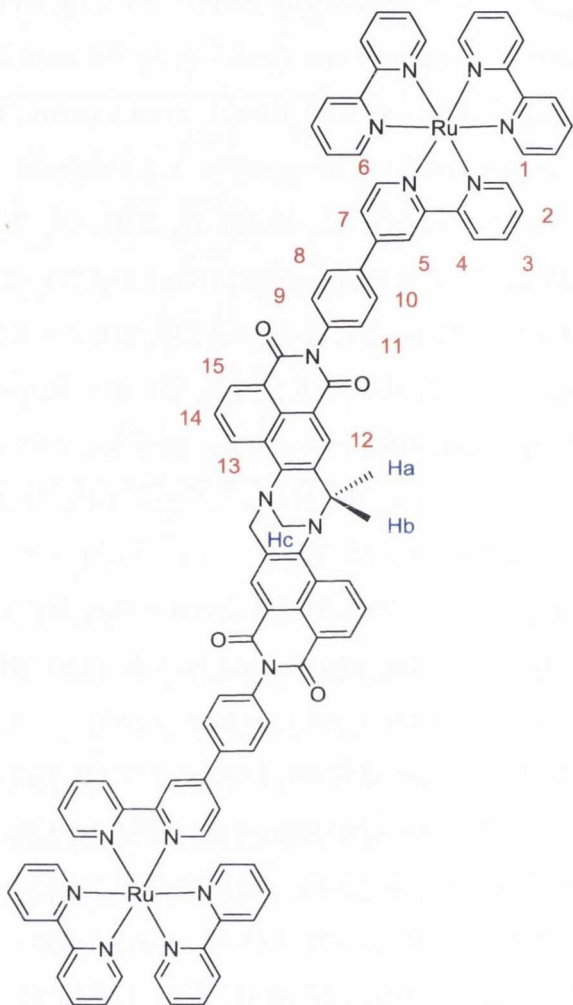
{[Ru(bipyridine)₂Cl₂]₂Bis-*N*-[3-(2,2'-dipyridin-4-yl)phenyl]}-9,18,-methano-1,8-naphthalimido[*b,f*][1,5]-diazocene} (91)



Complex **91** was synthesised according to **Procedure 8** using bis-*N*-[3-(2,2'-dipyridin-4-yl)phenyl]}-9,18,-methano-1,8-naphthalimido[*b,f*][1,5]-diazocene (0.05 g, 0.054 mmol, 1 eq.) and Ru(bpy)₂Cl₂ (0.053 g, 0.11 mmol, 2 eq.) giving the product as a red/brown solid (0.86 g, 68%). Calculated for C₉₉H₆₈F₂₄N₁₆O₄P₄Ru₂.NaCl.H₂O: C, 49.46; H, 2.93; N, 9.32. Found: C, 49.38; H, 3.02; N, 9.19. δ_{H} (600 MHz, CD₃CN): 8.87 (d, 2H, *J* = 8.4, H-13), 8.78 (s, 2H, H-5), 8.65 (d, 2H, *J* = 8.22, H-1), 8.58 (d, 2H, *J* = 7.32, H-15), 8.52 (m, 8H, 8 × Bpy-H), 8.18 (s, 1H, H-12), 8.08 (m, 10H, H-4 and 8 × Bpy-H), 7.98 (m, 4H, H-11 and H-14), 7.88 (br s, 2H, H-8), 7.77 (m, 14H, H-2/3, H-6, H-9 and 8 × Bpy-H), 7.65 (d, 2H, *J* = 6.12, H-7), 7.50 (d, 2H, *J* = 5.76, H-10), 7.43 (m, 10H, H-2/3 and 8 × Bpy-H), 5.25 (d, 2H, *J* = 17.28, H_A), 4.76 (m, 6H, H_B and H_C). δ_{C} (150 MHz, CD₃CN): 165.34 (Nap C=O), 164.72 (Nap C=O), 158.45, 157.96, 157.94, 157.91, 152.76, 152.67, 152.64, 152.60, 152.57, 150.88, 149.32, 138.80, 138.79 (Bpy-C), 138.69, 138.57, 137.66, 132.02 (C-10), 131.70 (C-12), 131.58 (C-15), 131.27, 130.66 (C-13), 129.39, 128.92 (C-8), 128.63, 128.61 (Bpy-C),

128.56 (Bpy-C), 128.52, 128.25 (C-11/14), 128.20 (C-11/14), 127.26 (C-2/3), 125.67 (C-7), 125.40 (C-1), 125.22, 124.20, 122.81 (C-5), 119.61, 67.33 (CH₂-C), 57.78 (CH₂-A/B). ν_{max} (film)/cm⁻¹: 3414 (Aromatic C-H stretch) 1700 (C=O), 1653 (C=O). HRMS (*m/z* -ES): Found: 2183.2554 (M + 3PF₆) C₉₉H₆₈N₁₆O₄F₁₈P₃Ru₂ Requires: 2183.2622.

{[Ru(bipyridine)₂Cl₂]₂Bis-*N*-[4-(2,2'-dipyridin-4-yl)phenyl]}-9,18-methano-1,8-naphthalimido[*b,f*][1,5]-diazocene (92)

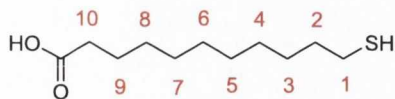


Complex **92** was synthesised according to **Procedure 8** using bis-*N*-[4-(2,2'-dipyridin-4-yl)phenyl]}-9,18-methano-1,8-naphthalimido[*b,f*][1,5]-diazocene (0.05 g, 0.054 mmol, 1 eq.) and Ru(bpy)₂Cl₂ (0.053 g, 0.11 mmol, 2 eq.) giving the product as a red/brown solid (0.079 g, 63%). Calculated for C₉₉H₆₈F₂₄N₁₆O₄P₄Ru₂·NaCl·H₂O: C, 49.22; H, 2.96; N, 9.32. Found: C, 49.46; H, 2.93; N, 9.38. δ_{H} (600 MHz, CD₃CN): 8.89 (d, 2H, *J* = 8.52, H-13), 8.83 (s, 2H, H-5), 8.70 (d, 2H, *J* = 8.76, H-1), 8.59 (d, 2H, *J* = 7.38, H-15), 8.55 (m, 8H, 8 × Bpy-H), 8.19 (s, 2H, H-12), 8.10 (m, 10H, H-4 and 8 × Bpy-H), 8.00 (m, 6H, H-9, H-11 and H-14), 7.79 (m, 12H, H-2/3, H-6 and 8 × Bpy-H), 7.73 (d, 2H, *J* = 4.86, H-7), 7.55 (d, 4H, *J* = 8.10, H-8 and H-10), 7.44 (m, 10H, H-2/3 and 8 × Bpy-H), 5.26 (d, 2H, *J* = 17.76, H_A),

4.77 (m, 6H, H_B and H_C). δ_{C} (150 MHz, CD₃CN): 164.18 (Nap C=O), 163.56 (Nap C=O), 157.30, 156.92, 156.90, 156.84, 156.82, 151.59 (Bpy-C), 151.48, 151.43, 149.72, 148.74, 138.35, 137.69 (Bpy-C), 137.61, 135.71, 130.53 (C-12), 130.40 (C-15), 129.98 (C-8 and C-10), 129.51 (C-13), 128.33, 128.06, 127.53 (Bpy-C), 127.46 (C-9 and C-11), 127.41, 127.05 (C-14), 126.11, 124.89 (C-7), 124.29 (C-1), 124.14 (Bpy-C), 123.20, 122.06 (C-5), 118.62, 65.12 (CH₂-C), 56.67 (CH₂-A/B). ν_{max} (film)/cm⁻¹: 3425 (Aromatic C-H stretch) 1707 (C=O), 1665 (C=O). HRMS (*m/z* -ES): Found: 2183.2517 (M + 3PF₆; C₉₉H₆₈N₁₆O₄F₁₈P₃Ru₂ Requires: 2183.2622).

8.10 Synthesis and characterisation of compounds described in Chapter 7

11-mercaptoundecanoic acid (109)²⁵⁰



Methyl 11-bromoundecanoate (3.3 g, 11.25 mmol, 1 eq.)

was added to a heated solution of thiourea (1.71 g, 22.5 mmol, 2 eq.) in H₂O:EtOH (1:1) (300 mL) before being

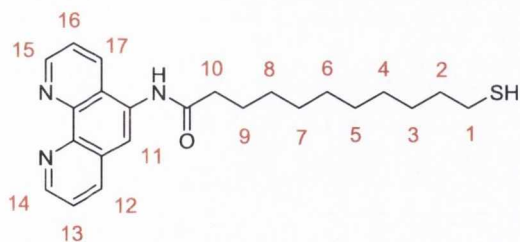
heated at reflux for 4 hrs. Addition of NaOH (1.12 g,

28.12 mmol, 2.5 eq.) was followed by a further 3 hrs heating at reflux before the solvent was removed under reduced pressure. A portion of H₂O (20 mL) was added and the resulting solution was acidified by addition of 6 N H₂SO₄ before the product was extracted into Et₂O (5 × 50 mL). The Et₂O layer was dried over MgSO₄ and the solvent was removed under vacuum.

The white product was recrystallised from EtOH to yield a bright white power which was collected by suction filtration and dried under high vacuum (1.90 g, 72%). m.p. 102-105 °C.

δ_{H} (400 MHz, [D₆] DMSO): 11.99 (s, 1H, -OH), 2.46 (t, 2H, $J = 10.9$, CH₂-1), 2.19 (t, 2H, $J = 11.0$, CH₂-10), 1.49 (m, 4H, CH₂-2 and 9), 1.34 – 1.25 (br s, 12H, CH₂-3, 4, 5, 6, 7 and 8). δ_{C} (100 MHz, [D₆] DMSO): 179.93 (qC), 38.87 (CH₂, 10), 36.29 (CH₂, 1), 34.33 (CH₂, 9), 34.12 (CH₂, 2), 34.08 (CH₂), 33.95 (CH₂), 33.79 (CH₂), 33.77 (CH₂), 33.40 (CH₂), 29.71 (CH₂). ν_{max} (film)/cm⁻¹: 2916 (alkane-C-H stretch), 2849 (alkane-C-H stretch), 1692 (C=O), 1470 (alkane-C-H bend), 718 (C-S).

11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide (108)



5-amino-1,10-phenanthroline (0.5 g, 2.56 mmol, 1

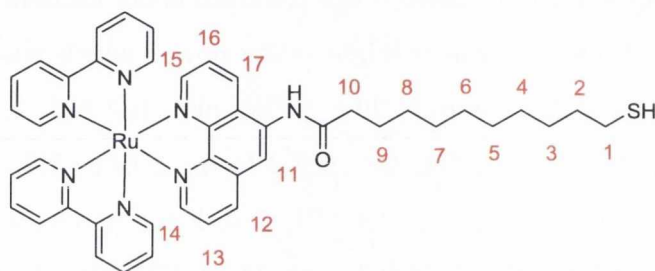
eq.) was dissolved in distilled CH₂Cl₂ (100 mL) before the solution was cooled to 0 °C. 11-

mercaptoundecanoic acid (0.56 g, 2.56 mmol, 1 eq.) was added followed by 1-Ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDCI) (1.23 g, 6.4 mmol, 2.5 eq.) and finally 4-dimethylaminopyridine (DMAP) (0.31 g, 2.56 mmol, 1 eq.) before the reaction mixture was stirred at 0 °C for a further 1 hr. The mixture was allowed to reach room temperature before being stirred for a further 24 hrs. The solvent was removed under reduced pressure before H₂O (50 mL) was added causing precipitation of a white solid which was isolated by centrifugation. The resulting solid was redispersed in MeCN (10 mL) and again centrifuged before being collected by suction filtration and dried under high vacuum to yield a beige/white solid. (0.8 g, 78%). m.p. 88-90 °C. δ_{H} (400 MHz, [D₆] DMSO): 10.12 (s, 1H, N-H), 9.13 (dd, 1H, $J = 1.56$, $J = 4.24$, CH-15), 9.04 (dd, 1H, $J = 1.72$, $J = 4.24$, CH-14), 8.61 (dd, H, $J = 1.48$, $J = 8.4$, CH-17), 8.44 (dd, 1H, $J = 1.72$, $J = 8.16$, CH-12), 8.18 (s, 1H, CH-11), 7.83

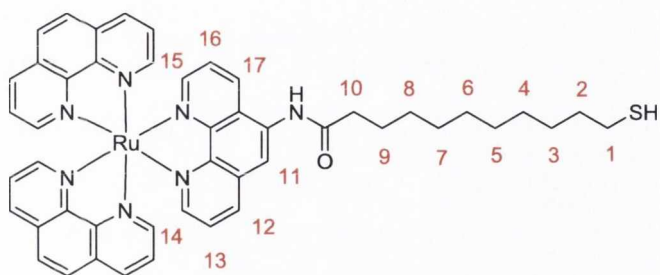
(dd, 1H, $J = 4.28$, $J = 8.4$, CH-16), 7.74 (dd, 1H, $J = 4.32$, $J = 8.08$, CH-13), 2.53 (from CH COSY) (t, 2H, CH₂-1), 2.43 (t, 2H, $J = 7.2$, CH₂-10), 1.69 (t, 2H, $J = 6.52$, CH₂-9), 1.47 (t, 2H, $J = 6.44$, CH₂-2), 1.42 – 1.20 (br s, 12H, CH₂-3, 4, 5, 6, 7 and 8). δ_{C} (100 MHz, [D₆] DMSO): 6132.25 (CH, 17), 132.05 (qC), 128.57 (qC), 125.09 (qC), 124.03 (CH, 13), 123.25 (CH, 16), 120.40 (CH, 11), 36.43 (CH₂, 1), 31.54 (CH₂, 10), 29.56 (CH₂, 2), 29.38 (CH₂), 29.36 (CH₂), 29.26 (CH₂), 29.20 (CH₂), 29.04 (CH₂), 28.64 (CH₂), 25.69 (CH₂, 2). ν_{max} (film)/cm⁻¹: 3253 (amide-N-H stretch), 2919 (alkane-C-H stretch), 2849 (alkane-C-H stretch), 1658 (C=O), 1533 (alkane-C-H bend), 721 (C-S).

Ru(11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide)(bipyridine)₂Cl₂ (105)



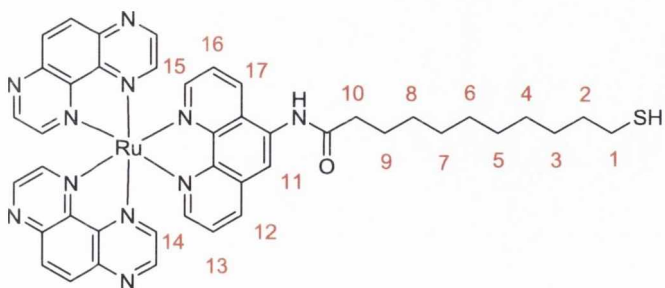
Complex **105** was synthesised according to **Procedure 9** using 11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide (0.1 g, 0.253 mmol, 1 eq.) and Ru(bpy)₂Cl₂ (0.123 g, 0.253 mmol, 1 eq.) giving the product as a red/brown

solid (0.167 g, 75%). Calculated for C₄₃H₄₅Cl₂N₇ORuS.1H₂O.1.25NaCl: C, 53.20; H, 4.88; N, 10.09. Found: C, 53.00; H, 4.99; N, 9.60. m.p. 115–120 °C. δ_{H} (600 MHz, MeOD): 8.77 (d, 1H, $J = 7.68$, H-15), 8.74 (d, 2H, $J = 8.22$, Bpy-H), 8.70 (d, 2H, $J = 8.16$, Bpy-H), 8.64 (d, 1H, $J = 7.5$, H-14), 8.48 (s, 1H, H-11), 8.22 (d, 1H, $J = 4.38$, H-17), 8.17 (t, 2H, $J = 7.92$, Bpy-H), 8.13 (d, 1H, $J = 5.16$, H-12), 8.07 (t, 2H, $J = 7.98$, Bpy-H), 7.93 (t, 2H, $J = 5.82$, Bpy-H), 7.86 (dd, 1H, $J = 5.28$, $J = 8.52$, H-16), 7.79 (dd, 1H, $J = 5.28$, $J = 8.28$, H-13), 7.64 (d, 2H, $J = 5.52$, Bpy-H), 7.55 (m, 2H, Bpy-H), 7.33 (m, 2H, Bpy-H), 2.76 (m, 2H, CH₂-1), 2.66 (t, 2H, $J = 7.44$, CH₂-10), 1.79 (m, 4H, CH₂-9 and 2), 1.49 (m, 2H, CH₂-8), 1.37 (s, 10H, CH₂-7, 6, 5, 4, 3). δ_{C} (150 MHz, MeOD): 174.66, 157.25, 157.23, 157.01, 157.00, 152.03 (C-H, 17), 151.33 (C-H, 12), 151.31 (C-H, Bpy), 151.21 (C-H, Bpy), 151.19, 147.78, 145.51, 137.79 (C-H, Bpy), 137.67 (C-H, Bpy), 136.30 (C-H, 14), 133.89, 132.58 (C-H, 15), 130.58, 127.44 (C-H, Bpy), 127.39 (C-H, Bpy), 127.36, 127.31, 127.28, 126.01 (C-H, 13), 125.43 (C-H, 16), 124.11 (C-H, Bpy), 124.09 (C-H, Bpy), 124.05, 124.03, 121.29, 51.30 (CH₂, 1), 35.94 (CH₂, 10), 28.99 (CH₂), 28.93 (CH₂), 28.91 (CH₂), 28.87 (CH₂), 28.77 (CH₂), 28.23 (CH₂, 8), 25.20 (CH₂, 9), 22.26 (CH₂, 2). ν_{max} (film)/cm⁻¹: 3366 (Aromatic C-H stretch), 2922 (alkane-C-H stretch), 2851 (alkane-C-H stretch), 1627 (C=O), 1421 (C-N stretch), 769 (C-S). HRMS (m/z -ES): Found: 954.2139 ((M+PF₆)⁺; C₄₃H₄₅F₆N₇OPRuS, Requires: 954.2139).

Ru(11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide)(phenanthroline)₂Cl₂ (106)

Complex **106** was synthesised according to **Procedure 9** using 11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide (0.1 g, 0.253 mmol, 1 eq.) and Ru(phen)₂ (0.135 g, 0.253 mmol, 1 eq.) giving the product as a red/brown solid (

0.152 g, 65%). Calculated for C₄₇H₄₅Cl₂N₇ORuS.1H₂O.1.25NaCl: C, 55.40; H, 4.65; N, 9.62. Found: C, 55.55; H, 4.71; N, 9.27. m.p. 115–120 °C. δ_{H} (600 MHz, MeOD): 8.74 (d, 1H, J = 8.4, H-15), 8.69 (t, 4H, J = 8.04, Phen-H), 8.61 (d, 1H, J = 8.22, H-14), 8.49 (s, 1H, H-11), 8.32 (s, 4H, Phen-H), 8.14 (m, 3H, Phen-H and H-17), 8.10 (dd, 2H, J = 5.22, J = 8.16, Phen-H), 8.06 (d, 1H, J = 5.1, H-12), 7.74 (m, 5H, Phen-H and H-16), 7.68 (dd, 1H, J = 5.28, J = 8.28, H-13), 2.76 (m, 2H, CH₂-1), 2.65 (t, 2H, J = 7.32, CH₂-10), 1.76 (m, 4H, CH₂-9 and 2), 1.48 (m, 2H, CH₂-8), 1.36 (s, 10H, CH₂-7, 6, 5, 4, 3). δ_{C} (150 MHz, MeOD): 174.67 (qC, amide), 152.38 (C-H, Phen), 152.36 (C-H, Phen), 152.26 (C-H, Phen), 152.23 (C-H, Phen), 151.58 (C-H, 12), 148.16, 147.66, 147.64, 147.63, 145.91, 136.75 (C-H, Phen), 136.27 (C-H, 14), 133.87, 132.55 (C-H, 15), 130.96, 130.54, 127.88 (C-H, Phen), 127.43, 125.88 (C-H, 13), 125.80, 125.78 (C-H, Phen), 125.75, 125.30 (C-H, 16), 121.30 (C-H, 11), 51.30 (C-H₂, 1), 35.93 (C-H₂, 10), 28.98 (C-H₂), 28.92 (C-H₂), 28.91 (C-H₂), 28.87 (C-H₂), 28.76 (C-H₂), 28.23 (C-H₂, 8), 25.20 (C-H₂, 9), 22.26 (C-H₂, 2). ν_{max} (film)/cm⁻¹: 3362 (Aromatic C-H stretch), 2922 (alkane-C-H stretch), 2850 (alkane-C-H stretch), 1627 (C=O), 1424 (C-N stretch), 720 (C-S). HRMS (m/z -ES): Found: 2147.3765 (2M + 3PF₆)⁺; C₉₄H₈₈F₁₈N₁₄O₂P₃Ru₂S₂, Requires: 2147.3669.

Ru(11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide)(tetraazaphenanthrene)₂Cl₂ (107)

Complex **107** was synthesised according to **Procedure 9** using 11-mercapto-N-(1,10-phenanthrolin-5-yl)undecanamide (0.1 g, 0.253 mmol, 1 eq.) and Ru(TAP)₂Cl₂ (0.135 g, 0.253 mmol, 1 eq.) giving the product as a red/brown

solid (0.127 g, 54%). Calculated for C₄₃H₄₁Cl₂N₁₁ORuS.1NaCl: C, 54.47; H, 5.90; N, 12.70. Found: C, 54.78; H, 5.03; N, 12.78. m.p. 115–120 °C. δ_{H} (600 MHz, MeOD): 9.03 (d, 4H, J = 2.58, TAP-H), 8.86 (dd, 1H, J = 0.96, J = 8.58, H-15), 8.71 (dd, 1H, J = 0.90, J = 8.28, H-14),

8.65 (s, 4H, TAP-H), 8.53 (s, 1H, H-11), 8.45 (dd, 2H, $J = 2.64$, $J = 6.42$, TAP-H), 8.30 (m, 3H, TAP-H and H-17), 8.20 (dd, 1H, $J = 0.96$, $J = 5.1$, H-12), 7.83 (dd, 1H, $J = 5.28$, $J = 8.58$, H-16), 7.76 (dd, 1H, $J = 5.28$, $J = 8.28$, H-13), 2.73 (m, 2H, CH₂-1), 2.66 (t, 2H, $J = 7.38$, CH₂-10), 1.80 (m, 2H, CH₂-9), 1.74 (m, 2H, CH₂-2), 1.47 (m, 2H, CH₂-8), 1.37 (s, 10H, CH₂-7, 6, 5, 4, 3). δ_{C} (150 MHz, MeOD): 174.61 (qC, amide), 153.32 (C-H, 17), 152.43 (C-H, 12), 149.40 (C-H, TAP), 149.37 (C-H, TAP), 149.25 (C-H, TAP), 149.23 (C-H, TAP), 148.57 (C-H, TAP), 148.55 (C-H, TAP), 148.05 (C-H, TAP), 148.02 (C-H, TAP), 147.27, 145.36, 145.34, 144.95, 142.16, 142.14, 142.12, 142.10, 137.65 (C-H, 14), 134.01 (C-H, 15), 132.58 (C-H, TAP), 132.51 (C-H, TAP), 130.79, 127.55, 126.31 (C-H, 13), 125.74 (C-H, 16), 121.15 (C-H, 11), 51.29 (C-H₂, 1), 35.94 (C-H₂, 10), 28.96 (C-H₂), 28.90 (C-H₂), 28.88 (C-H₂), 28.83 (C-H₂), 28.74 (C-H₂), 28.21 (C-H₂, 8), 25.18 (C-H₂, 9), 22.23 (C-H₂, 2). ν_{max} (film)/cm⁻¹: 3368 (Aromatic C-H stretch), 2922 (alkane-C-H stretch), 2851 (alkane-C-H stretch), 1629 (C=O), 1485 (C-N stretch), 723 (C-S). HRMS (m/z -ES): Found: 2155.3379 (2M + 3PF₆)⁺; C₈₆H₈₀F₁₈N₂₂O₂P₃Ru₂S₂, Requires: 2155.3288.

Chapter 9:

References

- (1) www.who.int/.
- (2) Rudden, R. W. *Cancer Biology*; 4th ed.; Oxford University Press, New York, 2007.
- (3) Forbes, I.; Leong, J. *Essential Oncology of the Lymphocyte*; 2nd ed., Springer-Verlag, Berlin, 1987.
- (4) Campbell, N.; Reece, J. B. *Biology*; 6th ed., San Francisco, Benjamin Cummings, 2003.
- (5) Hanahan, D.; Weinberg, R. A. *Cell* **2000**, 100, 57.
- (6) Persidis, A. *Nat. Biotech.* **1999**, 17, 94.
- (7) Hannon, M. J. *Chem. Soc. Rev.* **2007**, 36, 280.
- (8) Dervan, P. B. *Bioorg. Med. Chem.* **2001**, 9, 2215.
- (9) Watson, J. D.; Crick, F. H. *Nature* **1953**, 171, 737.
- (10) Bloomfield, V. A. C.; Tinoco Jr, D.M. *Nucleic Acids: Structures, Properties and Functions*; 1st ed., University Science Books, Sausalito, 2000.
- (11) Neidle, S. *Nucleic Acid Structure and Recognition*; 1st ed.; Oxford University Press, Oxford, 2002.
- (12) Blackburn, G. M.; Gait, M. J.; Loakes, D.; Williams, D. M. *Nucleic Acids in Chemistry and Biology*; 4th ed., Royal Society of Chemistry, Cambridge, 2006.
- (13) Radhakrishnan, I.; Patel, D. J. *Structure* **1994**, 2, 17.
- (14) Strobel, S. A.; Dervan, P. B. *Science* **1990**, 249, 73.
- (15) Clark, G. R.; Gray, E. J.; Neidle, S.; Li, Y.-H.; Leupin, W. *Biochemistry* **1996**, 35, 13745.
- (16) Westhof, E.; Beveridge, D. L. *Water. Sci. Rev.* **1990**, 5, 24.
- (17) Facon, T.; Mary, J. Y.; Hulin, C.; Benboubker, L.; Attal, M.; Pegourie, B.; Renaud, M.; Harousseau, J. L.; Guillermin, G.; Chaletix, C.; Dib, M.; Voillat, L.; Maisonneuve, H.; Troncy, J.; Dorvaux, V.; Monconduit, M.; Martin, C.; Casassus, P.; Jaubert, J.; Jardel, H.; Doyen, C.; Kolb, B.; Anglaret, B.; Grosbois, B.; Yakoub-Agha, I.; Mathiot, C.; Avet-Loiseau, H. *Lancet* **2007**, 370, 1209.
- (18) Zhang, C. X.; Lippard, S. J. *Curr. Opin. Chem. Bio.* **2003**, 7, 481.
- (19) Takahara, P. M.; Frederick, C. A.; Lippard, S. J. *J. Am. Chem. Soc.* **1996**, 118, 12309.
- (20) Jamieson, E. R.; Lippard, S. J. *Chem. Rev.* **1999**, 99, 2467.
- (21) Lerman, L. S. *J. Mol. Biol.* **1961**, 3, 18.

- (22) Waring, M. J. *Mol. Biol.* **1970**, *54*, 247.
- (23) Luedtke, N. W.; Hwang, J. S.; Nava, E.; Gut, D.; Kol, M.; Tor, Y. *Nucleic Acids Res.* **2003**, *31*, 5732.
- (24) Nuss, M. E.; Marsh, F. J.; Kollman, P. A. *J. Am. Chem. Soc.* **1979**, *101*, 825.
- (25) Kamitori, S.; Takusagawa, F. *J. Mol. Biol.* **1992**, *225*, 445.
- (26) Kielkopf, C. L.; Erkkila, K. E.; Hudson, B. P.; Barton, J. K.; Rees, D. C. *Nat. Struct. Biol.* **2000**, *7*, 117.
- (27) Kelly, J. M.; Tossi, A. B.; McConnell, D. J.; O' hUigin, C. *Nucleic Acids Res.* **1985**, *13*, 6017.
- (28) Clarke, M. J. *Coord. Chem. Rev.* **2002**, *232*, 69.
- (29) Brabec, V.; Novakova, O. *Drug Resist. Update* **2006**, *9*, 111.
- (30) Barton, J. K. *Science* **1986**, *233*, 727.
- (31) Vos, J. G.; Kelly, J. M. *Dalton Trans.* **2006**, 4869.
- (32) Moucheron, C.; Kirsch-De Mesmaeker, A.; Kelly, J. M. *J. Photochem. Photobiol. B* **1997**, *40*, 91.
- (33) Erkkila, K. E.; Odom, D. T.; Barton, J. K. *Chem. Rev.* **1999**, *99*, 2777.
- (34) Balzani, V. R. B. *Photochem. Photobiol.* **1990**, *52*, 409.
- (35) Friedman, A. E.; Chambron, J. C.; Sauvage, J. P.; Turro, N. J.; Barton, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 4960.
- (36) Barton, J. K. *Science* **1986**, *233*, 727. (37) Satyanarayana, S.; Dabrowiak, J. C.; Chaires, J. B. *Biochemistry* **1992**, *31*, 9319.
- (38) Chambron, J.-C.; Sauvage, J.-P.; Amouyal, E.; Koffi, P. *Nouv. J. Chim.* **1985**, 527.
- (39) Holmlin, R. E.; Stemp, E. D.; Barton, J. K. *Inorg. Chem.* **1998**, *37*, 29.
- (40) Amouyal, E.; Homsí, A.; Chambron, J.-C.; Sauvage, J.-P. *J. Chem. Soc. Dalton Trans.* **1990**, 1841.
- (41) Olofsson, J.; Önfelt, B.; Lincoln, P. *J. Phys. Chem. A* **2004**, *108*, 4391.
- (42) Olofsson, J.; Wilhelmsson, L. M.; Lincoln, P. *J. Am. Chem. Soc.* **2004**, *126*, 15458.
- (43) Hiort, C.; Lincoln, P.; Nordén, B. *J. Am. Chem. Soc.* **1993**, *115*, 3448.
- (44) Lincoln, P.; Broo, A.; Nordén, B. *J. Am. Chem. Soc.* **1996**, *118*, 2644.

- (45) Haq, I.; Lincoln, P.; Suh, D.; Nordén, B.; Chowdhry, B. Z.; Chaires, J. B. *J. Am. Chem. Soc.* **1995**, *117*, 4788.
- (46) O'Reilly, F.; Kelly, J.; Kirsch-De Mesmaeker, A. *Chem. Commun.* **1996**, 1013.
- (47) O'Reilly, F.M.; Kelly, J.M. *New J. Chem.* **1998**, *22*, 215.
- (48) O'Reilly, F. M.; Kelly, J. M. *J. Phys. Chem. B* **2000**, *104*, 7206.
- (49) Moucheron, C.; Kirsch-De Mesmaeker, A.; Choua, S. *Inorg. Chem.* **1997**, *36*, 584.
- (50) Demeunynck, M.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Tetrahedron Lett.* **2002**, *43*, 261.
- (51) MacDonnell, F. M.; Bodige, S. *Inorg. Chem.* **1996**, *35*, 5758.
- (52) Rajput, C.; Rutkaite, R.; Swanson, L.; Haq, I.; Thomas, J. A. *Chem. Eur. J.* **2006**, *12*, 4611.
- (53) Gill, M. R.; Garcia-Lara, J.; Foster, S. J.; Smythe, C.; Battaglia, G.; Thomas, J. A. *Nat. Chem.* **2009**, *1*, 662.
- (54) Önfelt, B.; Lincoln, P.; Nordén, B. *J. Am. Chem. Soc.* **1999**, *121*, 10846.
- (55) Önfelt, B.; Lincoln, P.; Nordén, B.; Baskin, J. S.; Zewail, A. H. *PNAS*. **2000**, *97*, 5708.
- (56) Önfelt, B.; Lincoln, P.; Nordén, B. *J. Am. Chem. Soc.* **2001**, *123*, 3630.
- (57) Pierard, F.; Kirsch-De Mesmaeker, A. *Inorg. Chem. Commun.* **2006**, *9*, 111.
- (58) Önfelt, B.; Gostring, L.; Lincoln, P.; Nordén, B.; Önfelt, A. *Mutagenesis* **2002**, *17*, 317.
- (59) Blasius, C. M.; Kirsch-De Mesmaeker, A. *Eur. J. Inorg. Chem.* **2004**, *2004*, 3971.
- (60) Herman, L.S.; Defrancq, E.; Kirsch-De Mesmaeker A. *J. Phys. Org. Chem.* **2008**, *21*, 670.
- (61) Triesscheijn, M.; Baas, P.; Schellens, J. H. M.; Stewart, F. A. *Oncologist* **2006**, *11*, 1034.
- (62) Celli, J. P.; Spring, B. Q.; Rizvi, I.; Evans, C. L.; Samkoe, K. S.; Verma, S.; Pogue, B. W.; Hasan, T. *Chem. Rev.* **2010**, *110*, 2795.
- (63) Smith, G.; McGimpsey, W. G.; Lynch, M. C.; Kochevar, I. E.; Redmond, R. W. *Photochem. Photobiol.* **1994**, *59*, 135.
- (64) Lipson, R. L.; Baldes, E. J.; Olsen, A. M. *J. Natl. Cancer I.* **1961**, *26*, 1.
- (65) Dougherty, T. J.; Grindey, G. B.; Fiel, R.; Weishaupt, K. R.; Boyle, D. G. *J. Natl. Cancer I.* **1975**, *55*, 115.

- (66) Zigler, D. F.; Brewer, K. J. *Metal Complex-DNA Interactions*; Hadjiliadis, N., Sletten, E., Eds.; Wiley: Chichester, U.K, 2009.
- (67) Gallagher, W. M.; Allen, L. T.; O' Shea, C.; Kenna, T.; Hall, M.; Gorman, A.; Killoran, J.; O' Shea, D. F. *Br. J. Cancer* **2005**, *92*, 1702.
- (68) Byrne, A. T.; O'Connor, A. E.; Hall, M.; Murtagh, J.; O'Neill, K.; Curran, K. M.; Mongrain, K.; Rousseau, J. A.; Lecomte, R.; McGee, S.; Callanan, J. J.; O'Shea, D. F.; Gallagher, W. M. *Br. J. Cancer* **2009**, *101*, 1565.
- (69) Fleisher, M. B.; Waterman, K. C.; Turro, N. J.; Barton, J. K. *Inorg. Chem.* **1986**, *25*, 3549.
- (70) Blazek, E. R.; Peak, J. G.; Peak, M. J. *Photochem. Photobiol.* **1989**, *49*, 607.
- (71) Kelly, J. M.; Mcconnell, D. J.; Ohuigin, C.; Tossi, A. B.; Kirsch-De Mesmaeker, A.; Masschelein, A.; Nasielski, J. *J. Chem. Soc., Chem. Commun.* **1987**, 1821.
- (72) Liu, Y.; Hammitt, R.; Lutterman, D. A.; Joyce, L. E.; Thummel, R. P.; Turro, C. *Inorg. Chem.* **2008**, *48*, 375.
- (73) Weishaupt, K. R.; Gomer, C. J.; Dougherty, T. J. *Cancer Res.* **1976**, *36*, 2326.
- (74) Kirsch-De Mesmaeker, A.; Nasielski-Hinkens, R.; Maetens, D.; Pauwels, D.; Nasielski, J. *Inorg. Chem.* **1984**, *23*, 377.
- (75) Masschelein, A.; Jacquet, L.; Kirsch-De Mesmaeker, A.; Nasielski, J. *Inorg. Chem.* **1990**, *29*, 855.
- (76) Jacquet, L.; Kirsch-De Mesmaeker, A. *J. Chem. Soc. Faraday Trans.* **1992**, *88*, 2471.
- (77) Lecomte, J.-P.; Kirsch-De Mesmaeker, A.; Feeney, M. M.; Kelly, J. M. *Inorg. Chem.* **1995**, *34*, 6481.
- (78) Lecomte, J.-P.; Kirsch-De Mesmaeker, A.; Kelly, J. M.; Tossi, A. B.; Görner, H. *Photochem. Photobiol.* **1992**, *55*, 681.
- (79) Elias, B.; Creely, C.; Doorley, G. W.; Feeney, M. M.; Moucheron, C.; Kirsch-De Mesmaeker, A.; Dyer, J.; Grills, D. C.; George, M. W.; Matousek, P.; Parker, A. W.; Towrie, M.; Kelly, J. M. *Chem. Eur. J.* **2008**, *14*, 369.
- (80) Coates, C. G.; Callaghan, P.; McGarvey, J. J.; Kelly, J. M.; Jacquet, L.; Kirsch-De Mesmaeker, A. *J. Mol. Struct.* **2001**, *598*, 15.
- (81) Gicquel, E.; Boisdenghien, A.; Defrancq, E.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Chem. Commun.* **2004**, 2764.

- (82) Gauthier, N.; De Winter, J.; Gerbaux, P.; Moucheron, C.; Luhmer, M.; Kirsch-De Mesmaeker, A. *Inorg. Chem.* **2010**, *49*, 6796.
- (83) Armitage, B. *Chem. Rev.* **1998**, *98*, 1171.
- (84) Hiroshi, U.-i.; Philippe, F.; De Schryver, F. C.; De Feyter, S.; Gicquel, E.; Etoc, A.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Chem. Eur. J.* **2006**, *12*, 758.
- (85) Jacquet, L.; Kelly, J. M.; Kirsch-De Mesmaeker, A. *J. Chem. Soc., Chem. Commun.* **1995**, 913.
- (86) Feeney, M. M.; Kelly, J. M.; Tossi, A. B.; Kirsch-De Mesmaeker, A.; Lecomte, J. P. *J. Photochem. Photobiol. B* **1994**, *23*, 69.
- (87) Jacquet, L.; Davies, R. J. H.; Kirsch-De Mesmaeker, A.; Kelly, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 11763.
- (88) Hambley, T. W. *J. Chem. Soc. Dalton T.* **2001**, 2711.
- (89) Takahara, P. M.; Rosenzweig, A. C.; Frederick, C. A.; Lippard, S. J. *Nature* **1995**, *377*, 649.
- (90) Del Guerzo, A.; Kirsch-De Mesmaeker, A.; Demeunynck, M.; Lhomme, J. *J. Phys. Chem. B* **1997**, *101*, 7012.
- (91) Lecomte, J. P.; Kirsch-De Mesmaeker, A.; Demeunynck, M.; Lhomme, J. *J. Chem. Soc. Faraday Trans.* **1993**, *89*, 3261.
- (92) Del Guerzo, A.; Kirsch-De Mesmaeker, A.; Demeunynck, M.; Lhomme, J. *J. Chem. Soc. Dalton* **2000**, 1173.
- (93) Del Guerzo, A.; Kirsch-De Mesmaeker, A. *Inorg. Chem.* **2002**, *41*, 938.
- (94) Del Guerzo, A.; Kirsch-De Mesmaeker, A. *Inorg. Chem.* **2002**, *41*, 938.
- (95) Pauly, M.; Kayser, I.; Schmitz, M.; Dicato, M.; Del Guerzo, A.; Kolber, I.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Chem. Commun.* **2002**, 1086.
- (96) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. *Chem. Soc. Rev.* **2010**, *39*, 3936.
- (97) Brana, M. F.; Ramos, A. *Curr. Med. Chem. Anticancer Agents* **2001**, *1*, 237.
- (98) Min, L.; Hui, X. *Curr. Med. Chem.* **2009**, *16*, 4797.
- (99) Braña, M. F.; Castellano, J. M.; Roldán, C. M.; Santos, A.; Vázquez, D.; Jiménez, A. *Cancer Chemoth. Pharm* **1980**, *4*, 61.

- (100) Waring, M. J.; Gonzalez, A.; Jimenez, A.; Vazquez, D. *Nucleic Acids Res.* **1979**, *7*, 217.
- (101) Liu, L. F. *Annu. Rev. Biochem.* **1989**, *58*, 351.
- (102) Ott, I.; Xu, Y.; Liu, J.; Kokoschka, M.; Harlos, M.; Sheldrick, W. S.; Qian, X. *Bioorg. Med. Chem.* **2008**, *16*, 7107.
- (103) Frimannsson, D. O. *Ph.D. Thesis*, University of Dublin, Trinity College, 2008.
- (104) Veale, E. B. *Ph.D. Thesis*, University of Dublin, Trinity College, 2007.
- (105) Gillespie, L. J. *Ph.D. Thesis*, University of Dublin, Trinity College, 2007.
- (106) Hussey, G. M. *Ph.D. Thesis*, University of Dublin, Trinity College, 2003.
- (107) Phelan, C. *Ph.D. Thesis*, University of Dublin, Trinity College, 2002.
- (108) Saito, I.; Takayama, M.; Kawanishi, S. *J. Am. Chem. Soc.* **1995**, *117*, 5590.
- (109) Saito, I.; Takayama, M.; Sugiyama, H.; Nakatani, K.; Tsuchida, A.; Yamamoto, M. *J. Am. Chem. Soc.* **1995**, *117*, 6406.
- (110) Sugiyama, H.; Saito, I. *J. Am. Chem. Soc.* **1996**, *118*, 7063.
- (111) Qian, X.; Huang, T.-B.; Wei, D.-z.; Zhu, D.-H.; Fan, M.-c.; Yao, W. *J. Chem. Soc. Perk. Trans. 2* **2000**, 715.
- (112) Tao, Z.-F.; Qian, X.; Wei, D. *Dyes Pigments* **1996**, *31*, 245.
- (113) Yao, W.; Qian, X. *Dyes Pigments* **2001**, *48*, 43.
- (114) Yang, Q.; Yang, P.; Qian, X.; Tong, L. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6210.
- (115) Duan, L.; Xu, Y.; Qian, X.; Zhang, Y.; Liu, Y. *Tetrahedron Lett.* **2009**, *50*, 22.
- (116) Li, Z.; Yang, Q.; Qian, X. *Bioorg. Med. Chem.* **2005**, *13*, 4864.
- (117) Giljohann, D. A.; Seferos, D. S.; Daniel, W. L.; Massich, M. D.; Patel, P. C.; Mirkin, C. A. *Angew. Chem. Int. Ed.* **2010**, *49*, 3280.
- (118) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547.
- (119) Daniel, M. C.; Astruc, D. *Chem. Rev.* **2004**, *104*, 293.
- (120) Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. *Nano Lett.* **2006**, *6*, 662.
- (121) Sandhu, K. K.; McIntosh, C. M.; Simard, J. M.; Smith, S. W.; Rotello, V. M. *Bioconjugate Chem.* **2002**, *13*, 3.
- (122) Rosi, N. L.; Giljohann, D. A.; Thaxton, C. S.; Lytton-Jean, A. K. R.; Han, M. S.; Mirkin, C. A. *Science* **2006**, *312*, 1027.

- (123) Gibson, J. D.; Khanal, B. P.; Zubarev, E. R. *J. Am. Chem. Soc.* **2007**, *129*, 11653.
- (124) Torchilin, V. P. *Adv. Drug Deliver. Rev.* **2006**, *58*, 1532.
- (125) Seferos, D. S.; Giljohann, D. A.; Hill, H. D.; Prigodich, A. E.; Mirkin, C. A. *J. Am. Chem. Soc.* **2007**, *129*, 15477.
- (126) Prigodich, A. E.; Seferos, D. S.; Massich, M. D.; Giljohann, D. A.; Lane, B. C.; Mirkin, C. A. *ACS Nano* **2009**, *3*, 2147.
- (127) Zheng, D.; Seferos, D. S.; Giljohann, D. A.; Patel, P. C.; Mirkin, C. A. *Nano Lett.* **2009**, *9*, 3258.
- (128) El-Sayed, I. H.; Huang, X.; El-Sayed, M. A. *Nano Lett.* **2005**, *5*, 829.
- (129) Loo, C.; Lowery, A.; Halas, N.; West, J.; Drezek, R. *Nano Lett.* **2005**, *5*, 709.
- (130) Hone, D. C.; Walker, P. I.; Evans-Gowing, R.; FitzGerald, S.; Beeby, A.; Chambrier, I.; Cook, M. J.; Russell, D. A. *Langmuir* **2002**, *18*, 2985.
- (131) Wieder, M. E.; Hone, D. C.; Cook, M. J.; Handsley, M. M.; Gavrilovic, J.; Russell, D. A. *Photochem. Photobiol. Sci.* **2006**, *5*, 727.
- (132) Wang, S.; Chen, K.-J.; Wu, T.-H.; Wang, H.; Lin, W.-Y.; Ohashi, M.; Chiou, P.-Y.; Tseng, H.-R. *Angew. Chem. Int. Ed.* **2010**, *49*, 3777.
- (133) Boris, K. *Nanotechnology* **2006**, *17*, 5167.
- (134) Kotaidis, V.; Dahmen, C.; von Plessen, G.; Springer, F.; Plech, A. *J. Chem. Phys.* **2006**, *124*, 184702.
- (135) Bartz, M.; Küther, J.; Seshadri, R.; Tremel, W. *Angew. Chem. Int. Ed.* **1998**, *37*, 2466.
- (136) Labande, A.; Ruiz, J.; Astruc, D. *J. Am. Chem. Soc.* **2002**, *124*, 1782.
- (137) James, D. E. T. W.-E. *Dalton Trans.* **2008**, 25.
- (138) Hallett, A. J.; Christian, P.; Jones, J. E.; Pope, S. J. A. *Chem. Commun.* **2009**, 4278.
- (139) Mohamed, S.; Nela, D.; Peter, G.; Hanadi, F. S. *Chem. Bio. Chem.* **2007**, *8*, 804.
- (140) Slim, M.; Sleiman, H. F. *Bioconjugate Chem.* **2004**, *15*, 949.
- (141) Mayer, C. R.; Dumas, E.; Secheresse, F. *J. Colloid Interf. Sci.* **2008**, *328*, 452.
- (142) Mayer, C. R.; Dumas, E.; Miomandre, F.; Meallet-Renault, R.; Warmont, F.; Vigneron, J.; Pansu, R.; Etcheberry, A.; Secheresse, F. *New J. Chem.* **2006**, *30*, 1628.
- (143) Huang, T.; Murray, R. W. *Langmuir* **2002**, *18*, 7077.
- (144) Cheng, P. P. H.; Silvester, D.; Wang, G.; Kalyuzhny, G.; Douglas, A.; Murray, R. W. *J. Phys. Chem. B* **2006**, *110*, 4637.

- (145) Thomas, K. G.; Kamat, P. V. *J. Am. Chem. Soc.* **2000**, *122*, 2655.
- (146) Gunnlaugsson, T.; Kruger, P. E.; Jensen, P.; Tierney, J.; Ali, H. D. P.; Hussey, G. M. *J. Org. Chem.* **2005**, *70*, 10875.
- (147) Pfeffer, F. M.; Buschgens, A. M.; Barnett, N. W.; Gunnlaugsson, T.; Kruger, P. E. *Tetrahedron Lett.* **2005**, *46*, 6579.
- (148) Duke, R. M.; Gunnlaugsson, T. *Tetrahedron Lett.* **2007**, *48*, 8043.
- (149) Parkesh, R.; Lee, T. C.; Gunnlaugsson, T. *Org. Biomol. Chem.* **2007**, *5*, 310.
- (150) Ali, H. D. P.; Kruger, P. E.; Gunnlaugsson, T. *New J. Chem.* **2008**, *32*, 1153.
- (151) Bonnet, C. S.; Devocelle, M.; Gunnlaugsson, T. *Chem. Commun.* **2008**, 4552.
- (152) Veale, E. B.; Frimannsson, D. O.; Lawler, M.; Gunnlaugsson, T. *Org. Lett.* **2009**, *11*, 4040.
- (153) Veale, E. B.; Gunnlaugsson, T. *J. Org. Chem.* **2010**, *75*, 5513.
- (154) Ryan, G. J. *Ph.D. Thesis*, University of Dublin, Trinity College, 2008.
- (155) Ryan, G. J.; Quinn, S.; Gunnlaugsson, T. *Inorg. Chem.* **2008**, *47*, 401.
- (156) Massue, J.; Quinn, S. J.; Gunnlaugsson, T. *J. Am. Chem. Soc.* **2008**, *130*, 6900.
- (157) Bonnet, C. S.; Massue, J.; Quinn, S. J.; Gunnlaugsson, T. *Org. Biomol. Chem.* **2009**, *7*, 3074.
- (158) Ortmans, I.; Elias, B.; Kelly, J. M.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Dalton Trans.* **2004**, 668.
- (159) Dupureur, C. M.; Barton, J. K. *Inorg. Chem.* **1997**, *36*, 33.
- (160) Dupureur, C. M.; Barton, J. K. *J. Am. Chem. Soc.* **1994**, *116*, 10286.
- (161) Coates, C. G.; McGarvey, J. J.; Callaghan, P. L.; Coletti, M.; Hamilton, J. G. *J. Phys. Chem. B* **2001**, *105*, 730.
- (162) Olson, E. J. C.; Hu, D.; Hörmann, A.; Jonkman, A. M.; Arkin, M. R.; Stemp, E. D. A.; Barton, J. K.; Barbara, P. F. *J. Am. Chem. Soc.* **1997**, *119*, 11458.
- (163) Coates, C. G.; Olofsson, J.; Coletti, M.; McGarvey, J. J.; Önfelt, B.; Lincoln, P.; Norden, B.; Tuite, E.; Matousek, P.; Parker, A. W. *J. Phys. Chem. B* **2001**, *105*, 12653.
- (164) Moucheron, C. *New J. Chem.* **2009**, *33*, 235.
- (165) Le Gac, S.; Foucart, M.; Gerbaux, P.; Defrancq, E.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Dalton Trans.* **2010**, *39*, 9672.

- (166) Nasielski-Hinkens, R.; Kotel, J.; Lecloux, T.; Nasielski, J. *Synthetic Commun.* **1989**, *19*, 511.
- (167) Nasielski-Hinkens, R.; Benedek-Vamos, M.; Maetens, D. *J. Heterocyclic Chem.* **1980**, *17*, 873.
- (168) Calucci, L.; Pampaloni, G.; Pinzino, C.; Prescimone, A. *Inorg. Chim. Acta.* **2006**, 359, 3911.
- (169) Sullivan, B. P. S., D. J. Meyer, T. J. *Inorg. Chem.* **1978**, *17*, 3334.
- (170) Clear, J. M.; Kelly, J. M.; Oconnell, C. M.; Vos, J. G.; Cardin, C. J.; Costa, S. R.; Edwards, A. J. *J. Chem. Soc.; Chem. Commun.* **1980**, 750.
- (171) Rau, S.; Schäfer, B.; Grüßing, A.; Schebesta, S.; Lamm, K.; Vieth, J.; Görls, H.; Walther, D.; Rudolph, M.; Grummt, U. W.; Birkner, E. *Inorg. Chim. Acta.* **2004**, 357, 4496.
- (172) Kappe, C. O. *Angew. Chem. Int. Ed.* **2004**, *43*, 6250.
- (173) Hartshorn, R. M.; Barton, J. K. *J. Am. Chem. Soc.* **1992**, *114*, 5919.
- (174) Schwalbe, M.; Karnahl, M.; Tschierlei, S.; Uhlemann, U.; Schmitt, M.; Dietzek, B.; Popp, J.; Groake, R.; Vos, J. G.; Rau, S. *Dalton. Trans.* **2010**, 39, 2768.
- (175) Rhys Williams, A. T.; Winfield, S. A.; Miller, J. N. *Analyst* **1983**, *108*, 1067.
- (176) Nakamaru, K. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 1639.
- (177) Nair, R. B.; Cullum, B. M.; Murphy, C. J. *Inorg. Chem.* **1997**, *36*, 962.
- (178) Metcalfe, C.; Thomas, J. A. *Chem. Soc. Rev.* **2003**, *32*, 215.
- (179) Fox, K. R. *Drug-DNA Interaction Protocols*; Humana Press: New Jersey, 1997, Vol. 90.
- (180) Kelly, J. M.; Tossi, A. B.; McConnell, D. J.; OhUigin, C. *Nucleic Acids Res.* **1985**, *13*, 6017.
- (181) Carter, M. T.; Rodriguez, M.; Bard, A. J. *J. Am. Chem. Soc.* **1989**, *111*, 8901.
- (182) Blasius, R.; Nierengarten, H.; Luhmer, M.; Constant, J.-F.; Defrancq, E.; Dumy, P.; van Dorsselaer, A.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Chem. Eur. J.* **2005**, *11*, 1507.
- (183) Fairley, T. A.; Tidwell, R. R.; Donkor, I.; Naiman, N. A.; Ohemeng, K. A.; Lombardy, R. J.; Bentley, J. A.; Cory, M. *J. Med. Chem.* **1993**, *36*, 1746.

- (184) Fairley, T. A.; Tidwell, R. R.; Donkor, I.; Naiman, N. A.; Ohemeng, K. A.; Lombardy, R. J.; Bentley, J. A.; Cory, M. J. *Med. Chem.* **1993**, *36*, 1746.
- (185) Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. *J. Am. Chem. Soc.* **2001**, *123*, 5878.
- (186) Lepecq, J. B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.
- (187) Wilson, W. D.; Tanious, F. A.; Fernandez, M. F.; Rigl, C. T. *Methods Mol. Biol.* **1997**, *90*, 219.
- (188) Pilch, D. S.; Poklar, N.; Baird, E. E.; Dervan, P. B.; Breslauer, K. J. *Biochem.* **1999**, *38*, 2143.
- (189) Ihmels, H.; Otto, D.; Dall 'Acqua, F.; Faccio, A.; Moro, S.; Viola, G. *J. Org. Chem.* **2006**, *71*, 8401.
- (190) Bengt Nordén, T. K. *J. Mol. Recognit.* **1994**, *7*, 141.
- (191) Garbett, N. C.; Ragazzon, P. A.; Chaires, J. B. *Nat. Protoc.* **2007**, *2*, 3166.
- (192) Carloni, P.; Damiani, E.; Greci, L.; Stipa, P.; Tanfani, F.; Tartaglini, E.; Wozniak, M. *Res. Chem. Intermediat.* **1993**, *19*, 395.
- (193) Frimannsson, D. O.; Grossi, M.; Murtagh, J.; Paradisi, F.; O'Shea, D. F. *J. Med. Chem.* **2010**, *53*, 7337.
- (194) Demas, J. N.; Harris, E. W.; McBride, R. P. *J. Am. Chem. Soc.* **1977**, *99*, 3547.
- (195) Armitage, B. *Chem. Rev.* **1998**, *98*, 1171.
- (196) Elias, B.; Kirsch-De Mesmaeker, A. *Coord. Chem. Rev.* **2006**, *250*, 1627.
- (197) De Vos, K.; Goossens, V.; Boone, E.; Vercammen, D.; Vancompernelle, K.; Vandenabeele, P.; Haegeman, G.; Fiers, W.; Grooten, J. *J. Biol. Chem.* **1998**, *273*, 9673.
- (198) Ogilby, P. R. *Photoch. Photobio. Sci.* **2010**, *9*, 1543.
- (199) Zhou, Q.-X.; Lei, W.-H.; Sun, Y.; Chen, J.-R.; Li, C.; Hou, Y.-J.; Wang, X.-S.; Zhang, B.-W. *Inorg. Chem.* **2010**, *49*, 4729.
- (200) Phillips, T.; Haq, I.; Meijer, A. J. H. M.; Adams, H.; Soutar, I.; Swanson, L.; Sykes, M. J.; Thomas, J. A. *Biochemistry* **2004**, *43*, 13657.
- (201) Dickeson, J. E.; Summers, L. A. *Aust. J. Chem.* **1970**, *23*, 1023.
- (202) Phillips, T.; Rajput, C.; Twyman, L.; Haq, I.; Thomas, J. A. *Chem. Commun.* **2005**, 4327.

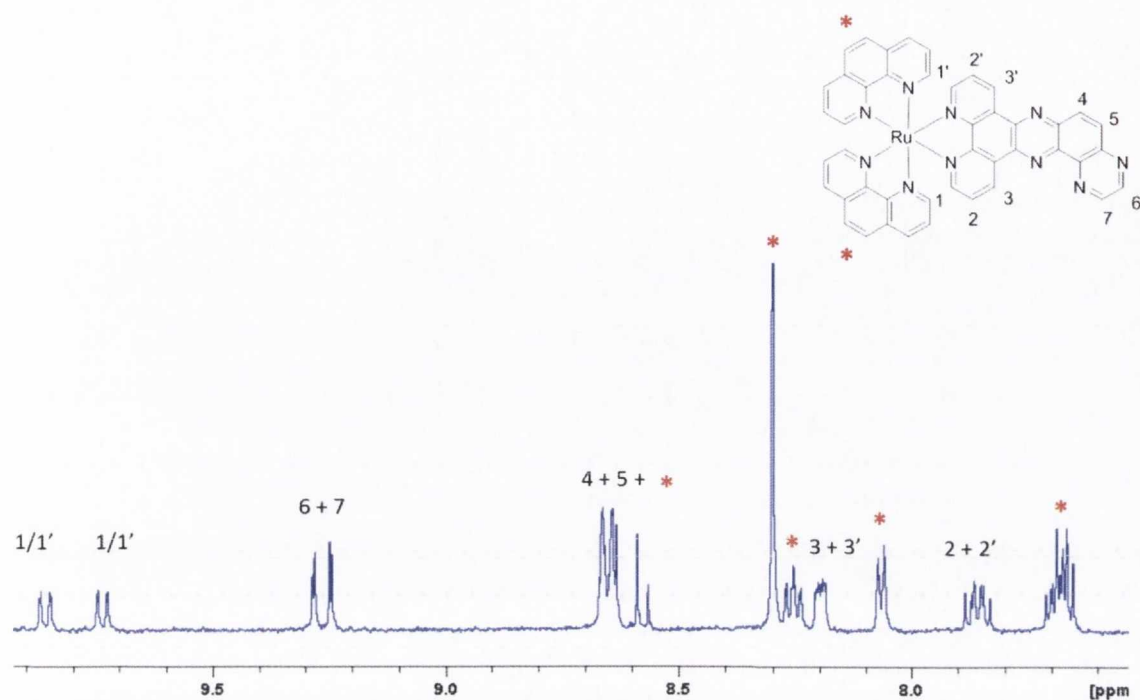
- (203) Demas, J. N.; Crosby, G. A. *J. Phys. Chem.* **1971**, 75, 991.
- (204) Knapp, C.; Lecomte, J. P.; Kirsch-De Mesmaeker, A.; Orellana, G. *J. Photoch. Photobio. B.* **1996**, 36, 67.
- (205) McGhee, J. D.; Hippel, P. H. V. *J. Mol. Biol.* **1974**, 86, 469.
- (206) Tuite, E.; Lincoln, P.; Nordén, B. *J. Am. Chem. Soc.* **1997**, 119, 239.
- (207) Jain, S. C.; Sobell, H. M. *J. Mol. Biol.* **1972**, 68, 1.
- (208) Johansson, O.; Borgstrom, M.; Lomoth, R.; Palmblad, M.; Bergquist, J.; Hammarstrom, L.; Sun, L.; Akermark, B. *Inorg. Chem.* **2003**, 42, 2908.
- (209) Elias, B.; Herman, L.; Moucheron, C.; Kirsch-De Mesmaeker, A. *Inorg. Chem.* **2007**, 46, 4979.
- (210) Del Guerzo, A.; Kirsch-De Mesmaeker, A.; Demeunynck, M.; Lhomme, J. *J. Phys. Chem. B* **1997**, 101, 7012.
- (211) Fox, K. R. *Drug-DNA Interaction Protocols*; Humana Press: New Jersey, 1997, Vol. 90.
- (212) Stevenson, K. A.; Yen, S. F.; Yang, N. C.; Boykin, D. W.; Wilson, W. D. *J. Med. Chem.* **1984**, 27, 1677.
- (213) O' Reilly, M.; Kelly, J. M. *New J. Chem.* **1998**, 215.
- (214) Puckett, C. A.; Barton, J. K. *J. Am. Chem. Soc.* **2007**, 129, 46.
- (215) Fernandez-Moreira, V.; Thorp-Greenwood, F. L.; Coogan, M. P. *Chem. Commun.*, **2010**, 46, 186.
- (216) DeRosa, M. C.; Crutchley, R. J. *Coord. Chem. Rev.* **2002**, 233-234, 351.
- (217) Redmond, R. W.; Gamlin, J. N. *Photochem. Photobiol.* **1999**, 70, 391.
- (218) Duke, R. M. *Ph.D. Thesis*, University of Dublin, Trinity College, 2009.
- (219) Ali, H. D. P. H., *Ph.D. Thesis*, University of Dublin, Trinity College, 2007.
- (220) Chen, F. M. *Biochemistry* **1988**, 27, 1843.
- (221) Xie, H.; Tkachenko, A. G.; Glomm, W. R.; Ryan, J. A.; Brennaman, M. K.; Papanikolas, J. M.; Franzen, S.; Feldheim, D. L. *Anal. Chem.* **2003**, 75, 5797.
- (222) Veale, E. B.; Gunnlaugsson, T. *J. Org. Chem.* **2008**, 73, 8073.
- (223) Valík, M.; Strongin, R. M.; Král, V. *Supramol. Chem.* **2005**, 17, 347.
- (224) Gunnlaugsson, T.; Glynn, M.; Tocci, G. M.; Kruger, P. E.; Pfeffer, F. M. *Coord. Chem. Rev.* **2006**, 250, 3094.

- (225) Tröger, J. *J. Prakt. Chem.* **1887**, 36, 225.
- (226) Spielman, M. A. *J. Am. Chem. Soc.* **1935**, 57, 583.
- (227) Wilcox, C. S. *Tetrahedron Lett.* **1985**, 26, 5749.
- (228) Adrian, J. C.; Wilcox, C. S. *J. Am. Chem. Soc.* **1989**, 111, 8055.
- (229) Adrian, J. C.; Wilcox, C. S. *J. Am. Chem. Soc.* **1991**, 113, 678.
- (230) Hansson, A. P.; Norrby, P.-O.; Wärnmark, K. *Tetrahedron Lett.* **1998**, 39, 4565.
- (231) Crossley, M. J.; Mackay, L. G.; Try, A. C. *J. Chem. Soc., Chem. Commun.* **1995**, 1925.
- (232) Yashima, E.; Akashi, M.; Miyauchi, N. *Chem. Lett.* **1991**, 20, 1017.
- (233) Van Gijte, O.; Tatibouët, A.; Demeunynck, M.; Lhomme, J.; Kirsch-De Mesmaeker, A. *Tetrahedron Lett.* **1997**, 38, 1567.
- (234) Bresson, C.; Luhmer, M.; Demeunynck, M.; Kirsch-De Mesmaeker, A.; Pierard, F. *Tetrahedron Lett.* **2004**, 45, 2863.
- (235) Claessens, N.; Pierard, F.; Bresson, C.; Moucheron, C.; Kirsch-De Mesmaeker, A. *J. Inorg. Biochem.* **2007**, 101, 987.
- (236) Veale, E. B. *Ph.D. Thesis*, University of Dublin, Trinity College, 2008.
- (237) Saha, S.; Samanta, A. *J. Phys. Chem. A* **2002**, 106, 4763.
- (238) Charras, G. T.; Coughlin, M.; Mitchison, T. J.; Mahadevan, L. *Biophys. J.* **2008**, 94, 1836.
- (239) Ozbay, E. *Science* **2006**, 311, 189.
- (240) Moloney, M. P.; Gun'ko, Y. K.; Kelly, J. M. *Chem. Commun.* **2007**, 3900.
- (241) Hasobe, T.; Imahori, H.; Kamat, P. V.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, 125, 14962.
- (242) Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, 277, 1078.
- (243) Pramod, P.; Sudeep, P. K.; Thomas, K. G.; Kamat, P. V. *J. Phys. Chem. B* **2006**, 110, 20737.
- (244) Zeglis, B. M.; Pierre, V. C.; Barton, J. K. *Chem. Commun.* **2007**, 4565.
- (245) FernAndez-Moreira, V.; Thorp-Greenwood, F. L.; Coogan, M. P. *Chem. Commun.* **2010**, 46, 186.
- (246) Neugebauer, U.; Pellegrin, Y.; Devocelle, M.; Forster, R. J.; Signac, W.; Moran, N.; Keyes, T. E. *Chem. Commun.* **2008**, 5307.

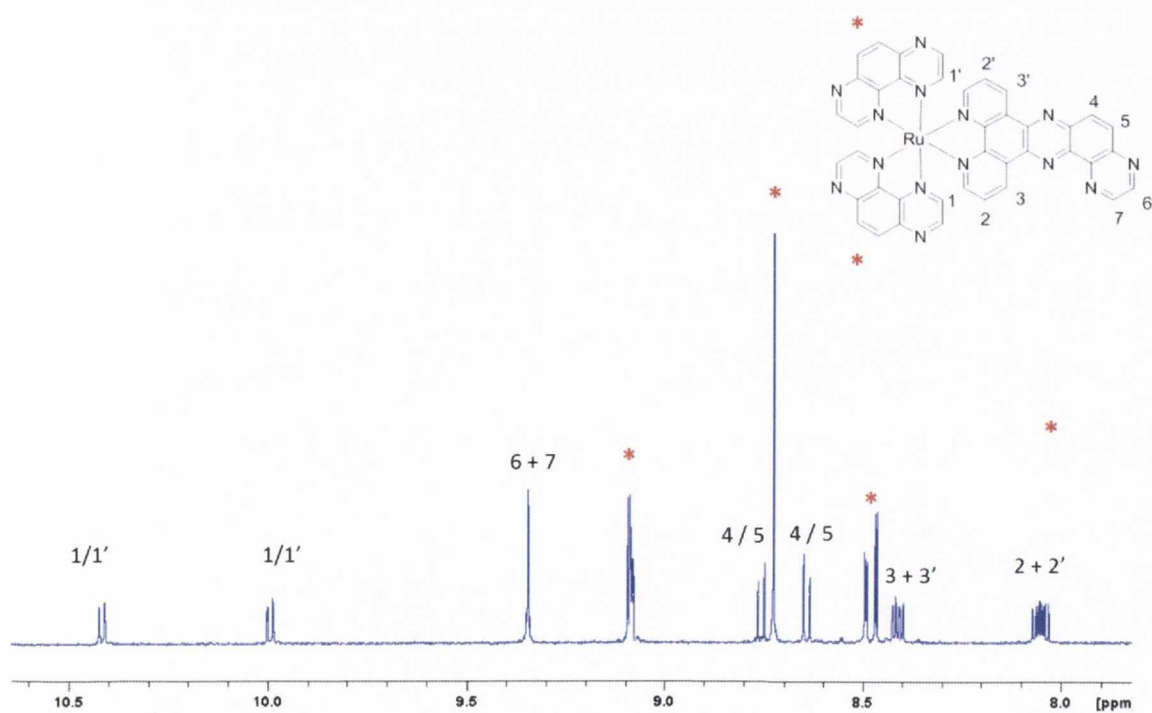
- (247) Puckett, C. A.; Barton, J. K. *J. Am. Chem. Soc.* **2009**, *131*, 8738.
- (248) Terasaki, N.; Akiyama, T.; Yamada, S. *Langmuir* **2002**, *18*, 8666.
- (249) Jebb, M.; Sudeep, P. K.; Pramod, P.; Thomas, K. G.; Kamat, P. V. *J. Phys. Chem. B* **2007**, *111*, 6839.
- (250) Zhu, M.; Schneider, M.; Papastavrou, G.; Akari, S.; Mohwald, H. *Langmuir*. **2001**, *17*, 6471.
- (251) Pyle, A. M.; Rehmann, J. P.; Meshoyrer, R.; Kumar, C. V.; Turro, N. J.; Barton, J. K. *J. Am. Chem. Soc.* **1989**, *111*, 3051.
- (252) Eriksson, M.; Leijon, M.; Hiort, C.; Norden, B.; Graeslund, A. *Biochemistry* **1994**, *33*, 5031.
- (253) Turkevich, J.; Stevenson, P. C.; Hillier, J. *J. Phys. Chem.* **1953**, *57*, 670.
- (254) Brust, M.; Walker, M.; Bethell, D.; Schiffrin, D. J.; Whyman, R. *J. Chem. Soc., Chem. Commun.* **1994**, 801.
- (255) Perrault, S. D.; Chan, W. C. W. *J. Am. Chem. Soc.* **2009**, *131*, 17042.
- (256) Martin, M. N.; Basham, J. I.; Chando, P.; Eah, S.-K. *Langmuir* **2010**, *26*, 7410.
- (257) Berne, B. J.; Pecora, R. *Dynamic Light Scattering: with applications to chemistry, biology, and physics*; Dover Publications: Mineola, N.Y., 2000.
- (258) Link, S.; El-Sayed, M. A. *J. Phys. Chem. B* **1999**, *103*, 8410.
- (259) Liu, X.; Atwater, M.; Wang, J.; Huo, Q. *Colloid. Surface. B* **2007**, *58*, 3.
- (260) Elias, B.; Creely, C.; Doorley, G. W.; Feeney, M. M.; Moucheron, C.; Kirsch-De Mesmaeker, A.; Dyer, J.; Grills, D. C.; George, M. W.; Matousek, P.; Parker, A. W.; Towrie, M.; Kelly, J. M. *Chem. Eur. J.* **2007**, *14*, 369.
- (261) Jin, R.; Wu, G.; Li, Z.; Mirkin, C. A.; Schatz, G. C. *J. Am. Chem. Soc.* **2003**, *125*, 1643.
- (262) Sullivan, B. P.; Salmon, D. J.; Meyer, T. J. *Inorg. Chem.* **1978**, *17*, 3334.
- (263) Albers, M. O. S., Eric; Yates, Janet E. *Inorg. Syn.* **1989**, 249.
- (264) Oki, A. R.; Morgan, R. J. *Synth. Commun.* **1995**, *24*, 4093.
- (265) Ghosh, A.; Mandoli, A.; Kumar, D. K.; Yadav, N. S.; Ghosh, T.; Jha, B.; Thomas, J. A.; Das, A. *Dalton. Trans.* **2009**, 9312.
- (266) Pourtois, G.; Beljonne, D.; Moucheron, C.; Schumm, S.; Kirsch-De Mesmaeker, A.; Lazzaroni, R.; Brédas, J-L. *J. Am. Chem. Soc.* **2004**, 683.

Appendices

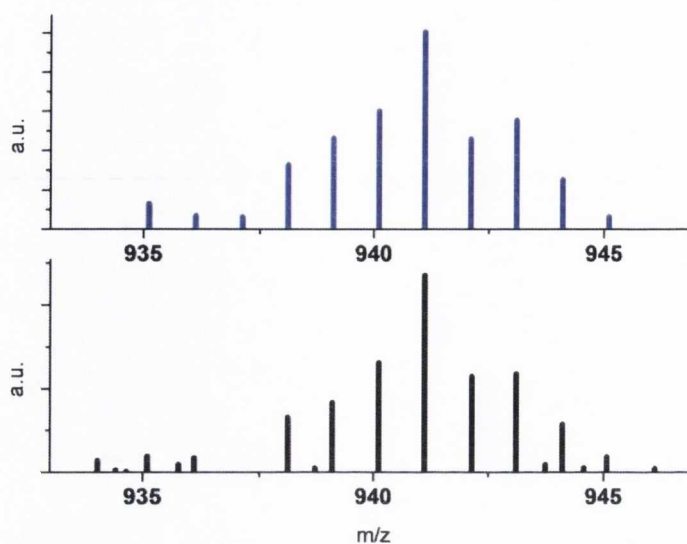
Appendix 2:



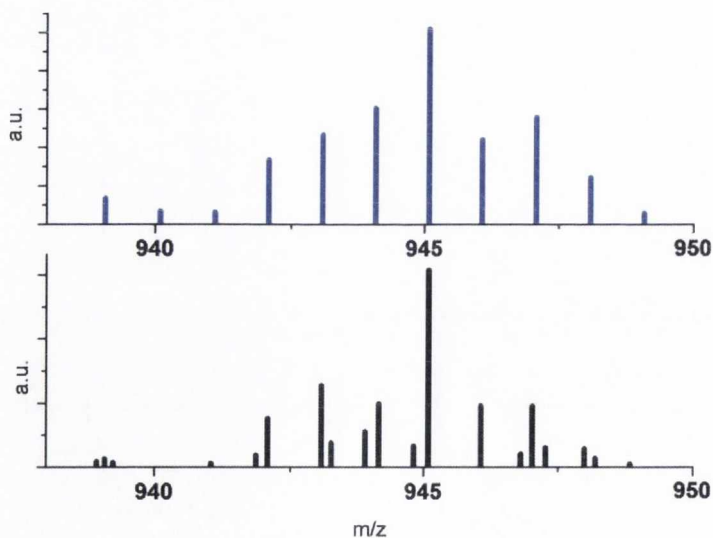
2A 1: The ^1H NMR spectra of **54** (600 MHz, $\text{DMSO}-d_6$)



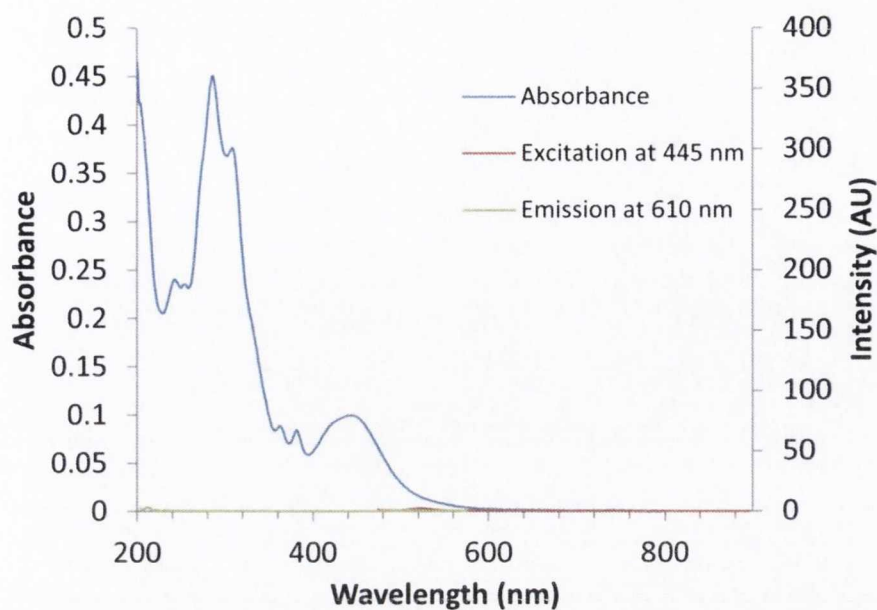
2A 2: The ^1H NMR spectra of **55** (600 MHz, $\text{DMSO}-d_6$)



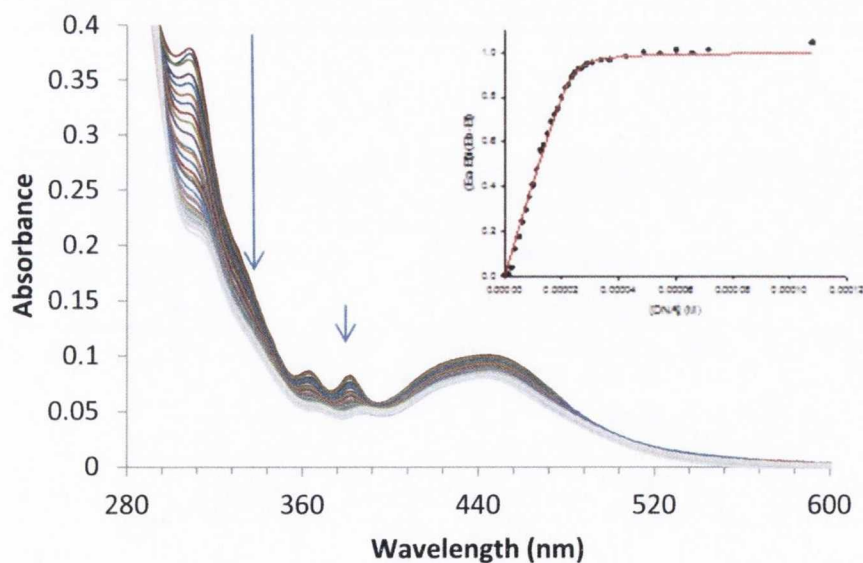
2A 3: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for 54 from high resolution mass spectrometry analysis



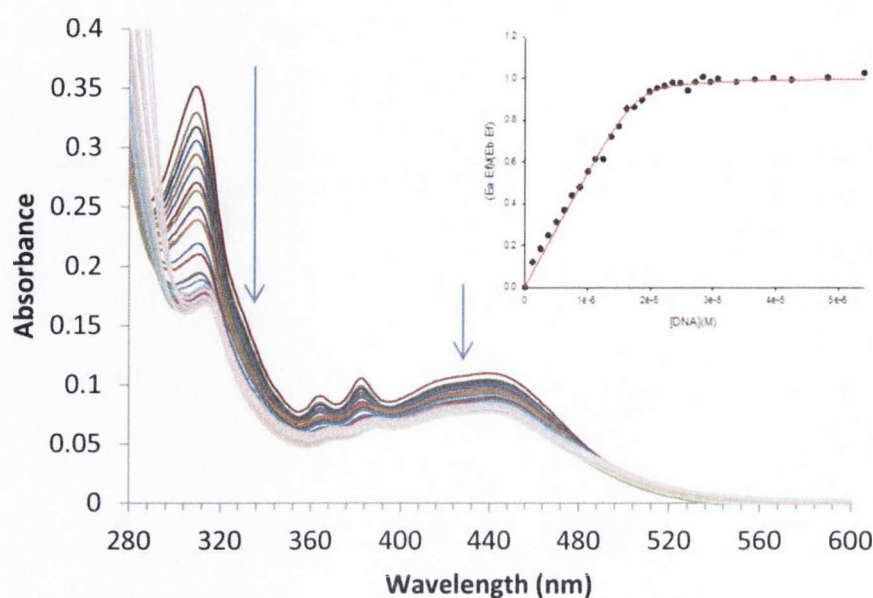
2A 4: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for 55 from high resolution mass spectrometry analysis



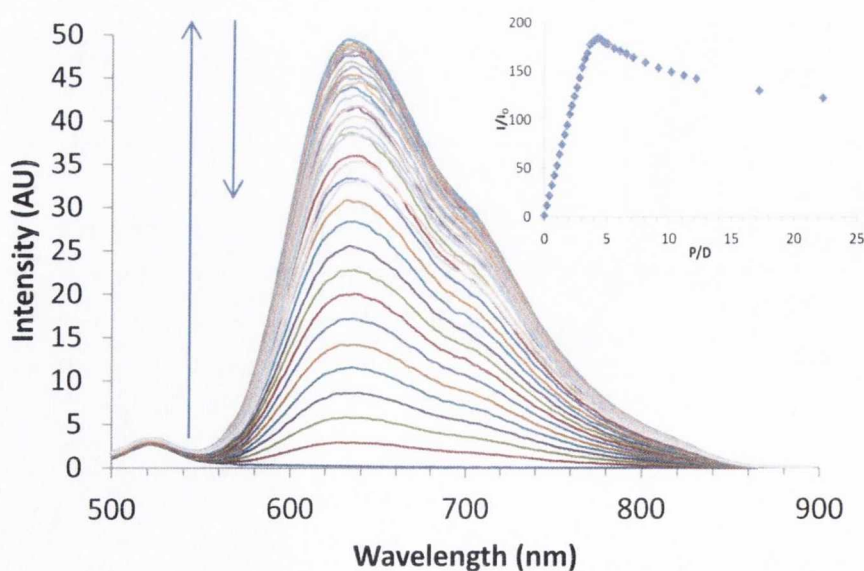
2A 5: UV/Visible, excitation and emission spectra of **53** ($6.9 \mu\text{M}$) in 10 mM phosphate buffer, pH 7.4.



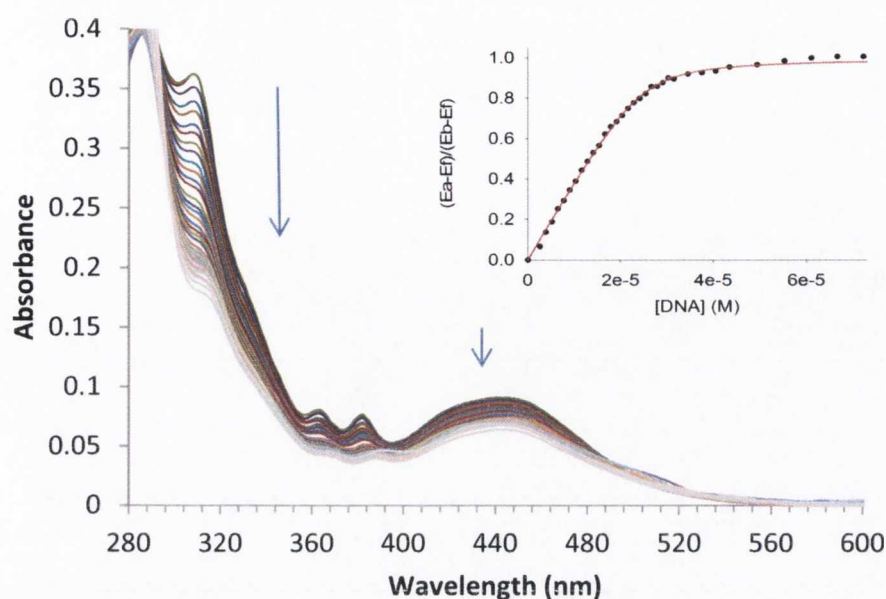
2A 6: Changes in the UV/Visible spectrum of **53** ($6.5 \mu\text{M}$) with increasing concentration of stDNA ($0\text{--}110 \mu\text{M}$). Inset: Plot of $(\epsilon_a - \epsilon_f) / \epsilon_b - \epsilon_f$ vs. $[\text{DNA}] (M^1, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



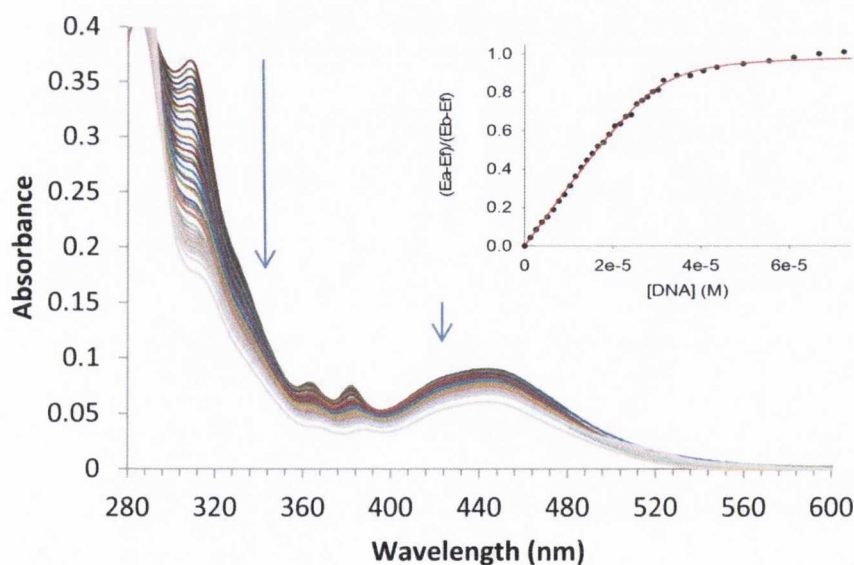
2A 7: Changes in the UV/Visible spectrum of **54** ($6.3\ \mu\text{M}$) with increasing concentration of stDNA (0 – $122\ \mu\text{M}$). Inset: Plot of $(\epsilon_a - \epsilon_f) / (\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}] (\text{M})$, P using data with a P/D between 0 – 12 and the best fit of the data (---) using the Bard Eqn.



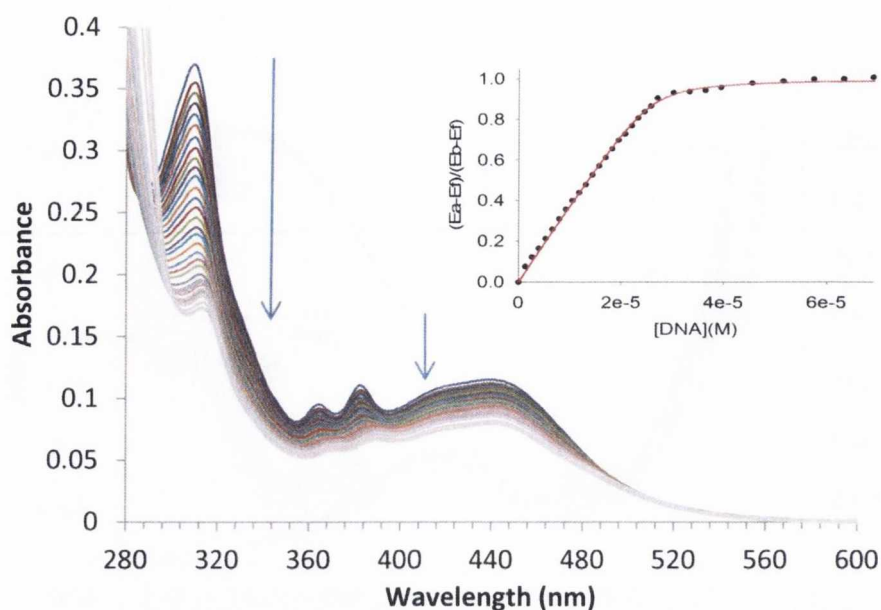
2A 8: Changes in the emission spectrum of **53** ($6.5\ \mu\text{M}$) ($\lambda_{\text{ex}}\ 445\ \text{nm}$) with increasing concentration of stDNA (0 – $110\ \mu\text{M}$). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



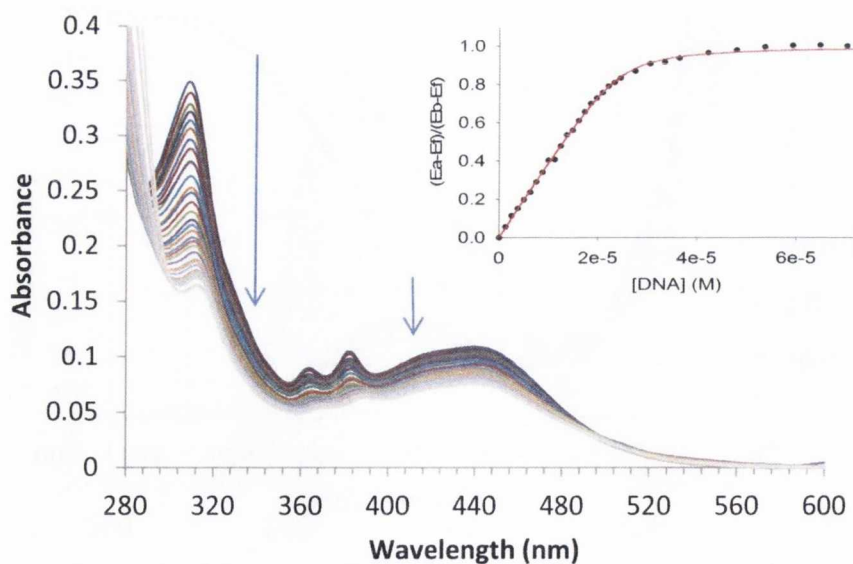
2A 9: Changes in the UV/Visible spectrum of **53** ($6.5\mu\text{M}$) with increasing concentration of stDNA ($0\text{--}122\mu\text{M}$) at 50 mM NaCl concentration. Inset: Plot of $(\epsilon_a-\epsilon_f)/\epsilon_b-\epsilon_f$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}12$ and the best fit of the data (---) using the Bard Eqn.



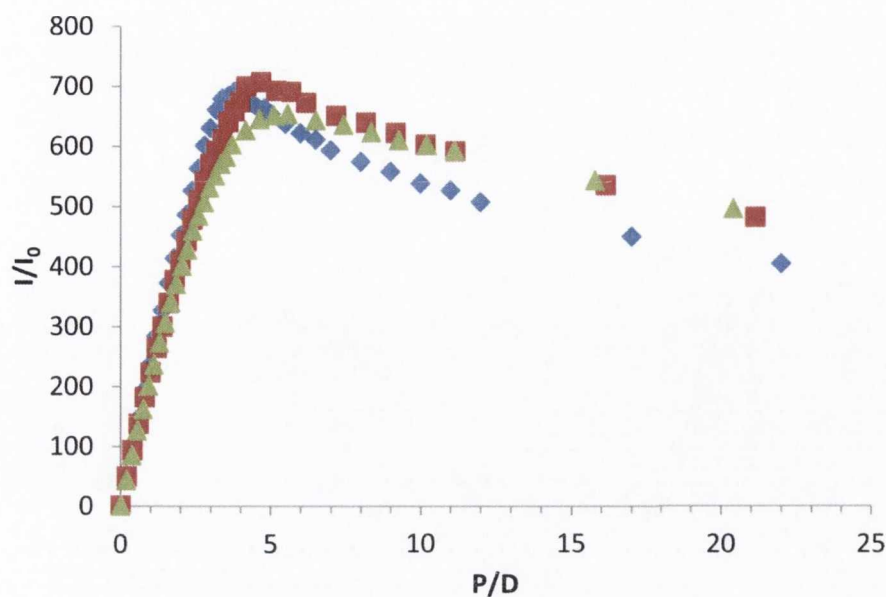
2A 10: Changes in the UV/Visible spectrum of **53** ($6.5\mu\text{M}$) with increasing concentration of stDNA ($0\text{--}122\mu\text{M}$) at 100 mM NaCl concentration. Inset: Plot of $(\epsilon_a-\epsilon_f)/\epsilon_b-\epsilon_f$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}12$ and the best fit of the data (---) using the Bard Eqn.



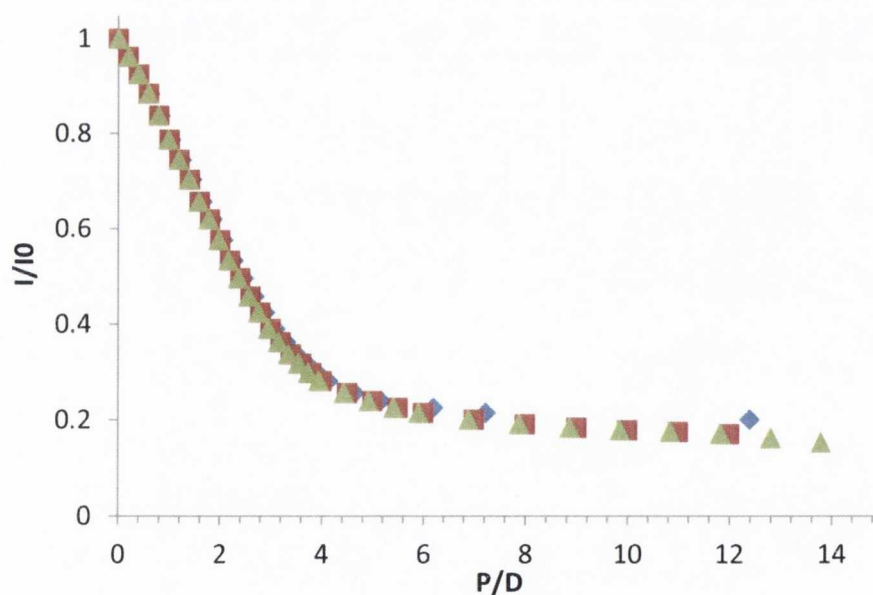
2A 11: Changes in the UV/Visible spectrum of **54** ($6.6\mu\text{M}$) with increasing concentration of stDNA ($0\text{--}122\mu\text{M}$) at 50 mM NaCl concentration. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}12$ and the best fit of the data (---) using the Bard Eqn.



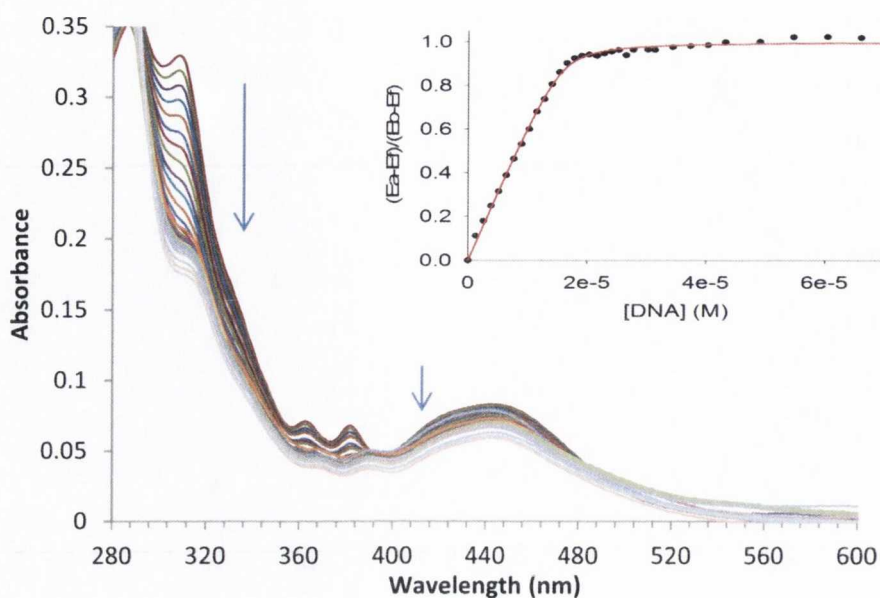
2A 12: Changes in the UV/Visible spectrum of **54** ($6.3\mu\text{M}$) with increasing concentration of stDNA ($0\text{--}122\mu\text{M}$) at 100 mM NaCl concentration. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}12$ and the best fit of the data (---) using the Bard Eqn.



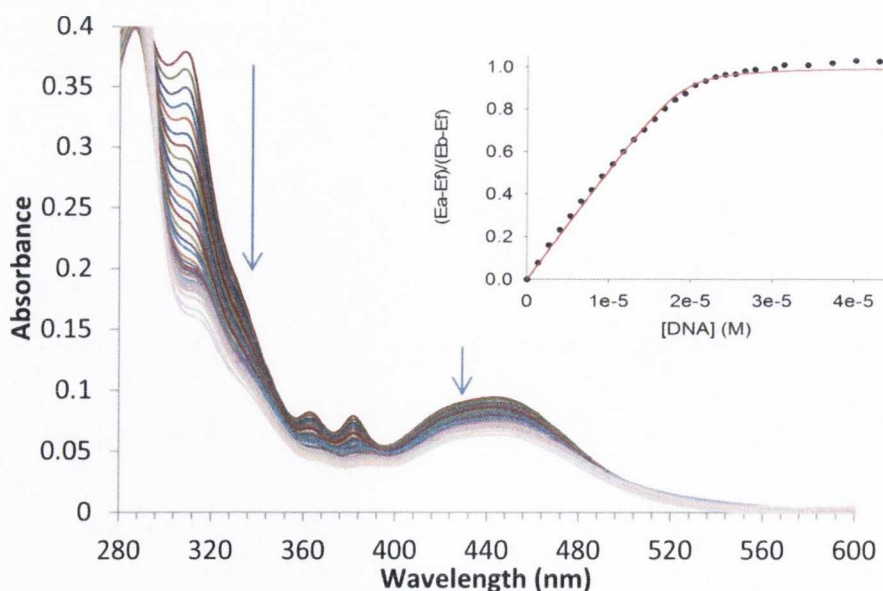
2A 13: Relative changes in the integrated emission intensity of **54** ($6.5 \mu\text{M}$) ($\lambda_{\text{ex}} 445 \text{ nm}$) with increasing concentration of st-DNA ($0 - 125 \mu\text{M}$), in 10 mM phosphate buffer (♦), 10 mM phosphate buffer + 50 mM NaCl (■) and 10 mM phosphate buffer + 100 mM NaCl (▲).



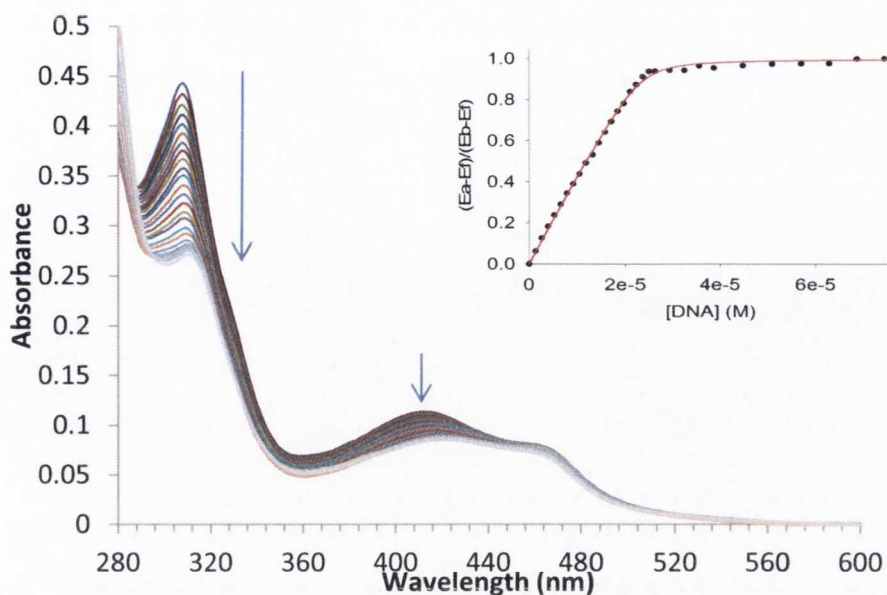
2A 14: Relative changes in the integrated emission intensity of **55** ($6.5 \mu\text{M}$) ($\lambda_{\text{ex}} 445 \text{ nm}$) with increasing concentration of st-DNA ($0 - 124 \mu\text{M}$), in 10 mM phosphate buffer (♦), 10 mM phosphate buffer + 50 mM NaCl (■) and 10 mM phosphate buffer + 100 mM NaCl (▲).



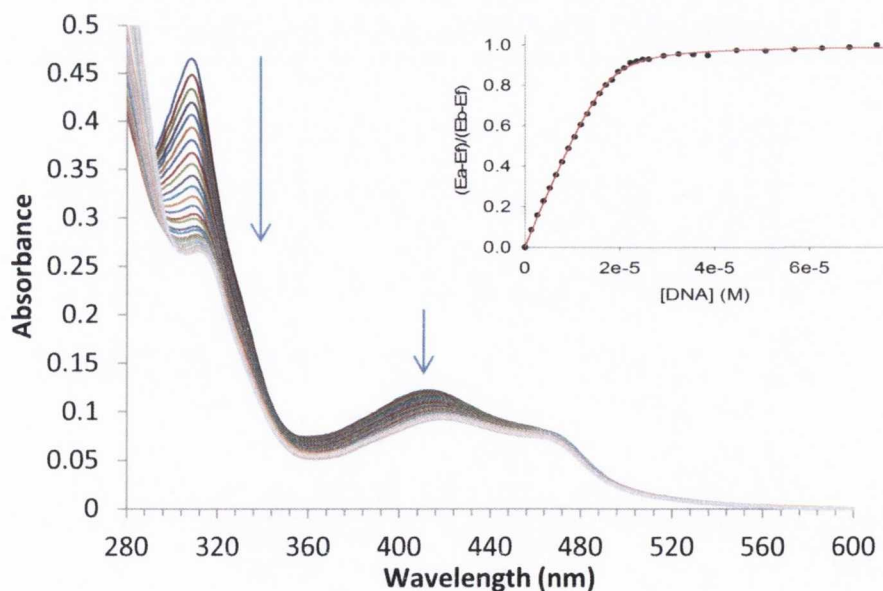
2A 15: Changes in the UV/Visible spectrum of **53** ($6.4\mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dAdT})]_2$ ($0\text{--}122\mu\text{M}$) at pH 7.4. **Inset:** Plot of $(\epsilon_a - \epsilon_b)/\epsilon_b - \epsilon_b$ vs. $[\text{DNA}](\text{M}^1, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



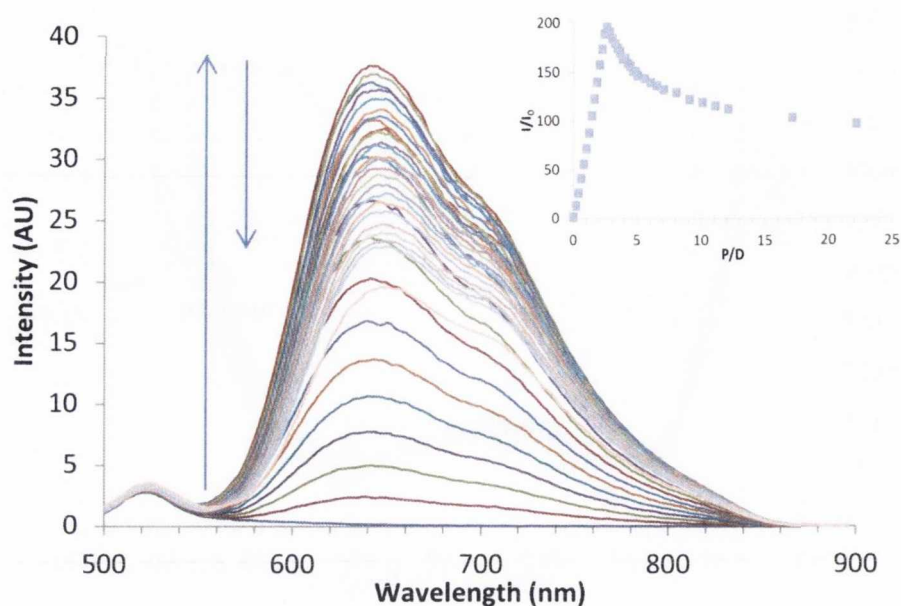
2A 16: Changes in the UV/Visible spectrum of **53** ($6.8\mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dGdC})]_2$ ($0\text{--}117\mu\text{M}$) at pH 7.4. **Inset:** Plot of $(\epsilon_a - \epsilon_b)/\epsilon_b - \epsilon_b$ vs. $[\text{DNA}](\text{M}^1, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



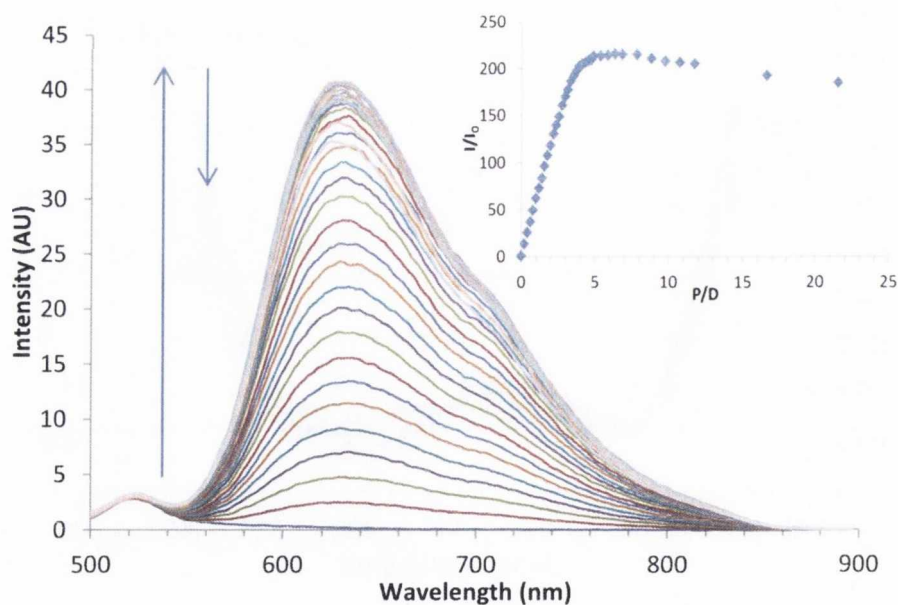
2A 17: Changes in the UV/Visible spectrum of **55** ($6.5\mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dAdT})]_2$ ($0\text{--}75\mu\text{M}$) at pH 7.4. **Inset:** Plot of $(\epsilon_a - \epsilon_b)/(\epsilon_b - \epsilon_c)$ vs. $[\text{DNA}](M^I, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



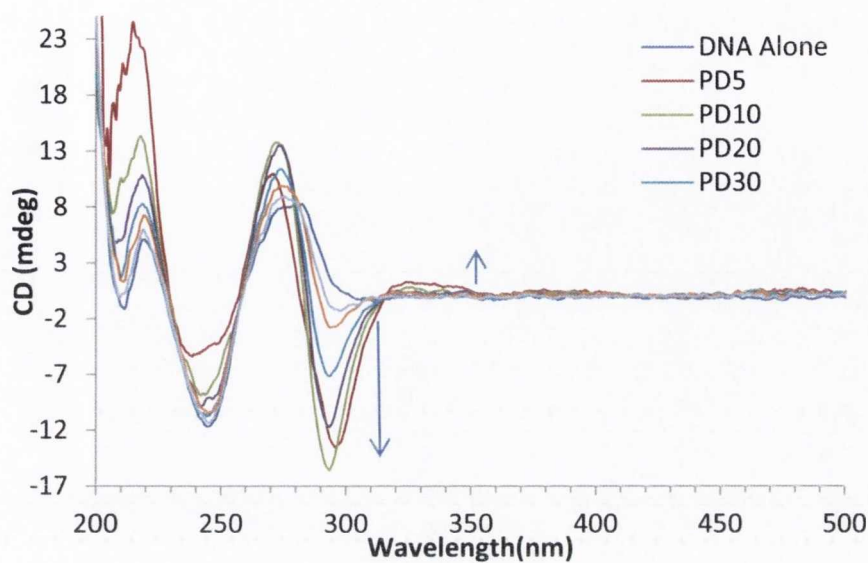
2A 18: Changes in the UV/Visible spectrum of **55** ($6.8\mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dGdC})]_2$ ($0\text{--}74\mu\text{M}$) at pH 7.4. **Inset:** Plot of $(\epsilon_a - \epsilon_b)/(\epsilon_b - \epsilon_c)$ vs. $[\text{DNA}](M^I, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



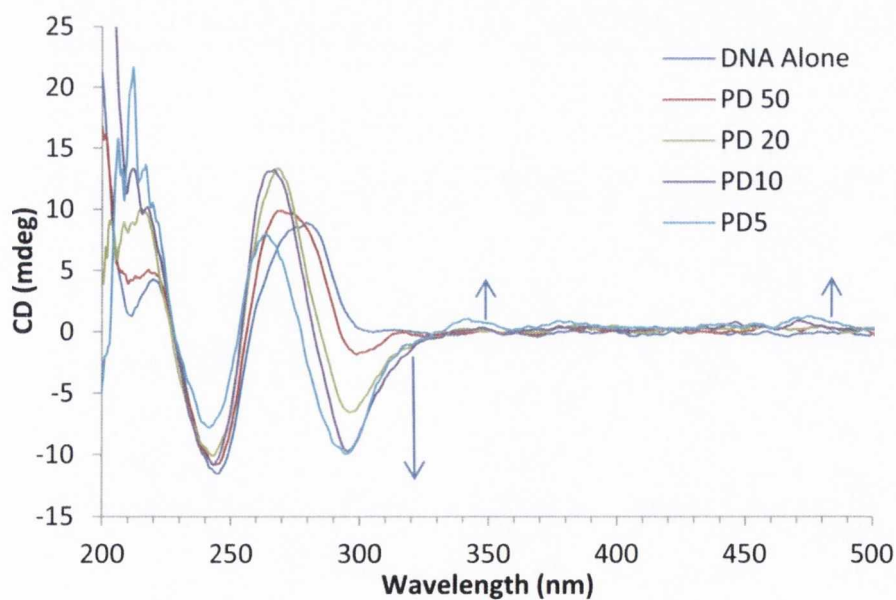
2A 19: Changes in the fluorescence emission spectrum of **53** ($6.4\mu\text{M}$) (λ_{ex} 445 nm) with increasing concentration $[\text{poly}(\text{dAdT})]_2$ (0-100 μM) at pH 7.4 **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



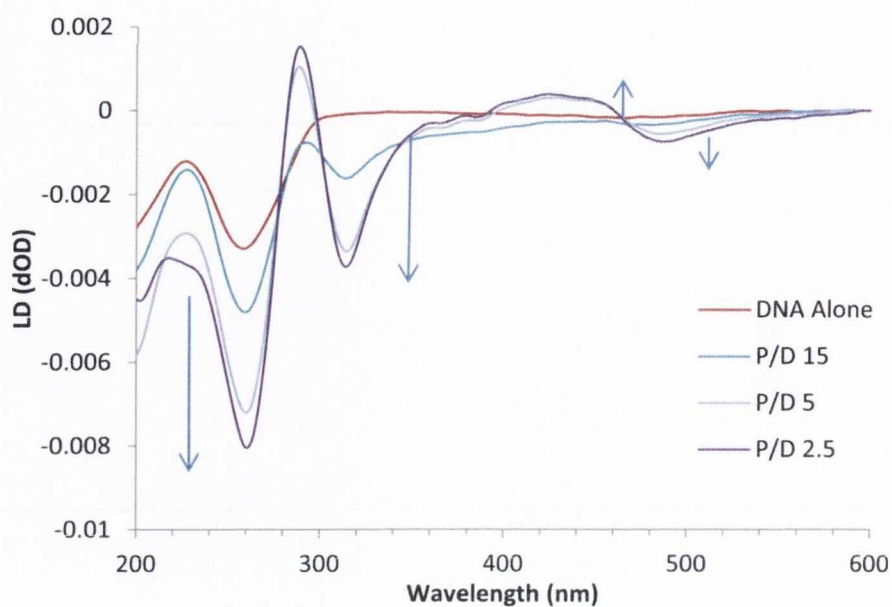
2A 20: Changes in the fluorescence emission spectrum of **53** ($6.8\mu\text{M}$) (λ_{ex} 445 nm) with increasing concentration $[\text{poly}(\text{dGdC})]_2$ (0-116 μM) at pH 7.4 **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



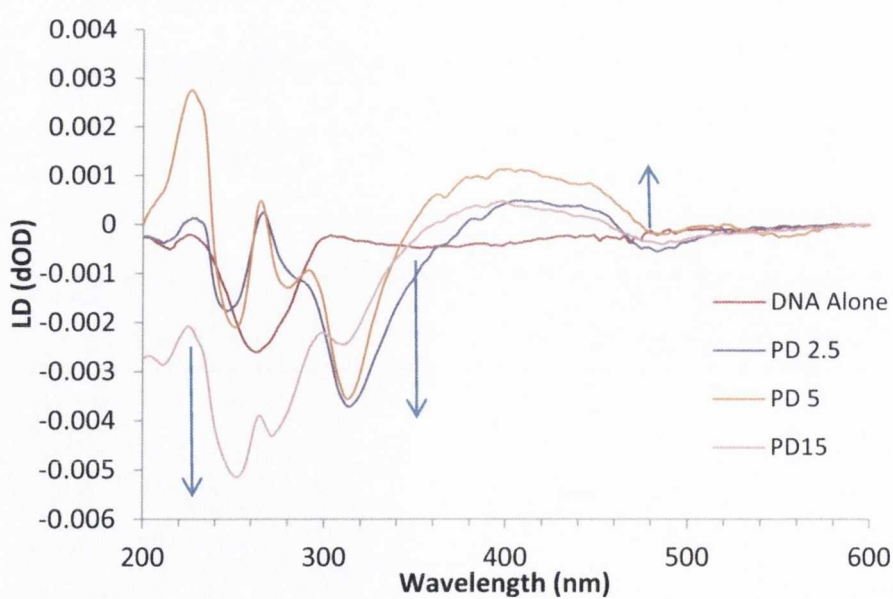
2A 21: Circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **53** at varying ratios.



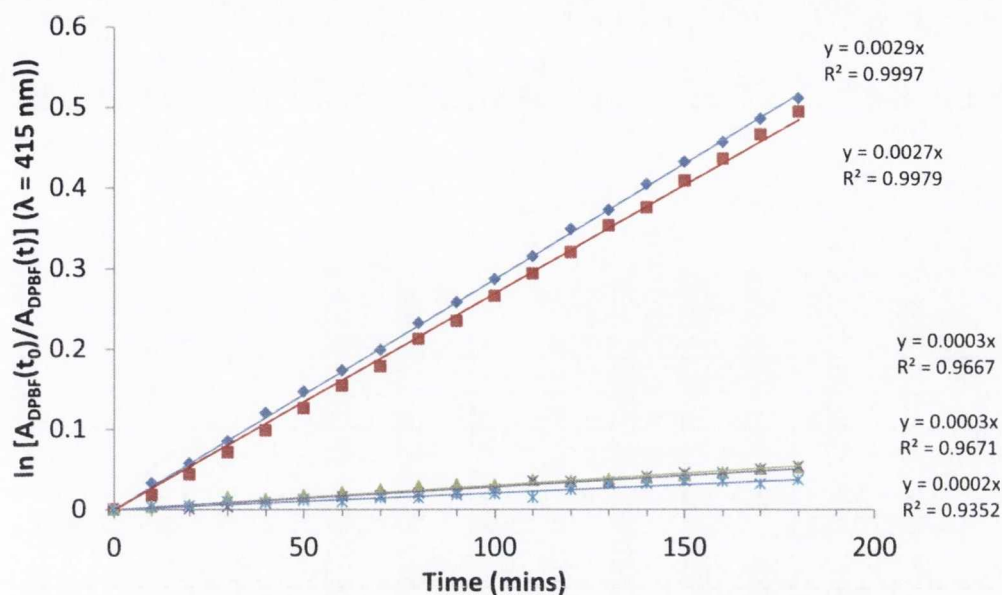
2A 22: Circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **55** at varying ratios.



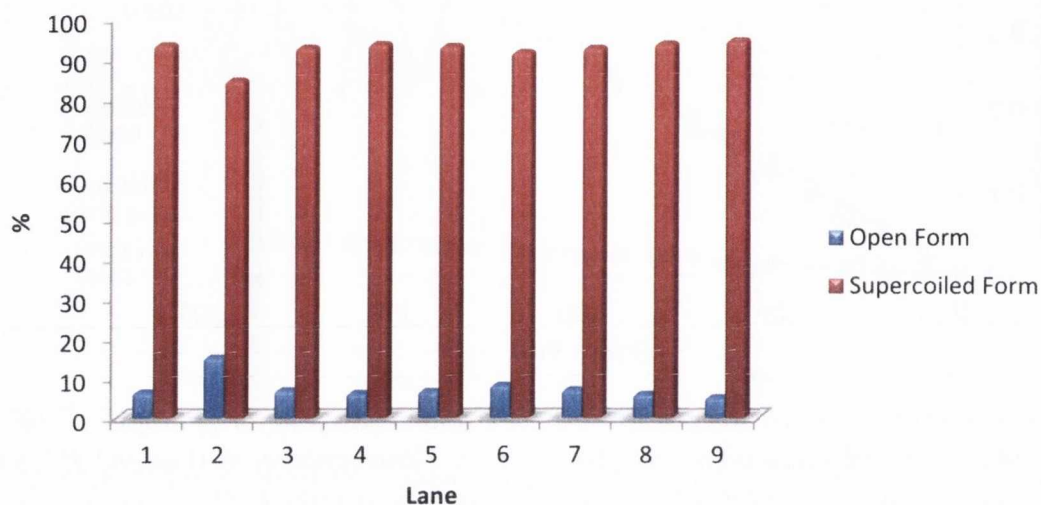
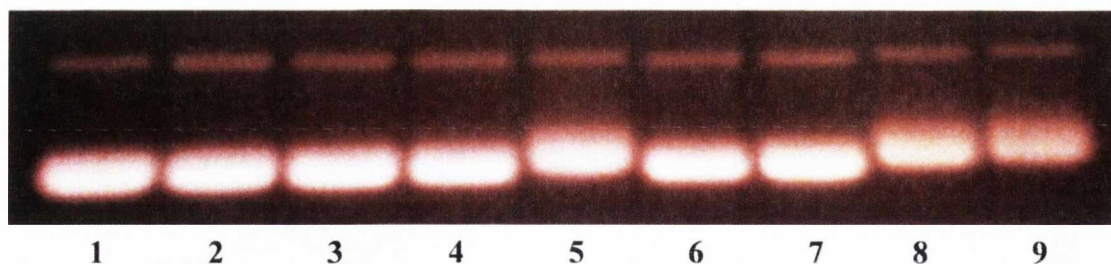
2A 23: Linear dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **53** at varying ratios.



2A 24: Linear dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **54** at varying ratios.

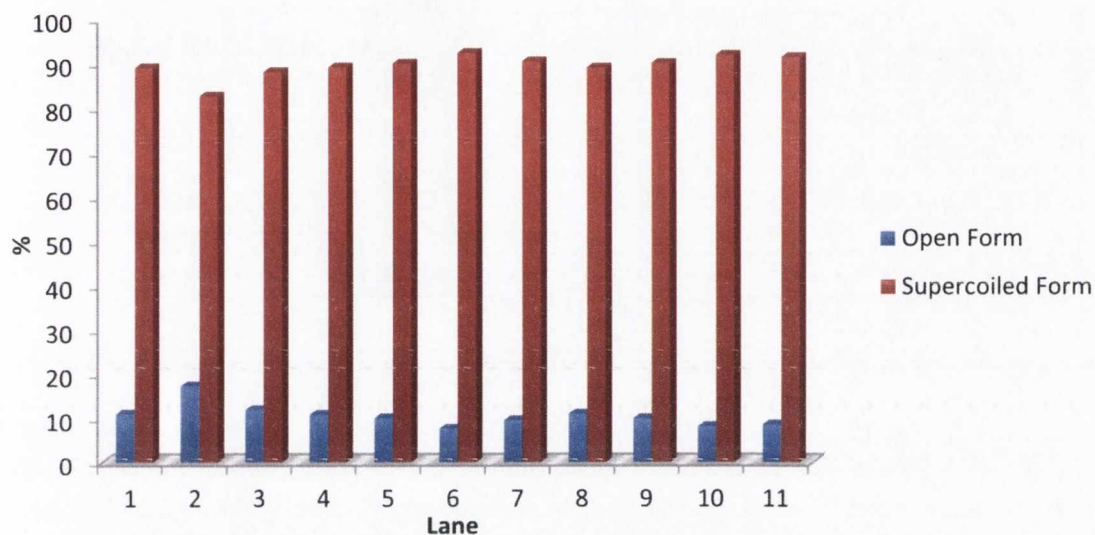
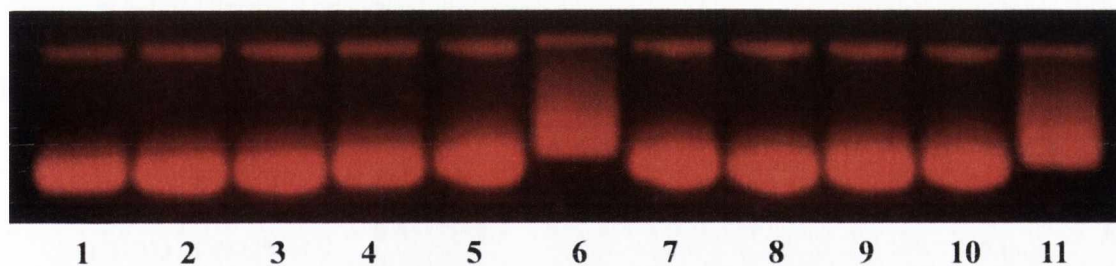


2A 25: Comparative reactive oxygen species generation rates plot for $[Ru(bpy)_3]^{2+}$ (■), 53 (×), 54 (▲), 55 (◆) and no photosensitizer (*). Concentration of $[Ru(bpy)_3]^{2+}$, 53, 54 and 55 at 1×10^{-8} M and DPBF initially at 5×10^{-5} M in MeOH. The slopes (m) and the R^2 coefficients of the lines are determined by linear regression.



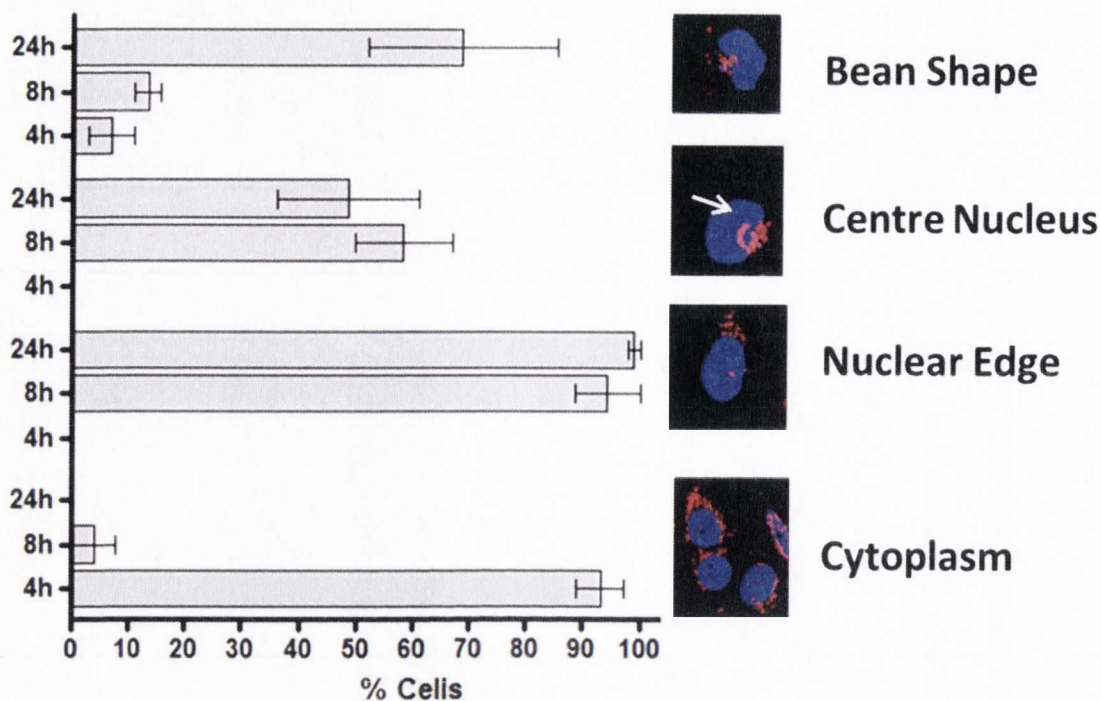
Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	6.5	93.5	6	8.3	91.7
2	15.4	84.6	7	7.2	92.8
3	7.1	92.9	8	6.1	93.9
4	6.3	93.7	9	5.3	94.7
5	6.8	93.2			

2A 26: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after irradiation at 2 J cm^{-2} ($\lambda > 390 \text{ nm}$) in 10 mM phosphate buffer, pH 7. 4 Lane 1: Plasmid DNA control; Lane 2: $[\text{Ru}(\text{bpy})_3]^{2+}$ (P/D 250); Lanes 3-5: **53** (P/D 250, 150, 50); Lanes 6-8: **53** + 10mM NaN_3 (P/D 250, 150, 50); Lane 9: **53** (P/D 50) in the dark.



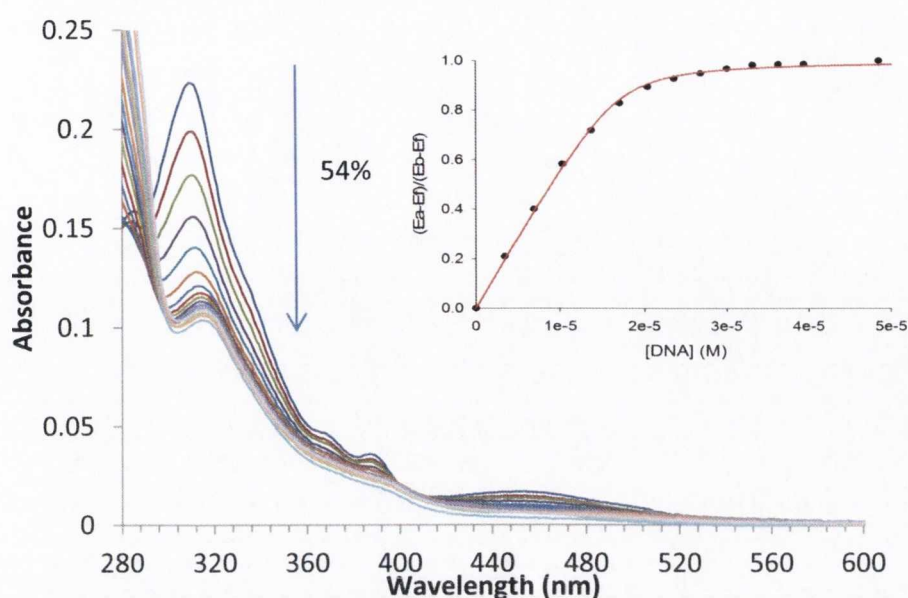
Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	11.0	89.0	7	9.6	90.4
2	17.4	82.6	8	11.0	89.0
3	11.9	88.1	9	10.0	90.0
4	10.9	89.1	10	8.2	91.8
5	10.1	89.9	11	8.6	91.4
6	7.7	92.3			

2A 27: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after irradiation at 2 J cm⁻² ($\lambda > 390$ nm) in 10 mM phosphate buffer, pH 7. 4 Lane 1: Plasmid DNA control in the dark; Lane 2: Plasmid DNA control; Lane 3: [Ru(bpy)₃]²⁺ (P/D 250); Lanes 4-6: **54** (P/D 250, 150, 50); Lane 7 : **54** in the dark (P/D 250); Lane 8: 10mM NaN₃ control; Lanes 9-11: **54** + 10mM NaN₃ (P/D 250, 150, 50).

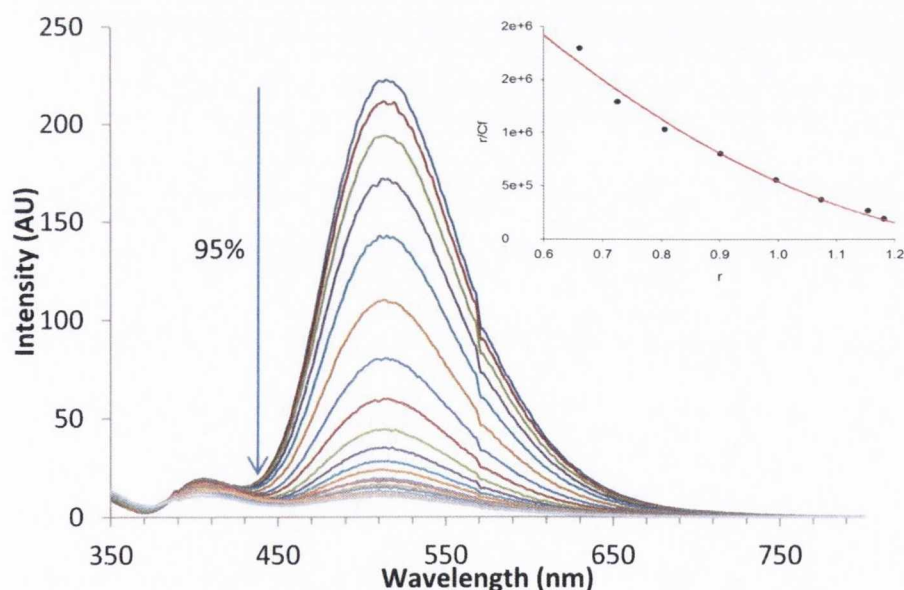


2A 28: Cellular distribution and uptake of 55 into HeLa cells at 4, 8 and 24 hr time points. Percentages are expressed over the total amount of cells (approximately 200) per field of view.

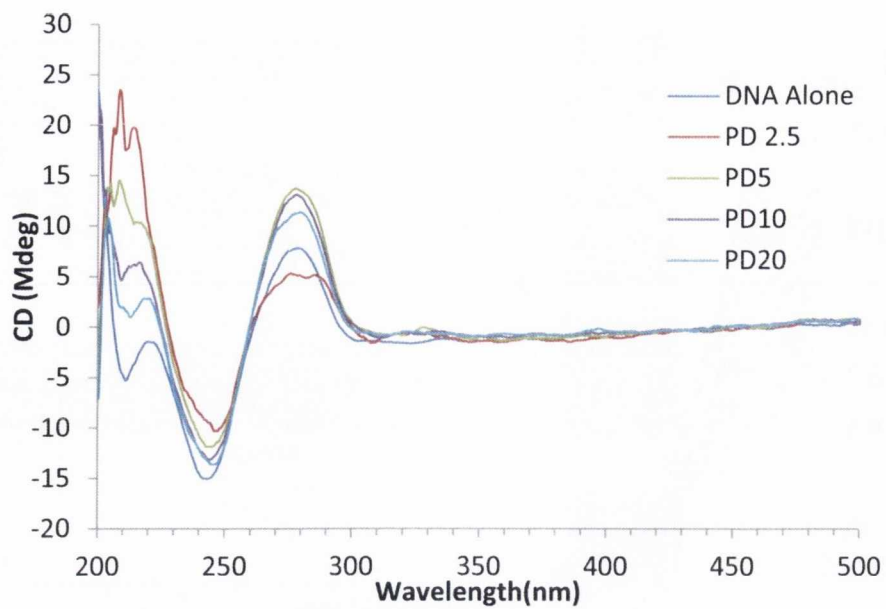
Appendix 3



3A 1: Changes in the UV/Visible spectrum of **67** (10 μ M) with increasing concentration of $[\text{poly}(\text{dGdC})]_2$ (0-63 μ M) at pH 7.4. **Inset:** Plot of $(\epsilon_a - \epsilon_f)/\epsilon_b - \epsilon_f$ vs. $[\text{DNA}]$ (M^I , P) using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.

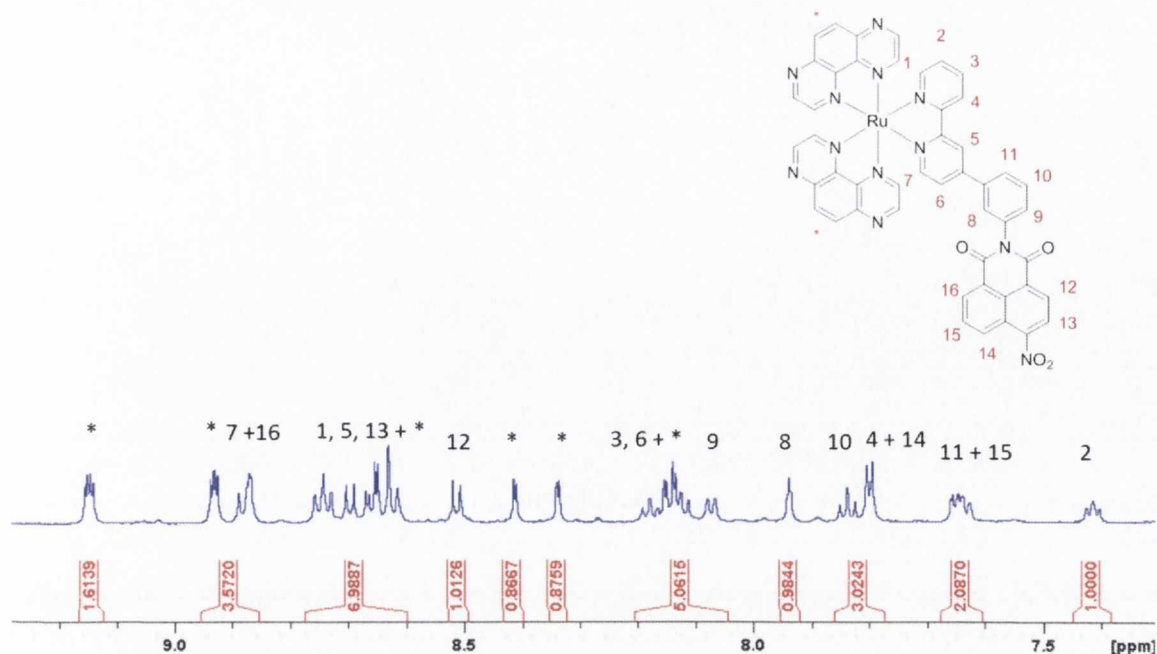


3A 2: Changes in the fluorescence emission spectrum of **67** (10 μ M) (λ_{ex} 310 nm) with increasing concentration of $[\text{poly}(\text{dGdC})]_2$ (0-63 μ M) at pH 7.4. **Inset:** Plot of r/C_f vs. r and the best fit of the data (---) using the McGhee Von Hippel equation.

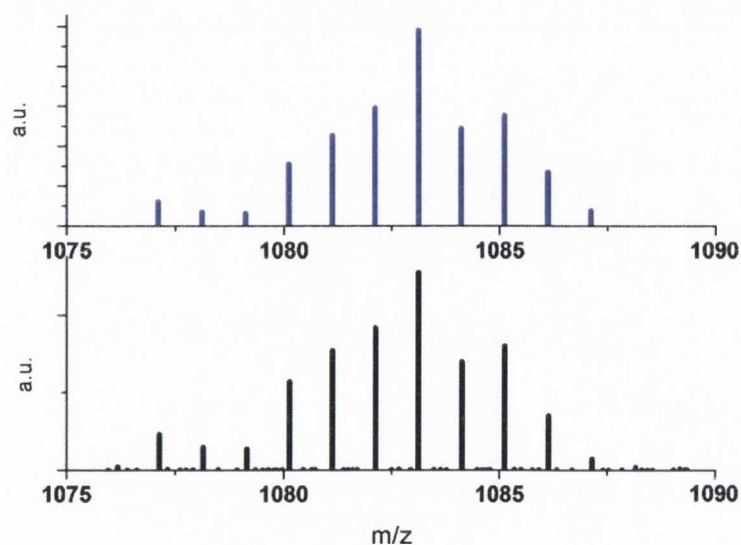


3A 3: The circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **67** at varying P/D.

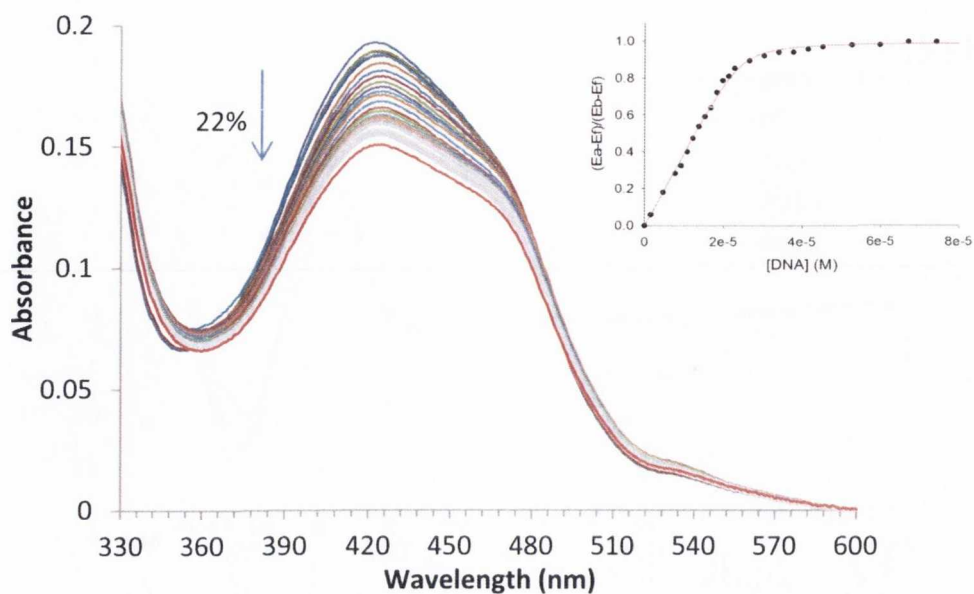
Appendix 4:



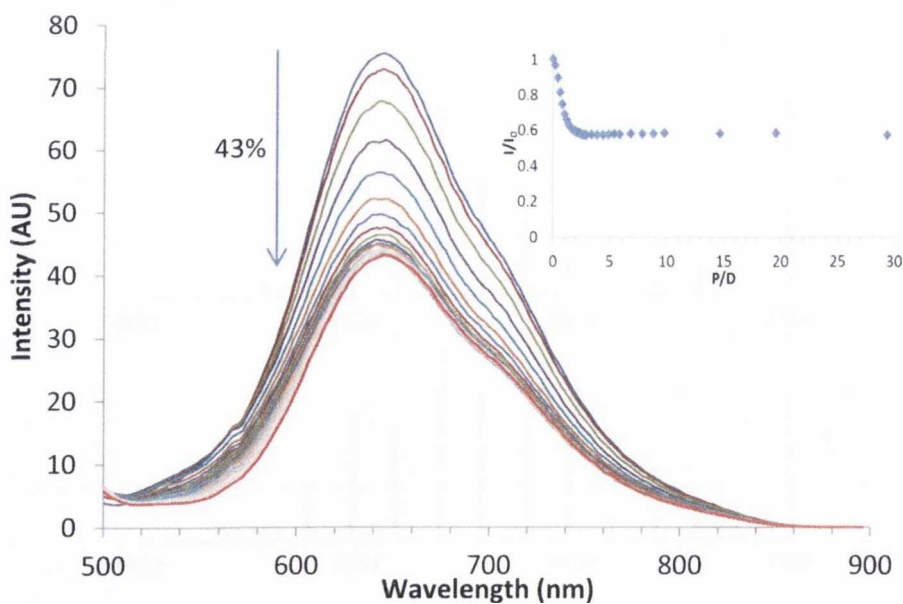
4A 1: The ^1H NMR spectra of **70** (600 MHz, $\text{DMSO}-d_6$)



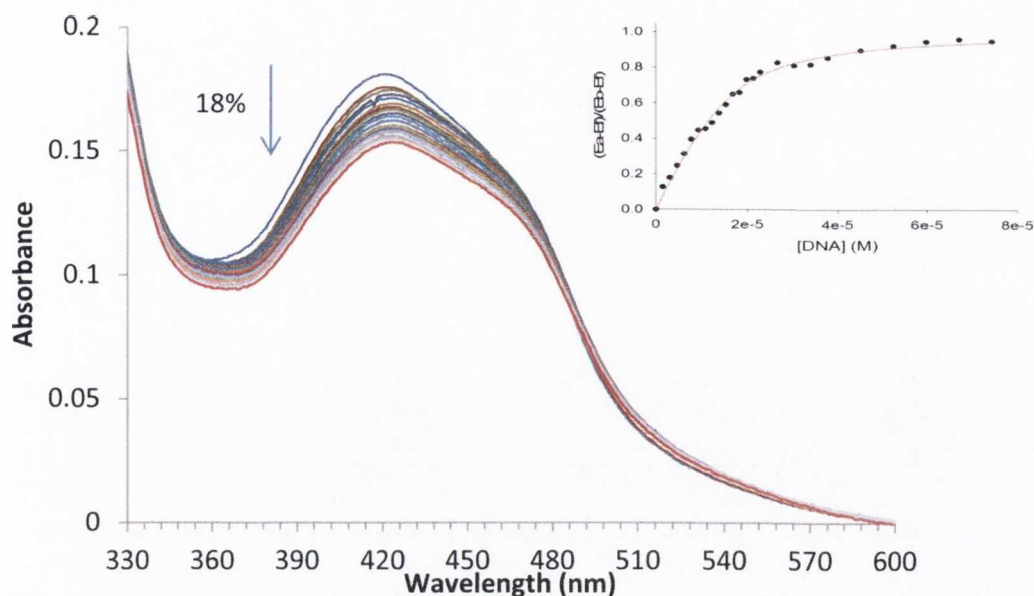
4A 2: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for **70** from high resolution mass spectrometry analysis



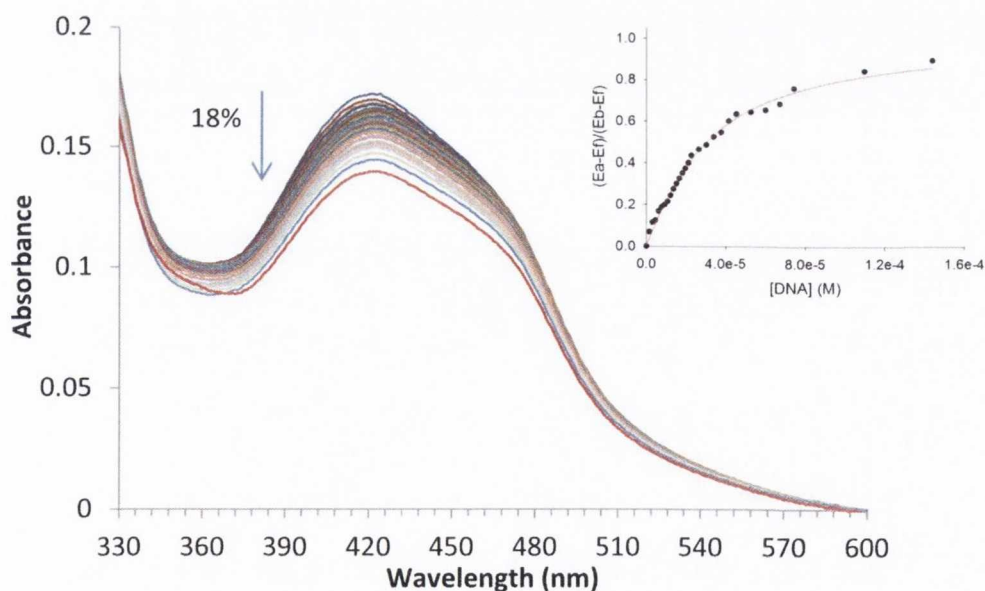
4A 3: Changes in the UV/Visible spectrum of **7I** (8 μM) with increasing additions of stDNA (0-210 μM) in 10 mM phosphate buffer, pH 7.4). Inset: Plot of $(\epsilon_a - \epsilon_f) / (\epsilon_b - \epsilon_f)$ vs. $[DNA] (M^1, P)$ using data with a P/D between 0-10 and the best fit of the data (---) using the Bard Eqn.



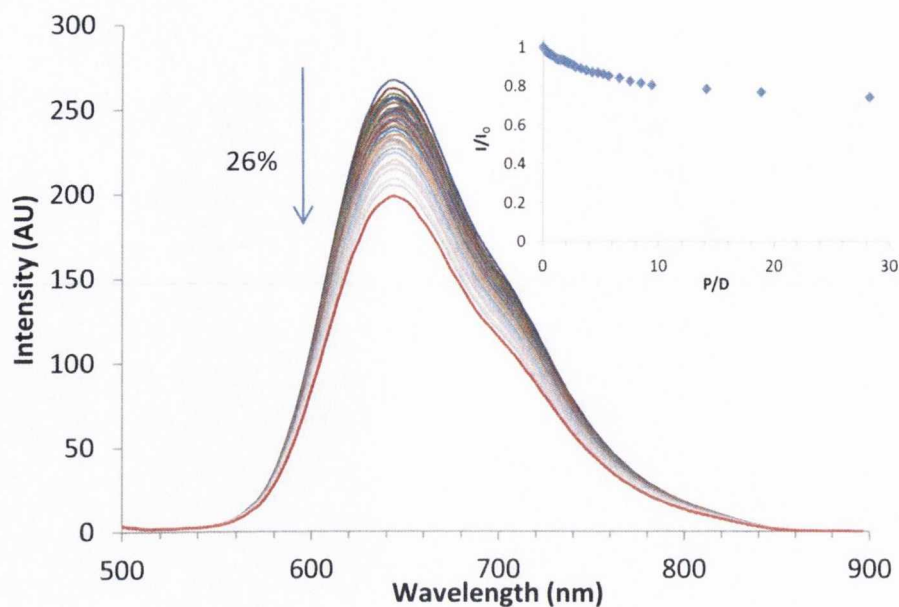
4A 4: Changes in the emission spectrum of **7I** (8 μM) (λ_{ex} 425 nm) with increasing concentration of stDNA (0-210 μM). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



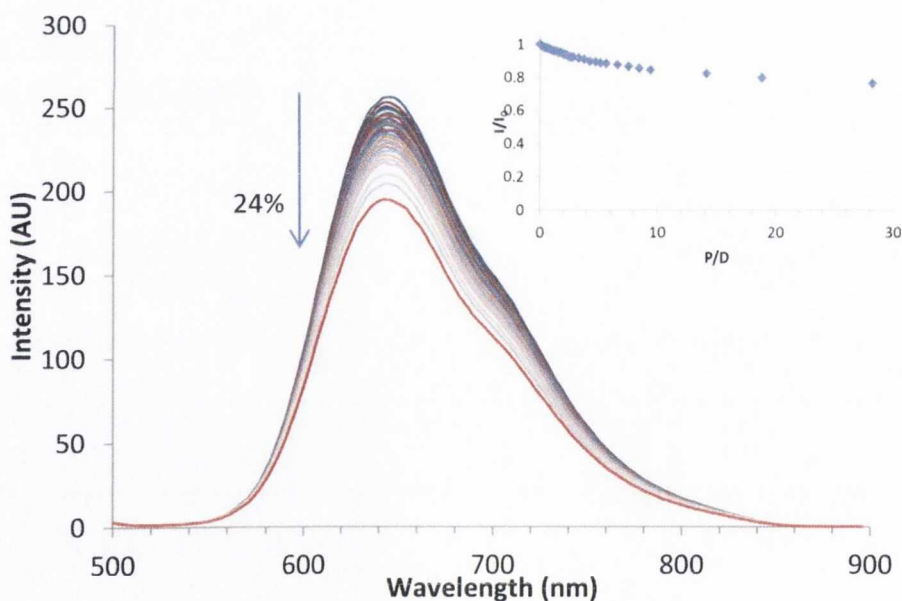
4A 5: Changes in the UV/Visible spectrum of **71** ($8\ \mu\text{M}$) with increasing concentration of stDNA ($0\text{--}210\ \mu\text{M}$) at $50\ \text{mM}$ NaCl concentration. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}10$ and the best fit of the data (---) using the Bard Eqn.



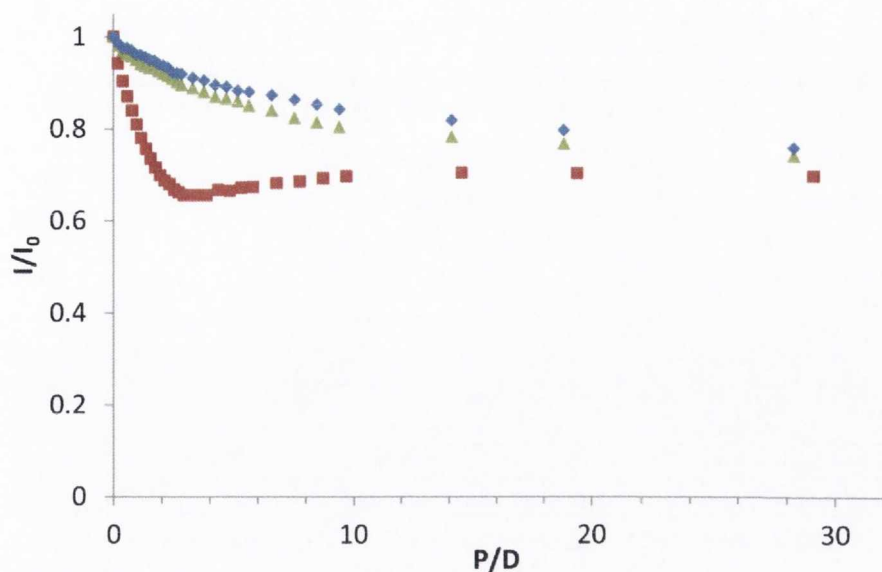
4A 6: Changes in the UV/Visible spectrum of **71** ($8\ \mu\text{M}$) with increasing concentration of stDNA ($0\text{--}210\ \mu\text{M}$) at $100\ \text{mM}$ NaCl concentration. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}](M^1, P)$ using data with a P/D between $0\text{--}10$ and the best fit of the data (---) using the Bard Eqn.



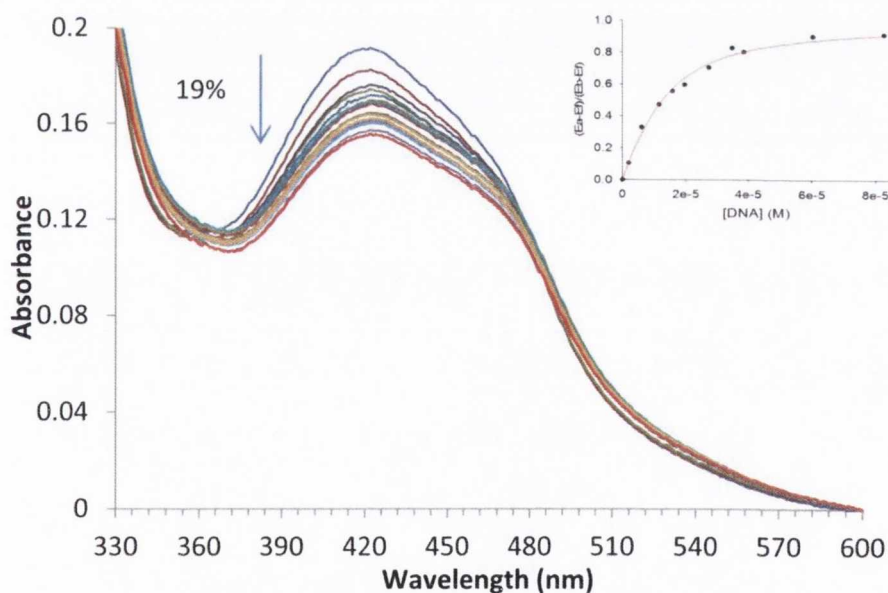
4A 7: Changes in the emission spectrum of **70** ($8\mu\text{M}$) (λ_{ex} 415 nm) with increasing concentration of stDNA (0-210 μM) at 50 mM NaCl concentration. **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio



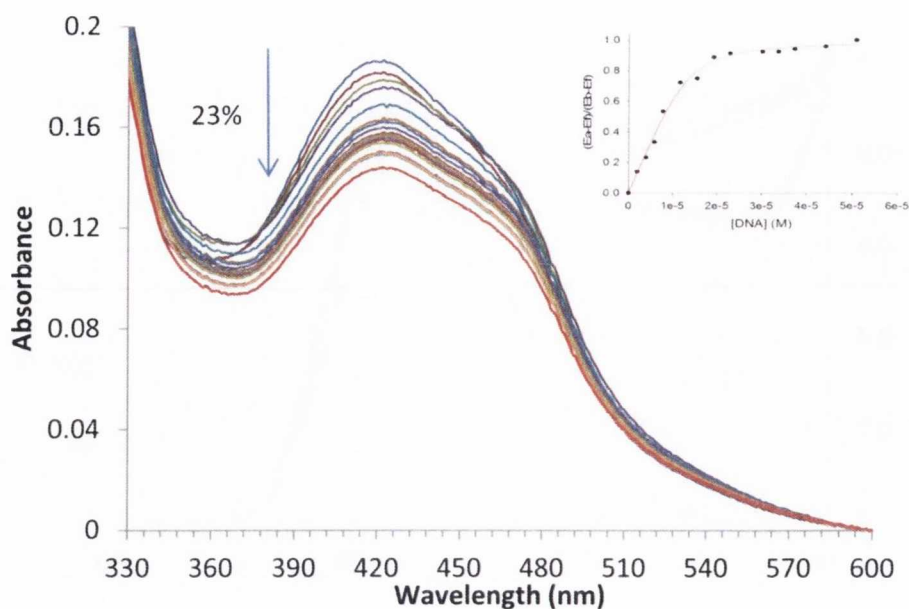
4A 8: Changes in the emission spectrum of **70** ($8\mu\text{M}$) (λ_{ex} 415 nm) with increasing concentration of stDNA (0-210 μM) at 100 mM NaCl concentration. **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio



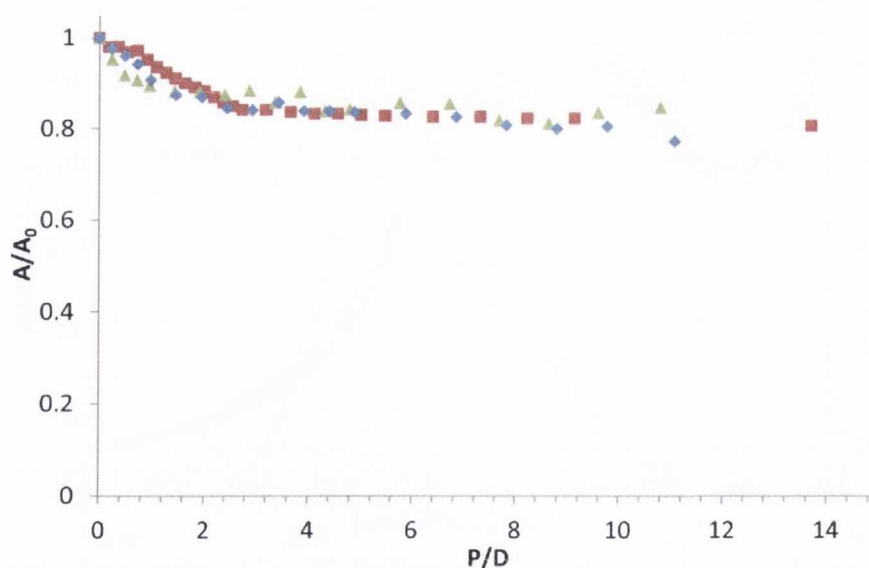
4A 9: Relative changes in the integrated emission intensity of **70** ($8 \mu\text{M}$) (λ_{ex} 415 nm) at 645 nm with increasing concentration of st-DNA ($0 - 210 \mu\text{M}$), in 10 mM phosphate buffer (■), 10 mM phosphate buffer + 50 mM NaCl (▲) and 10 mM phosphate buffer + 100 mM NaCl (◆)



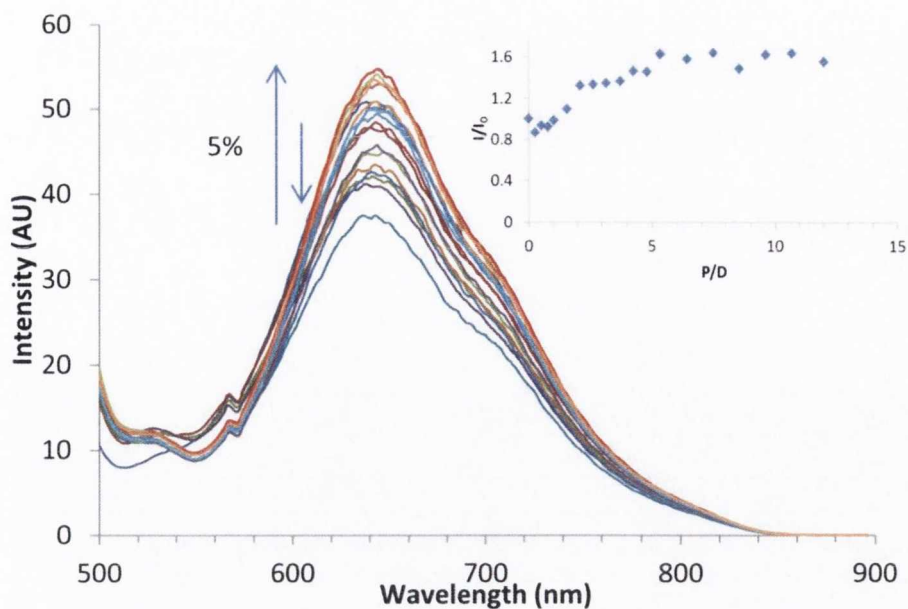
4A 10: Changes in the UV/Visible spectrum of **71** ($7.5 \mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dAdT})]_2$ ($0-83 \mu\text{M}$) at pH 7.4. Inset: Plot of $(\epsilon_a - \epsilon_b) / (\epsilon_b - \epsilon_b)$ vs. $[\text{DNA}] (M^I, P)$ using data with a P/D between 0-12 and the best fit of the data (---) using the Bard Eqn.



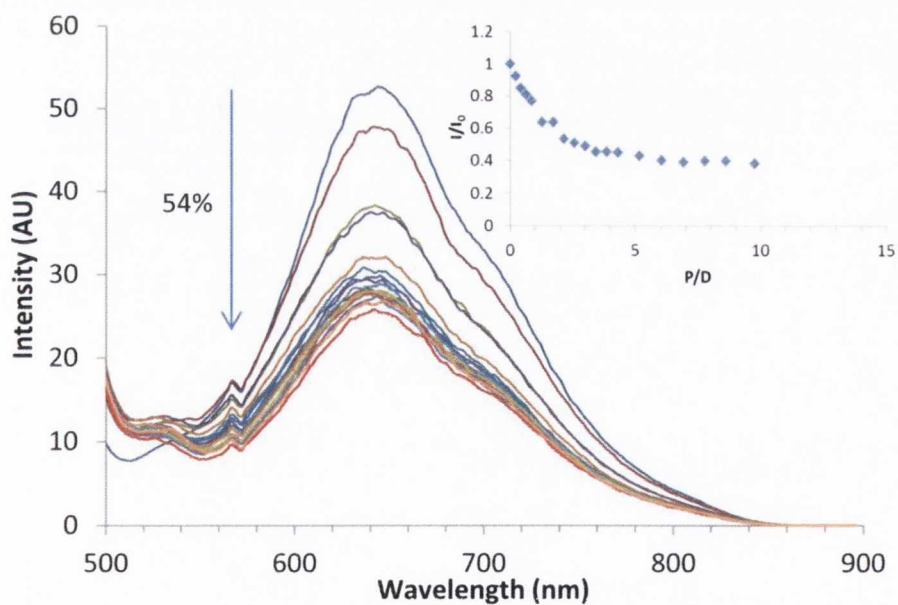
4A 11: Changes in the UV/Visible spectrum of **71** ($7.5 \mu\text{M}$) with increasing concentration of $[\text{poly}(\text{dGdC})]_2$ ($0\text{--}78 \mu\text{M}$) at pH 7.4. Inset: Plot of $(\epsilon_a - \epsilon_f)/(\epsilon_b - \epsilon_f)$ vs. $[\text{DNA}] (\text{M})$, P using data with a P/D between 0–12 and the best fit of the data (---) using the Bard Eqn.



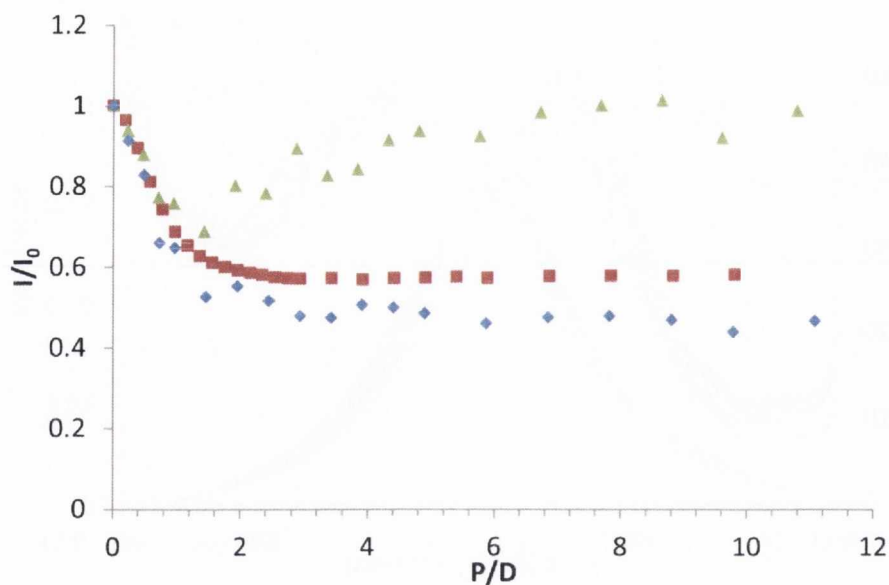
4A 12: Changes in the absorption of **71** ($8 \mu\text{M}$) in 10 mM phosphate buffer, at pH 7.4 upon addition of stDNA (■), $[\text{poly}(\text{dAdT})]_2$ (▲) and $[\text{poly}(\text{dGdC})]_2$ (◆).



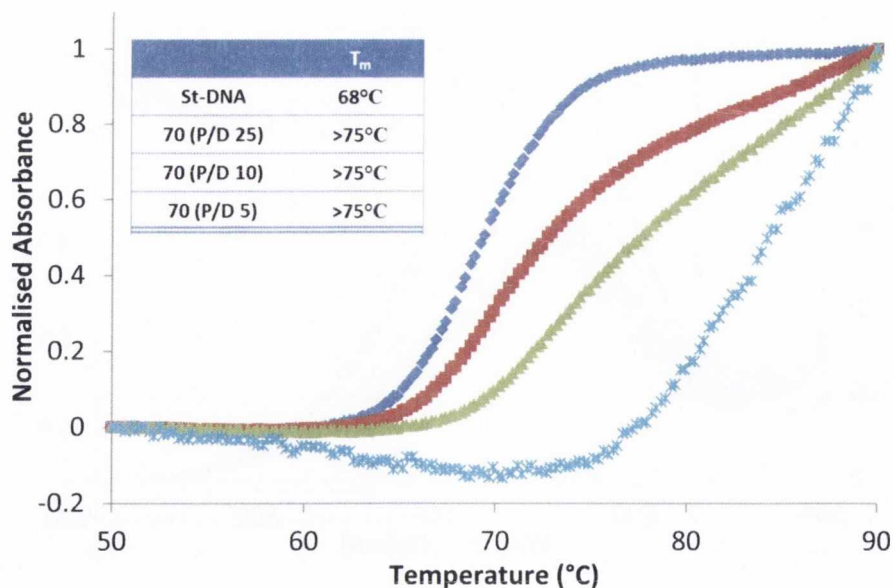
4A 13: Changes in the fluorescence emission spectrum of **7I** ($8\mu\text{M}$) (λ_{ex} 415 nm) with increasing concentration $[\text{poly}(\text{dAdT})]_2$ (0-83 μM) at pH 7.4 Inset: Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



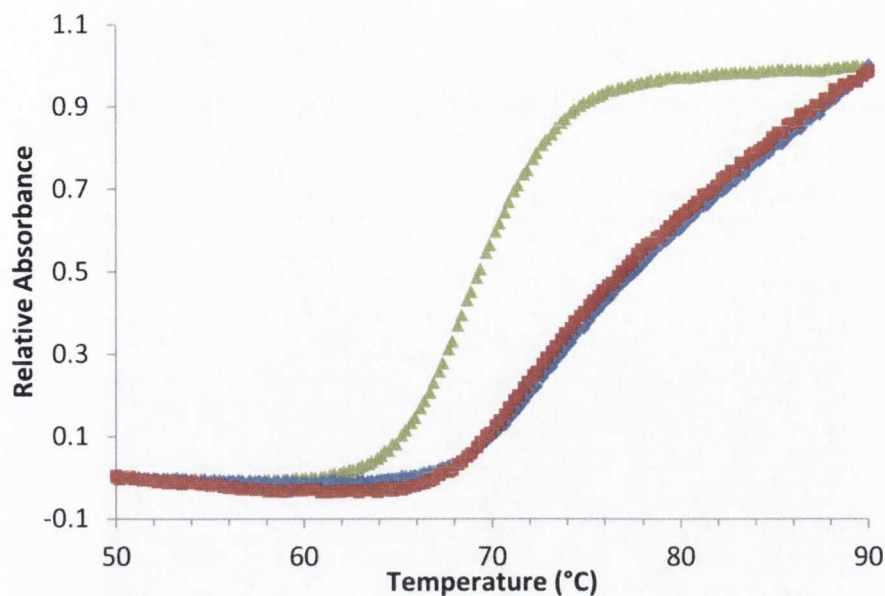
4A 14: Changes in the fluorescence emission spectrum of **7I** ($8\mu\text{M}$) (λ_{ex} 415 nm) with increasing concentration $[\text{poly}(\text{dGdC})]_2$ (0-79 μM) at pH 7.4 Inset: Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



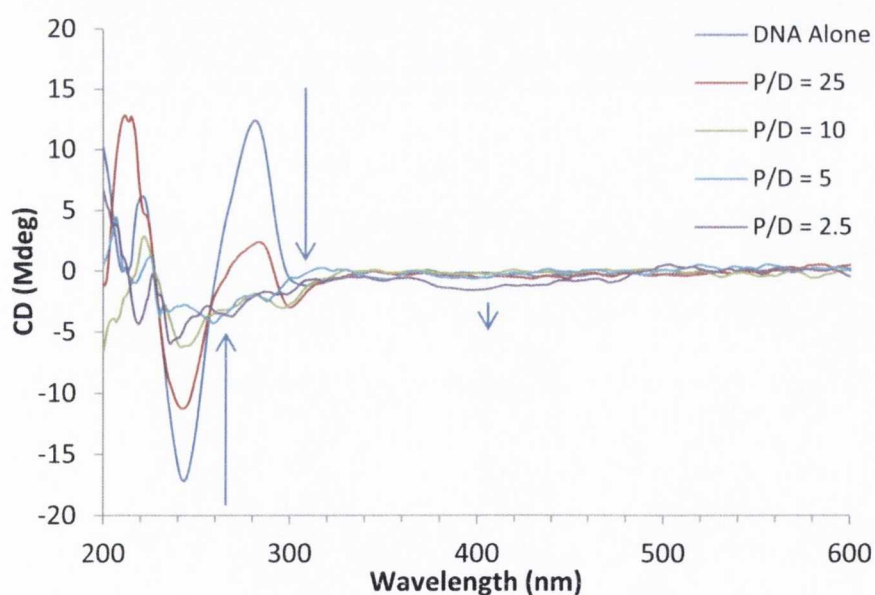
4A 15: Relative changes in the integrated emission intensity of **71** ($8\ \mu\text{M}$) ($\lambda_{\text{ex}}\ 415\ \text{nm}$) with increasing concentration of stDNA (■), $[\text{poly}(\text{dAdT})]_2$ (▲) and $[\text{poly}(\text{dGdC})]_2$ (◆) in 10 mM phosphate buffer at pH 7.4.



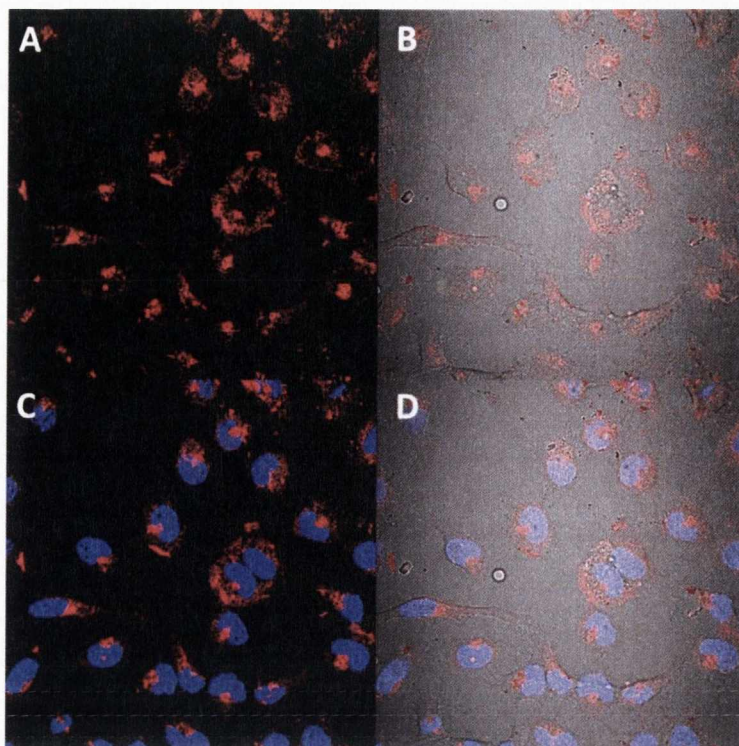
4A 16: Thermal denaturation curves of stDNA ($150\ \mu\text{M}$) in 10 mM phosphate buffer, pH 7.4, in the absence (◆) and presence of **70** at P/D = 25 (■) P/D = 10 (▲) and P/D = 5 (*). **Inset:** The T_m values from first derivative plots of the melting curve.



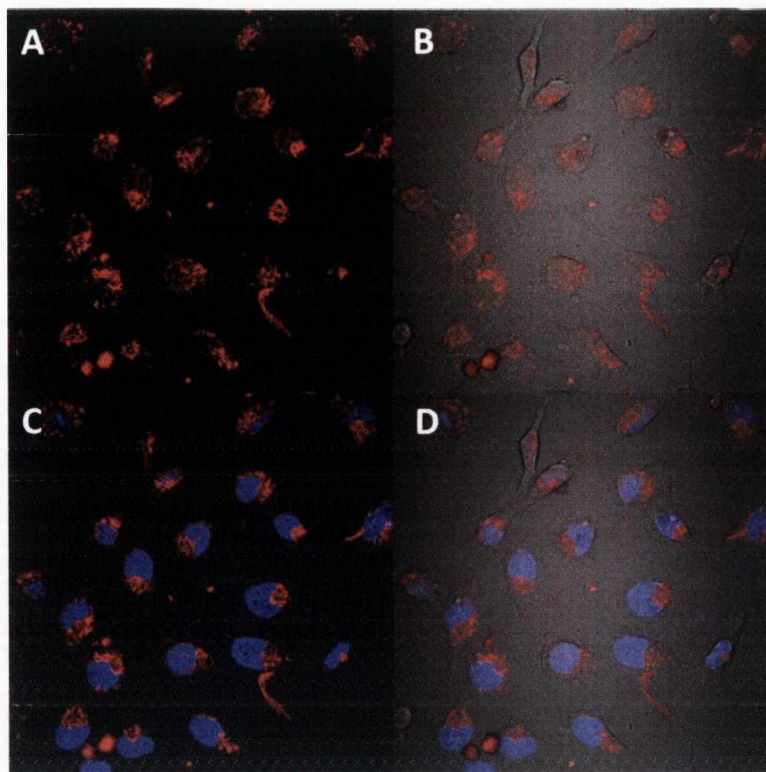
4A 17: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\blacktriangle$) and presence of **70** ($\blacksquare$) and **71** ($\blacklozenge$) at a P/D ratio of 10.



4A 18: Circular dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **71** at varying ratios.

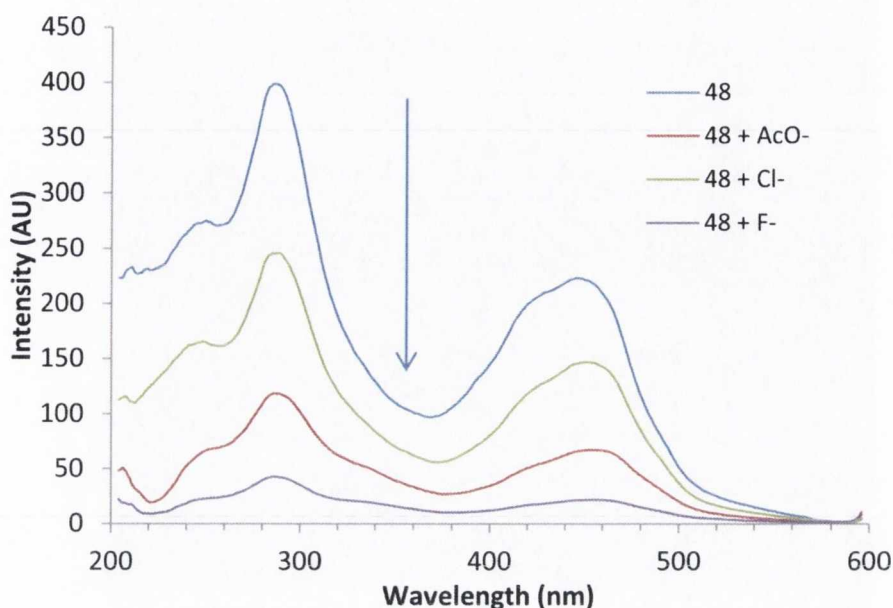


4A 19: Confocal Laser Scanning Microscopy live cell images of **70** (50 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **70** (red), (B) overlay of **70** (red) and the bright field view, (C) overlay of **70** (red) and nuclear co-stain DAPI (blue) and (D) overlay of **70** (red) , nuclear co-stain DAPI (blue) and the bright field view after 4 hrs of incubation.

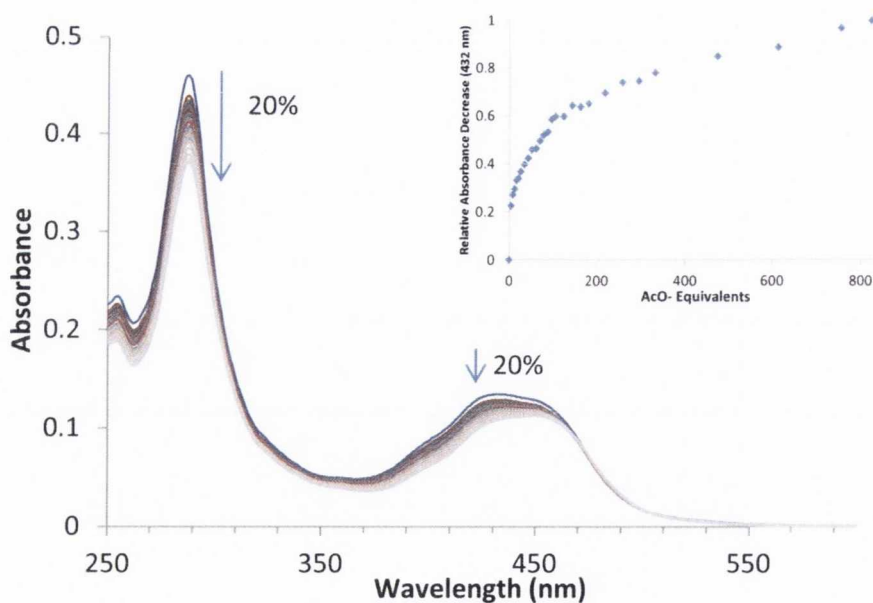


4A 20: Confocal Laser Scanning Microscopy live cell images of **71** (50 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **71** (red), (B) overlay of **71** (red) and the bright field view, (C) overlay of **71** (red) and nuclear co-stain DAPI (blue) and (D) overlay of **71** (red) , nuclear co-stain DAPI (blue) and the bright field view after 4 hrs of incubation.

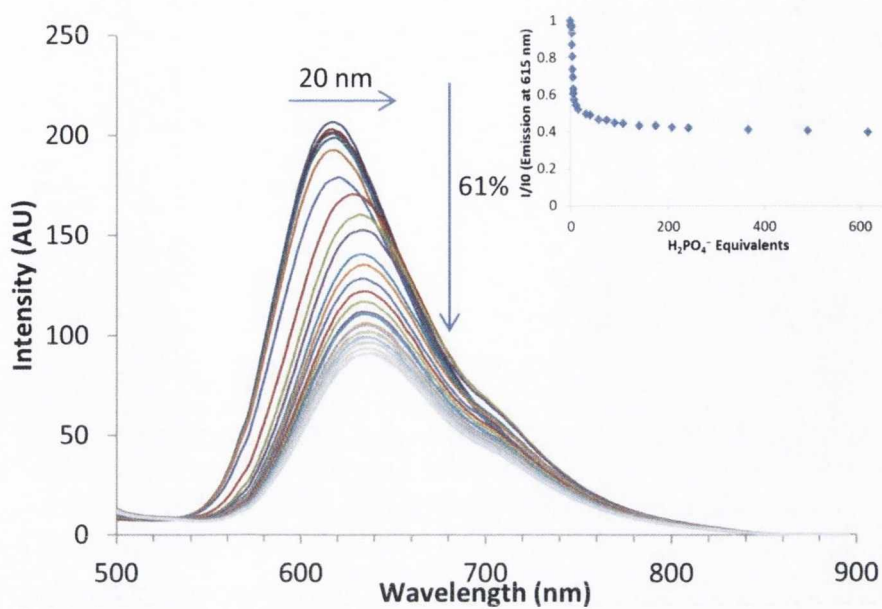
Appendix 5:



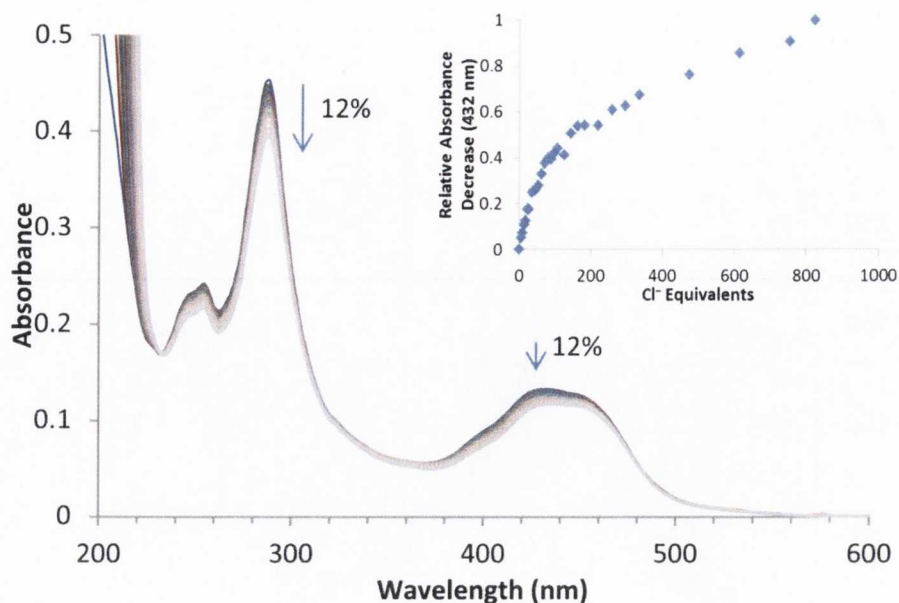
5A 1: The changes in the excitation spectra of **48** ($\lambda_{ex} = 615$ nm) in MeCN in the absence and presence of AcO^- , Cl^- and F^- as their TBA salts



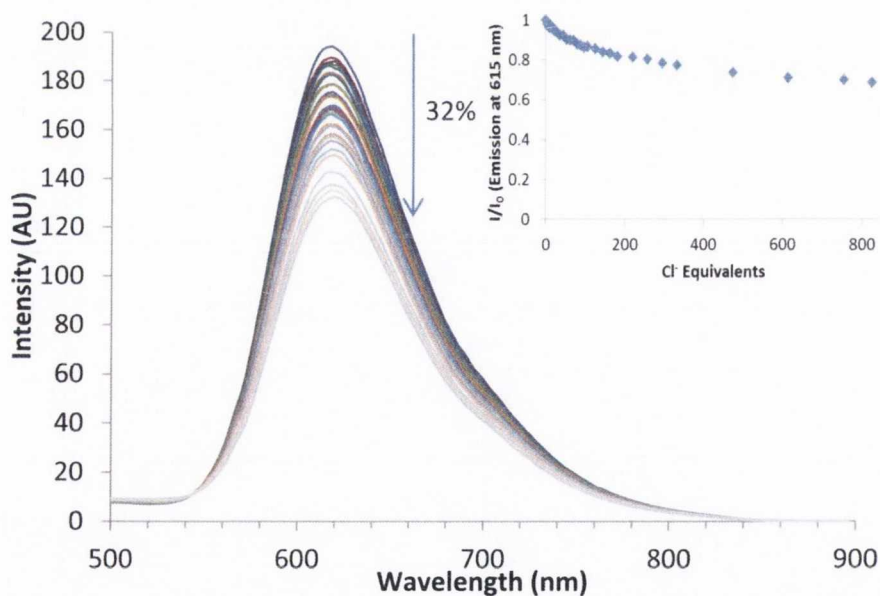
5A 2: The changes in the absorption spectrum of **48** (1.15×10^{-5} M) in MeCN upon titration with AcO^- ($0 \rightarrow 850$ eq., [9 mM]). Inset: The relative absorption decrease at 432 nm as a function of AcO^- concentration.



5A 3: The changes in the emission spectrum of **48** ($1.18 \times 10^{-5} \text{ M}$) in MeCN upon titration with H_2PO_4^- ($0 \rightarrow 615 \text{ eq.}$, $[6.6 \text{ mM}]$). Inset: The integrated fluorescence decrease measured at 615 nm

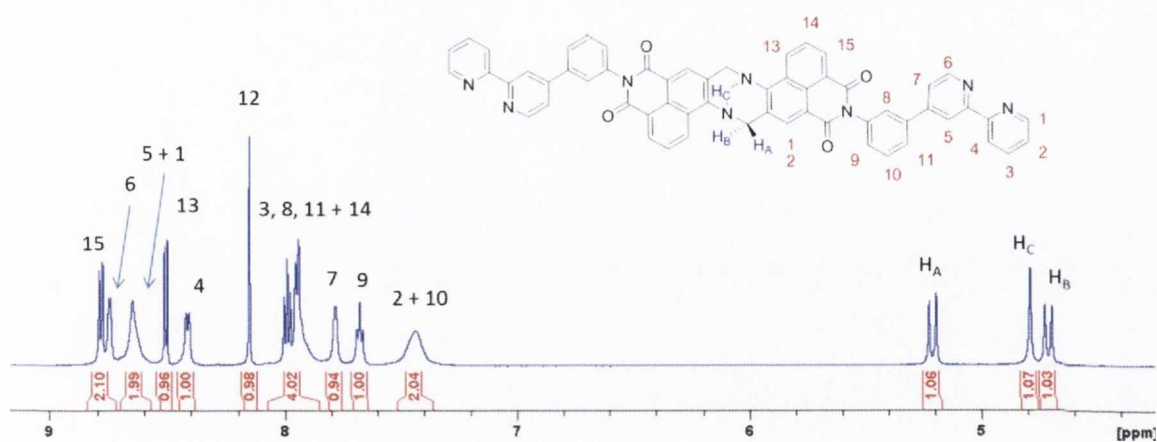


5A 4: The changes in the absorption spectrum of **48** ($1.13 \times 10^{-5} \text{ M}$) in MeCN upon titration with Cl^- ($0 \rightarrow 825 \text{ eq.}$, $[8.5 \text{ mM}]$). Inset: The relative absorption decrease at 432 nm as a function of Cl^- concentration.

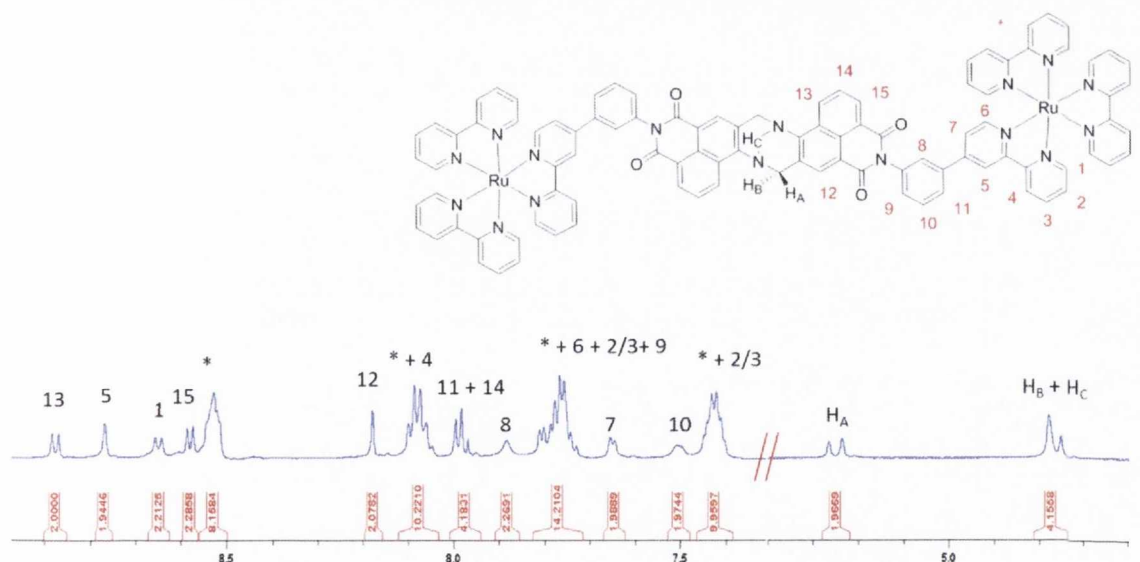


5A 5: The changes in the emission spectrum of **48** ($1.13 \times 10^{-5} \text{ M}$) in MeCN upon titration with Cl^- ($0 \rightarrow 825 \text{ eq.}$, $[8.5 \text{ mM}]$). Inset: The integrated fluorescence decrease measured at 615 nm

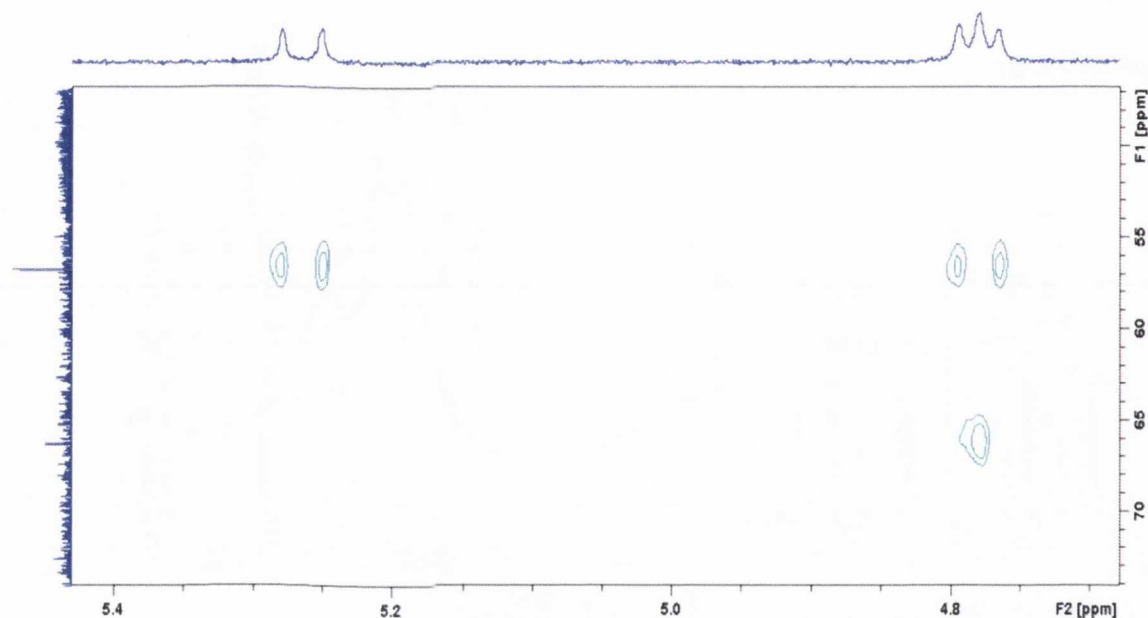
Appendix 6:



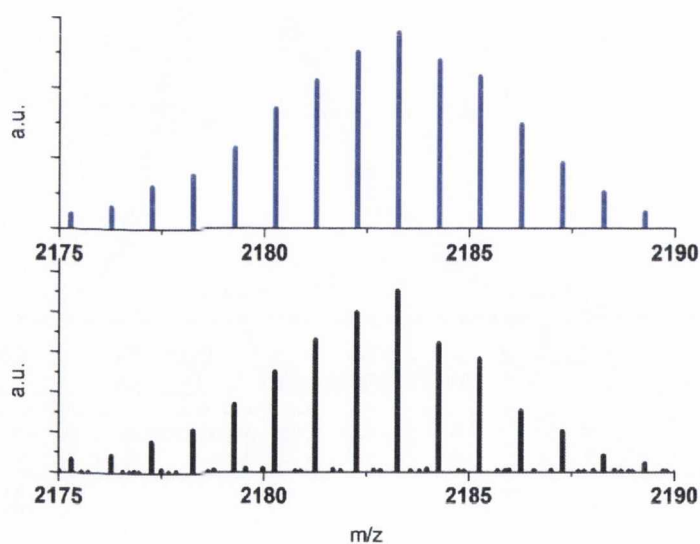
6A 1: The ^1H NMR spectra of **100** (600 MHz, $\text{DMSO}-d_6$)



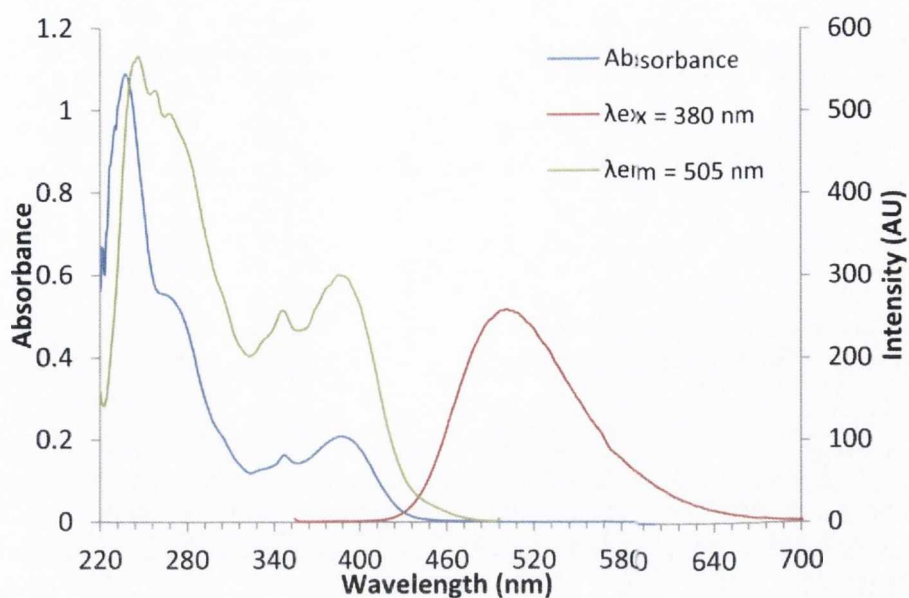
6A 2: The ^1H NMR spectrum of **89** (600 MHz, CD_3CN .)



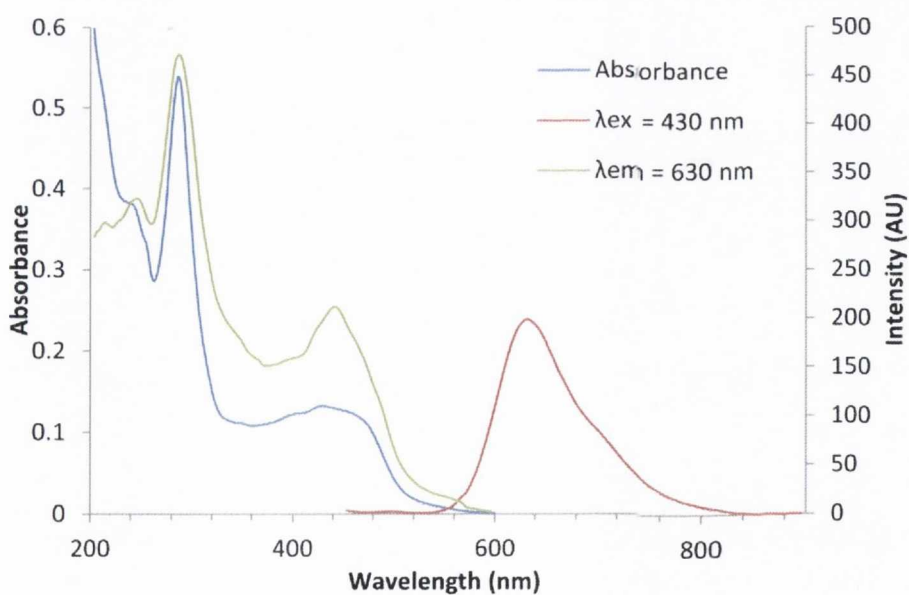
6A 3: The C-H COSY spectrum of **90** (600 MHz, CD_3CN) showing the interaction of the methylene protons of the diazocene bridge with their corresponding ^{13}C resonances



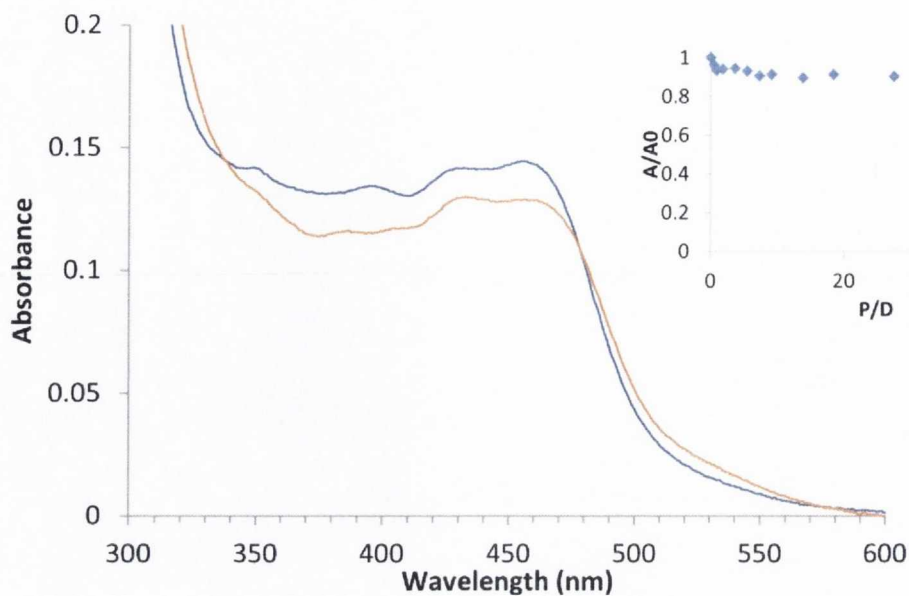
6A 4: Comparison between the calculated (blue) and obtained (black) isotopic distribution pattern for **90** from high resolution mass spectrometry analysis.



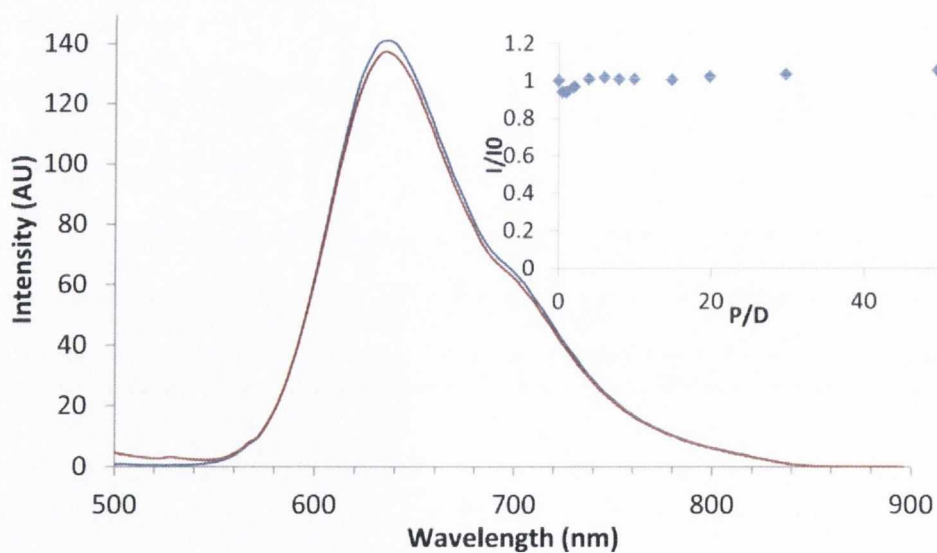
6A 5: The UV/Visible, excitation and emission spectra of **100** in CH_2Cl_2 .



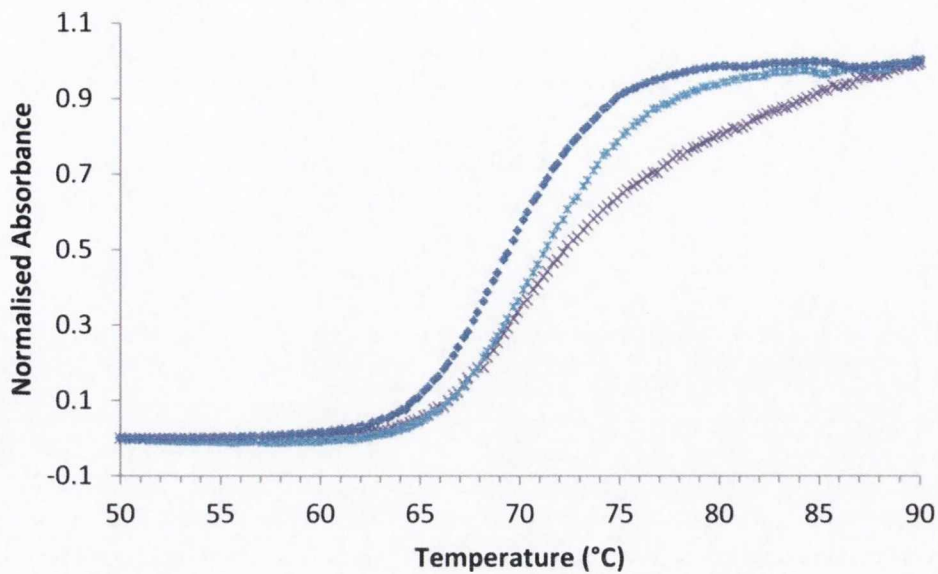
6A 6: The UV/Visible, excitation and emission spectra of **89** in 10 mM phosphate buffer at pH 7.4.



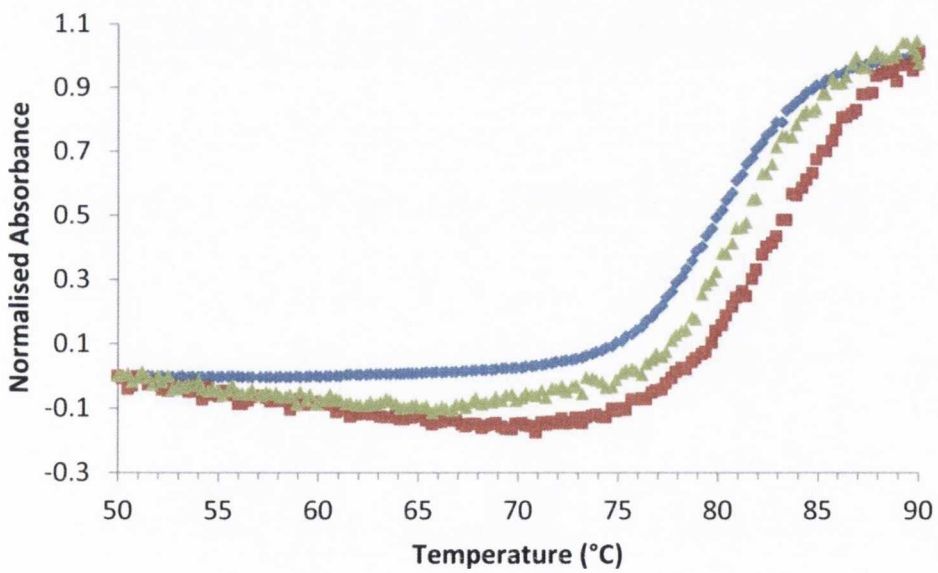
6A 7: Change in the UV/Visible spectrum of **90** ($4\mu\text{M}$) upon addition of stDNA ($0-187\mu\text{M}$). Inset: Plots of the change in integrated MLCT absorption as a function of P/D ratio.



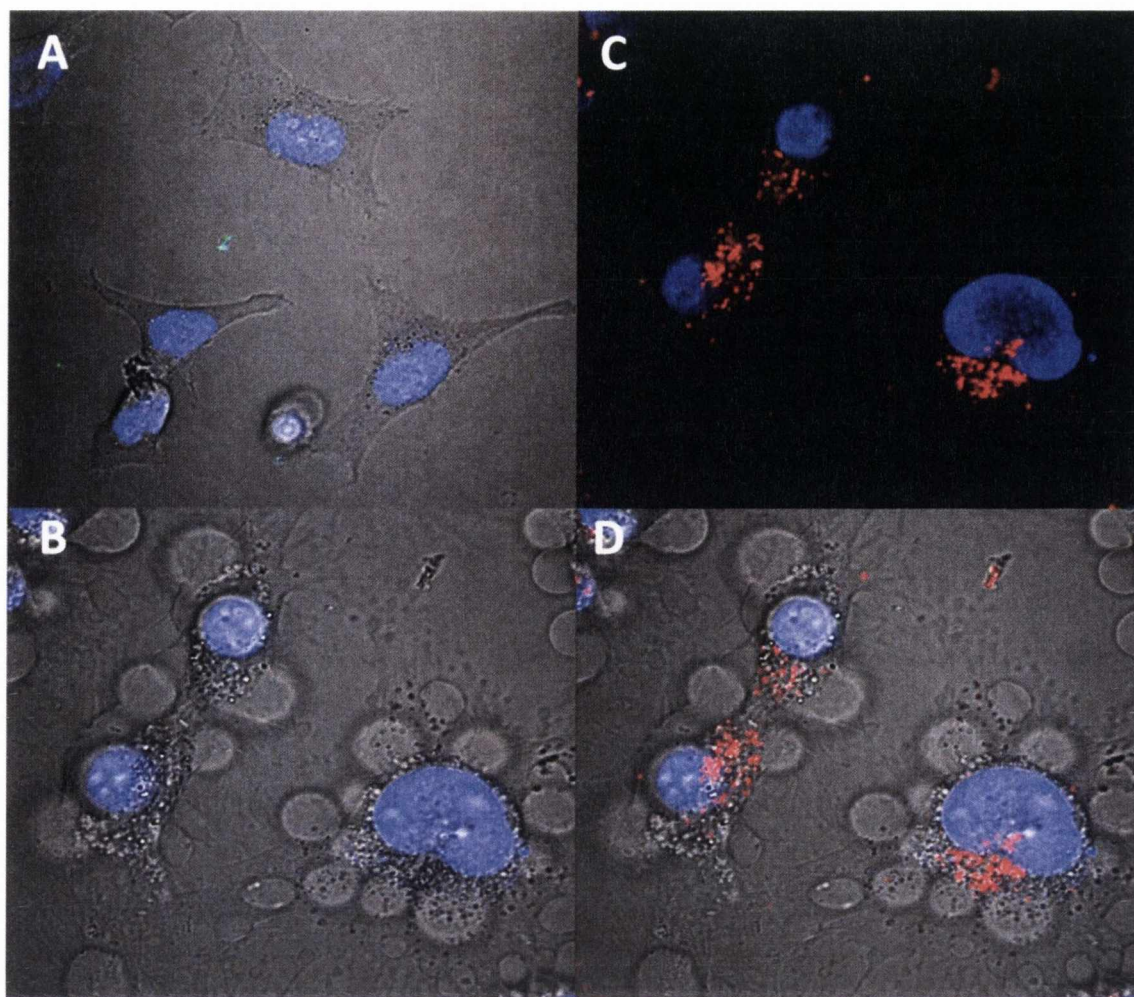
6A 8: Change in the fluorescence emission spectrum of **89** ($\lambda_{\text{ex}} 460\text{ nm}$) ($4\mu\text{M}$) upon addition of stDNA ($0-187\mu\text{M}$). Inset: Plots of the change in integrated MLCT emission as a function of P/D ratio.



6A 9: Thermal denaturation curves of stDNA (150 μM) in 10 mM phosphate buffer, pH 7.4, in the absence (♦) and presence of 89 (×) and 90 (*) at P/D = 25.

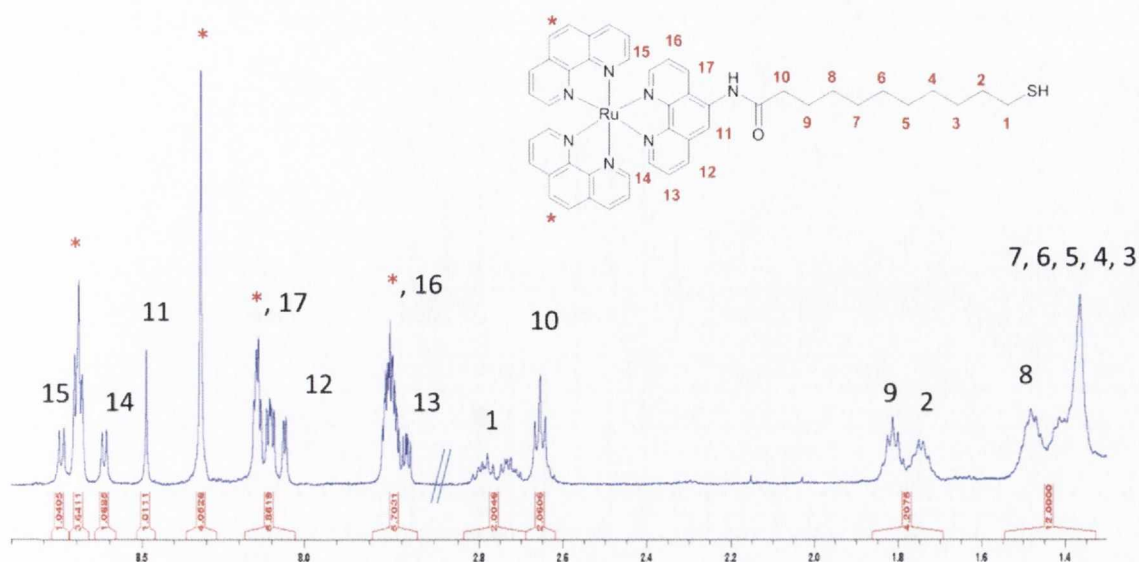
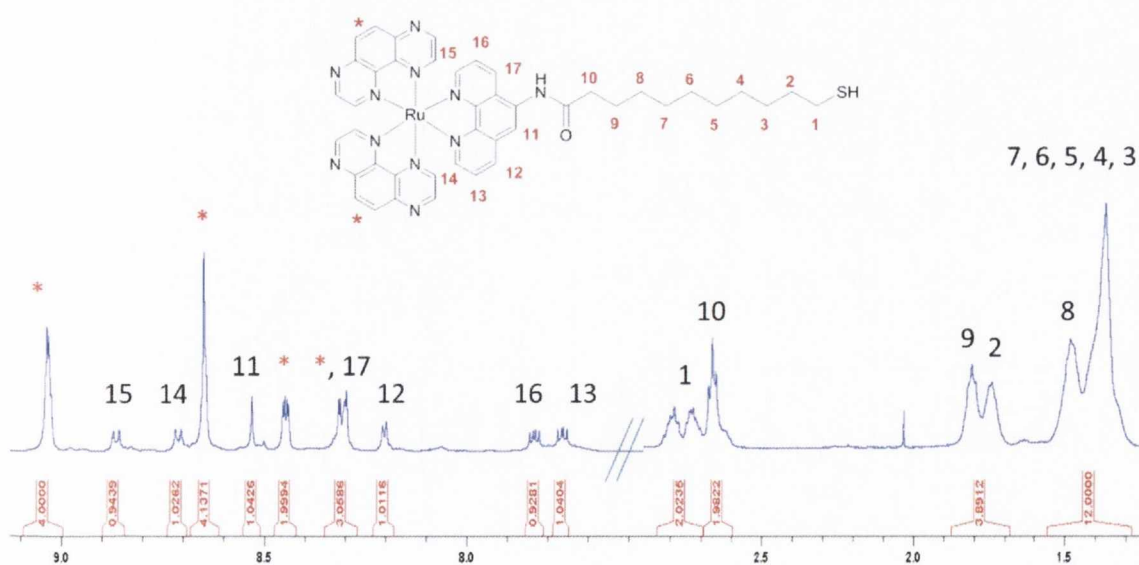


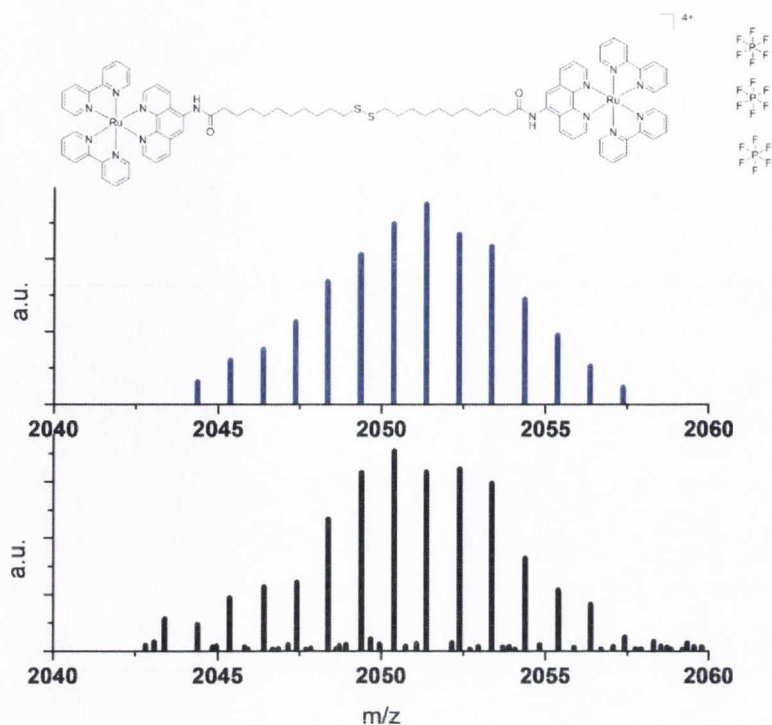
6A 10: Thermal denaturation curves of stDNA (150 μM) in 10 mM phosphate buffer + 50 mM NaCl, pH 7.4, in the absence (♦) and presence of 89 (■) and 90 (▲) at P/D = 10



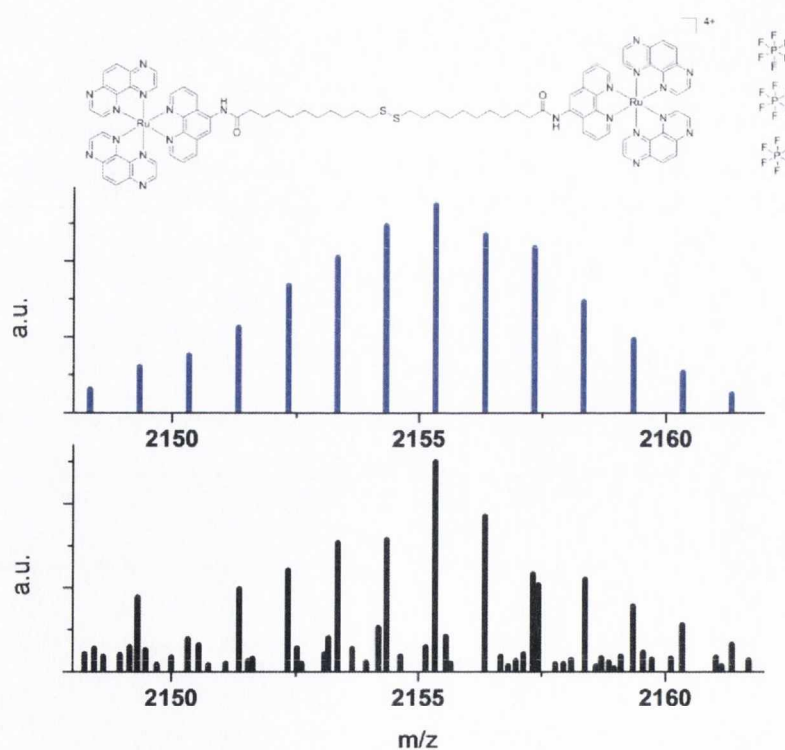
6A 11: Confocal Laser Scanning Microscopy live cell images of **90** (25 μ M) with HeLa cells. Shown is the image obtained with (A) control cells and (B) the bright field view of cells treated with **90** after 4 hrs incubation, stained with DAPI (blue), (C) overlay of **90** (red) and nuclear co-stain DAPI (blue) and (D) overlay of **90** (red) , nuclear co-stain DAPI (blue) and the bright field view.

Appendix 7:

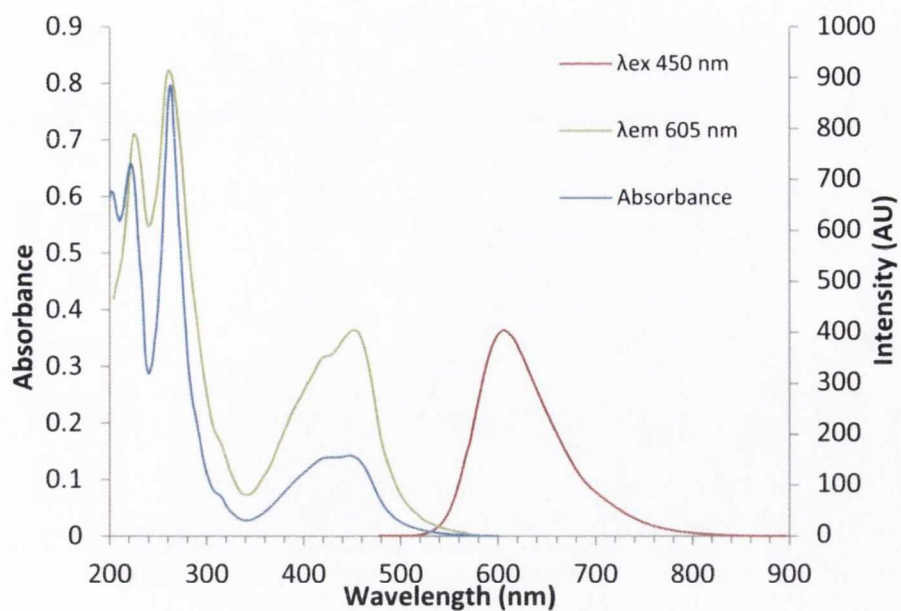
7A 1: ^1H NMR spectra of **106** (600 MHz, MeOD)7A 2: ^1H NMR spectra of **107** (600 MHz, MeOD)



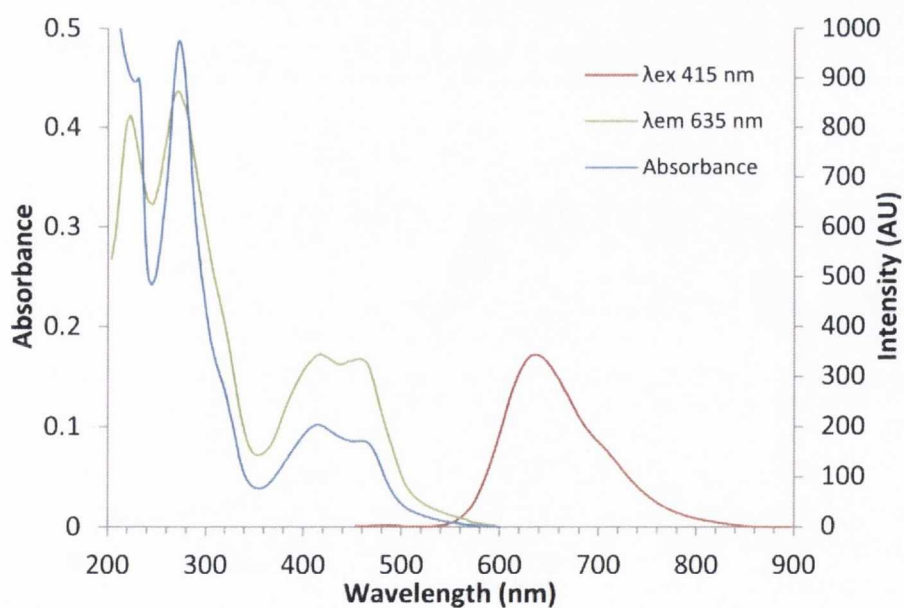
7A 3: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for **105** from high resolution mass spectrometry analysis.



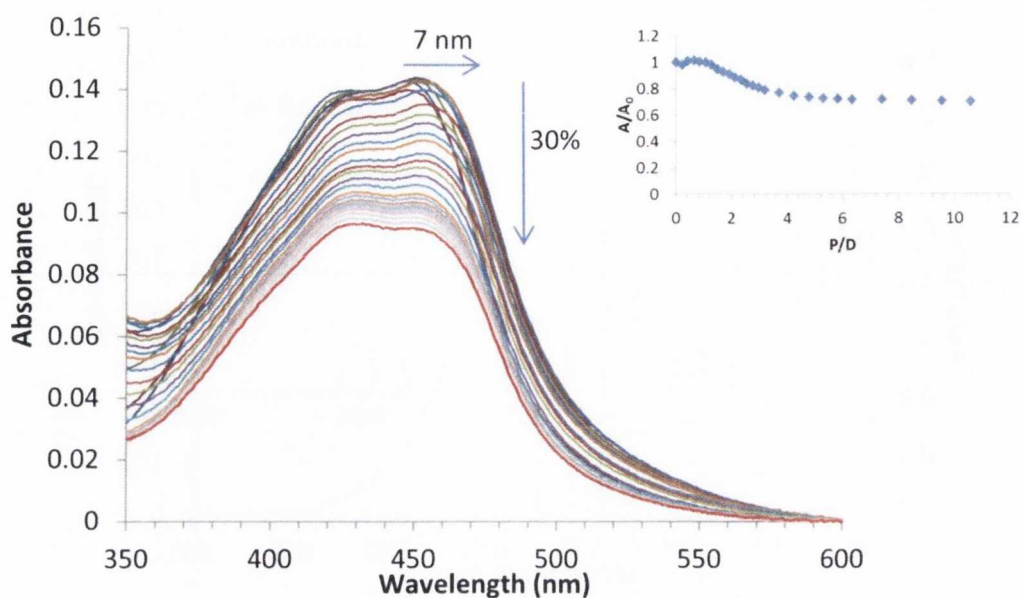
7A 4: Comparison between the estimated (blue) and actual (black) isotopic distribution pattern for **107** from high resolution mass spectrometry analysis.



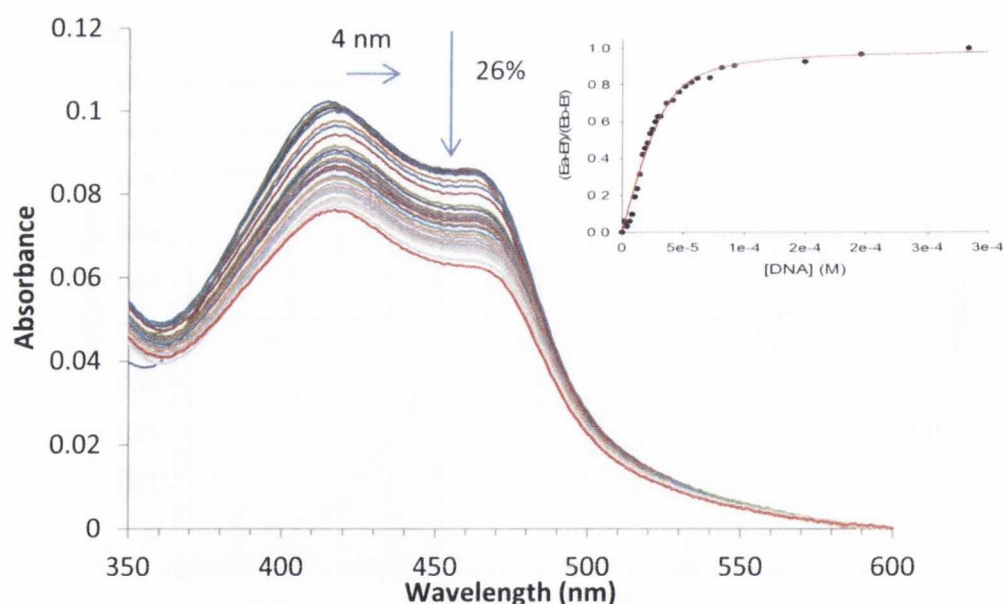
7A 5: UV/Visible, excitation and emission spectra of **106** (10 μ M) in 10 mM phosphate buffer, pH 7.4.



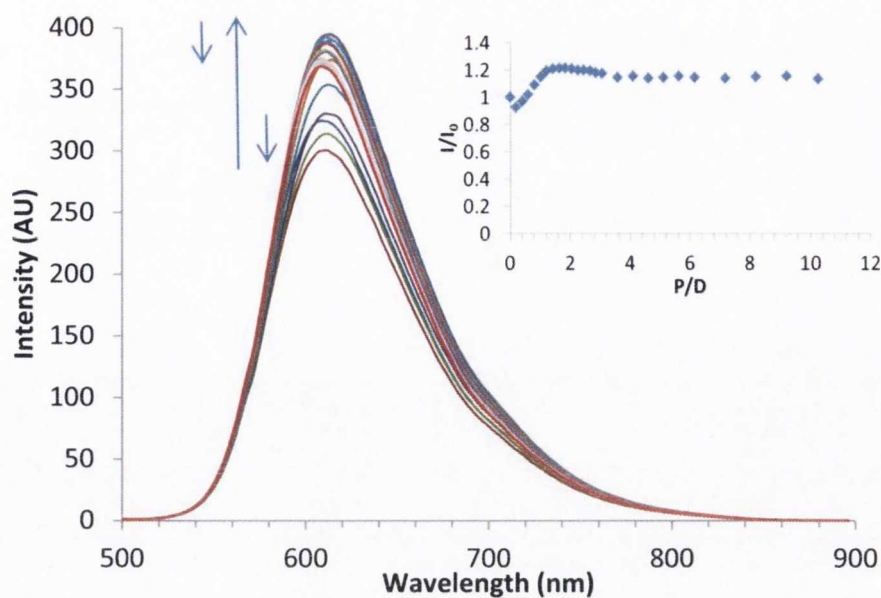
7A 6: UV/Visible, excitation and emission spectra of **107** (10 μ M) in 10 mM phosphate buffer, pH 7.4.



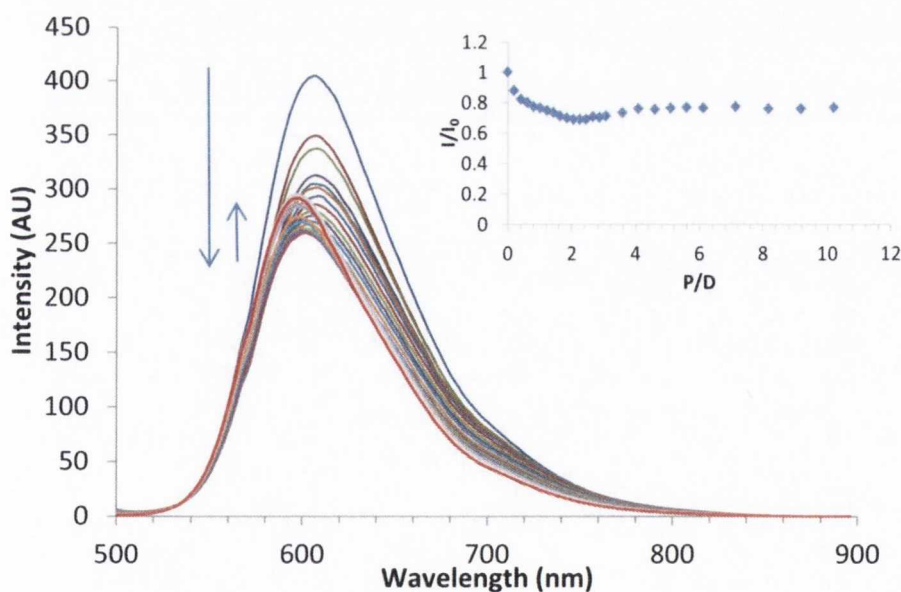
7A 7: Changes in the UV/Visible spectrum of **106** ($10\ \mu\text{M}$) with increasing additions of *stDNA* ($0\text{--}285\ \mu\text{M}$) in 10mM phosphate buffer, ($\text{pH}\ 7.4$). Inset: Plots of the change in integrated MLCT absorption using data with a P/D between $0\text{--}10$.



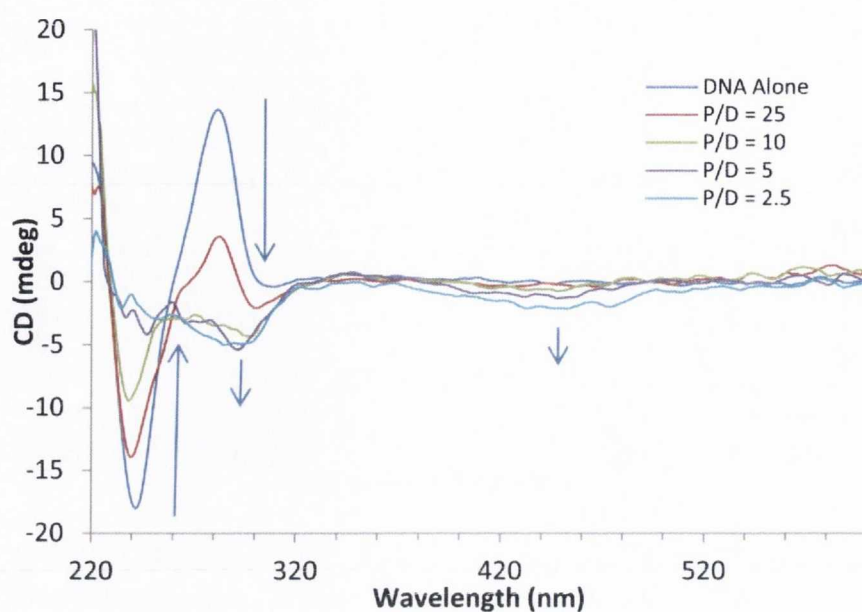
7A 8: Changes in the UV/Visible spectrum of **107** ($10\ \mu\text{M}$) with increasing additions of *stDNA* ($0\text{--}285\ \mu\text{M}$) in 10mM phosphate buffer, ($\text{pH}\ 7.4$). Inset: Plot of $(\epsilon_a - \epsilon_b)/\epsilon_b - \epsilon_b$ vs. $[\text{DNA}] (M^1, P)$ using data with a P/D between $0\text{--}15$ and the best fit of the data (---) using the Bard Eqn.



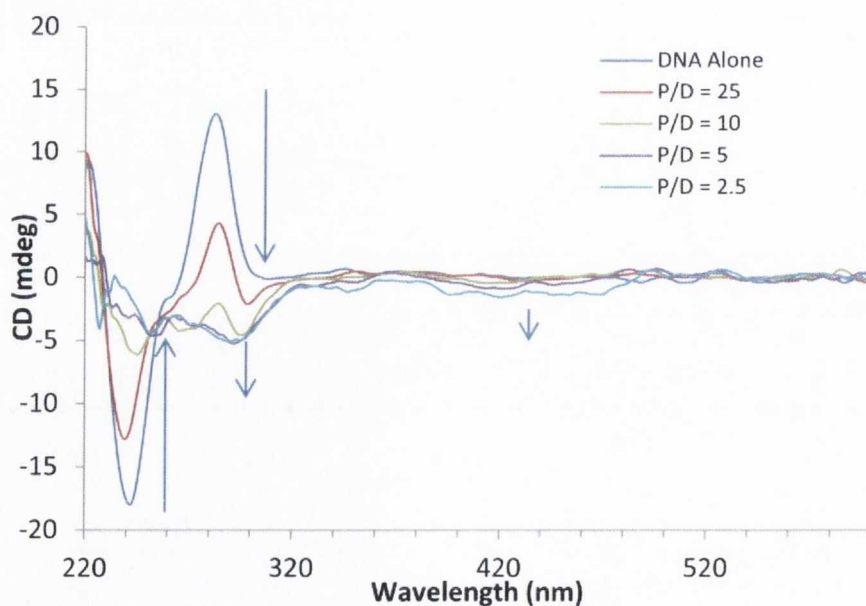
7A 9: Changes in the emission spectrum of **105** ($10\ \mu\text{M}$) ($\lambda_{\text{ex}}\ 450\ \text{nm}$) with increasing concentration of stDNA ($0\text{--}290\ \mu\text{M}$). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



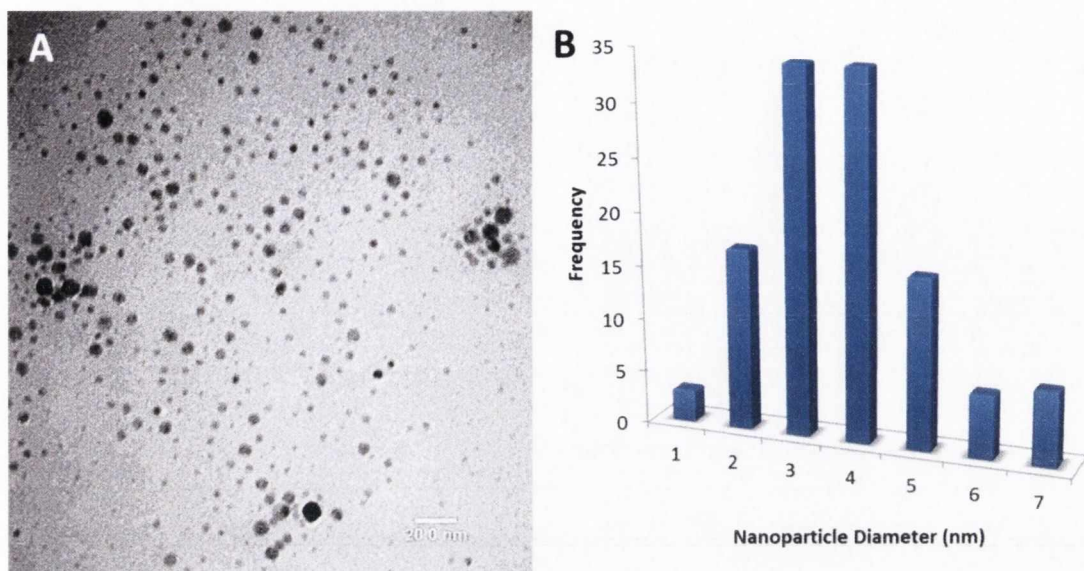
7A 10: Changes in the emission spectrum of **106** ($10\ \mu\text{M}$) ($\lambda_{\text{ex}}\ 450\ \text{nm}$) with increasing concentration of stDNA ($0\text{--}285\ \mu\text{M}$). **Inset:** Plot of the change in integrated MLCT emission intensity as a function of P/D ratio.



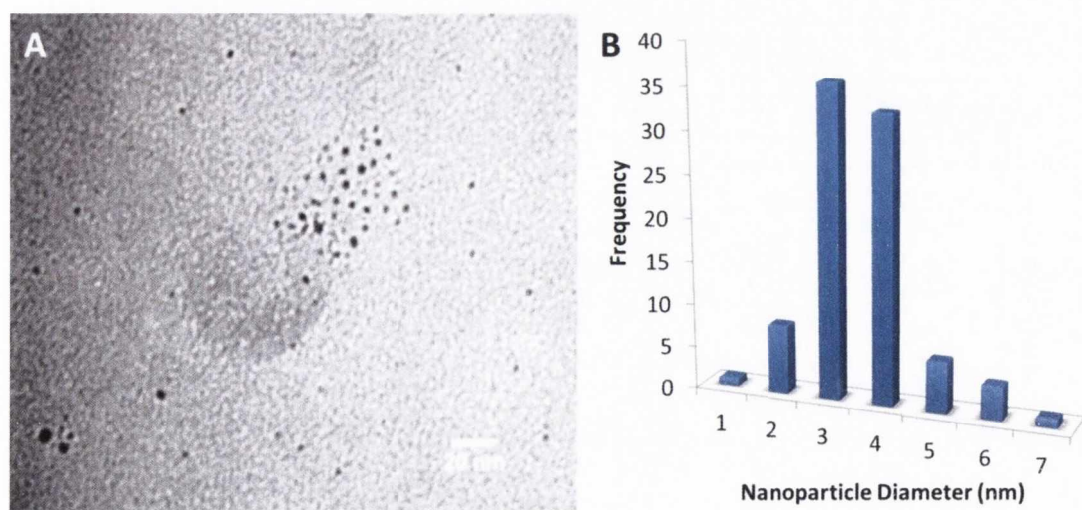
7A 11: Circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **105** at varying ratios.



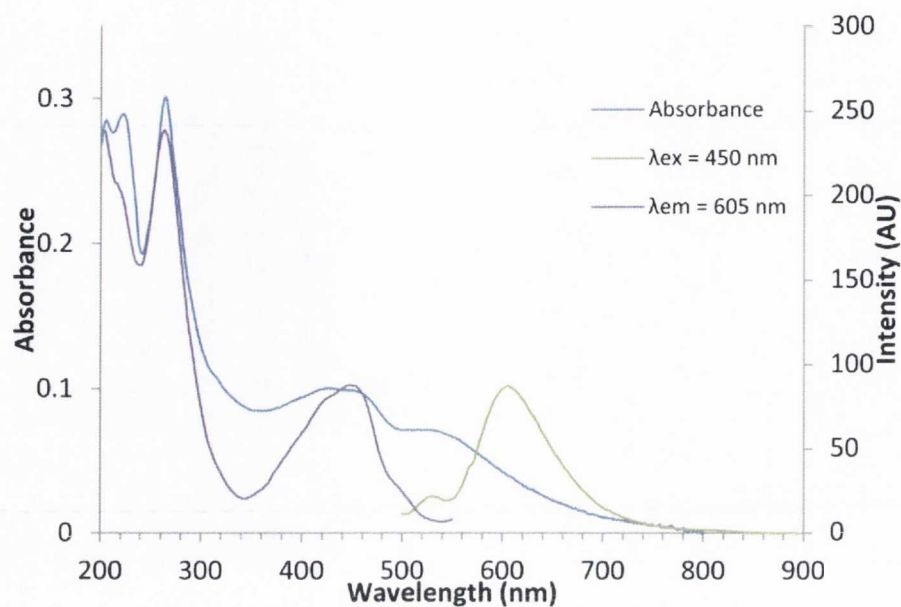
7A 12: Circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **106** at varying ratios.



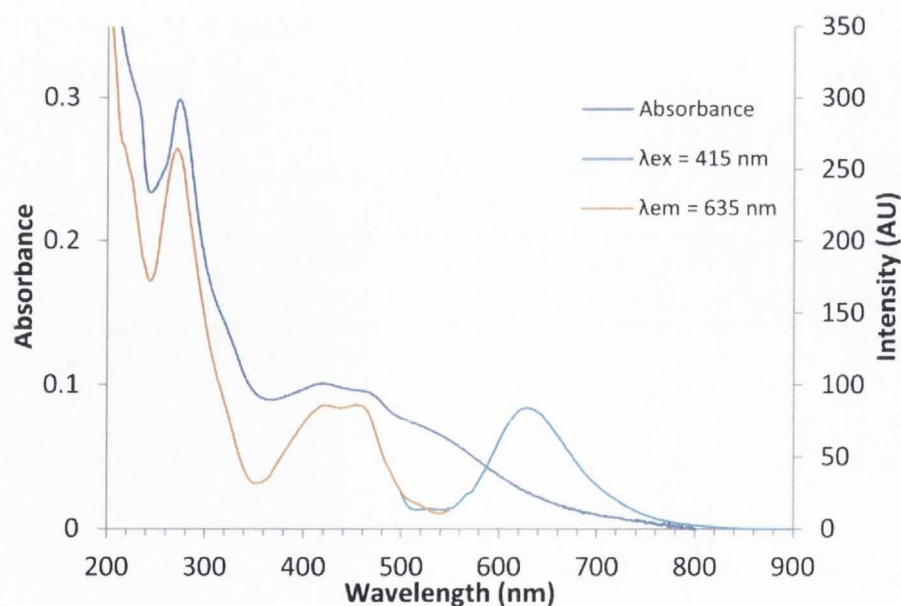
7A 13: (A) TEM image of AuNP-106 after deposition on copper grids and (B) The corresponding particle size distribution calculated from the TEM data.



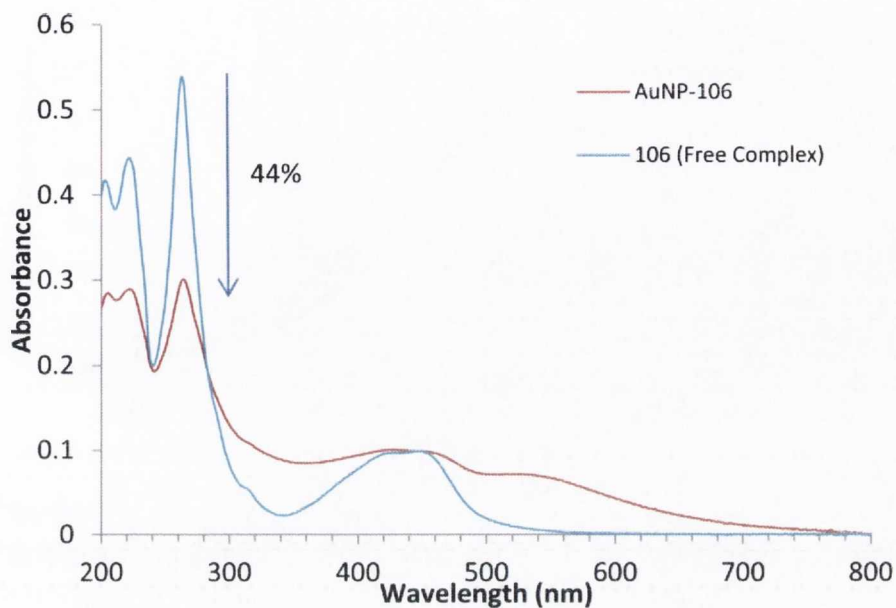
7A 14: (A) TEM image of AuNP-107 after deposition on copper grids and (B) The corresponding particle size distribution calculated from the TEM data.



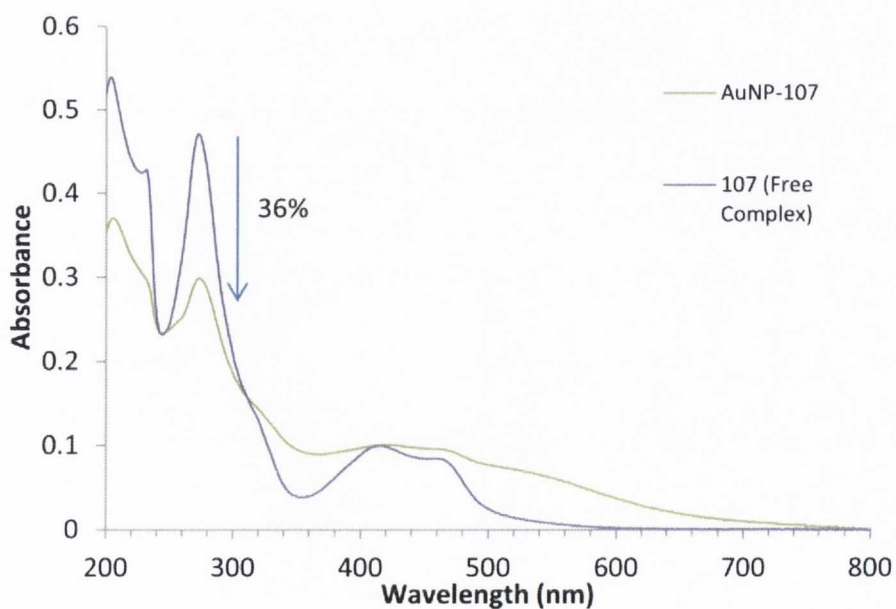
7A 15: UV/Visible, excitation and emission spectra of **AuNP-106** in 10 mM phosphate buffer at pH 7.4



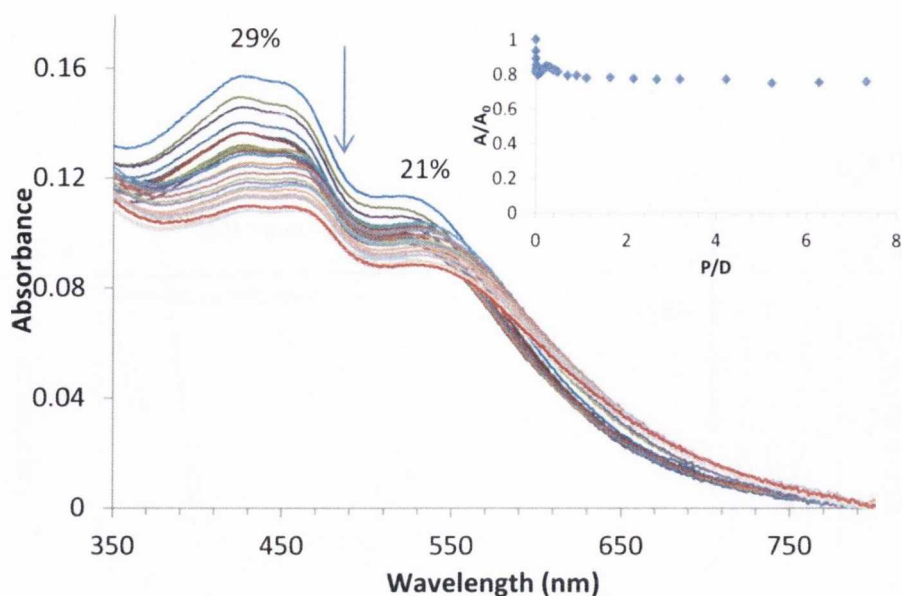
7A 16: UV/Visible, excitation and emission spectra of **AuNP-107** in 10 mM phosphate buffer at pH 7.4



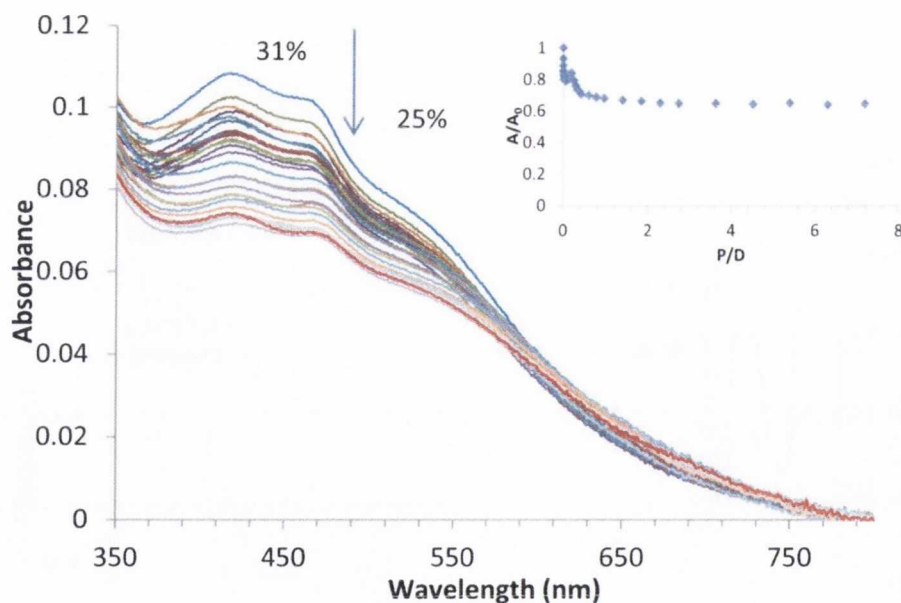
7A 17: UV/Visible spectra showing the comparison between the free complex **106** and the gold conjugates **AuNP-106**



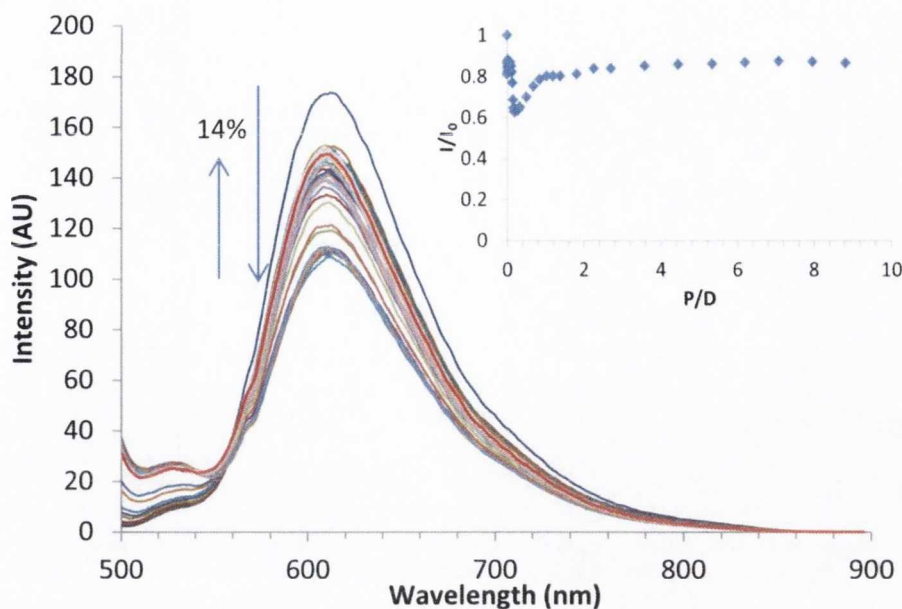
7A 18: UV/Visible spectra showing the comparison between the free complex **107** and the gold conjugates **AuNP-107**



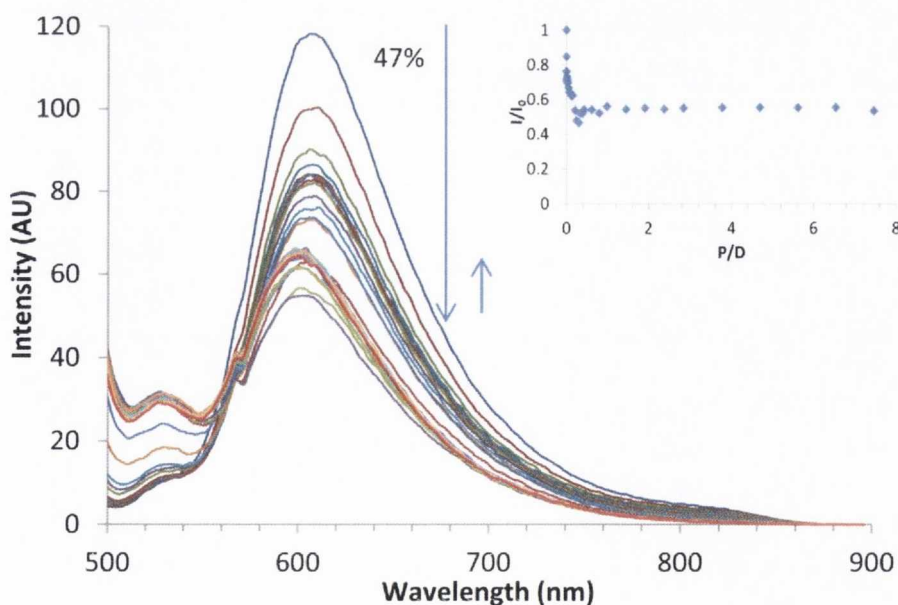
7A 19: Changes in the UV/Visible spectrum of **AuNP-106** ($1.79 \times 10^{-8} M^l$) with increasing additions of stDNA (0-245 μM) in 10mM phosphate buffer, (pH 7.4). Inset: Plot of the change in integrated MLCT absorption as a function of P/D ratio.



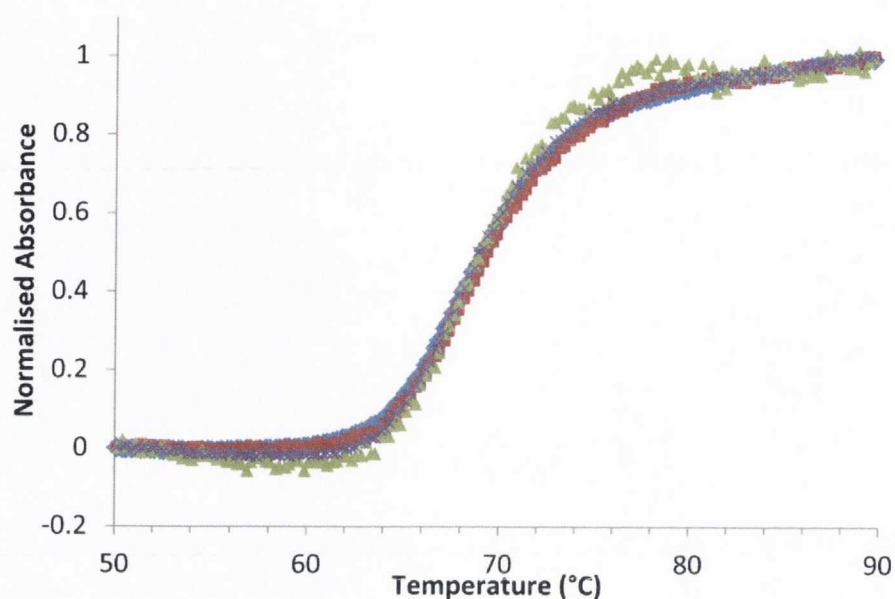
7A 20: Changes in the UV/Visible spectrum of **AuNP-107** ($2.54 \times 10^{-8} M^l$) with increasing additions of stDNA (0-77 μM) in 10mM phosphate buffer, (pH 7.4). Inset: Plot of the change in integrated MLCT absorption as a function of P/D ratio.



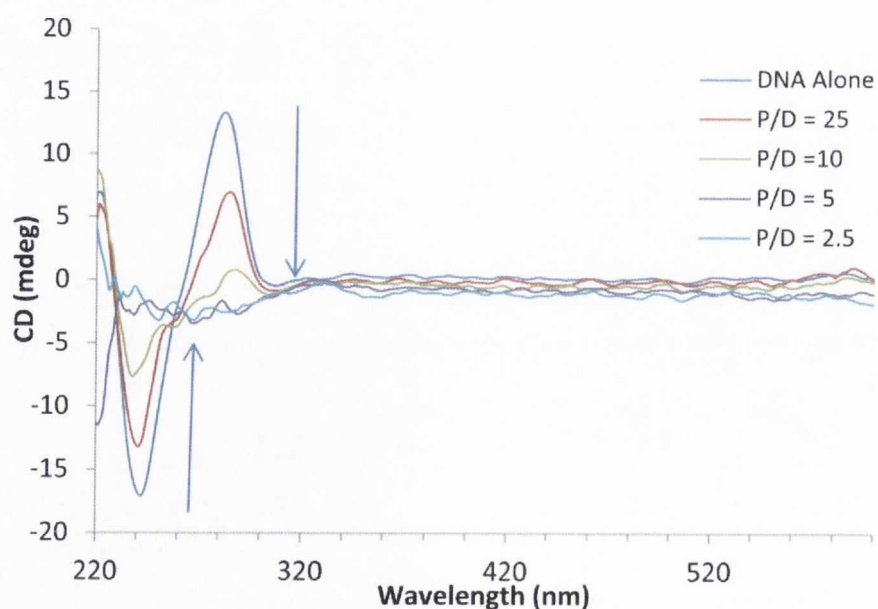
7A 21: Changes in the fluorescence emission spectrum of **AuNP-105** ($1.98 \times 10^{-8} M^I$) with increasing additions of stDNA (0-185 μM) in 10mM phosphate buffer, (pH 7.4). Inset: Plot of the change in integrated MLCT absorption as a function of P/D ratio.



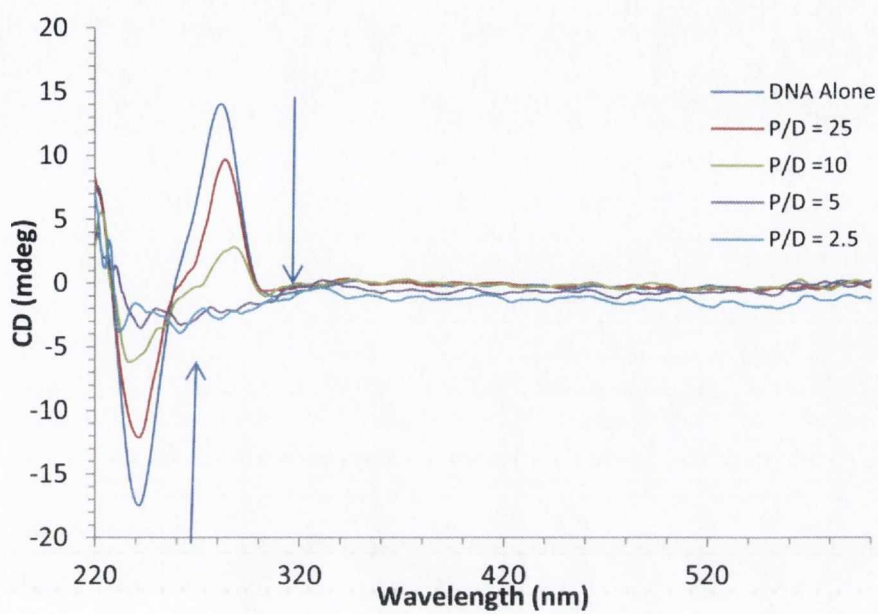
7A 22: Changes in the fluorescence emission spectrum of **AuNP-106** ($1.79 \times 10^{-8} M^I$) with increasing additions of stDNA (0-77 μM) in 10mM phosphate buffer, (pH 7.4). Inset: Plot of the change in integrated MLCT absorption as a function of P/D ratio.



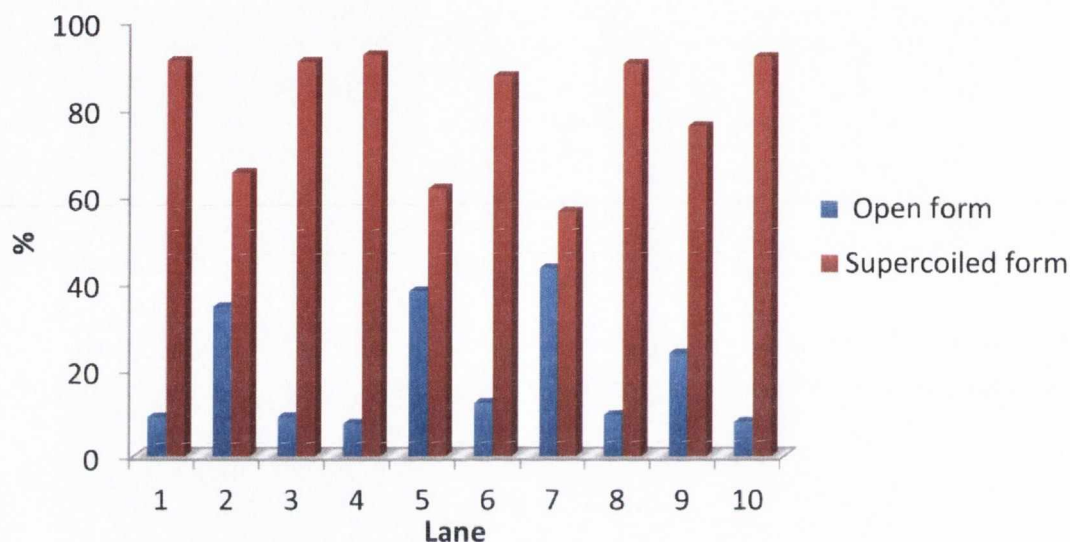
7A 23: Thermal denaturation curves of stDNA (150 μ M) in 10 mM phosphate buffer, pH 7.4, in the absence ($\diamond$) and presence of AuNP-105 ($\blacksquare$), AuNP-106 ($\blacktriangle$) and AuNP-107 ($\times$) at P/D = 5.



7A 24: Circular dichroism spectra of stDNA (150 μ M) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of AuNP-105 at varying ratios.

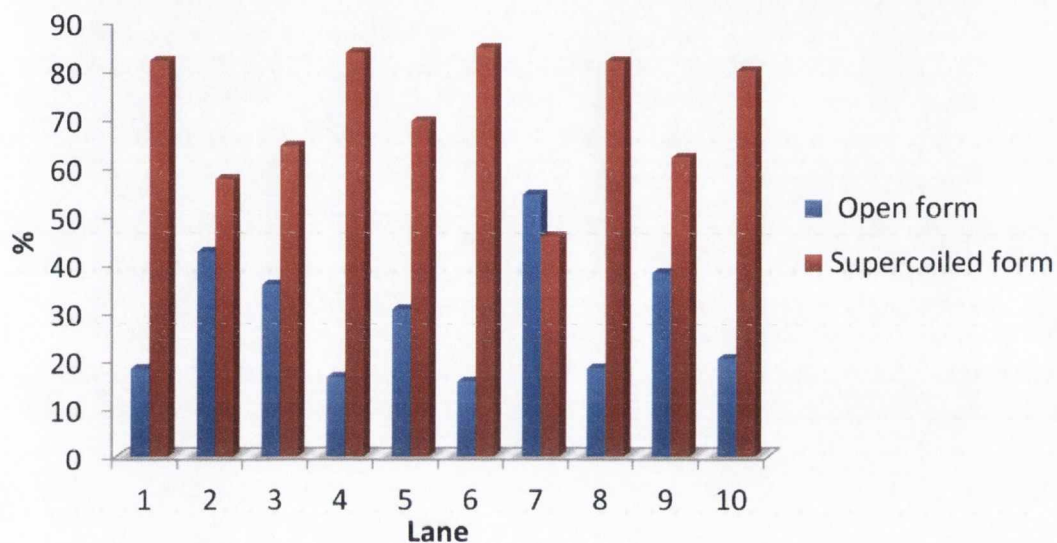
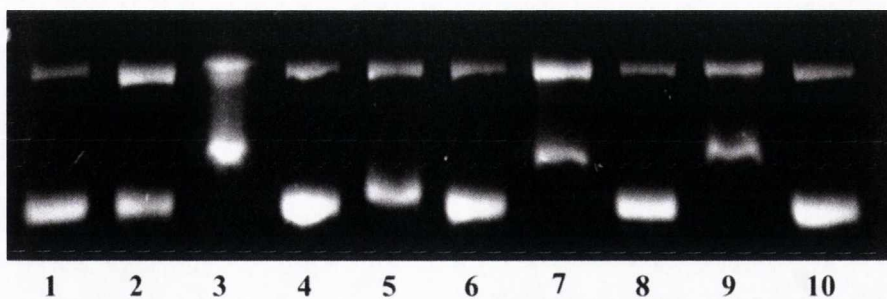


7A 25: Circular dichroism spectra of stDNA (150 μM) in 10 mM phosphate buffer, at pH 7.4, in the absence and presence of **AuNP-107** at varying ratios.



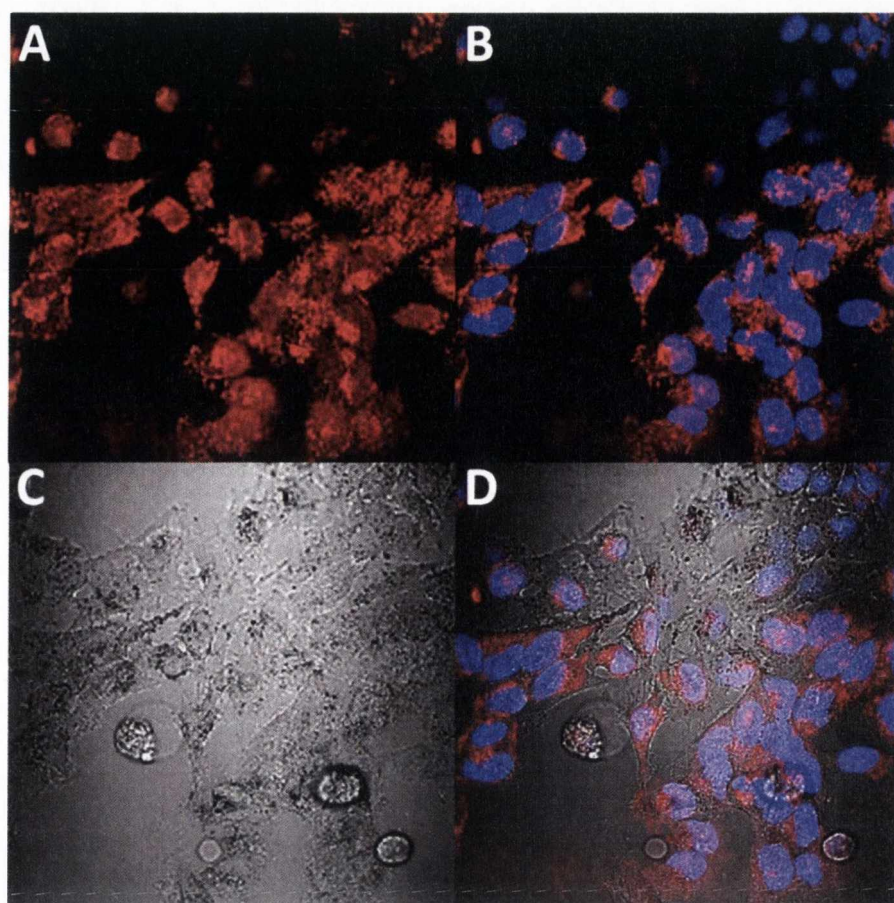
Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	9.1	90.9	6	12.3	87.7
2	34.5	65.6	7	43.5	56.5
3	9.2	90.8	8	9.6	90.4
4	7.6	92.4	9	23.8	76.2
5	38.2	61.8	10	8.1	91.9

7A 26: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after 30 min irradiation (2 J cm^{-1}) in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: $\text{Ru}(\text{bpy})_3^{2+}$ (P/D 20); Lane 3: **105** in the dark (P/D 10); Lane 4 : **AuNP-105** in the dark (P/D 10); Lanes 5-6: **105** and **AuNP-105** (P/D 20); Lanes 7-8: **105** and **AuNP-105** (P/D 10); Lanes 9-10: **105** and **AuNP-105** + 10mM NaN_3 (P/D 10).

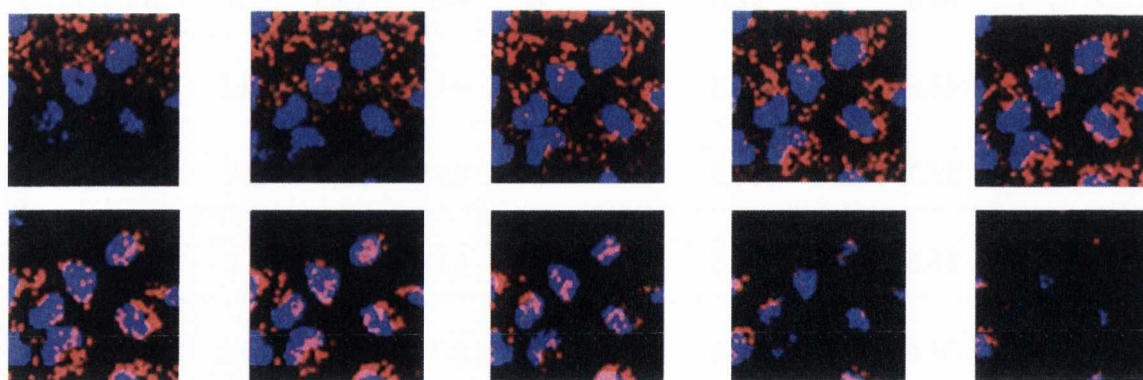


Lane	% Open	% Supercoiled	Lane	% Open	% Supercoiled
1	18.2	81.8	6	15.5	84.5
2	42.5	57.5	7	54.3	45.7
3	35.7	64.3	8	18.3	81.7
4	16.5	83.5	9	38.1	61.9
5	30.6	69.4	10	20.3	79.7

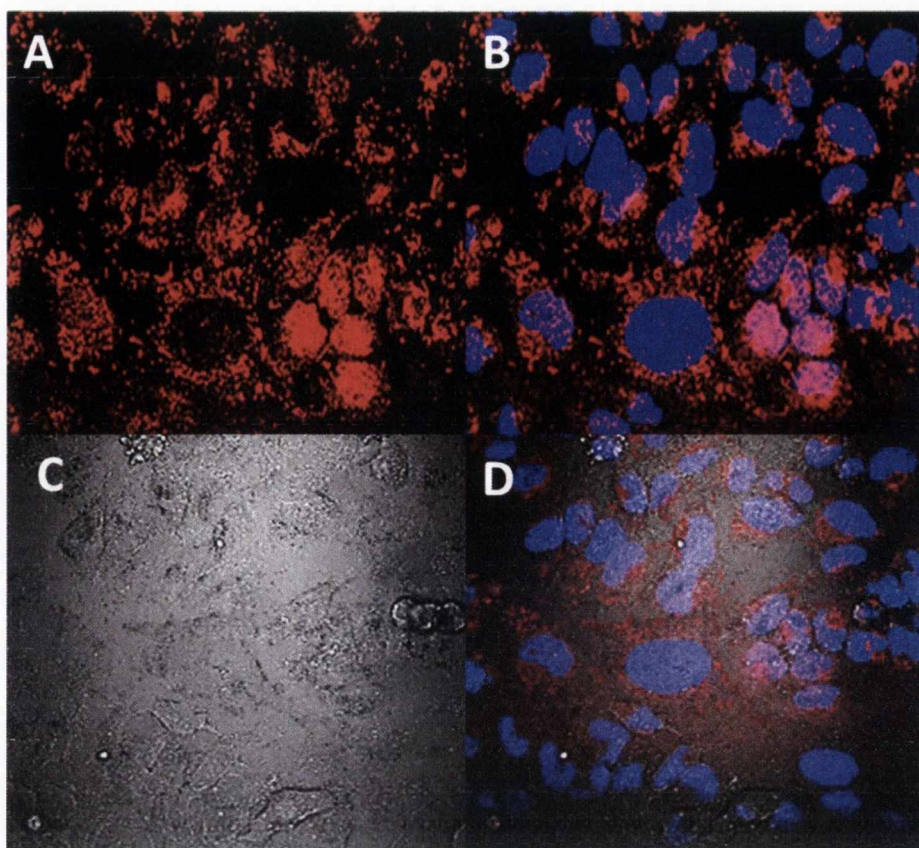
7 A27: Agarose gel electrophoresis of pBR322 DNA (1mg/ml) after 30 min irradiation (2 J cm^{-1}) in 10 mM phosphate buffer, pH 7. 4; Lane 1: Plasmid DNA control ;Lane 2: Ru(bpy)_3^{2+} (P/D 20); Lane 3: **106** in the dark (P/D 10); Lane 4 : **AuNP-106** in the dark (P/D 10); Lanes 5-6: **106** and **AuNP-106** (P/D 20); Lanes 7-8: **106** and **AuNP-106** (P/D 10); Lanes 9-10: **106** and **AuNP-106** + 10mM NaN_3 (P/D 10).



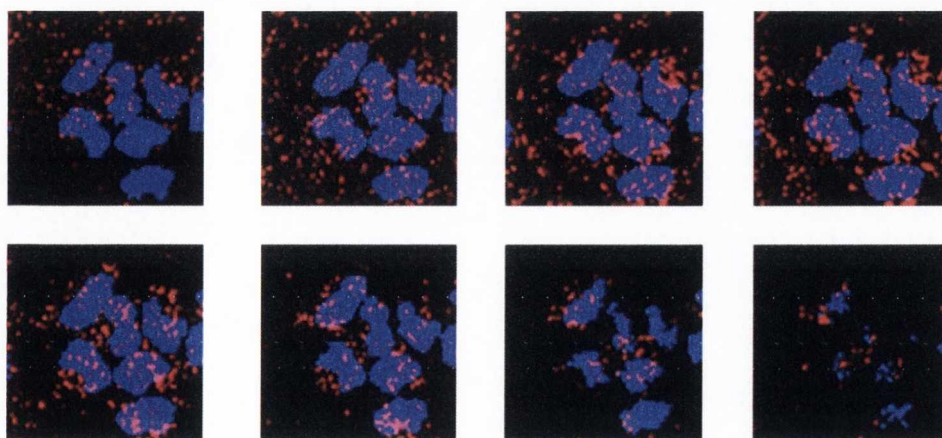
7A 28: Confocal Laser Scanning Microscopy live cell images of **AuNP-106** (20 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **AuNP-106** (red), (B) overlay of **AuNP-106** (red) and nuclear co-stain DAPI (blue), (C) the bright field view and (D) overlay of **AuNP-106** (red), nuclear co-stain DAPI (blue) and the bright field view.



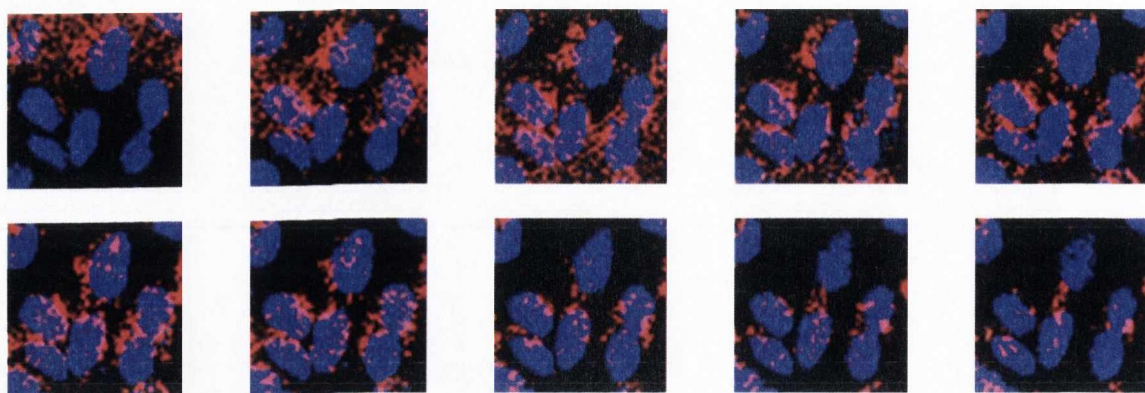
7A 29: Confocal z-section live cell images of **AuNP-106** (20 μ M) with HeLa cells showing luminescence in 3 dimensions. Shown is the image obtained with cells co-stained with nuclear stain DAPI (blue) and **AuNP-106** (red)



7A 30: Confocal Laser Scanning Microscopy live cell images of **AuNP-107** (20 μ M) with HeLa cells. Shown is the image obtained with cells (A) stained with **AuNP-107** (red), (B) overlay of **AuNP-107** (red) and nuclear co-stain DAPI (blue), (C) the bright field view and (D) overlay of **AuNP-107** (red), nuclear co-stain DAPI (blue) and the bright field view.



7A 31: Confocal z-section live cell images of **AuNP-107** (20 μ M) with HeLa cells showing luminescence in 3 dimensions. Shown is the image obtained with cells co-stained with nuclear stain DAPI (blue) and **AuNP-107** (red)



7A 32: Confocal z-section live cell images of **AuNP-105** (20 μ M) with HeLa cells showing luminescence in 3 dimensions. Shown is the image obtained with cells co-stained with nuclear stain DAPI (blue) and **AuNP-105** (red)

Publications