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Nanoparticle & Luminescent Ruthenium Oligonucleotide Conjugates



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

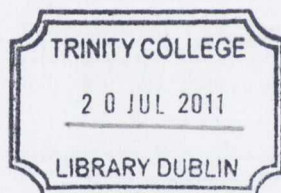
by

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March 2011



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Declaration

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*To my parents, Bernie and Pat
for their love, support and encouragement*

Summary

The field of plasmonics is a rapidly growing area of research and holds much promise for the improvement of diagnostics. The work in this thesis outlines the advances that have been made to assemble the components needed to prepare a potential sensitive DNA detection probe. This entails combining systems involving metal complexes (ruthenium complexes) with nanoparticles (triangular silver nanoplates) using DNA (oligonucleotides).

Chapter 2 details the conjugation of a ruthenium complex at both the 5' and 3' ends of oligonucleotides (ODNs) using oxime ligation. A Ru (II) complex bearing an oxyamine modified phenanthroline ligand was prepared and subsequently coupled with aldehyde modified ODNs. The method improved on previous reports in the literature and is a very efficient, high yielding strategy requiring only small amounts of material. The method was extended, by incorporating an aromatic aldehyde, to remove the harsh oxidation step, thereby allowing the incorporation of other sensitive functional groups. The preparation of a bifunctionalised ODN with a Ru complex at the 5' end and a disulfide at the 3' end is detailed in Chapter 4.

Chapter 3 deals with the preparation of triangular silver nanoplates. The triangular silver nanoplates are shown to degrade in the presence of certain anions and therefore are a challenge to directly functionalise with ODNs. The successful stabilisation of the triangular silver nanoplates using either 16-mercaptohexadecanoic acid or titania coating is detailed. The functionality introduced offers a route for ODN conjugation and Chapter 5 deals with the preparation of ODNs to use with these stabilised nanoplates. ODNs modified with a long-chain alkyl thiol and a catechol are described.

Chapter 4 details the conjugation of gold nanoparticles with thiolated ODNs. Both spherical gold nanoparticles (Au-ODN) and triangular AuAg nanoboxes (AuAg-ODN) were functionalised with ODN sequences. The assemblies formed from hybridisation of each of the systems with its complement were investigated and analysed. Interesting nanoassemblies were formed by combining the Au-ODN with complementary AuAg-ODN. Spherical gold nanoparticles were functionalised with a bifunctionalised ODN, with a 5'-Ru and a 3'-disulfide.

Acknowledgements

The work detailed in this thesis could not have been achieved without the help and support of many people and to each of you I am deeply grateful.

First and foremost, I would like to thank my supervisor Prof. John M. Kelly for giving me the opportunity to carry out this PhD. Your help, guidance and support throughout my studies, has been greatly valued. I can only hope that I have acquired some of your vast knowledge and enthusiasm for chemistry.

The DNA synthesis and oximation reactions described in this thesis were carried out as a collaborative effort with Prof. Eric Defrancq in Université Joseph Fourier, Grenoble. I would like to thank Prof. Defrancq for all his help and guidance over the last few years. I also wish to acknowledge the members of the Defrancq research team; Sumana, Pierre and Romaric for making my visits to Grenoble so productive and enjoyable.

Dr. Ger Doorley and Dr. Deirdre Ledwith deserve special mention for their help and advice on all things chemistry. Even long after their departure from the Kelly group they have obliged in answering my many questions. Dr. Sibin Padmanabhan and Dr. Damian Aherne are acknowledged for their advice and input into the titania coating and nanoparticle ODN conjugation work, respectively. Dr. Susan Quinn has always provided me with good advice and useful literature.

I would like to thank all the technical and administrative staff in the School of Chemistry. Dr. John O'Brien and Dr. Manuel Ruether are acknowledged for the running of all my NMR samples. A special mention goes to Dr. Martin Feeney for the running of all mass spectrometry samples, especially his assistance and patience with acquiring the MALDI MS of all ODNs. I am very grateful to Neal Leddy in the CMA for his assistance with TEM. Leanne Moore in the Department of Genetics is thanked for her help with, and access to, the fluorescence microplate reader. Raman measurements were carried out in Queens University Belfast. Dr. Steven Bell and his students are acknowledged for their assistance and useful discussions.

As the Kelly group office empties it is with great fondness that I can remember busier days. I would like to extend my thanks to all the past and present Kelly Group members: Dave, Deirdre, Damian, Ger, Sibú, Páraic, Kyra, Marie-Helene, Suni, Michal, Matthew, Swagata, Jayden and Michael for creating such a great atmosphere and ensuring that there was never a dull moment. For all the chat, antics, coffee breaks, after work drinks and general abuse. Cheers guys! It will be sometime before I forget our escapades to Sicily and Poland.

I am very grateful for all the help and support my friends have given me over the years. A special mention must go to Leanne and Lisa who have always obliged in lending me an ear. Your advice and support has been constant and it is greatly appreciated. Thanks for all the well timed lunches, coffee breaks, gigs, movies and dinners, ensuring I kept my sanity, particularly towards the end. We've had many memorable adventures and I'm sure there are many more to come.

Last, but by no means least, I would like to thank my family. My brothers who forever endeavor to keep me grounded and my parents for their constant support and encouragement. When things were toughest you were always there and I will be eternally grateful for that.

Table of Contents

Declaration	i
Dedication	ii
Summary	iii
Acknowledgements	iv
Table of contents	vi
List of Figures	xv
List of Schemes	xxv
List of Tables	xxviii
Abbreviations	xxix

Chapter 1: Introduction

1.1 Preface	1
1.2 Motivation - A Potential Sensitive Molecular Beacon	2
1.3 Ruthenium Polypyridyl Complexes – Our Choice of Fluorophore	3
1.4 DNA – The Ideal Molecular Ruler	5
1.4.1 DNA Structure	5
1.4.2 Assembly of Nanostructures using DNA	6
1.5 Metal Nanoparticles	8
1.5.1 Optical Properties of Metal Nanoparticles - LSPR	8
1.5.2 Mie Theory and Discrete Dipole Approximation	9
1.5.3 Why Silver?	10
1.5.4 Why Shaped?	11
1.5.5 Triangular Silver Nanoplates – Our Choice of Nanoparticle	12
1.5.5.1 Nanoparticle Preparation–Focus on Triangular Silver Nanoplates	12
1.5.5.2 Selected Triangular Silver Nanoplates – Aherne <i>et al.</i>	13
1.6 Metal Nanoparticle ODN Conjugates	15
1.7 Fluorescence	15
1.8 Fluorescence Resonance Energy Transfer (FRET)	16
1.9 Quenching of Fluorescence by Metals	17
1.10 Metal Enhanced Fluorescence	18

1.11 Molecular Beacons	19
1.11.1 Molecular Beacons – Improvement of Sensitivity with the Incorporation of Metal Nanoparticles	20
1.12 Aims and Overview of Thesis	22
1.13 References	23

Chapter 2: Preparation of Ruthenium Oligonucleotide Conjugates

2.1 Introduction	27
2.2 DNA Synthesis	27
2.3 Preparation of Modified Oligonucleotides	28
2.4 Oligonucleotide Fluorophore Conjugates	28
2.5 Ruthenium Oligonucleotide Conjugates	29
2.5.1 ‘On-Column’ Preparation of Ruthenium Oligonucleotide Conjugates	29
2.5.2 ‘Off-Column’ Preparation of Ruthenium Oligonucleotide Conjugates	34
2.5.3 Stability of Ruthenium Modified Oligonucleotides	39
2.6 Advantages and Disadvantages of ‘On-Column’/ ‘Off-Column’ Approaches	40
2.7 The Advent of ‘Click Chemistry’– An ‘Off-Column’ Approach	40
2.8 Oxime Ligation	41
2.9 Overview of Results	43
2.10 Synthesis of an Oxyamine Modified Phenanthroline Ligand	43
2.11 Synthesis of a Ruthenium Complex	45
2.12 Synthesis of Aldehyde Modified Oligonucleotides	49
2.13 Synthesis of a Phosphoramidite for the 5’ Introduction of an Aldehyde	49
2.14 Automated DNA Synthesis of 5’-Diol Oligonucleotide	51
2.15 Conjugation of 3’-Aldehyde-ODNs with an Oxyamine Modified Ruthenium Complex	52
2.15.1 Automated DNA Synthesis of 3’-diol Modified Oligonucleotides	52
2.15.2 Deprotection of the Reactive Oxyamine on the Ruthenium Complex	53
15	
2.15.3 Oxidation of 3’-Diol to 3’-Aldehyde	54
2.15.4 Conjugation of the 3’-Aldehyde ODN with the Ru Complex	54
2.15.5 Purification and Analysis of 3’ Ru-ODN Conjugates	55
2.16 The use of Aromatic Aldehyde Modified ODNs	59

2.17 Conjugation of 5'-Aldehyde-ODNs with Oxyamine Modified Ruthenium complex	60
2.17.1 Automated DNA Synthesis of 5'-Aldehyde Oligonucleotides	60
2.17.2 Removal of the Acetal Protecting Group	61
2.17.3 Conjugation of the 5'-Benzaldehyde ODNs with the Ru Complex	61
2.17.4 Purification and Analysis of 5'-Ruthenium Oligonucleotide Conjugates	61
2.18 Conclusions	63
2.19 References	65

Chapter 3: Preparation, Stability and Functionalisation of Triangular

Silver Nanoplates

3.1 Introduction	68
3.2 Aims	68
3.3 Stabilisation of Metal Nanoparticles	68
3.4 Instability of Silver Nanoparticles	70
3.5 Etching of Silver Nanoparticles by Halide Ions	70
3.6 Methods of Stabilisation of Silver Nanoparticles	75
3.6.1 Thiol Capping of Silver Nanoparticles	75
3.6.2 Silica Coating	76
3.6.3 Gold Coating	76
3.6.4 Titania Coating	77
3.6.4.1 Why TiO ₂ Coating?	77
3.6.4.2 Titania Coating of Nanoparticles	78
3.6.4.2.1 Characterisation of Titania Coated Nanoparticles	80
3.7 Results	81
3.8 Preparation of Triangular Silver Nanoplates	81
3.8.1 Seed Preparation	82
3.8.2 Triangular Nanoplate Growth	84
3.9 Concentration of a Typical Triangular Ag Nanoplate Preparation	86
3.10 Stability of 'As-Prepared' Triangular Ag Nanoplates	87
3.10.1 Stability of 'As-Prepared' Triangular Nanoplates to Halide Anions	89
3.11 Thiols for Stabilising Triangular Silver Nanoplates	91

3.11.1 16-Mercaptohexadecanoic Capping	91
3.11.2 Capping with Other Thiol Molecules	98
3.11.3 Discussion on Thiol Stabilisation of Triangular Silver Nanoplates	99
3.12 TiO ₂ Coating of Triangular Silver Nanoplates	100
3.12.1 Coating of Citrate Stabilised Silver Nanoplate Sol	100
3.12.2 Development of a Concentrated Triangular Ag Nanoplate Preparation	101
3.12.2.1 Preparation of a Ten Times Concentrated Triangular Silver Nanoplate Sol	101
3.12.2.2 Preparation of a Five Times Concentrated Triangular Silver Nanoplate Sol	102
3.12.3 Titania Coating of Concentrated Citrate Stabilised Ag Nanoplate Sol	103
3.12.3.1 Summary and Discussion of Titania Coating of Citrate Stabilised Ag Nanoplates	108
3.12.4 Titania Coating of Concentrated MHA Capped Ag Nanoplate Sol	109
3.12.4.1 Analysis of Titania Coated MHA Capped Silver Nanoplates	110
3.12.4.2 Discussion on Titania Coating of MHA Capped Silver Nanoplates	116
3.13 Conclusions	117
3.14 References	119

Chapter 4: Nanoparticle Oligonucleotide Conjugates

4.1 Introduction	122
4.2 General Strategies for the Preparation of Nanoparticle ODN Conjugates	122
4.3 Gold Nanoparticle ODN Conjugates	122
4.4 Properties of Conjugates	125
4.4.1 Melting Temperature of DNA	125
4.4.2 Melting Temperature of Nanoparticle-ODN Conjugate Assemblies	126
4.4.3 Preparation and Adjustments in the Preparation of Au-ODN Conjugates	127
4.4.4 Measurement of Surface Loading and Hybridisation	128
4.4.5 Stability of Nanoparticle-ODN Conjugates	129
4.4.6 Size and Shape of Particles	129

4.5 Silver nanoparticle ODN conjugates	130
4.6 Assemblies of Nanoparticle-ODN Conjugates	135
4.7 Use of Nanoparticle-ODN Conjugates for Detection and Medicine	135
4.8 Overview of Results	136
4.9 Spherical Gold Nanoparticle ODN Conjugates	137
4.9.1 Preparation of Spherical Gold Nanoparticles	137
4.9.2 Choice of ODN Sequences	139
4.9.3 Synthesis of 5'-Sulfhydryl Modified ODNs	139
4.9.4 Cleavage of Trityl Protecting Group	141
4.9.5 Calculation of Required Amount of ODN for Functionalisation	141
4.9.6 Preparation of ODN Nanoparticle Conjugates	142
4.9.7 Hybridisation of Complementary Conjugates	143
4.9.8 Analysis of the Hybridisation by UV-Vis Spectroscopy	144
4.9.9 Analysis of the Hybridisation by Dynamic Light Scattering	145
4.9.10 Analysis of the Hybridised Conjugates by TEM	146
4.9.11 Analysis of the Hybridised Conjugates by Thermal Denaturation	147
Experiments	
4.9.12 Quantification of the Number of ODNs on Gold Nanoparticles	148
4.9.13 Summary and Discussion of Gold Nanoparticle-ODN Conjugates	150
4.10 Bifunctionalised ODN for Nanoparticle Labelling	150
4.10.1 Preparation of a Bifunctional ODN with a 3'-Disulfide and 5'-Benzaldehyde	151
4.10.2 Removal of the Acetal Protecting Group	151
4.10.3 Conjugation of the 5'-Benzaldehyde of 37A with a Ru Complex	152
4.10.4 Purification and Analysis of Ru-ODN-SS	153
4.10.5 Investigation of Effect of Gold Nanoparticles on the Emission of Ru(bpy) ₃	154
4.10.6 Conjugation of Ru-ODN with Spherical Gold Nanoparticles	156
4.10.7 Analysis of the Ru-ODN-Au Conjugate	158
4.10.8 Discussion of the Ru-ODN-Au Conjugate Section	162
4.11 AuAg Nanobox ODN Conjugates	163
4.11.1 Preparation of Hollow AuAg Nanoboxes	163
4.11.2 Amount of ODN Needed	165

4.11.3 Functionalisation of AuAg Nanoboxes with 5'-Thiol Modified ODNs	165
4.11.4 Hybridisation of Two Complementary Nanobox Conjugates	167
4.11.5 Analysis of the Thermal Denaturation of the Nanobox ODN Assembly	168
4.11.6 TEM Analysis of the Nanobox ODN Assembly	170
4.11.7 Effect of Increasing Salt Concentration on the Nanobox ODN Assembly	171
4.11.8 Summary and Discussion of Nanobox-ODN Conjugate Assemblies	172
4.12 Non-Crosslinking Melting of AuAg Nanobox-ODN Conjugate	173
4.13 Assembly of Spherical Gold Nanoparticles with AuAg Nanoboxes	174
4.13.1 Preparation and Analysis of Nanobox-ODN and Au-ODN Assembly	175
4.13.2 TEM Analysis	177
4.13.3 Discussion of Assembly of Nanobox-ODN and Au-ODN	177
4.14 Overall Summary and Conclusions	178
4.15 References	180
4.16 Appendix	183
 Chapter 5: Modified Oligonucleotides for Nanoparticle Conjugation	
5.1 Introduction	185
5.2 Synthesis of ODNs Modified with a Long Sulfhydryl Alkyl Chain	185
5.2.1 Incorporation of a C ₁₆ Sulfhydryl Unit on the 5' End of ODNs by an 'On-Column' Approach	186
5.2.2 Protection and Activation of 16-mercaptohexadecanoic acid	186
5.2.3 Preparation of 5'-Amino Modified Oligonucleotides and Choice of Sequences	187
5.2.4 Confirmation of Successful 5'-Amino Modification	189
5.2.5 Deprotection of the MMT Protecting Group	190
5.2.6 Coupling	193
5.2.6.1 Coupling with Test Sequence 42A	193
5.2.6.2 Coupling of 41B and 41C	195
5.2.7 Discussion and Conclusion	197
5.3 Catechol Modified ODN	197

5.3.1 Initial Coupling Reaction	199
5.3.2 Synthesis of tert-butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate	199
5.3.3 Removal of the Boc Protecting Group	201
5.3.4 Choice of ODN Sequences	202
5.3.5 Preparation of ODNs	202
5.3.6 Oxidation of Diol to Aldehyde	203
5.3.7 Deprotection of tert-butyl-2-(3,4-dihydroxyphenethylamino)-2-oxoethoxycarbamate	203
5.3.8 Coupling of ODN 58 with 59	203
5.3.9 Analysis and Purification of Catechol ODN Conjugate 52	204
5.3.10 Summary on Catechol Modified ODN	205
5.4 Future Work	205
5.5 Conclusions	206
5.6 References	206
 Chapter 6: Experimental	
6.1 Reagents and Solvents	207
6.2 Instrumentation	207
6.2.1 Raman	207
6.2.2 UV-Vis Spectroscopy	207
6.2.3 Photoluminescence	207
6.2.4 Infrared Spectroscopy	207
6.2.5 Mass Spectrometry	208
6.2.6 Nuclear Magnetic Resonance Spectroscopy	208
6.2.7 DNA Synthesis	208
6.2.8 Transmission Electron Microscopy	208
6.2.9 Dynamic Light Scattering	208
6.2.10 Zeta Potential	209
6.2.11 Speed-vac	209
6.2.12 HPLC	209
6.3 Methods	210
6.3.1 5-amino-1,10-phenanthroline (6)	210

6.3.2 N-(tert-butoxycarbonyl)glycine anhydride	210
6.3.3 2-Boc-amino-N-[1,10]-phenanthroline-5-yl-acetamide (7)	211
6.3.4 2-amino-N-[1,10]-phenanthroline-5-yl-acetamide (8)	211
6.3.5 N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine	212
6.3.6 NHS ester of N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (10)	212
6.3.7 2-Boc-aminoxy-N-[(1,10)-phenanthroline-5-ylcarbamoylmethyl]-acetamide.(PF ₆) ₂ (1)	213
6.3.8 Ru(bpy) ₂ Cl ₂ .2H ₂ O (12)	213
6.3.9 Attempted Synthesis of Ru(bpy) ₂ (acetone) ₂ . (13)	214
6.3.10 Ru(bpy) ₂ [2-Boc-aminoxy-N-(1,10)phenanthroline-5-ylcarbamoylmethyl] acetamide.(PF ₆) ₂ (15)	214
6.3.11 4-[2-Phenyl-(1,3)-dioxolan-4-yl]-butanol-1-ol (17)	215
6.3.12 Phosphoramidite (18)	215
6.3.13 Automated ODN Synthesis in Dublin (19)	216
6.3.14 Desalting of Oligonucleotide (19) in Preparation for HPLC	216
6.3.15 Ru(bpy) ₂ [2-aminoxy-N-(1,10)phenanthroline-5-ylcarbamoylmethyl]acetamide.(PF ₆) ₂ (2)	216
6.3.16 General Procedure for Automated ODN Synthesis (Grenoble)	217
6.3.17 General Procedure for Oxidation of 3' diol-ODN to 3' aldehyde-ODN	217
6.3.18 General Procedure for Coupling Reaction Between Aldehyde-ODN and Ru Complex (2)	217
6.3.19 General Procedure for Polyacrylamide Gel Electrophoresis (PAGE) Purification of ODNs	217
6.3.20 General Procedure for MALDI-TOF MS Analysis of Oligonucleotides	218
6.3.21 Triangular Silver Nanoplate Preparation	218
6.3.21.1 Preparation of Silver Seed Particles	218
6.3.21.2 Preparation of Triangular Silver Nanoplates	218
6.3.21.3 Five Times Concentrated Preparation of Triangular Silver Nanoplates	219
6.3.22 Anion Etching Experiments	219
6.3.23 MHA Capping of Triangular Silver Nanoplates	219

6.3.24 TiO ₂ Coating of Triangular Silver Nanoplates	219
6.3.25 Preparation of Spherical Gold Nanoparticles	219
6.3.26 General Procedure for Cleavage of Trityl Protected Sulfhydryl ODN	220
6.3.27 General Procedure for Preparation of Gold-ODN Conjugates	220
6.3.28 Oligreen Assay	220
6.3.29 Thermal Denaturation of Nanoparticle ODN Assemblies	221
6.3.30 Thermal Denaturation of Free Duplex ODN	221
6.3.31 Preparation of Hollow Triangular AuAg Nanoboxes	221
6.3.32 Triangular AuAg Nanobox ODN Conjugates	221
6.3.33 16-tritylmercaptohexadecanoic acid (39)	222
6.3.34 Succinimidyl 16-tritylmercaptohexadecanoate (40)	222
6.3.35 'On-Column' ODN Coupling Reaction	223
6.3.36 Preparation of tert-butyl-2-(3,4-dihydroxyphenethylamino)-2 oxoethoxycarbamate (53)	223
6.3.37 Deprotection of tert-butyl-2-(3,4-dihydroxyphenethylamino)-2 oxoethoxycarbamate	223
6.4 References	224
 Chapter 7: Conclusions and Future Work	
7.1 Conclusions	225
7.2 Future Work	226

List of Figures

Figure 1.1 Schematic illustration of a variety of biodetection strategies using biofunctionalised nanomaterials. Taken from reference ⁷	1
Figure 1.2 Common heteroaromatic ligands	4
Figure 1.3 A representation of a multifunctional Ru polypyridyl complex	5
Figure 1.4 DNA helix showing nanometric dimensions (left) and Watson-Crick base pairing (right) of B- DNA	6
Figure 1.5 Holliday junction- intermediate in genetic recombination	7
Figure 1.6 Examples of supramolecular DNA assemblies (a) 3 ODN arm based 2D network ³⁶ (b) DNA triangle ³⁷ (c) Tetrahedral DNA assembly ³⁸ (d) 6 ODN based supramolecular assembly ³⁹ (e) Cyclic DNA ⁴⁰	7
Figure 1.7 Schematic drawing showing the oscillation of the free electron cloud upon interaction with an electromagnetic field. Image taken from reference. ⁴³	8
Figure 1.8 Calculated UV-Vis extinction (black), absorption (red) and scattering (blue) spectra of a variety of different shaped silver nanoparticles illustrating the effect of the particle shape on the spectral characteristics. Adapted from reference ⁴⁶	10
Figure 1.9 E-field enhancement contours external to shaped silver nanoparticles (a) a triangular nanoplate (b) a nanorod. The arrows indicate the region of maximum field intensity. Adapted from reference ⁵¹	11
Figure 1.10 Picture of triangular nanoplate with the particle dimensions marked.	12
Figure 1.11 Extinction spectrum of nanoprism (100 nm edge length and 16 nm thick) with snips of 0, 10 and 20 nm obtained using DDA calculations. Taken from reference ⁴²	13
Figure 1.12 (a) Photograph and UV-Vis spectra of silver seeds and corresponding triangular silver nanoplates prepared with different volumes of seed particles 1) 650 μ l, 2) 500 μ l, 3) 400 μ l, 4) 260 μ l, 5) 200 μ l, 6) 120 μ l, 7) 90 μ l, 8) 60 μ l, 9) 40 μ l, 10) 20 μ l. (b) TEM image of flat lying triangular silver nanoplates. (c) TEM image of stacked triangular silver nanoplates. (d) UV-Vis spectrum of triangular nanoplates shown in B. The λ_{max} is at 825 nm. Images taken from reference. ⁶⁶	14
Figure 1.13 Jablonski diagram illustrating the molecular processes involved in absorption and emission of light by a fluorophore. k_f is the emissive rate constant	16

of the fluorophore, k_{isc} is the rate constant for the intersystem crossing and k_{nr} is the non-radiative decay rate constant.

Figure 1.14 Spectral overlap required for fluorescence resonance energy transfer.	17
Figure 1.15 (a) Graphical representation of molecular beacon system in its closed and open conformations. (b) Schematic of the working principle of a molecular beacon. Taken from reference ¹⁵	20
Figure 1.16 Schematic of molecular beacon immobilised on a metal film. The metal acts as the quencher. Taken from reference ⁴⁹	21
Figure 1.17 Schematic of multi-analyte molecular beacon. Adapted from reference ⁸³	22
Figure 2.1 A general cycle for automated DNA synthesis	27
Figure 2.2 Normalized absorbance and emission spectra ($\lambda_{ex} = 450$ nm) of $Ru(bpy)_2$ [2-Boc-aminoxyl-N-(1,10) phenanthroline-5-ylcarbamoylmethyl]-acetamide].(PF ₆) ₂ 15 (1×10^{-4} M) in MeOH at room temperature.	48
Figure 2.3 ³¹ P NMR of phosphoramidite 18	51
Figure 2.4 Commercially available CPG solid support 20 and the four 3' diol modified ODN sequences prepared, 21A , 21B , 21C and 21D	53
Figure 2.5 RP-HPLC profile of crude reaction mixture of 23A – Detection at 260 nm (top) and at 420 nm (bottom).	56
Figure 2.6 RP-HPLC profile after purification of 23A , also characteristic of 23C and 23D – Detection at 260 nm (top) and at 420 nm (bottom).	57
Figure 2.7 RP-HPLC profile after purification of 23B – Detection at 260 nm (top) and at 420 nm (bottom).	57
Figure 2.8 MALDI-TOF mass spectrum for 23A using 3-hydroxypicolinic acid as matrix. Calculated for C ₁₂₆ H ₁₄₄ O ₅₇ N ₄₂ P ₉ Ru = 3538.6394, Found 3538.4092 [M-H] ⁻	58
Figure 2.9 a) UV-Vis spectrum of 22A and b) Emission spectrum ($\lambda_{ex} = 450$ nm) of 22A in H ₂ O at room temperature (4.2×10^{-5} M).	59
Figure 2.10 Commercial phosphoramidites for incorporation of aromatic aldehydes	59
Figure 2.11 ODNs with 5' aromatic aldehyde modification	60
Figure 2.12 RP-HPLC profile of crude reaction mixture for Ru-ODN conjugate	63

28A after 3 hours – Detection at 260 nm (top) and 420 nm (bottom).	
Figure 2.13 a) UV-Vis spectrum of 28A and b) Emission spectrum ($\lambda_{\text{ex}} = 450$ nm) of 28A in H ₂ O at room temperature (3.5×10^{-5} M).	63
Figure 3.1 Electrostatic stabilisation of nanoparticles. Taken from reference ²	69
Figure 3.2 Steric stabilisation of nanoparticles. Taken from reference ²	69
Figure 3.3 Upper schematic shows the complexation of a nucleophile (N) on the nanoparticle surface followed by the oxidation of the particle by O ₂ . Taken from reference ¹³	71
Figure 3.4 HRTEM image of Ag@TiO ₂ prepared via a one-pot method. Image taken from reference ⁴⁸	81
Figure 3.5 UV-Vis spectrum of silver seed particles	83
Figure 3.6 Zeta potential measurement of citrate stabilised silver seed particles	83
Figure 3.7 Size distribution by volume DLS measurement for citrate stabilised silver seed particles	84
Figure 3.8 UV-Vis spectrum of a typical triangular silver nanoplate sol prepared using 100 μ l of seed particles.	85
Figure 3.9 (a) Size distribution by intensity DLS measurement for a typical triangular silver nanoplate sol prepared with 100 μ l of seed particles. (b) Zeta potential measurement for a typical citrate stabilised triangular silver nanoplate sol	86
Figure 3.10 UV-Vis spectra of triangular Ag nanoplates with no added citrate (a) after 1 day and (b) after 7 days.	88
Figure 3.11 UV-Vis spectra of triangular Ag nanoplate sol (a) with citrate added monitored for 18 days. The sample was stored in the dark at 4 °C. (b) after centrifuging at 6000 rpm for 30 mins.	89
Figure 3.12 (a) UV-Vis spectra of triangular silver nanoplates taken immediately after addition of 10 mM KBr, KCl and KI. (b) Solutions after reacting overnight.	90
Figure 3.13 UV-Vis spectra of triangular Ag nanoplate sol (a) before and after capping with MHA. (b) MHA capped sol before and after centrifugation at 6000 rpm for 30 mins.	92
Figure 3.14 Size distribution by intensity DLS measurement of triangular Ag nanoplate before and after capping with MHA.	92
Figure 3.15 Zeta potential of MHA capped triangular Ag nanoplates.	93

Figure 3.16 IR spectrum of 16-mercaptohexadecanoic acid (MHA) and MHA capped triangular silver nanoplate sol	94
Figure 3.17 Raman spectra of the MELLFs prepared of citrate stabilised and MHA capped triangular silver nanoplates.	95
Figure 3.18 UV-Vis spectra of MHA capped triangular silver nanoplates in the presence of 10 and 100 mM NaCl.	96
Figure 3.19 (a) UV-Vis spectra of triangular silver nanoplates before and after addition of MHA of various final concentrations (20, 10, 5 and 2.5 μ M) (b) UV-Vis spectra of the various MHA capped particles in the presence of 10 mM NaCl.	97
Figure 3.20 UV-Vis spectrum of MHA capped triangular silver nanoplate sol after addition of DTT (1 M). Scanned every 2 mins for 60 cycles. Final scan after 13 hours	98
Figure 3.21 UV-Vis spectra of a five times concentrated preparation of triangular silver nanoplates prepared using various seed quantities, 100 μ l - 500 μ l.	102
Figure 3.22 Size distribution by intensity measured by DLS for a five times concentrated preparation of triangular silver nanoplates.	103
Figure 3.23 UV-Vis spectra of citrate stabilised concentrated triangular silver nanoplates before and after treating with titania coating mixture.	104
Figure 3.24 Size measurement by intensity measured by DLS for citrate stabilised concentrated triangular silver nanoplates before and after coating	104
Figure 3.25 Zeta potential measurements for citrate stabilised concentrated triangular silver nanoplates before and after coating.	105
Figure 3.26 TEM of Ag@TiO ₂ sample prepared with concentrated citrate stabilised triangular Ag nanoplates.	105
Figure 3.27 UV-Vis spectra of Ag@TiO ₂ sample prepared with citrate stabilised concentrated triangular silver nanoplates after addition of 10 mM NaCl after 30 minutes.	106
Figure 5.28 (a) UV-Vis spectra of 'as-prepared' citrate stabilised triangular silver nanoplates before and after addition of nitric acid. The sample was monitored for 8 hours. (b) UV-Vis spectra of Ag@TiO ₂ particles prepared with citrate stabilised concentrated triangular silver nanoplates before and after	107

addition of nitric acid.

Figure 3.29 UV-Vis spectra of Ag@TiO₂ sample on day 1 and day 25. 107

Figure 3.30 UV-Vis spectra of triangular Ag nanoplates in the presence of (a) 108
0.35 M acetic acid (b) 0.07 M titanium tetraisopropoxide (TTIP)

Figure 3.31 UV-Vis spectra for the original sol (black), MHA capped sol (red) 110
and titania coated sol (blue)

Figure 3.32 (a) Size distribution by intensity measured by DLS for the citrate 111
stabilised (red), MHA capped (blue) titania coated (green) triangular silver
nanoplate samples. (b) Zeta potential measurement of MHA capped (red) and
titania coated (green) triangular silver nanoplates.

Figure 3.33 TEM images of Ag@TiO₂ prepared with concentrated MHA capped 112
triangular silver nanoplate preparation

Figure 3.34 UV- Vis spectra of MHA capped particles before and after addition 113
of acetic acid (0.35 M, pH of 3.5).

Figure 3.35 UV-Vis spectra of Ag@TiO₂ particles prepared from concentrated 113
MHA capped triangular silver nanoplate preparation after addition of nitric acid.

Figure 3.36 UV-Vis spectrum (a) of Ag@TiO₂ particles prepared from 114
concentrated MHA capped triangular silver nanoplates in the presence of 10 mM
NaCl (b) in the presence of 100 mM NaCl

Figure 3.37 UV-Vis of Ag@TiO₂ prepared with concentrated MHA capped 115
triangular silver nanoplate sol after addition of DTT (1M). The sample was
scanned every 5 minutes for 100 minutes.

Figure 3.38 UV-Vis spectrum of reaction supernatant after centrifuging the 116
Ag@TiO₂ sample before and after addition of dopamine

Figure 4.1 Schematic representation (top) of the colour change induced on 123
addition of a complementary target to DNA functionalised gold nanoparticles.
Photograph (bottom left) of the sample before and after addition of a DNA target
strand. Melting curve (bottom right) for the free duplex compared with the
nanoparticle duplex. Adapted from references^{2,18}

Figure 4.2 Representation of the different conformation of ODNs on a gold 127
nanoparticle surface dependent on the number of ODNs conjugated. Taken from
reference⁴³

Figure 4.3 (a) UV-Vis spectrum of silver nanoparticle ODN conjugate 132

monitored after addition of complementary target ODN. (b) Photograph of silver nanoparticle ODN conjugate in the presence of a complementary ODN target (pale red) and a non-complementary ODN target (yellow). Taken from reference⁶⁶

Figure 4.4 TEM image of triangular silver nanoplates before (left) and after (right) conjugation with thiolated ODNs. Taken from reference⁷⁰ 133

Figure 4.5 Thermal denaturation curves for silver nanoprism-ODN conjugate aggregates assembled at (a) room temperature and (b) -20 °C and then at 4 °C and the corresponding TEM images showing particles assembled (c) edge-to-edge/face and (d) face-to face. Taken from reference⁷⁰ 134

Figure 4.6 Photograph of the silver nanoprism-ODN conjugates before and after hybridisation with a complementary target strand. Taken from reference⁷⁰ 134

Figure 4.7 TEM images of (a) 8 nm gold and 31 nm gold particle assembly and (b) a satellite structure obtained from mixing the 8 nm and 31 nm particles in a ratio of 120:1. A linking ODN is added in each case. Taken from reference⁷² 135

Figure 4.8 UV-Vis spectrum of citrate stabilised spherical gold nanoparticles with a λ_{max} at 519 nm. 137

Figure 4.9 (a) TEM image of citrate stabilised spherical gold nanoparticles and (b) Histogram showing the particle size distribution for a population of 200 nanoparticles. Particle size of $13 \text{ nm} \pm 1 \text{ nm}$. 138

Figure 4.10 Zeta potential measurement for the ‘as-prepared’ citrate stabilised spherical gold nanoparticles. 138

Figure 4.11 Commercial phosphoramidite for the 5’ modification of ODNs with an alkyl thiol **34** and the prepared ODN sequences **35A** and **35B** 139

Figure 4.12 Reverse phase HPLC analysis of ODN **35A** before purification. Top at 260 nm, Bottom at 280 nm. 140

Figure 4.13 Citrate stabilised ‘as-prepared’ particles (left) and ODN functionalised particles **Au-35C** (right), after addition of 0.1 M NaCl, 10 mM sodium phosphate and 0.01% SDS. The ‘as-prepared’ particles are observed to aggregate. 142

Figure 4.14 UV-Vis spectra of Au and AuNP-ODN conjugates, **Au-35C** and **Au-35D**. The inset shows the 5 nm red shift, from 519 nm to 524 nm, observed on conjugation with the ODN. 143

Figure 4.15 Photograph of Au-35C conjugate (left) and hybridised Au-35C and Au-35D after mixing for 5 mins	144
Figure 4.16 UV-Vis spectrum of the resuspended aggregate ($\lambda_{\text{max}} = 595 \text{ nm}$) formed from hybridisation of Au-35C and Au-35D in 0.3 M NaCl, 10 mM sodium phosphate buffer, 0.01% SDS compared with the individual conjugate spectra.	144
Figure 4.17 DLS measurements of ‘as-prepared’ gold nanoparticles Au (red), Au-35C (green), Au-35D (black), hybridised conjugates Au-35C and Au-35D (blue). A size increase of 4 to 6 nm was observed for Au-35C and Au-35D relative to the Au particles. A large aggregate with a size of 5.5 μm was observed on mixing Au-35C and Au-35D .	145
Figure 4.18 Dynamic light scattering of Au-ODN hybridised conjugates as a function of temperature. An unfunctionalised gold nanoparticle sample was monitored as a control (red).	146
Figure 4.19 Transmission Electron Microscopy image showing a 3D assembly formed from the hybridisation of equimolar Au-35C and Au-35D in 0.3 M PBS.	147
Figure 4.20 Thermal melting curve for the aggregate formed from hybridised conjugates Au-35C and Au-35D monitored at 260 nm (where DNA absorbs) and at 525 nm (the maximum LSPR band of the gold nanoparticles) and the free duplex DNA (8 μM , monitored at 260 nm). The denaturation curves are normalised for clarity.	148
Figure 4.21 Example standard curve obtained for ODN sequence Au-35C with Oligreen reagent.	149
Figure 4.22 Simplified diagram illustrating the aim of this section, to functionalise gold nanoparticles with a Ru labelled ODN.	151
Figure 4.23 Commercially available phosphoramidite 25 and CPG support 36 for ODN modification and the sequence of the prepared ODN 37	151
Figure 4.24 HPLC trace of crude coupling of 37B . Top at 260 nm, Bottom at 420 nm.	152
Figure 4.25 a) UV-Vis spectrum of 37B (5.8 μM) and b) Emission spectrum ($\lambda_{\text{ex}} = 450 \text{ nm}$) of 37B (2.9 μM) in H_2O at room temperature.	154
Figure 4.26 Diagram depicting the adsorption of ruthenium complexes on the gold nanoparticle surface.	154

Figure 4.27 UV-Vis of 1×10^{-7} M ruthenium tris(bipyridine) in the presence of gold nanoparticles (13 nm) with final nanoparticle concentrations of 4×10^{-9} M and 2×10^{-9} M. No flocculation was observed.	155
Figure 4.28 Emission spectrum of 1×10^{-7} M ruthenium tris(bipyridine) ($\lambda_{\text{ex}} = 450$ nm) in the presence of gold nanoparticles with final nanoparticle concentrations of 4×10^{-9} M and 2×10^{-9} M. The band at 530 nm is the raman band of water. Slits of 20 nm.	156
Figure 4.29 (a) Normalised UV-Vis of gold nanoparticles before conjugation (black line) and after conjugation (red line). A red shift of 5 nm was observed, from 519 nm to 524 nm. (b) TEM image of the functionalised particles. Average particle size of 13 nm.	158
Figure 4.30 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450$ nm) of 37C Ru-ODN-Au conjugate (black line) and after addition of DTT (coloured lines). An increase in the luminescence was observed over 30 mins. The band at 530 nm is the raman band of water. Slits of 20 nm and a high PMT voltage were used.	159
Figure 4.31 Single photo counting lifetime analysis of the Ru-ODN-Au conjugate before (black trace) and after (blue trace) addition of DTT. The straight lines represent the data fitted to a single exponential decay. (Note the background is due to the long (18 hour) acquisition time)	160
Figure 4.32 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450$ nm) of 37C gold nanoparticle conjugate before (black line) and after (red line) addition of the complementary ODN.	161
Figure 4.33 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450$ nm) of 37C gold nanoparticle conjugate before (black line) and after (red line) addition of the complementary ODN. The sample was centrifuged and the supernatant (green line) and pellet (blue line) analysed.	162
Figure 4.34 UV-Vis of triangular silver nanoplates (black line) and the corresponding AuAg nanoboxes (red line) prepared from addition of ascorbic acid (825 μ l, 10 mM) and HAuCl ₄ (10 ml, 0.5 mM).	164
Figure 4.35 TEM image of triangular silver nanobox	165
Figure 4.36 UV-Vis of the nanoboxes before and after conjugation with the ODNs. A red shift of 18 nm, from 744 nm to 762 nm, was observed on functionalisation	166

Figure 4.37 DLS measurement of AuAg nanobox before (red) and after (green) functionalisation with ODN 35C .	167
Figure 4.38 (a) Photograph of the nanoboxes before (left) and after (right) hybridisation. (b) UV-Vis/NIR spectrum of nanobox-ODN conjugate and the hybridised nanobox conjugates.	168
Figure 4.39 Thermal melting curve for the hybridised conjugates 35C-Nanobox and 35D-Nanobox (0.3 M PBS, monitored at 760 nm) and the free duplex ODN (8 μ M, monitored at 260 nm). The citrate stabilised particles (blue line) show no behaviour.	169
Figure 4.40 Graph showing the particle size of the nanobox assembly as a function of temperature. Data shown is an average of three measurements.	170
Figure 4.41 (a) TEM image of the assembly formed on hybridisation of 35C-Nanobox and 35D-Nanobox in 0.3 M PBS (b) TEM image of 35C-Nanobox , no large aggregates were observed.	171
Figure 4.42 The effect of salt concentration on the melting of nanobox aggregates. Sample in different concentrations of PBS monitored at 760 nm.	171
Figure 4.43 Plot of T_m for hybridised nanoboxes as a function of salt concentration	172
Figure 4.44 Photograph of 35D-Nanobox with (right) and without(left) ODN 38	174
Figure 4.45 Melting curve obtained for 35D-nanobox and ODN 38 in 0.6 M PBS. The absorbance was monitored at 760 nm.	174
Figure 4.46 UV-Vis spectra of the assembly of 35C-Nanobox and 35D-Au in 0.3 M PBS (red) and control samples, 35C-Nanobox and 35C-Au (black) and 35C-Nanobox and citrate stabilised gold (blue).	176
Figure 4.47 Normalised melting curve obtained for the assembly of 35C-nanobox and 35D-Au in 0.3 M PBS, monitored at 760 nm.	176
Figure 4.48 TEM images of the assembly of 35C-Nanobox and 35D-Au in 0.3 M PBS.	177
Figure 4.49 UV-Vis spectrum of nanobox-ODN dehybridisation monitored every 5 $^{\circ}$ C from 20 – 70 $^{\circ}$ C	183
Figure 4.50 Plot of temperature vs extinction @ 760 nm for nanobox-ODN	184
Figure 5.1 Commercial phosphoramidite for 5'-amino incorporation 6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-	188

phosphoramidite 41 and ODN sequences prepared (41A , 41B and 41C)	
Figure 5.2 Structure of phosphoramidites dA , dC , dG and dT . The exocyclic amines of dA and dC are protected with a benzoyl group and the amine of dG with an isobutyryl group.	188
Figure 5.3 HPLC trace of the CPG resin of 41A treated with ammonia. Top at 260 nm, Bottom at 280 nm. (Note background due to low concentration of ODN injected)	190
Figure 5.4 HPLC trace of 42A after deprotection of 41A with 80% acetic acid. Top at 260 nm, Bottom at 280 nm.	192
Figure 5.5 HPLC trace of 44A coupling reaction after 3.5 hours. Top at 260 nm, Bottom at 280 nm	193
Figure 5.6 HPLC trace of 44A coupling reaction after 24 hours. Top at 260 nm, Bottom at 280 nm.	194
Figure 5.7 ESI-MS of 44A coupling reaction. 1226.5 [M^2], 1106.3 [MMT-NH-ODN $^{2-}$], 817.4 [M^3], 737.6 [MMT-NH-ODN $^{3-}$], 613.5 [M^4]. M = Required ODN, Tr-S-C ₁₆ -ODN	195
Figure 5.8 HPLC trace of 42B after deprotection of 41B with 3% TCA in DCM. Top at 260 nm, Bottom at 280 nm	196
Figure 5.9 HPLC trace of 44B conjugate. Top at 260 nm, Bottom at 280 nm.	197
Figure 5.10 Catechol modified ODN	199
Figure 5.11 Boc protected oxyamine derivative of dopamine	199
Figure 5.12 1H NMR spectrum of <i>tert</i> -butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate (53) Note peaks at 8.02 (DMF), 7.28 (CHCl ₃), 2.98 (DMF) and 2.91(DMF) are due to residual solvent.	201
Figure 5.13 MALDI-TOF MS of the more slowly migrating band excised from the gel in negative ionisation mode.	205

List of Schemes

Scheme 2.1 Overall schematic for the results presented in this Chapter. A phenanthroline ligand bearing an oxyamine (1) was prepared, subsequently incorporated into a Ru complex (2) and reacted with various aldehyde modified ODNs (3A & 3B) to give Ru-ODN conjugates (4).	43
Scheme 2.2 Preparation of 2-Boc-aminoxy-N-[(1,10) phenanthroline-5-ylcarbamoylmethyl]-acetamide (1). (i) N ₂ H ₄ , EtOH, Pd/C, 70 °C, 4 h (ii) N-Boc-Glycine anhydride prepared in situ – [DCC, N-Boc-Glycine, MeCN, 1 h (16 equivalents)], MeCN, 42 h (iii) DCM/TFA (50/50, v/v), 2 h (iv) (a) (Boc) ₂ O, NaOH, H ₂ O:1,4-dioxane (50/50, v/v), (b) NHS, DCC, MeCN (v) DMF, DIPEA	44
Scheme 2.3 Synthesis of Ru(bpy) ₂ Cl ₂ ·2H ₂ O (12) and attempted synthesis of 14 via an acetone intermediate (13). (i) 2,2'-bipyridyl, LiCl, DMF, 9 h (ii) Acetone, AgClO ₄ ⁶⁹	46
Scheme 2.4 Synthesis of Ru(bpy) ₂ [2-Boc-aminoxy-N- (1,10) phenanthroline-5-ylcarbamoylmethyl]-acetamide] .(PF ₆) ₂ 15 . (i) MeOH, reflux.	48
Scheme 2.5 Synthesis of phosphoramidite (18), for the 5' introduction of an aldehyde to an ODN and subsequent DNA synthesis to give the modified ODN (19). ^{53,71} (i) PPTS, benzaldehyde dimethyl acetal, DMF, 55 °C, 24 h, (ii) DIPEA, 2-cyanoethyl-diisopropylchlorophosphoramidite, dry DCM (iii) Automated DNA synthesis, 28% ammonia for 2 h, 16 h at 55 °C	50
Scheme 2.6 Removal of Boc group (i) HCl (1 M), 2.5 h	54
Scheme 2.7 Example of oxidation of 21A (i) H ₂ O, NaIO ₄ (20 equivalents)	54
Scheme 2.8 Coupling of ruthenium complex 2 with aldehyde modified ODN 22A to give conjugate 23A . (i) Ammonium acetate buffer (0.4 M, pH 4.5)	55
Scheme 2.9 Deprotection of acetal protecting group and coupling of aldehyde modified ODN 27A & 27 B with an oxyamine modified ruthenium complex 2 to give conjugate 28A & 28B . (i) 80% acetic acid, 1 h, (ii) Ammonium acetate buffer (0.4 M, pH 4.5)	62
Scheme 3.1 Schematic representation (I) of the proposed mechanism for chloride etching (II) of the redeposition of silver atoms. Taken from reference ¹⁹	73
Scheme 3.2 Diagram showing the growth of triangular AuAg nanoboxes from triangular Ag nanoplates. A cross-sectional illustration of the growth process with different amounts of gold added is shown. Taken from reference ³⁸	77

Scheme 3.3 Schematic for the typical preparation of a blue triangular silver nanoplate sol with a λ_{max} of 750 nm.	82
Scheme 3.4 General procedure for the preparation of Ag@TiO ₂ using a sol gel approach	100
Scheme 4.1 Simplified schematic of a two-strand system. The mixing of two complementary ODN conjugates results in the formation of an aggregated assembly.	124
Scheme 4.2 Simplified schematic of a three-stand system. A complementary crosslinking segment is used to induce the assembly of two distinct nanoparticle ODN conjugates.	124
Scheme 4.3 Simplified schematic of a non-crosslinking system. Addition of the complementary ODN to a nanoparticle ODN conjugate induces aggregation under high salt conditions.	125
Scheme 4.4 Cleavage of trityl group of 35A and 35B to give sulfhydryl 35C and 35D	141
Scheme 4.5 Simplified schematic showing the assembly of two complementary gold nanoparticle-ODN conjugates	143
Scheme 4.6 Removal of acetal protecting group on ODN 37 to give 37A	152
Scheme 4.7 Coupling of ODN 37A with an oxyamine modified ruthenium complex 2 to give conjugate 37B	153
Scheme 4.8 Reduction of disulfide of 37B to the corresponding thiol 37C using dithiothreitol	157
Scheme 4.9 Simplified schematic depicting the release of the ODN from the nanoparticle surface on addition of dithiothreitol.	159
Scheme 4.10 Simplified schematic showing the switching on of the Ru luminescence on hybridisation and formation of the DNA duplex	161
Scheme 4.11 Schematic representation for the assembly formed from the mixing of two complementary ODN conjugates.	167
Scheme 4.12 Diagram depicting the non-crosslinking experiment on 35D-nanobox	173
Scheme 4.13 Simplified schematic for the assembly of 35C-Nanobox and 35D-Au	175
Scheme 5.1 Synthesis of 16-tritylmercaptohexadecanoic acid (39) and	187

succinimidyl 16-tritylmercaptohexadecanoate (40). (i) Trityl chloride, DMF, N ₂ , room temperature, 48 hours (ii) NHS, EDC, DMAP, DCM, room temperature, 2 hours	
Scheme 5.2 Simplified representation of the possible systems A and B that could be created with the selected ODN conjugates 41B and 41C .	189
Scheme 5.3 Preparation of MHA modified ODN via ‘on-column’ coupling and subsequent use to prepare nanoparticle conjugate. (i) 80% AcOH, (ii) Anhydrous DCM, DIPEA, (iii) 28% ammonia, (iv) (a) DTT, (b) triangular Ag nanoplates	191
Scheme 5.4 Reattachment of the MMT protection group on the confines of the CPG solid support	192
Scheme 5.5 Schematic showing the possible preparation of a bifunctionalised ODN, with a 3’-catechol and a 5’-Ru complex using two oximation steps.	198
Scheme 5.6 Schematic for synthesis of <i>tert</i> -butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate (53).	200
Scheme 5.7 Schematic showing the removal of the Boc group on the oxyamine derivative 53 with TFA and subsequent formation of the acetone oxime 56 .	202
Scheme 5.8 Schematic representation of the potential use of the ODNs 57 and 23B	202
Scheme 5.9 Oxidation of diol 57 with sodium periodate to give the corresponding aldehyde 58	203
Scheme 5.10 Schematic for the removal of the Boc group on the oxyamine derivative 53 to give 59	203
Scheme 5.11 Oxime coupling of ODN 58 and oxyamine 59 to give catechol modified ODN 52 .	204

List of Tables

Table 1.1 Assignment of LSPR peaks in triangular nanoplate extinction spectrum ⁵⁹	13
Table 2.1 ‘On-column’ - Approach 1	29
Table 2.2 ‘Off-column’ - Approach 2	35
Table 2.3 Commonly employed ligation reactions that can be classed as ‘click’ reactions. R ₁ and R ₂ represent either an ODN or reporter group.	41
Table 2.4 MALDI-TOF MS analysis of Ru-ODN conjugates in negative mode	58
Table 2.5 MALDI-TOF MS analysis of Ru-ODN conjugates in negative mode	62
Table 3.1 The solubility constants for silver halide compounds in water ¹⁸	72
Table 3.2 Previous reports of the preparation of Au@TiO ₂ and Au@TiO ₂ nanoparticles	78
Table 3.3 Comparison of the halide etching order of various nanoplate preparations	90
Table 3.4 Structures of thiolated compounds used for nanoparticle capping	98
Table 4.1 MALDI-TOF MS analysis of 5’-sulfhydryl modified ODNs in negative mode	140

Abbreviations

A	adenine
(Boc) ₂ O	di- <i>tert</i> -butyl-dicarbonate
AcOH	acetic acid
AE-HPLC	anion exchange high performance liquid chromatography
AFM	atomic force microscopy
Ag@TiO ₂	silver core titania shell
APS	ammonium persulfate
ATR	attenuated total reflectance
Au@TiO ₂	gold core titania shell
Boc	<i>tert</i> -butoxycarbonyl
bpy	2,2'-bipyridine
C	cytosine
CD	circular dichroism
CPG	controlled pore glass
CTAB	cetyltrimethylammonium bromide
Cy5	cyanine
DABCYL	4-(4'-dimethylaminophenylazo)benzoic acid
DCC	dicyclohexyldicarbodimide
DCM	dichloromethane
DCU	dicyclohexylurea
DDA	discrete dipole approximation
DIPEA	N,N-diisopropylethylamine
DLS	dynamic light scattering
DMAP	dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
dppz	dipyridophenazine
DTT	dithiothreitol
EDANS	5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid
EDC	ethylcarbodimide
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive X-ray analysis
EELS	electron energy loss spectroscopy
E-field	electric-field
em	emission
ESI-MS	electrospray ionisation mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
ex	excitation
FAM	carboxyfluorescein

FDA	food and drug administration
FRET	fluorescence resonance energy transfer
FTIR	fourier transform infrared
G	guanine
GP	general practitioner
h	hour
hat	hexaazatriphenylene
HPLC	high performance liquid chromatography
HRTEM	high resolution transmission electron microscopy
ICP-MS	inductively coupled plasma-mass spectrometry
IR	infrared
ISC	intersystem crossing
K _{sp}	solubility constant
LBL	layer-by-layer
LC	ligand centred
lcaa	long chain alkyl amino
LSPR	localised surface plasmon resonance
MALDI-TOF	matrix assisted laser desorption ionisation-time of flight
max	maximum
MeCN	acetonitrile
MEF	metal enhanced fluorescence
MELLF	metal liquid-like film
MeOH	methanol
MHA	16-mercaptohexadecanoic acid
MLCT	metal to ligand charge transfer
MMT	monomethoxytrityl
MMT	monomethoxytrityl
MS	mass spectrometry
MUA	11-mercaptopundecanoic acid
NaAOT	sodium bis(2-ethylhexyl) sulfosuccinate
NHS	N-hydroxy succinimide
NMR	nuclear magnetic resonance
NP	nanoparticle
OD	optical density
ODN	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PAH	poly(allylamine hydrochloride)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pd/C	palladium on charcoal
PEG	polyethylene glycol
phen	1,10-phenanthroline
PPTS	pyridinium paratoluene sulfonate

PSSS	poly(sodium 4-styrenesulfonate)
PVDF	polyvinylidene fluoride
PVP	polyvinylpyrrolidone
py	pyridine
ref	reference
Rox	carboxyrhodamine
RP-HPLC	reverse phase-high performance liquid chromatography
SAM	self assembled monolayer
SDS	sodium dodecyl sulfate
SERS	surface enhanced raman scattering
SET	surface energy transfer
SPC	single photon counting
T	thymine
TALH	titanium (IV) bis(ammonium lactato) dihydroxide
tap	tetraazaphenanthrene
TBE	trizma, boric acid, edta buffer
TCA	trichloroacetic acid
TEAA	triethylammonium acetate
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
T _m	melting temperature
TOAA	titanium oxide acethylacetate
TSU	N,N,N',N'-tetramethyl(succinimido)uranium tetrafluoroborate
TTIP	Titanium tetraisopropoxide
U	uracil
UV-Vis	Ultraviolet-Visible
UV-Vis/NIR	Ultraviolet-Visible/near infrared
XRD	X-ray diffraction

Chapter 1

Introduction

1.1 Preface

In the present day, as science and technology advance rapidly, the approach to the diagnosis and treatment of disease and illness is progressing. There is an ever present need to diagnose a patient early and efficiently. The vision has been to have very sensitive point-of-care diagnostics, whereby a simple test by a GP could verify if one is ill and needs treatment, eliminating the need for time consuming, expensive resources and lengthy waiting periods. The sequencing of the human genome a decade ago¹⁻² and an unravelling of the genetic basis of disease, mean that the disease targets and biomarkers for a variety of illnesses have been readily identified. These biological developments, coupled with advances in nanotechnology, have seen a surge in the possibilities for a revolutionised approach to the detection, and indeed, treatment of disease.³ The field of plasmonics offers many possibilities for sensitive detection owing to a variety of recently observed phenomena such as surface enhanced raman scattering (SERS)⁴⁻⁵ and metal enhanced fluorescence (MEF)⁶⁻⁸ (Figure 1.1).

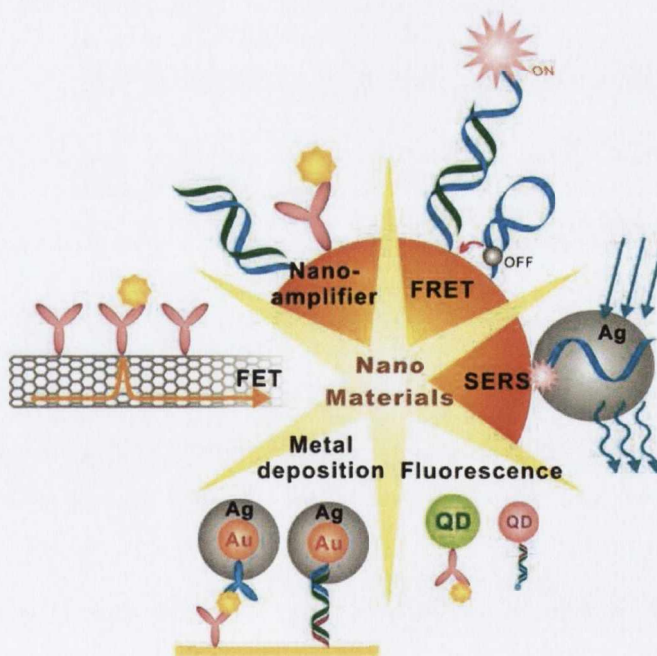


Figure 1.1 Schematic illustration of a variety of biodetection strategies using biofunctionalised nanomaterials. Taken from reference⁷

The use of the optical properties of nanoparticles, namely the localised surface plasmon resonance (LSPR) phenomenon, has much potential in the development of a variety of

sensors.⁹ These can be divided into categories based on the origin of the effect that is used, aggregation¹⁰ and refractive index¹¹ sensors are two examples. This is a rapidly growing area and it is exciting to think of the prospects of implementing the developed technologies from the research lab into practical devices for use in the clinic. Nanosphere's Verigene[®] system is one successful example of a nanoparticle based diagnostic system that has emerged. It currently has a number of FDA approvals for the efficient analysis of a variety of biomarkers, nucleic acids and proteins, across a range of areas including genetics, pharmacogenetics and infectious diseases.

1.2 Motivation - A Potentially Sensitive Molecular Beacon

There have been many advances in DNA detection technology in the past decade, but there is still a need for improvement. Sensitivity is the key issue that needs to be addressed. Selectivity is also important. Many approaches have been applied to the creation of DNA biosensors, including both optical and electrochemical.¹²⁻¹³ Fluorescence is one of the most useful and established approaches for DNA detection and sequencing.¹⁴ Advances in selectivity were made with the discovery of the molecular beacon in the 1990's, whereby a specific nucleic acid sequence could be detected.¹⁵ Fluorescence technology still poses a viable approach to the creation of biosensors for the early detection of disease, but improvements in sensitivity are needed.

Research has prompted the use of nanoparticles in conjunction with fluorophores as a new approach to creating sensitive components for devices. Fluorophores are efficiently quenched when near metal surfaces by energy transfer and based on this idea, it has been demonstrated that nanoparticle-fluorophore conjugates can be more effective than molecular beacon systems.¹⁶⁻¹⁷ By contrast, the phenomenon of metal enhanced fluorescence (MEF) poses a possible way of increasing sensitivity, whereby when a fluorescent molecule is located at a short distance from a noble-metal nanoparticle, its fluorescence can be enhanced.¹⁸ This occurrence should hold promise as a means to creating more sensitive systems and thus great potential in the field of detection technology.

In an effort to study this occurrence and to learn more about the factors that determine the phenomenon of MEF and to potentially create a sensitive molecular beacon, a series of systems needs to be synthesised and studied. A system involving nanoparticles and fluorophores with a controllable, distance dependent, spacer is needed. We have selected DNA as our spacer, ruthenium complexes as our luminescent moiety and triangular silver nanoplates as our nanoparticle system of choice. This study necessitates the preparation of ruthenium complex and triangular silver nanoplate oligonucleotide (ODN) conjugates, as well as bifunctionalised ODN strands.

The potential use of these individual components and combined systems is varied. The ODN conjugates could be used as building blocks for the ‘bottom up’ route to the creation of optoelectronic devices. The fluorophore could be used as a geometry defining node for assembling networks of the conjugates, allowing precisely defined motifs of nanoparticles to be prepared.

Some background information for each component and reasons for our selection will be outlined below, including an introduction to metal enhanced fluorescence and molecular beacons. Finally, an overview and the main aims of this thesis will be given.

1.3 Ruthenium Polypyridyl Complexes – Our Choice of Fluorophore

Ruthenium polypyridyl complexes have been studied for many years.¹⁹⁻²⁰ Vast numbers of complexes have been prepared with a diverse range of properties and purposes. Ruthenium complexes have an extensive organic chemistry with well documented, established synthetic routes. Ruthenium is a second row transition metal and thus has a larger ligand field than first row metals such as iron. This results in higher temperatures being necessary for reaction, but the complexes that are obtained are very stable. The stability of these complexes is due to the symmetrical t_{2g}^6 electronic configuration. Both homoleptic and heteroleptic complexes have been synthesised including the more challenging tris heteroleptic complexes.²¹ It is found that starting from different precursors and with the scope to remove ligands in a systematic manner, retaining some selectively over others, that a great variety of complexes can be prepared. Another key

factor in favour of ruthenium complexes, is the diverse array of heteroaromatic ligands that have been used and the possibilities of modifying these ligands. Common ligands include 2,2'-bipyridine (bpy), 1,10-phenanthroline (phen), tetraazaphenanthrene (tap), hexaazatriphenylene (hat), and dipyridophenazine (dppz) (Figure 1.2).

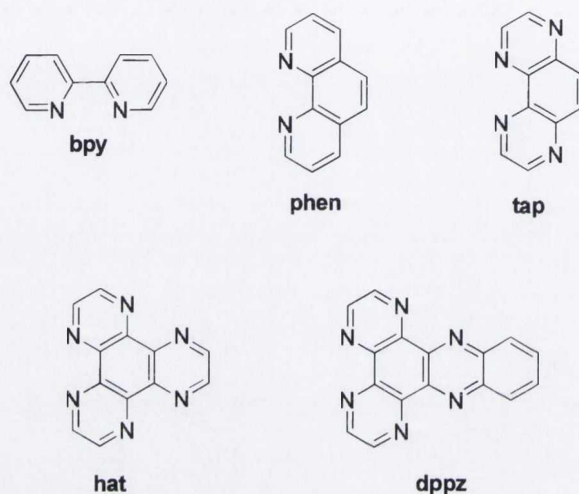


Figure 1.2 Common heteroaromatic ligands

Ruthenium complexes have well defined electrochemical and physical properties, which can be adjusted with 'tunable' ligands. They have high chemical, thermal and photochemical stability. Metal complexes favour different geometries (tetrahedral, square-planar, trigonal bipyrimidal and octahedral) depending on their ground state electronic configuration and the chelating nature of the ligands (mono, bi or tri-dentate). Ruthenium polypyridyl complexes, with three bi-dentate ligands, have 3 dimensional geometry defining nodes and thus are ideal precursors for the creation of 3D networks (Figure 1.3). Nanometric dimensions, along with network directing properties, make them ideal units for creating networks.

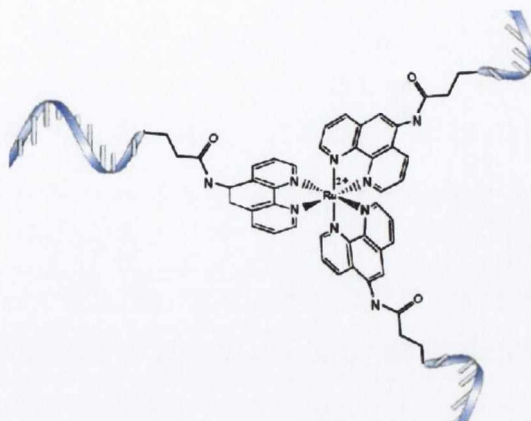


Figure 1.3 A representation of a multifunctional Ru polypyridyl complex

Although the attachment of fluorophores, in particular, ruthenium complexes to oligonucleotides is well documented in the literature,²²⁻²³ including within our own group,²⁴ there is still scope for providing better conjugation strategies, whereby less material is necessary, purification is easy and yields are high. This will be the focus of the results presented in Chapter 2. A more detailed discussion on the previous conjugation strategies will be detailed in the introduction of that chapter.

1.4 DNA – The Ideal Molecular Ruler

1.4.1 DNA Structure

The primary structure of DNA consists of phosphodiester linked nucleoside units that contain a deoxyribose sugar ring and an aromatic nucleobase. Each nucleoside is linked to the next by the 3' and 5' positions of the sugar moieties as phosphodiester to form a polymer. The nucleobases found in DNA include the purines, adenine (A) and guanine (G) and the pyrimidines, cytosine (C) and thymine (T). The predominant DNA structure found under physiological conditions is referred to as B-form. The structure is a right handed helix. This conformation of DNA contains two antiparallel strands of nucleic acid connected by Watson-Crick A:T and G:C base pairs that spiral around a central polymer axis. The base pairing of A with T, and C with G, results from the hydrogen bonding interactions between them. The NH_2 groups of the bases are good hydrogen bond donors, and the sp^2 hybridised electron pairs on the oxygen of the base carbonyl

groups and on the ring nitrogens are good hydrogen bond acceptors. This base pairing is known as Watson and Crick pairing. There are two hydrogen bonds in an A:T pair and three in a C:G pair (Figure 1.4). The sugar phosphate backbones are on the outside and therefore the purine and pyrimidine bases lie on the inside of the helix. The bases are nearly perpendicular to the helical axis and adjacent bases are separated by 3.4 Å (0.34 nm). The helical structure repeats every 34 Å so there are 10 bases per turn of the helix. There is a rotation of 36 degrees per base. The diameter of the helix is ~20 Å (2 nm). The π - π stacking interactions that occur between these aromatic planes, provide a substantial stabilising force, that helps to maintain the duplex nature of the DNA.²⁵⁻²⁸

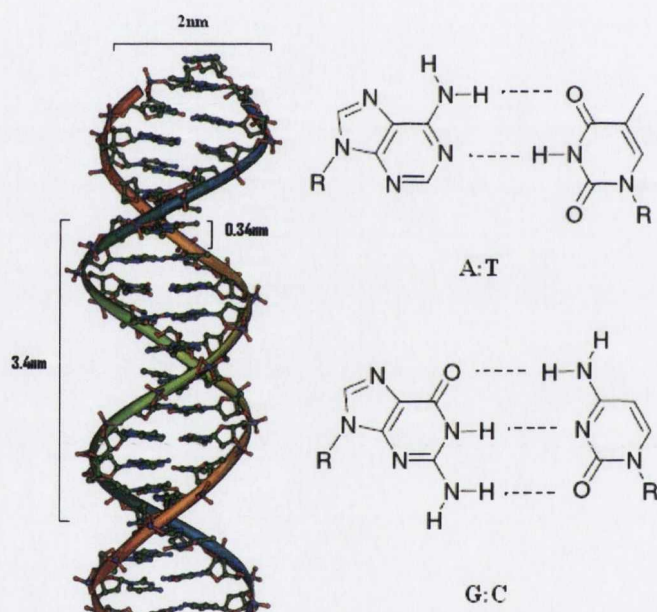


Figure 1.4 DNA helix showing nanometric dimensions (left) and Watson-Crick base pairing (right) of B-DNA

1.4.2 Assembly of Nanostructures using DNA

Synthetic defined sequence DNA is the ideal molecule to use as an assembly material for the creation of nanostructures using both nanoparticles and metal complexes.²⁹⁻³⁰ The key factor that makes DNA an ideal molecule for assembly, is its inbuilt self recognition properties through Watson-Crick base pairing. DNA's nanometric dimensions (molecular ruler) and stability at room temperature are also important. Seeman has used 'sticky end' junction-forming DNA to create networks.³¹⁻³² This

methodology has stemmed from the genetic recombination intermediate, the Holliday junction (Figure 1.5), and is a promising approach to the ‘bottom up’ creation of nanostructures.

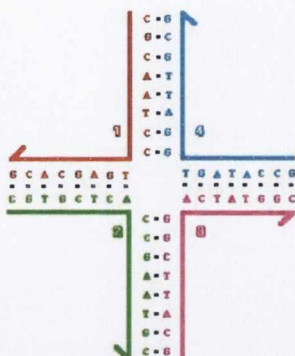


Figure 1.5 Holliday junction - intermediate in genetic recombination

DNA-DNA hybridisation has been used in the supramolecular assembly of nanostructures using metal complexes (Figure 1.6).³³ Nanoparticle assemblies have also been created with a variety of gold nanoparticles.³⁴⁻³⁵

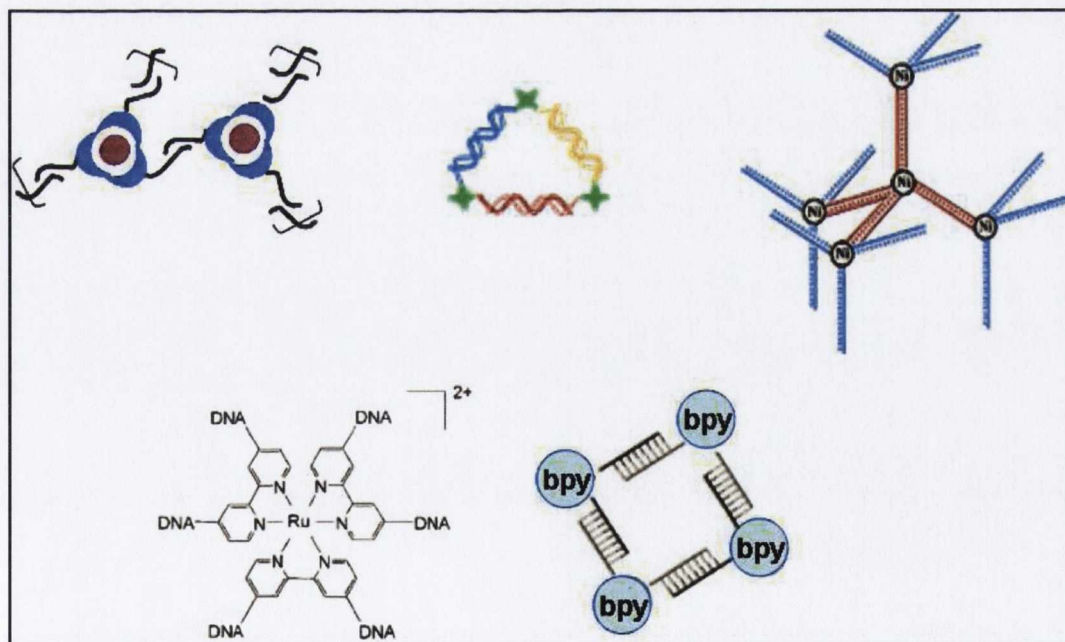


Figure 1.6 Examples of supramolecular DNA assemblies (a) 3 ODN arm based 2D network³⁶ (b) DNA triangle³⁷ (c) Tetrahedral DNA assembly³⁸ (d) 6 ODN based supramolecular assembly³⁹ (e) Cyclic DNA⁴⁰

1.5 Metal Nanoparticles

1.5.1 Optical Properties of Metal Nanoparticles - LSPR

Metal nanoparticles have unique optical properties when compared with the bulk metal, with absorption bands obtained in the visible. An example of the utilisation of this optical property of nanoparticles can be found as far back as the 4th century AD in the colour of the glass of the Lycurgus cup. The colour has since been shown to be due to the presence of small Au and Ag particles. Michael Faraday investigated this optical occurrence in 1857, when he ascribed the bright ruby red colour of a gold sol to the size of the gold particles.⁴¹ The origin of this absorption band can be assigned to the interaction of the nanoparticles conduction electrons with an electromagnetic field. The phenomenon is known as the localised surface plasmon resonance (LSPR) or the dipole plasmon resonance.⁴² When a spherical nanoparticle is irradiated with light, the oscillating electric field, results in the coherent oscillation of the conduction electrons. This induces a dipole in the nanoparticle as the electron cloud is displaced relative to the nuclei. The columbic force of attraction between the electrons and nuclei results in the oscillation of the electron cloud relative to the nuclear framework. The frequency of this oscillation is determined by: the density of the electrons, the effective electron mass, and the size and shape of the charge distribution. This process is depicted in Figure 1.7. Higher modes of plasmon excitation can occur. For example the quadrupole mode results from half of the electron cloud moving parallel to the applied electric field and the other half moving antiparallel to the electric field.

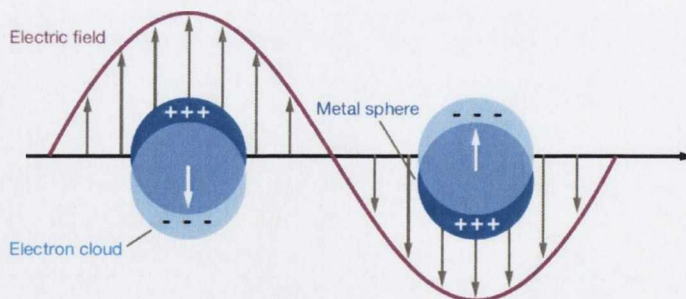


Figure 1.7 Schematic drawing showing the oscillation of the free electron cloud upon interaction with an electromagnetic field. Image taken from reference.⁴³

1.5.2 Mie Theory and Discrete Dipole Approximation

In 1908 Gustav Mie presented a solution to Maxwell's equations describing the extinction spectra of small spherical particles, whereby the extinction is the sum of the absorption and scattering.⁴⁴ A simple expression for the extinction (E) can be given as,⁴⁵

$$E = \frac{24\pi^2 N_A a^3 \epsilon_m^{\frac{3}{2}}}{\lambda \ln(10)} \left[\frac{\epsilon_i}{(\epsilon_r + \chi \epsilon_m)^2 + \epsilon_i^2} \right] \quad \text{Equation 1.1}$$

where N_A is the areal density of nanoparticles, a is the radius of the metallic nanospheres, λ is the wavelength of the absorbing radiation, χ is the nanoparticles shape factor and ϵ_m is the dielectric constant of the surrounding medium, ϵ_i and ϵ_r are the real and imaginary parts of the materials dielectric function. Mie theory remains of importance to this day as it is the only simple exact solution to Maxwell's equations that is relevant to particles. However, it only holds for spherical nanoparticles. Numerical methods have been developed such as the Discrete Dipole Approximation (DDA) to describe the extinction of non-spherical metal nanoparticles. DDA involves dividing the particle into a number (N) of polarisable points.^{42,46} The LSPR band is found to be determined by the size, shape, dielectric constant of the nanoparticle and the dielectric constant of the surrounding medium. The LSPR of larger particles (>30 nm) has been shown to have a significant light scattering component. The strong dependence of the LSPR on shape is illustrated in Figure 1.8.

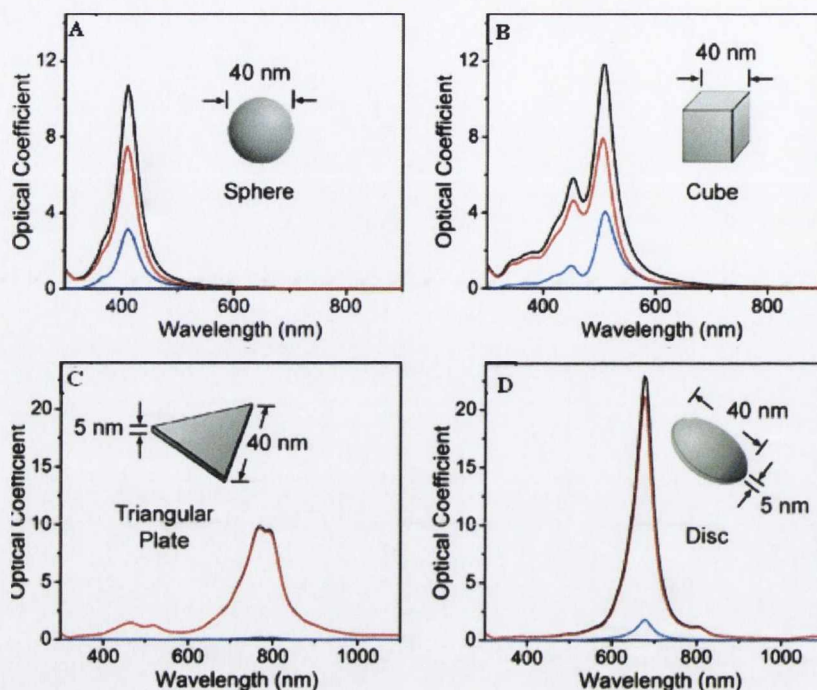


Figure 1.8 Calculated UV-Vis extinction (black), absorption (red) and scattering (blue) spectra of a variety of different shaped silver nanoparticles, illustrating the effect of the particle shape on the spectral characteristics. Adapted from reference⁴⁶

1.5.3 Why Silver?

Silver nanoparticles have a higher extinction coefficient than gold nanoparticles and thus offer a route for the creation of more sensitive systems.⁴⁷ This has been shown by Miller, whereby a molecular beacon based device prepared with silver nanoparticles⁴⁸ showed a 10 fold increase in sensitivity when compared with the analogous gold system.⁴⁹ Graham has also shown an increased detection limit with silver nanoparticles compared with gold.⁵⁰ Silver has been shown to be a good substrate for metal enhanced fluorescence. It has been shown to show sharper and more intense LSPR bands than gold. Silver nanoparticles have been shown to scatter light and enhance near-fields an order of magnitude more strongly than gold.⁴⁶ The disadvantage and challenge of silver is its instability, particularly the ease by which it oxidises. Many methods have been utilised to stabilise silver particles, whilst maintaining their desirable properties, and this will form the basis of the results that are outlined in Chapter 3.

1.5.4 Why Shaped?

Improvements in nanoparticle synthesis in the past decade mean that a wide variety of shaped noble metal nanoparticles can be prepared in high yield. The key here is that the position of the LSPR band can be tuned throughout the visible and into the near-infrared region. This is an important factor for the study of metal enhanced fluorescence (MEF) whereby the overlap of the particle SPR band and the emission of the luminophore, is thought to result in enhanced emission. Excitation of the nanoparticle resonances creates an external electric field around the nanoparticle. Shaped particles show a greater localised electric field enhancement.⁵¹ Triangular particles accumulate the electric charge at their tips, which is known as the lightning rod effect, and thus have been shown to be better substrates than spherical particles for SERS and MEF. The distribution of the electric field for a triangular silver nanoplate and silver nanorod is shown in Figure 1.9, the largest field intensities occur at the tips of the nanoplate and at the ends of the nanorod.

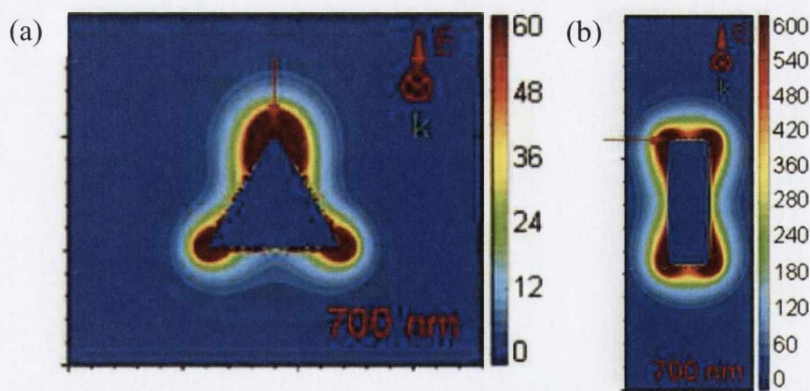


Figure 1.9 E-field enhancement contours external to shaped silver nanoparticles (a) a triangular nanoplate (b) a nanorod. The arrows indicate the region of maximum field intensity. Adapted from reference⁵¹

1.5.5 Triangular Silver Nanoplates – Our Choice of Nanoparticle

1.5.5.1 Nanoparticle Preparation – Focus on Triangular Silver Nanoplates

The increased interest in metal nanoparticles, in recent times, can be attributed to the advances in nanoparticle preparation, with noble metal nanoparticles of varying size and shape readily achievable.⁵²⁻⁵⁶ The focus of this thesis is on the preparation of triangular silver nanoplates. A variety of methods for the preparation of gold and silver triangular nanoplates exist, including the ‘top-down’ method of lithography⁵⁷⁻⁵⁸ and the ‘bottom-up’ solvent based approaches, either by photochemical⁵⁹ or thermal methods.⁶⁰ Each approach has its advantages and disadvantages. Lithography is expensive and low yielding, but great control over particle structure can be achieved. Photochemical methods generally result in high quality, monodisperse samples, but the method is generally slow. Thermal methods have the advantage of being fast, but earlier preparations resulted in particles with mixed morphologies. Recently there have been considerable advances in the preparation of monodisperse samples by thermal aqueous methods. The intricate details of metal nanoplate preparation and the varying growth theories are beyond the scope of this thesis. The reader is directed to a number of review articles and book chapters for a detailed discussion on the preparation and growth of metal nanoplates.^{18,61-65}

Triangular silver nanoplates (nanoprisms) have a large flat basal plane and a large aspect ratio (Figure 1.10). The large flat face has been shown to be a $\{111\}$ crystal facet with the edges consisting of higher energy $\{100\}$ or $\{110\}$ facets.

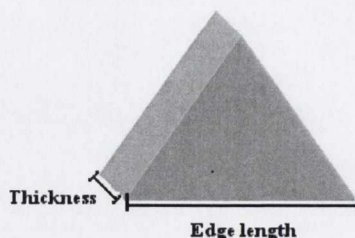


Figure 1.10 Picture of triangular nanoplate with the particle dimensions marked.

DDA has proven particularly useful in determining the optical properties of triangular silver nanoplates,⁴² and it has been shown that the sharp tips contribute greatly to their optical properties (Figure 1.11).

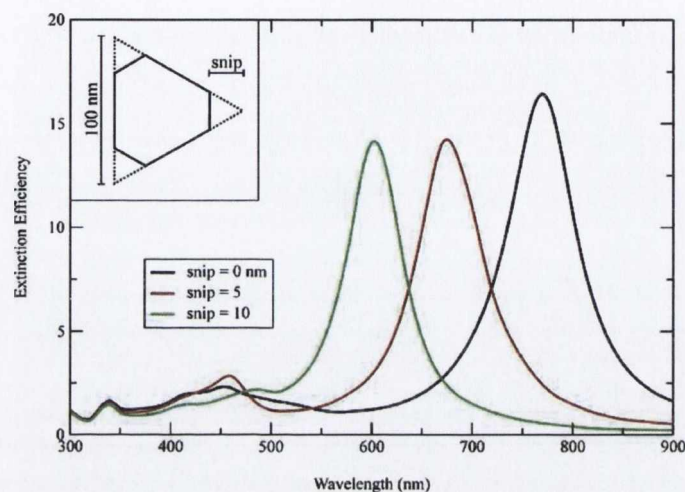


Figure 1.11 Extinction spectrum of nanoprism (100 nm edge length and 16 nm thick) with snips of 0, 10 and 20 nm obtained using DDA calculations. Taken from reference⁴²

The position of the LSPR is dependent on the edge length, thickness and tip morphology of the triangular nanoplate. The main LSPR band (λ_{max}) can be tuned throughout the visible and into the near-infrared. Triangular nanoplates generally have four observed LSPR bands (Table 1.1).⁵⁹

Table 1.1 Assignment of LSPR peaks in triangular nanoplate extinction spectrum⁵⁹

Peak Position (nm)	Description	Peak Assignment
600-1000	Sharp, Strong	In-plane dipole
~470	Broad, Weak	In-plane quadrupole
~410	Very broad & Very weak	Out-of- plane dipole
~340	Sharp, Weak	Out-of-plane quadrupole

1.5.5.2 Selected Triangular Silver Nanoplates – Aherne et al. Nanoplates

A highly selective procedure that produces (>95%) silver nanoprisms was developed by Dr. Damian Aherne building on previous work within the group.⁶⁶ This method improves on previous preparations within the group as the amount of spherical particles

produced is kept to a minimum.⁶⁷⁻⁶⁸ The challenge with nanoparticle synthesis is to produce particles of one shape and one size and in effect this has been accomplished. The preparation is a rapid, highly reproducible seed based method, yielding high quality triangular silver nanoplates in high yield. The success here is that by simply altering the amount of seed used in the growth step, nanoplates of varying size and hence varying colour can be achieved easily (Figure 1.12). The position of the main LSPR band for the particles can be carefully controlled and varied.

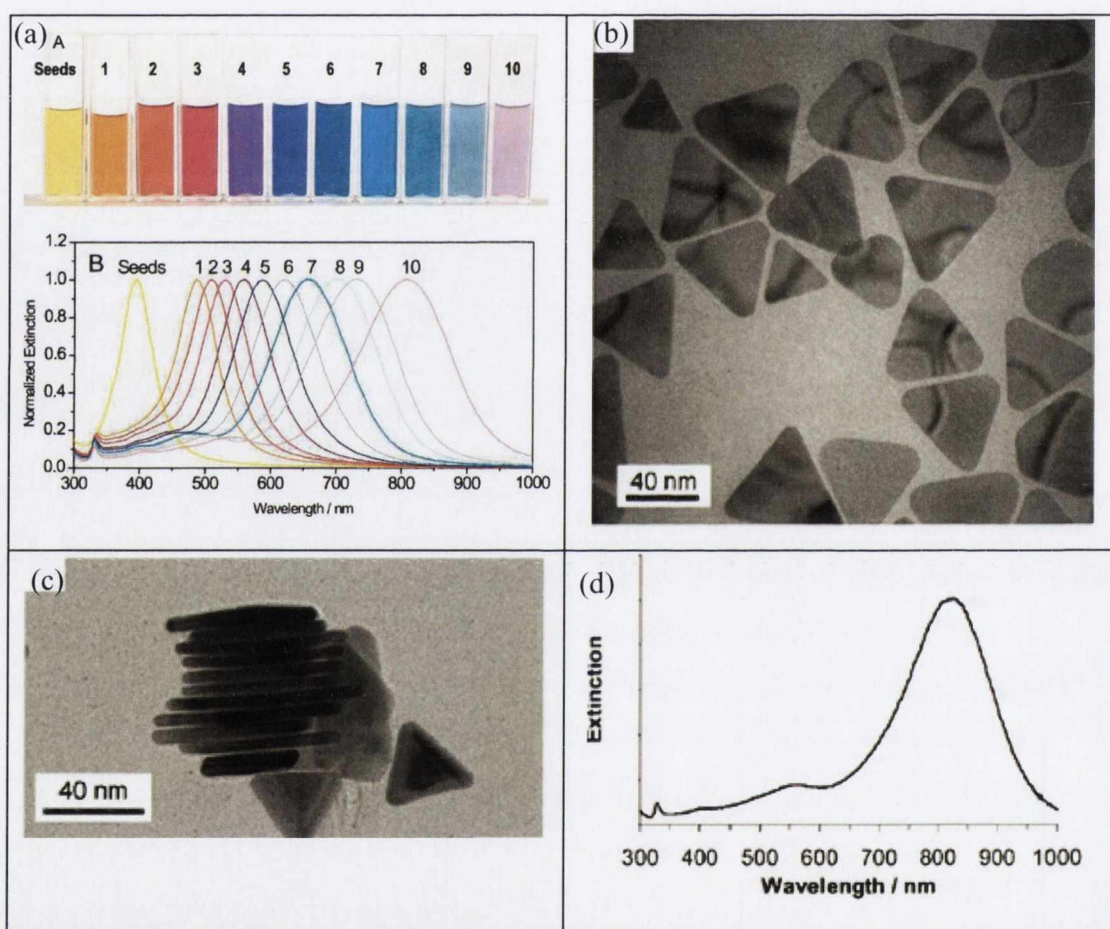


Figure 1.12 (a) Photograph and UV-Vis spectra of silver seeds and corresponding triangular silver nanoplates prepared with different volumes of seed particles 1) 650 μl , 2) 500 μl , 3) 400 μl , 4) 260 μl , 5) 200 μl , 6) 120 μl , 7) 90 μl , 8) 60 μl , 9) 40 μl , 10) 20 μl . (b) TEM image of flat lying triangular silver nanoplates. (c) TEM image of stacked triangular silver nanoplates. (d) UV-Vis spectrum of triangular nanoplates shown in B. The λ_{max} is at 825 nm. Images taken from reference⁶⁶

1.6 Metal Nanoparticle ODN Conjugates

1996 saw the pioneering work of the Mirkin and Alivisatos groups report the successful attachment of ODNs to gold nanoparticles.⁶⁹⁻⁷⁰ The area has seen a considerable number of developments over the last few years and these systems have been used for a vast array of purposes. Much of the work to this day has utilised gold nanoparticles, as silver nanoparticles have proven more difficult to functionalise. The background to this area and a discussion on the challenge of functionalising silver nanoparticles will form the introduction of Chapter 4.

1.7 Fluorescence

Fluorescence describes the emission of energy by a molecule as it relaxes back to its ground state after being electronically excited.⁷¹ The process involves the excitation of a species from its ground electronic state (S_0) to a vibrational state in an excited electronic state by absorption of a photon. The species loses its excess energy by radiationless decay and returns to the lowest vibrational level of the first excited electronic state (S_1). The species then returns to the ground electronic state (S_0) emitting a photon in the process. Alternatively, the molecule may undergo intersystem crossing (ISC), a radiationless process, to a lower lying triplet state. From this state, it may emit a photon of energy (P, phosphorescence) or lose its energy by a radiationless process. These processes are depicted in the Jablonski diagram (Figure 1.13).

The fluorescence quantum yield (Φ) is the ratio of the emitted photons to absorbed photons. k_f is the emissive rate constant of the fluorophore and k_{nr} is sum of the non-radiative decay rate constants ($k_{nr} + k_{isc}$).

$$\Phi = \frac{k_f}{k_f + k_{nr}}$$

Equation 1.2

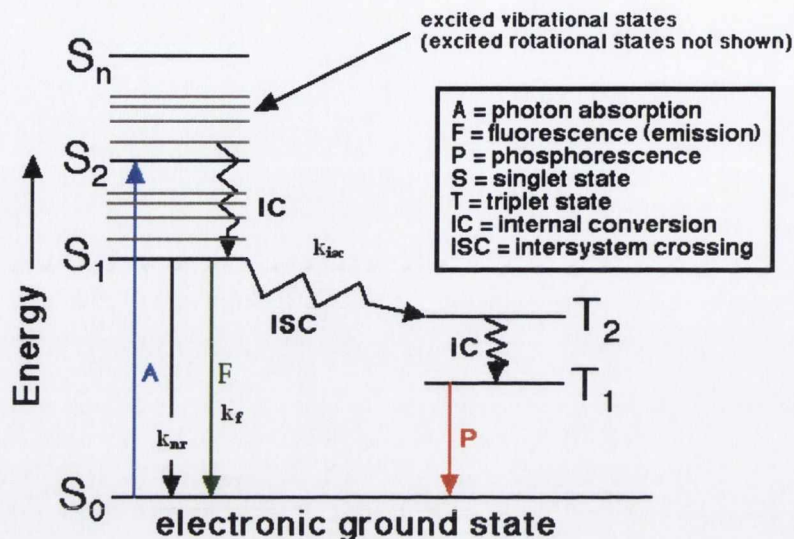


Figure 1.13 Jablonski diagram illustrating the molecular processes involved in absorption and emission of light by a fluorophore. k_f is the emissive rate constant of the fluorophore, k_{isc} is the rate constant for the intersystem crossing and k_{nr} is the non-radiative decay rate constant.

The fluorescence lifetime (τ) is the time the molecule stays in its excited state before returning to the ground state, emitting a photon. Its value is determined by the rate constants of all processes involving S_1 .

$$\tau = \frac{1}{k_f + k_{nr}}$$

Equation 1.3

It should be noted that ruthenium complexes (our luminophore of choice) are triplet emitters and the emission is termed phosphorescence. Fluorescence has been used extensively for DNA sequencing and detection of DNA hybridisation with the process of fluorescence resonance energy transfer (FRET) being widely used.

1.8 Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET) or simply RET is a process involving the transfer of energy from an excited state donor to a ground state acceptor, without the

emission of a photon. A spectral overlap between the acceptor absorbance and the donor emission is required (Figure 1.4) and the energy transfer depends on the distance between the donor and acceptor. The efficiency (E) of the resonance energy transfer is dependent on the Förster distance (R_0) given by:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

Equation 1.4

where r is the distance between the donor and the acceptor and R_0 is the Förster distance, the distance at which RET is 50% efficient. For FRET to occur the donor and acceptor must be in fairly close proximity to each other, usually < 10 nm. FRET forms the basis for a variety of DNA sequence probes, including molecular beacons, which will be discussed below. In a closed state form, where the donor and acceptor are close together, the fluorescence of the donor is quenched, but in the open form, when the donor and acceptor are significantly separated, the fluorescence of the donor is observed.

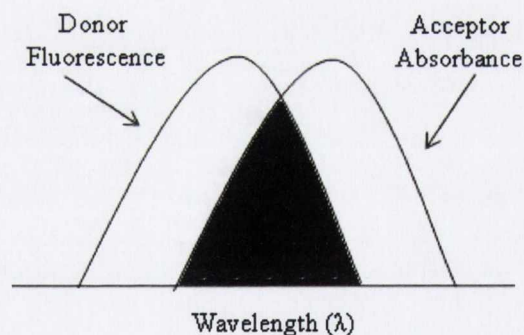


Figure 1.14 Spectral overlap required for fluorescence resonance energy transfer.

1.9 Quenching of Fluorescence by Metals

Metal nanoparticles have been shown to be very efficient quenchers of fluorescence. It is thought that quenching probably occurs by RET to the metal surface, but other mechanisms may also be present.⁷² The non-radiative energy transfer decreases the quantum yield and correspondingly the lifetime. The energy transfer from a donor fluorescent molecule, to a metallic surface is known as surface energy transfer (SET) and has been shown to enhance the quenching efficiency by a factor of 10^4 - 10^5 , compared with organic quenchers.

1.10 Metal Enhanced Fluorescence

It has been observed that when a fluorophore is located some distance (5-20 nm) from the surface of a metal that its fluorescence can be enhanced. This phenomenon is known as metal enhanced fluorescence (MEF).^{18,71,73-74} It is thought to have two causes, an increase in both the excitation and the emission rates. The increase in the excitation rate is attributed to the increased electric fields around the nanoparticle. The increase in the emission is as a result of altering the radiative and non-radiative decay rates of the fluorophore. It is thought to involve the non-radiative coupling of the excited state of the fluorophore with the induced surface plasmons of the metal nanoparticle, which is subsequently radiated by the nanoparticle. An increased quantum yield and a shorter lifetime are obtained. MEF depends on a number of factors including the nanoparticle shape, size and metal type. The orientation of the fluorophore, the distance of the fluorophore from the metal, the fluorophore quantum efficiency, the overlap of the LSPR of the nanoparticle with the emission spectrum of the fluorophore, all affect whether MEF is observed. The theory of MEF is complex and there has been some controversy surrounding the mechanism of MEF. Initial theory attributed the phenomenon to the ability of the nanoparticle to offer additional non-radiative pathways for the decay of the excited state fluorophore, thereby increasing the quantum yield of fluorescence.⁷⁵ The more recent radiative plasmon model equates the scattering component of the extinction as the responsible factor for the origin of MEF.⁷⁶⁻⁷⁷

In classical fluorescence the quantum yield (Φ) and the lifetime (τ) (Equations 1.2 and 1.3) depend on the non-radiative decay rate constant k_{nr} . The values either both increase or both decrease. Fluorophores near metal particles or surfaces have been shown to have an increased quantum yield, with a corresponding decreased lifetime. The equations for the quantum yield and lifetime in the presence of metals are given below, where k_f is the emissive rate constant of the fluorophore, k_{nr} is the non-radiative decay rate constant to S_0 and k'_{nr} is the additional rate constant due to the metal.

$$\Phi = \frac{k_f + k'_{nr}}{k_f + k_{nr} + k'_{nr}}$$

Equation 1.5

$$\tau = \frac{1}{k_f + k_{nr} + k_{nr}'}$$

Equation 1.6

The study of MEF is still in its infancy. A better understanding of the fundamentals that govern this phenomenon are needed for its true potential and uses to be realised. Recent literature reports endorse the idea that MEF will result in the creation of sensitive detection systems.⁷⁸

1.11 Molecular Beacons

The molecular beacon system, a single-stranded DNA fluorescent probe, was first described in 1996 by Tyagi and Kramer.¹⁵ A molecular beacon typically consists of a stem-loop structure. The loop is generally composed of a 15-30 base probe sequence that is complementary to a specific target nucleic acid sequence. The stem is composed of two short, self complementary sequences that maintain the system in a hairpin conformation in the absence of a target sequence. A fluorophore and quencher are covalently linked to the probe strand at opposite ends, and in the absence of a target, when the system adopts a hairpin structure, the fluorophore is located close to the quencher and its emission is quenched by fluorescence resonance energy transfer (FRET). In the presence of a target, the system undergoes a conformational change upon hybridisation of the probe loop sequence with the target sequence. The quencher and fluorophore are separated by the relatively rigid double helix and when illuminated with UV light fluorescence is observed. This is depicted in Figure 1.15. Molecular beacons are found to be very specific. The addition of a non-complementary sequence, even with just a single base mismatch, does not result in any increase in fluorescence signal. Molecular beacons have been the subject of many studies and efforts have been made to improve their design and extend their scope of application.⁷⁹

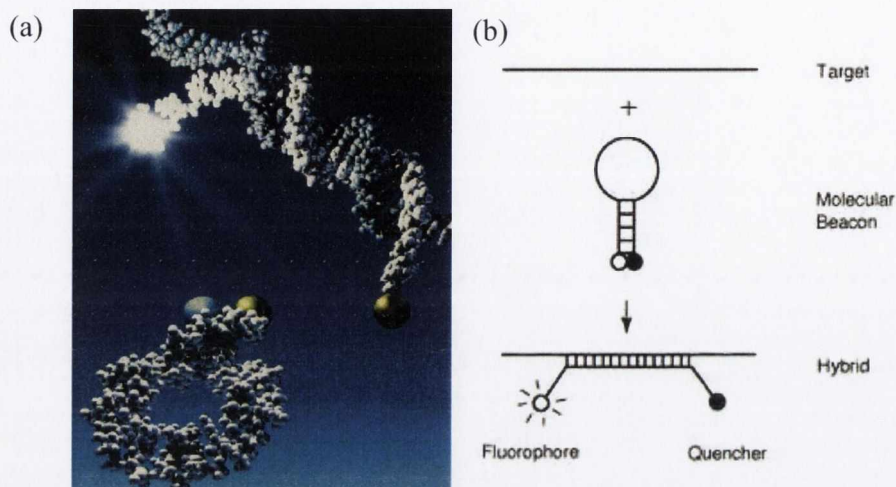


Figure 1.15 (a) Graphical representation of molecular beacon system in its closed and open conformations. (b) Schematic of the working principle of a molecular beacon. Taken from reference¹⁵

1.11.1 Molecular Beacons – Improvement of Sensitivity with the Incorporation of Metal Nanoparticles

The original molecular beacon system used 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS) as the fluorophore and 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) as the quencher. The replacement of DABCYL, an organic quencher, with a small gold nanoparticle (1.4 nm) resulted in a system that was over 100 times more sensitive because of the extremely high quenching efficiency of the nanoparticle.¹⁶⁻¹⁷ This observation was significant in the development of sensitive molecular beacons by using nanoparticles or nanofilms as the quencher.

Maxwell *et al.* showed that a gold nanoparticle (2.5 nm) based molecular beacon did not require a stem whilst still undergoing a target induced conformational change because of the strong dye adsorption on the nanoparticle surface.⁸⁰ The attachment of a thiolated molecular beacon onto a gold film was achieved by Du *et al.* (Figure 1.16). Similar quenching efficiencies were achieved for the immobilised system when compared with the solution based assay.^{49,81} Very recently, the use of silver nanoparticles to prepare an analogous system resulted in even further increased sensitivities. A 10-fold larger

response was obtained by attaching thiolated molecular beacons to silver nanoparticles embedded on glass chips when compared with the previous gold substrate.⁴⁸ This was attributed to the phenomenon of metal enhanced fluorescence. By adjusting the surface roughness, the amount of silver and the length of the DNA probe, the signal could be optimised. A study into the aging of the silver system showed that a further increase was achieved.⁸²

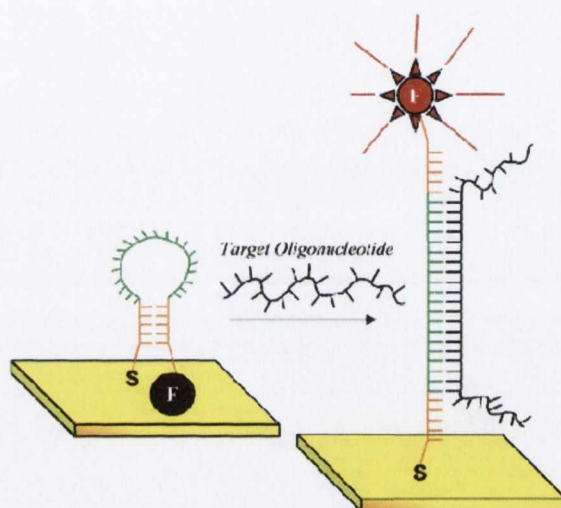


Figure 1.16 Schematic of molecular beacon immobilised on a metal film. The metal acts as the quencher. Taken from reference⁴⁹

Recently there have been some reports using larger gold nanoparticles, where many probes can be anchored on each individual nanoparticle because of the increased surface area. This has led to the creation of a multi-analyte nanobeacon using 15 nm gold nanoparticles.⁸³ Three probes were designed for the detection of three different tumour suppressor genes (p16, p21 and p53 genes). Each one was labelled with a different fluorophore, FAM, Cy5 and Rox respectively. The systems showed sequence specificity and were shown to undergo a conformational change in the presence of a target resulting in an observed signal (Figure 1.17). This approach was further extended for the detection of the non-nucleic acid targets, adenosine, potassium and cocaine, by using fluorophore tagged aptamers.⁸⁴

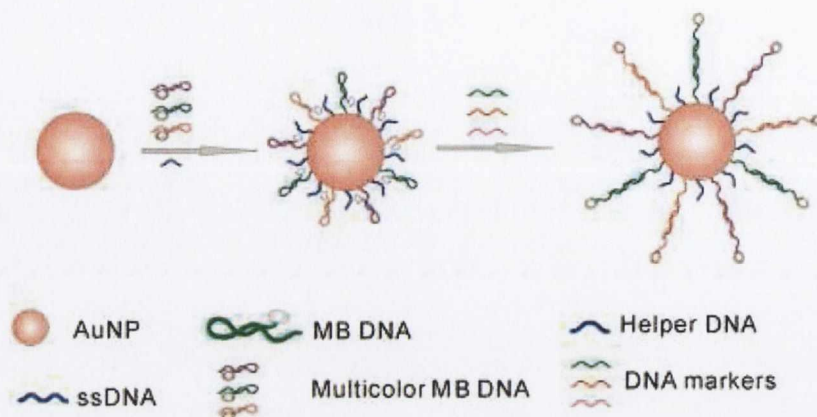


Figure 1.17 Schematic of multi-analyte molecular beacon. Adapted from reference⁸³

The gold nanoparticle size and the DNA length were varied in a study to investigate the effect on a DNA sensing system. Efficient quenching was found for all particle sizes, 20 nm, 50 nm and 100 nm respectively. A slight enhancement was observed for the 100 nm particle if the distance to the fluorophore was over 13 nm.⁸⁵

The potential use of nanoparticles in the area of detection has been clearly outlined above, with specific reference to DNA detection. A large amount of research has been carried out in the area in recent years, particularly during the course of this PhD. This is an exciting area of research and it is thought that future advancements in diagnostics will almost inevitably rely on nanotechnology.

1.12 Aims and Overview of Thesis

The overall aims of this thesis were:

- To prepare ruthenium complex oligonucleotide conjugates by an efficient approach, improving on previous strategies.
- To prepare triangular silver nanoplate oligonucleotide conjugates – maintaining the stability of the particle structure.
- To prepare a sensitive molecular beacon, a bifunctionalised strand, with a nanoparticle conjugated at one ODN terminus, and a Ru complex at the opposite end, by combining two compatible chemistries.
- To combine the nanoparticle and ruthenium ODN systems to study MEF.

This chapter serves as a general introduction to this thesis. Due to the diverse nature of the topics involved, a more detailed literature analysis will be given in the individual introductory sections of Chapters 2, 3 and 4 as outlined below.

Chapter 2 – Preparation of Ruthenium Oligonucleotide Conjugates

Chapter 3 – Preparation, Stability and Functionalisation of Triangular Silver Nanoplates

Chapter 4 – Nanoparticle Oligonucleotide Conjugates

Chapter 5 – Modified Oligonucleotides for Nanoparticle Conjugation

Chapter 6 – Experimental

Chapter 7 – Conclusions and Future Work

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Chapter 2

Preparation of Ruthenium Oligonucleotide Conjugates

2.1 Introduction

One of the main aims of the work presented in this thesis was to prepare luminescent oligonucleotide (ODN) conjugates. As outlined in Chapter 1, we selected polypyridyl ruthenium complexes as our luminescent moiety of choice. This chapter deals with the preparation of ruthenium complex ODN (Ru-ODN) conjugates by covalent attachment of the ruthenium complex at the 3' or 5' end of an ODN.

2.2 DNA Synthesis

We have come a long way since the advent of DNA synthesis in the 1950's.¹⁻⁴ A large range of DNA synthesizer units are available for the automated solid phase synthesis of ODN sequences. Although many methods for the formation of internucleosidic linkages have been utilised throughout the years (H-phosphonate, phosphodiester and phosphotriester), the phosphoramidite approach remains the forerunner and most common approach today. A general cycle applies to all automated systems involving four steps (Figure 2.1).

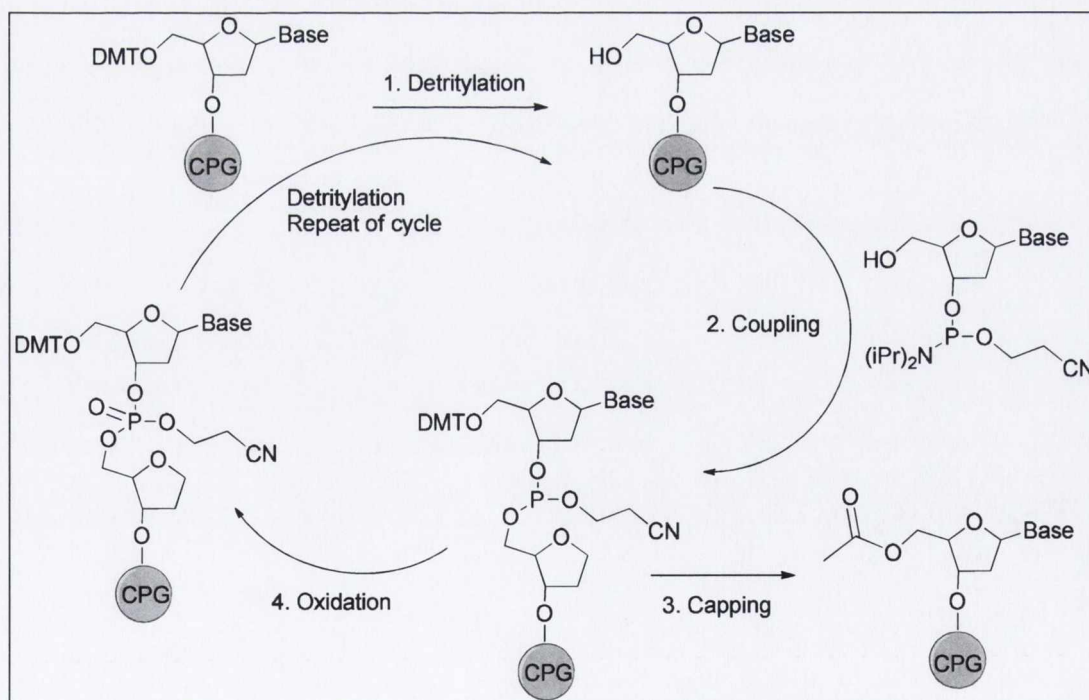


Figure 2.1 A general cycle for automated DNA synthesis

Automated DNA synthesis entails the 3'-5' directional synthesis of ODNs attached to a solid support, usually controlled pore glass (CPG). **Detritylation** involves the removal of the acid labile dimethoxytrityl (DMT) group, from the 5'-hydroxyl of the 3'-terminal nucleoside using trichloroacetic acid. The 5'-hydroxyl group is then free to react with the cyanoethyl phosphoramidite derivatives of subsequent nucleosides. **Coupling** then occurs whereby tetrazole reacts with the phosphoramidite, to form a reactive intermediate, which couples with the exposed 5'-hydroxyl, attaching to the support bound chain. A certain number of 5'-hydroxyls fail to react and need to be blocked so they do not react in the next cycle. This is called **capping** and involves an acetylating intermediate being formed from acetic anhydride and N-methylimidazole, which then reacts with the free hydroxyl groups to form acetyl groups rendering them unreactive in the cycle. Phosphorous (III) is unstable as it is very susceptible to acids and bases so it is oxidised to the more stable P (V). **Oxidation** is carried out using iodine in water. This completes one cycle and another cycle begins with the removal of the 5'-DMT from the previously added nucleoside. At the end of the synthesis, the final trityl can be removed, 'trityl-off', or left on, 'trityl-on', to aid purification. The ODN is cleaved from the solid support by treating the CPG resin with concentrated ammonium hydroxide. Removal of the protecting groups on the heterocyclic bases and elimination of the cyanoethyl group on the phosphorous is achieved by placing the ammonia solution at 55 °C for 16 hours.

2.3 Preparation of Modified Oligonucleotides

A vast amount of work has been carried out on the preparation of modified ODNs.⁵ A large number of commercially available solid supports and phosphoramidites are available for the introduction of a variety of functional groups.⁶ More elaborate ODNs can be made using synthetically prepared phosphoramidites. A primary or secondary hydroxyl group is a prerequisite.

2.4 Oligonucleotide Fluorophore Conjugates

The conjugation of fluorophores with ODNs is well documented in the literature.⁷ Attachment of the label can take place at any 5'-terminus, 3'-terminus, nucleobase, ribose, or phosphate backbone. The methods and locations of attachment apply to both

organic fluorophores and also metal complexes.⁸⁻⁹ There are two general approaches for the preparation of modified ODNs, the 'on-column' approach, and the 'off-column' approach. By 'on-column' we mean still attached to the solid support of the DNA synthesis. It is also known as solid-phase coupling. 'Off-column' is where the ODN has been cleaved from the solid support before coupling in solution and is sometimes referred to as solution-phase coupling. The reader is directed to a number of reviews detailing the conjugation of a variety of fluorescent and non-fluorescent compounds to ODNs.¹⁰⁻¹² My focus will be on the attachment of ruthenium complexes to ODNs.

2.5 Ruthenium Oligonucleotide Conjugates

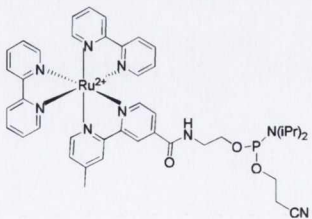
Both 'on-column' and 'off-column' approaches have been utilised. Ru-ODN conjugates have been prepared for a variety of purposes such as luminescent DNA probes,¹³ to study long-range photoinduced electron transfer,¹⁴⁻¹⁵ and for DNA photocleavage¹⁶⁻¹⁷ and photocrosslinking.¹⁸⁻²⁰

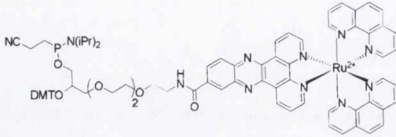
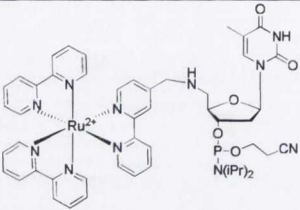
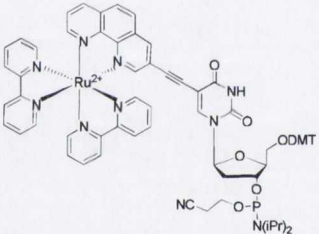
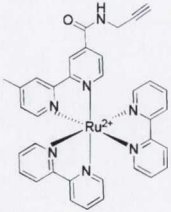
2.5.1 'On-Column' Preparation of Ruthenium Oligonucleotide Conjugates

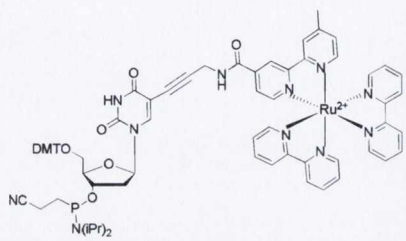
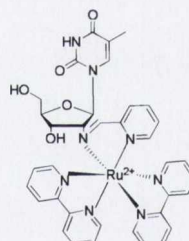
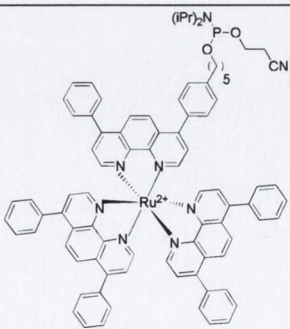
The 'on-column' approach or solid-phase coupling can be divided into two methods:

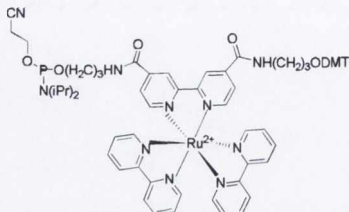
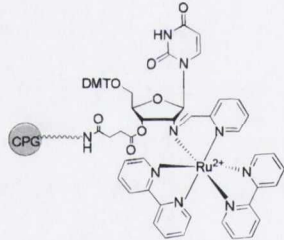
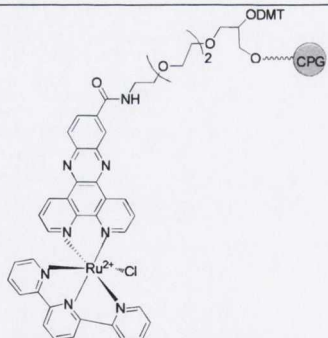
(**Method 1**) The modification of the ODN by introduction of a modified nucleoside/phosphoramidite derivative to which the metal group is already bound (Table 2.1).

Table 2.1 'On-column' - Approach 1

Author ^{ref}	Method of attachment	Attachment site	Complex
Khan <i>et al.</i> ²¹	Directly during automated DNA synthesis as Ru complex phosphoramidite	5' - terminus	

Author ^{ref}	Method of attachment	Attachment site	Complex
Ossipov <i>et al.</i> ²²	Directly during automated DNA synthesis as Ru complex phosphoramidite or as a CPG support	3', 5'-terminus & mid-strand	
Hu <i>et al.</i> ²³	Ru modified nucleoside phosphoramidite prepared and introduced by DNA synthesis	5'-terminus	
Hurley & Tor ²⁴	Sonogashira Pd (0) cross coupling with Br Ru complex & alkynyl uridine nucleobase. Incorporated directly by automated DNA synthesis	5'-terminus & mid-strand	
Khan & Grinstaff ²⁵	Introduction via coupling of alkynyl substituted Ru complex to iodo uridine. Nucleobase introduced during automated synthesis, paused, Sonogashira reaction carried out and automated synthesis continued	5'-terminus & mid-strand	

Author ^{ref}	Method of attachment	Attachment site	Complex
Khan <i>et al.</i> ²⁶⁻²⁷	Fully automated incorporation of Ru complexed uridine phosphoramidite. Phosphoramidite prepared by coupling iodo substituted uridine and alkynyl Ru complex	5'-terminus & mid-strand	
Rack <i>et al.</i> ²⁸	A chelating nucleoside was synthesised by reacting the 2'-amino on the ribose of a uridine with 2-pyridine carboxaldehyde. Further reaction with a Ru complex, and conversion to the succinate derivative allowed attachment to the solid support and incorporation into the ODN	3'-terminus	
Bannwarth <i>et al.</i> ²⁹⁻³¹	Ru phosphoramidite incorporated by automated DNA synthesis	5'-terminus & mid-strand	

Author ^{ref}	Method of attachment	Attachment site	Complex
Lewis <i>et al.</i> ³²	Automated DNA synthesis carried out to introduce initial bases. Synthesis paused and incorporation of the phosphoramidite was carried out in a glove box. Column replaced on synthesizer and further bases incorporated as normal	Mid-strand DNA hairpin	
Krider <i>et al.</i> ³³	Synthesised Ru CPG used for automated DNA synthesis	3'-terminus	
Ossipov <i>et al.</i> ¹⁹	Synthesised Ru CPG used for automated DNA synthesis	3'-terminus	

Author ^{ref}	Method of attachment	Attachment site	Complex
Barton ³⁴⁻³⁶	ODN with amino alkyl prepared and coupled with NHS activated ester of Ru complex whilst still attached to the CPG solid support	5'-terminus	

Khan *et al.*²¹ and Ossipov *et al.*²² described the introduction of ruthenium complexes directly by automated DNA synthesis as phosphoramidites. Ru complexes were incorporated at the 5'-terminus, 3'-terminus and central strand of the ODN. Similarly, a Ru(bpy)₃²⁺ modified thymidine phosphoramidite was introduced onto the 5' end of an ODN. The Ru complex was incorporated into the 5' position of the ribose.²³ An approach employing the use of Sonogashira Pd (0) cross coupling has also proven popular. Hurley and Tor²⁴ attached a brominated ruthenium complex to 5-ethynyldeoxyuridine using this approach. Following conversion to the corresponding phosphoramidite, the ruthenium complex was incorporated into the 5' and central positions of the ODN strands. Khan and Grinstaff have numerous reports where the alkynyl group was incorporated into the ligand of the complex. The complex was reacted with an iodo-substituted uridine phosphoramidite. Standard automated DNA synthesis was carried out as normal until incorporation of 5'DMT-3'-cyanoethyl-N,N'-diisopropylphosphoramidite-2'-deoxy-5-iodouridine. The synthesis was paused and the column was flushed with N₂ before being attached to a syringe and reacted for 3 hours with the alkyne derivatised Ru complex using Pd(Ph₃P)₄, CuI and DMF:Et₃N. It was then washed, placed back on the synthesizer and additional bases added.²⁵⁻²⁶ The fully automated incorporation of this Ru complex-uridine derivative was accomplished by reaction between the iodo-substituted uridine and the alkynyl ligand of the complex, followed by incorporation as a phosphoramidite.²⁷ Rack *et al.*²⁸ described the incorporation of a Ru complex into the 3' end of an ODN. A chelating nucleoside was synthesised by reacting the 2'-amino group on the ribose of a uridine with 2-pyridine

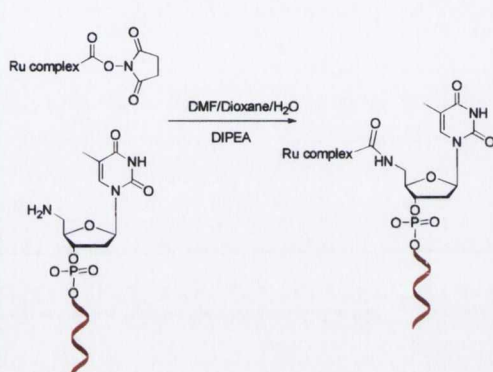
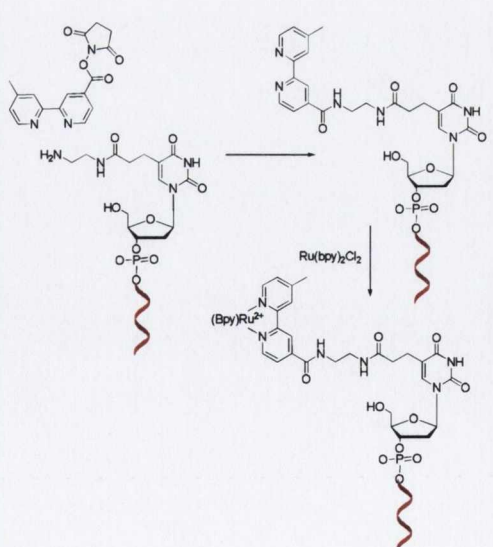
carboxaldehyde. Further reaction with a Ru complex, and conversion to the succinate derivative, allowed attachment to the solid support and incorporation into the ODN. Bannwarth *et al.*²⁹⁻³¹ prepared a ruthenium complex phosphoramidite and incorporated it into the ODN by automated DNA synthesis. A ruthenium complex phosphoramidite was incorporated into the middle of an ODN to create a bridged hairpin.³² The coupling of the modified phosphoramidite was carried out in a glove box because of its moisture sensitivity. Krider *et al.*³³ and Ossipov *et al.*¹⁹ incorporated ruthenium complexes into the 3' end of ODNs by preparing ruthenium complex CPG solid supports.

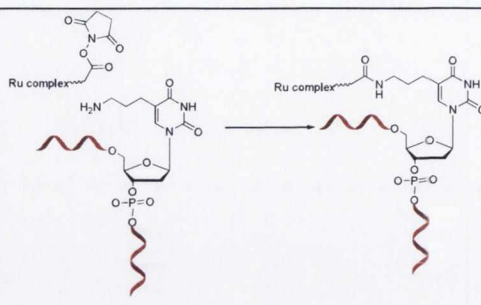
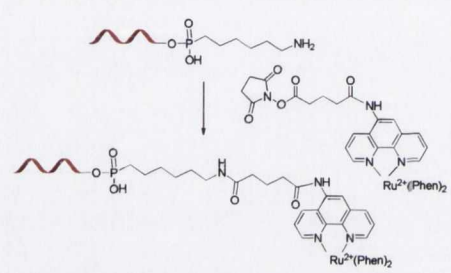
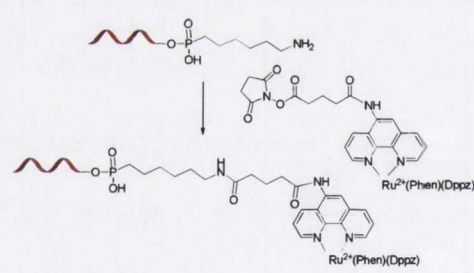
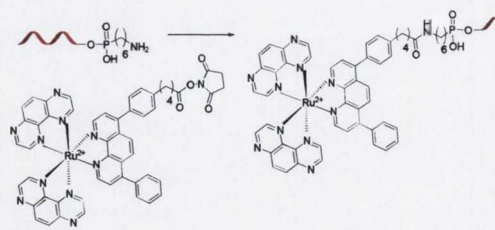
(Method 2) This method involves the incorporation of a functional group into the ODN during automated DNA synthesis, followed by conjugation to the metal complex, while still protected and attached to the solid support column. An example of this approach, involved the introduction of a ruthenium complex modified with an activated carboxylic acid group to an amino substituted ODN while still attached to the CPG support.³⁴⁻³⁶ The amino group was initially incorporated by activating the 5'-hydroxyl of the ribose with carbonyldiimidazole and subsequent reaction with 1,9-diaminononane. The N-succinimidyl ester of the complex, with an excess of DIPEA, was then coupled with an amino modified ODN-CPG resin for 12 hours. This synthetic strategy was initially used by the Barton group to conjugate a rhodium complex to the 5' end of an ODN.³⁷

2.5.2 'Off-column' Preparation of Ruthenium Oligonucleotide Conjugates

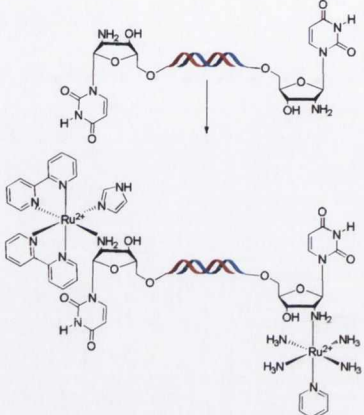
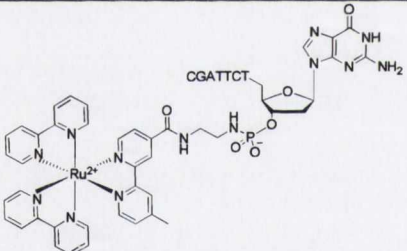
The 'off-column' approach, or solution-phase coupling, involves the introduction of a functional group to the ODN during automated synthesis, followed by cleavage from the solid support, deprotection and purification. A mutually reactive functional group is introduced into the metal complex. Conjugation then takes place between the functionalised ODN and the metal complex in solution. The majority of these preparations involve the reaction between an amino functionalised ODN and an activated acid (NHS ester) derivative of a Ru complex to form an amide linkage (Table 2.2).

Table 2.2 'Off-column' - Approach 2

Author ^{ref}	Method of attachment	Attachment site	Complex
Bannwarth <i>et al.</i> ³⁸	5'-amino-5'-deoxythymidine phosphoramidite incorporated during automated DNA synthesis. Reacted with TSU activated ester of carboxy group on ligand of Ru complex	5'-terminus	
Telser <i>et al.</i> ³⁹	C and T modified nucleobases with alkyl amino linkers were incorporated into ODN. ODN reacted with NHS bipyridine followed by reaction with Ru (bpy) ₂ Cl ₂	Mid-strand	

Author ^{ref}	Method of attachment	Attachment site	Complex
Ortmans <i>et al.</i> ¹⁷	Uracil modified phosphoramidite bearing an amino group. Reacted with TSU ester of Ru complex	Mid-strand	
Crean <i>et al.</i> ¹⁶	Hexylamino linker attached to the 5' end of ODN. Coupled with TSU ester of Ru complex	5'-terminus	
Barton ¹³⁻¹⁴	Hexylamino linker attached to the 5' end of ODN. Coupled with TSU ester of Ru complex	5'-terminus	
Garcia-Fresnadillo <i>et al.</i> ⁴⁰ Lentzen <i>et al.</i> ^{18,41}	Amino introduced mid strand using a uridine phosphoramidite or a commercial aminohexyl phosphoramidite. Coupled with activated ester of Ru complex	5'-terminus & mid-strand	

Author ^{ref}	Method of attachment	Attachment site	Complex
Ossipov <i>et al.</i> ¹⁹	Amino functionalised phosphoramidite linker incorporated into ODN and reacted with NHS ester of Ru complex	5'-terminus & mid-strand	
Grimm <i>et al.</i> ⁴²	Two approaches: (1) Amino-Ru complex with activated 5' phosphate & (2) Bromo modified Ru complex with thiolated ODN	5'-terminus	
Wiederdolt & McLaughlin ⁴³	Involved the synthesis of a 2-2'bipyridine phosphoramidite. After cleavage and deprotection the ODN was reacted with Ru(bpy) ₂ Cl ₂ to form the ODN conjugate	Mid-strand	

Author ^{ref}	Method of attachment	Attachment site	Complex
Meade & Kayyem ¹⁵	Uracil phosphoramidite 2' amino incorporated on the 5' end. Duplexes formed. Reacted each with [Ru(bpy) ₂ CO ₃] and [Ru(NH ₃) ₄ (py)] ²⁺ respectively.	5'-terminus	
Bichenkova <i>et al.</i> ⁴⁴	Amino Ru complex with activated phosphate of ODN	5' & 3' terminus	

Bannwarth *et al.*³⁸ attached bathophenanthroline Ru (II) complexes successfully to ODNs, whereby one of the phenanthroline ligands had a linker arm terminating in a carboxyl group. This was activated using N,N,N',N'-tetramethyl(succinimido)uranium tetrafluoroborate (TSU), which was found to be much cleaner than the N-hydroxysuccinimide (NHS) and dicyclohexyldicarbodimide (DCC) usually used. An amino group was introduced onto the 5' position of the terminal ribose of a thymidine base by the introduction of a 5'-amino-5'-deoxythymidine phosphoramidite during automated DNA synthesis. The ODN was cleaved from the support and deprotected. The ruthenium complex was then conjugated with the ODN, with a reaction time of 24 hours and a 25 fold excess of the complex being used. Purification proved difficult because of the large excess of the complex. Similar approaches have been reported with the main variance being in the position of the amino group within the ODN. C and T

modified nucleobases with alkyl amino linkers³⁹ and a uracil modified phosphoramidite bearing and amino group were reported.^{18,40-41} Hexylamino linkers attached to the 5' ribose have been used including within the Kelly group.^{13-14,16} Ossipov *et al.*¹⁹ incorporated an amino linker phosphoramidite into the ODN and subsequently coupled it with the activated ester of an Ru complex. Grimm *et al.*⁴² used two approaches. Firstly, they coupled an amino modified Ru complex with an activated 5' phosphate. Secondly, they coupled a bromo Ru complex derivative with a thiolated ODN. An interesting approach by Wiederholt & McLaughlin⁴³ involved the synthesis of a 2'-2'bipyridine phosphoramidite. After cleavage and deprotection, the ODN was reacted with Ru(bpy)₂Cl₂ to form the Ru-ODN conjugate. Another unusual approach was carried out by Meade & Kayyem¹⁵ where they inserted a uracil phosphoramidite with an amino group on the 2' position of the ribose at the 5' end of two individual complementary strands. They then formed duplexes with each individual strand, with its corresponding non modified strand, and reacted each with [Ru(bpy)₂CO₃] and [Ru(NH₃)₄(py)]²⁺, respectively. The strands were then denatured using 7 M urea and purified by HPLC. The two ruthenium modified strands were annealed to give a duplex with a ruthenium complex at each end. Bichenkova *et al.* prepared Ru and Os ODN conjugates by activating the 3' or 5' phosphate group of the ODN and subsequently coupling it with amino derivatives of the metal complexes.⁴⁴

2.5.3 Stability of Ruthenium Modified Oligonucleotides

In the above examples, the stability of the modified ODN strands was accessed by duplex melting curve experiments and circular dichroism (CD). Quite a variance was observed depending on the site of ligation, with the general trend resulting in a few conclusions. Attachment at the terminus of the ODN strands, as opposed to mid strand, resulted in more stable duplex formation, comparable with the unmodified duplex control. A further degree of stability was found if a linker was inserted, so that the complex did not interfere as much with the base pairing. It was found in the case of nucleobase modifications that those that did not disrupt the hydrogen bonding capabilities i.e., T or U as opposed to C, resulted in much more stable duplexes and can account for their frequent use.

2.6 Advantages and Disadvantages of ‘On-Column’/ ‘Off-Column’ Approaches

There are many advantages and disadvantages to both approaches. In the case of the ‘on-column’ approach, less side reactions and purification steps make it quite favourable, but the complex must be able to withstand both the mildly acidic and strongly basic conditions of the automated DNA synthesis procedure. To prepare a phosphoramidite or CPG support, the compound must have an unprotected hydroxyl group and be suited to P(III) chemistry. The synthesis of the appropriate phosphoramidite, which is moisture sensitive and can be poorly soluble in common solvents, is often time consuming and problematic. Problems with the ‘off-column’ approach include the low coupling yield, requiring a large excess of material, and thus tedious purification, resulting in low yields. Side reactions can pose problems in some instances. The key advantage to the ‘off-column’ approach is that there are many commercially available precursors for the introduction of functional groups to ODNs. Once the coupling has been carried out, the product is only exposed to mild conditions, such as the purification conditions of HPLC or PAGE.

2.7 The Advent of ‘Click Chemistry’– An ‘Off-Column’ Approach

The term ‘click chemistry’ was coined by Sharpless⁴⁵ and although generally used in reference to Huisgen’s 1,3 dipolar cycloaddition, the concept can be applied across a range of efficient reactions that have been developed for the incorporation of reporter groups into ODNs (Table 2.3). It refers to a set of efficient reactions that occur under mild conditions, which are chemoselective, high yielding and easily purified. The reactions generally involve the formation of carbon-heteroatom bonds, with the reactions being energetically favourable. Click reactions have a high thermodynamic driving force ($>20 \text{ kcal mol}^{-1}$). Click chemistry was originally set out for the advancement of drug discovery, but it has since seen much use in the field of material sciences.⁴⁶ The advent of click reactions has made the ‘off-column’ approach much more attractive. The need to factor in both the mildly acidic and especially the strongly basic conditions of automated DNA synthesis in the planning of functionalised ODNs has been removed.

Table 2.3 Commonly employed ligation reactions that can be classed as ‘click’ reactions. R_1 and R_2 represent either an ODN or reporter group.

Name of chemical reaction	Schematic
Huisgen 1,3-dipolar cycloaddition ⁴⁷⁻⁴⁸	
Diels-Alder cycloaddition ⁴⁹	
Staudinger reaction ⁵⁰	
Reductive amination via imine ⁵¹	
Hydrazone reaction ⁵¹	
Oxime reaction ^{11-12,51}	

The Huisgen’s 1,3 dipolar cycloaddition has seen a lot of use for the efficient modification of ODNs.⁴⁷⁻⁴⁸ However, this method involves a copper catalysis step, which could interfere with the incorporation of metal complexes into ODNs. Ruthenium being a d^6 metal complex, and thus fairly inert, may be compatible with the copper catalysis step. We wanted a system that could be applied across a wide range of metal complexes and that could be extended to include systems such as porphyrins, with a variety of metal centres.

2.8 Oxime Ligation

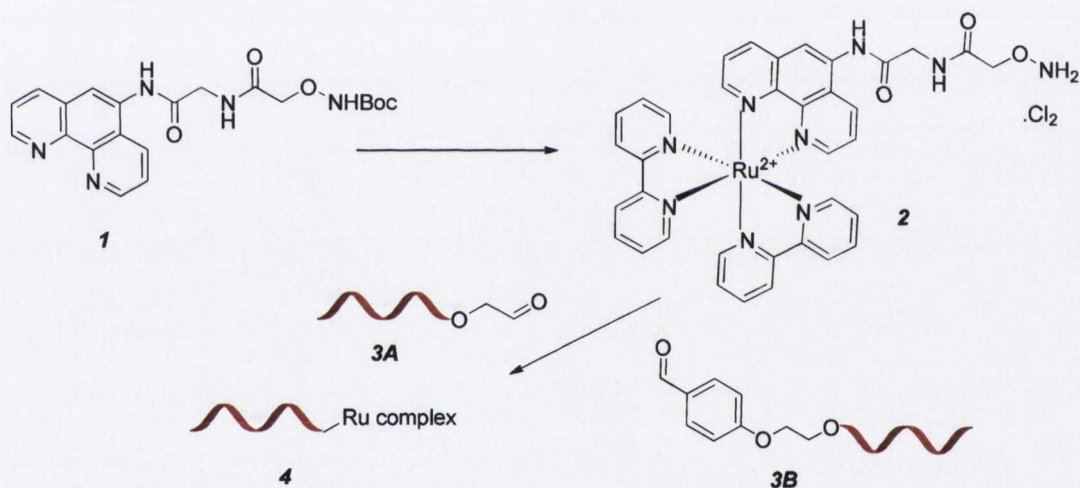
Carbonyl group addition–elimination reactions form the basis for the conjugation of many molecules to ODNs with ligations such as imine, hydrazone and more recently oxime being used.⁵² An oxime bond forms from reaction of an oxyamine with an aldehyde or ketone. This reaction has been used to conjugate many compounds to ODNs including peptides, carbohydrates, a modified phenanthroline derivative and most recently a ruthenium complex.⁵³⁻⁵⁶ The ideal conjugation reaction takes place between unprotected moieties, with little chemical manipulation, minimal material, easy work up and high yield. Oxime ligation is close to fitting all these requirements. It is chemoselective. It requires no coupling reagent or chemical manipulation for its

introduction, simply stirring the components in buffered solution is sufficient. Only a slight excess of material is required, improving greatly on the activated acid method previously used by our group.¹⁶ It is efficient with high yields. Reactions are clean with purification being achieved simply by HPLC or preparative PAGE.

The incorporation of aldehydes and aminooxy groups, to both the ODN, and the reporter group have been described. The aminooxy group is highly reactive, and thus is generally incorporated into the reporter group, as it is available in larger quantities, and subsequently reacted with an aldehyde modified ODN. The high reactivity of aminooxy groups can be attributed to an α -effect. The presence of a lone pair of electrons on the oxygen next to the amino group makes it a strong nucleophile.⁵⁷ A selection of aminooxy phosphoramidites have been prepared and incorporated into ODNs.⁵⁸⁻⁶¹ Aldehyde modified ODNs are generally prepared by incorporating a 1,2-diol that necessitates oxidation to the corresponding aldehyde,⁵³ or as an acetal, which after treatment reveals the required aldehyde. Ketones have also been successfully used but longer reaction times are required.⁶² The bis conjugation of ODNs has been carried out employing an aldehyde at both ends of the ODN.⁶³ A recent report by the Defrancq group has shown the preparation of a bifunctionalised ODN using successive oxime and 1,3-Huisgen cycloaddition reactions.⁶⁴

2.9 Overview of Results

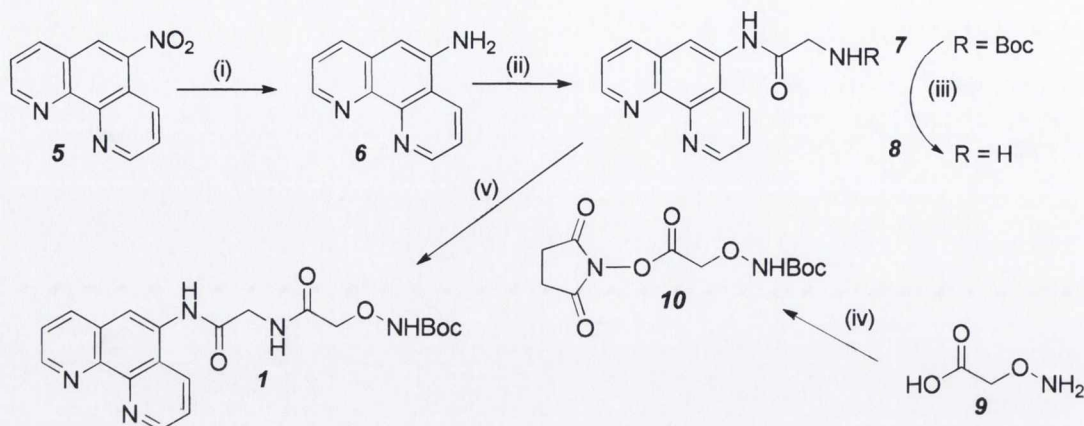
The preparation of Ru-ODN conjugates via oxime ligation is described and discussed below. An overall schematic for the reactions is shown in Scheme 2.1. The preparation of an oxyamine modified phenanthroline ligand (**1**) is described and its subsequent incorporation into a ruthenium complex (**2**). Ru conjugation was carried out at both the 5' and 3' ends of ODNs. The introduction of diols on the 5' and 3' end of ODNs is discussed. The preparation of a phosphoramidite for the introduction of a diol on the 5' terminus is detailed. The synthesis of 3'-diol modified ODNs is described and the subsequent preparation of Ru-ODN conjugates with these strands is outlined. The use of an aromatic aldehyde rather than an aliphatic aldehyde for the preparation of Ru-ODN conjugates is described.



Scheme 2.1 Overall schematic for the results presented in this Chapter. A phenanthroline ligand bearing an oxyamine (**1**) was prepared, subsequently incorporated into a Ru complex (**2**) and reacted with various aldehyde modified ODNs (**3A** or **3B**) to give Ru-ODN conjugates (**4**).

2.10 Synthesis of an Oxyamine Modified Phenanthroline Ligand

The synthesis of an oxyamine modified phenanthroline ligand (**1**) (Scheme 2.2) was carried out by largely following literature procedures, but introducing modifications to optimise the process.⁵⁵



Scheme 2.2 Preparation of 2-Boc-aminoxy-N-[(1,10) phenanthroline-5-ylcarbamoylmethyl]-acetamide (**1**). (i) N_2H_4 , EtOH, Pd/C, 70 °C, 4 h (ii) N-Boc-Glycine anhydride prepared in situ – [DCC, N-Boc-Glycine, MeCN, 1 h (16 equivalents)], MeCN, 42 h (iii) DCM/TFA (50/50, v/v), 2 h (iv) (a) $(\text{Boc})_2\text{O}$, NaOH, H_2O :1,4-dioxane (50/50, v/v), (b) NHS, DCC, MeCN (v) DMF, DIPEA

The 5-nitro-1,10-phenanthroline (**5**) was reduced to the 5-amino-1,10-phenanthroline (**6**) using Pd/C and hydrazine in ethanol, in excellent yield (94%), following well documented procedures.⁶⁵⁻⁶⁷

The exocyclic amine of **6** is very unreactive and thus an excess of the N-Boc-Glycine anhydride was needed for the synthesis of **7**. The N-Boc-glycine anhydride was prepared by reacting two equivalents of N-Boc-glycine with dicyclohexylcarbodiimide (DCC) in acetonitrile for 1 hour, followed by filtration to remove the DCU side product. It was found, deviating from the reported synthesis, that 16 equivalents of the anhydride were necessary for complete reaction. It was also found that the anhydride was moisture sensitive. All filtrations were carried out using a filter stick, under N_2 , and subsequent concentration of the reaction mixture was carried out under vacuum, using schlenk techniques. The starting compound **6** was insoluble in acetonitrile, whereas the product **7** was completely soluble. This was a good indication of the end point of the reaction, along with monitoring by TLC (DCM/MeOH/ NH_4OH , 4:1:0.05). TLC plates were visualised by UV and stained using a ferrous chloride solution. Bright red spots were observed on complexation with the phenanthroline derivatives. Purification of **7** proved

difficult because of the large excess of the anhydride used, but was achieved with 70% recovery, using silica gel column chromatography with a solvent system of DCM:MeOH (9:1) + NH₄OH (2%). A pale yellow solid was obtained.

The *tert*-butoxycarbonyl (Boc) group was then removed by treatment of **7** with dichloromethane/trifluoroacetic acid (50/50, v/v) at room temperature for 2 hours. The successful deprotection was confirmed by ESI-MS. The product **8** was obtained in quantitative yield and used without further purification in the next step. It should be noted that there is the possibility of forming the TFA salt of the compound, and the use of a strong base (DIPEA) in the next step ensures neutralisation to the amino compound.

Compound **10** was synthesised in two steps from *O*-(carboxymethyl)hydroxylamine hemi hydrochloride (**9**).⁶⁸ Boc protection was carried out using di-*tert*-butyldicarbonate and NaOH in H₂O/1,4 dioxane (50/50, v/v) for 3 hours. A white crystalline solid was obtained in 70% yield. The carboxylic acid group was converted to the corresponding activated ester **10**, using N-hydroxysuccinimide (NHS) and the coupling reagent dicyclohexylcarbodiimide (DCC) in anhydrous acetonitrile. A white crystalline solid was obtained in 51% yield after recrystallisation with carbon tetrachloride. It was found that this product was not stable for long periods of time and was best prepared fresh when needed. (Boc-aminoxy)-acetic acid is commercially available and was used in repeat efforts of the synthesis, reducing this to one step.

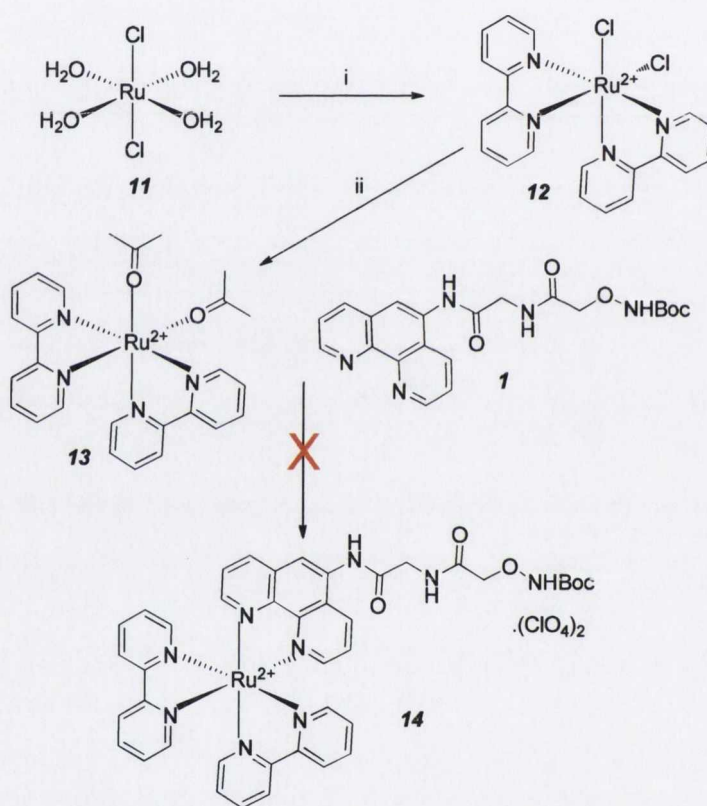
The required ligand 2-Boc-aminoxy-N-[(1,10) phenanthroline-5-ylcarbamoylmethyl]-acetamide (**1**) was then prepared by combining **8** and **10**, in anhydrous DMF with DIPEA at room temperature for 1 hour. Purification by silica gel column chromatography yielded a white solid in 42% yield. All compounds were fully characterised using ¹H and ¹³C NMR and high resolution mass spectrometry.

2.11 Synthesis of a Ruthenium Complex

The *tert*-butoxycarbonyl group was left on the phenanthroline ligand, due to the high reactivity of the oxyamine group with traces of carbonyl groups. The high reactivity of this group is due to an α -effect. The lone pair of electrons on the adjacent oxygen atom

to the nitrogen increases the nucleophilicity of the oxamine. It was found that the Boc group is quite susceptible to cleavage when the ligand is attached to the ruthenium metal centre and thus the mildest conditions possible were sought for the synthesis of the complex.

The first compound used was $[\text{Ru}(\text{bpy})_2(\text{acetone})_2]^{2+}$ **13**, but the results were not consistent and the desired product **14** was not obtained (Scheme 2.3).



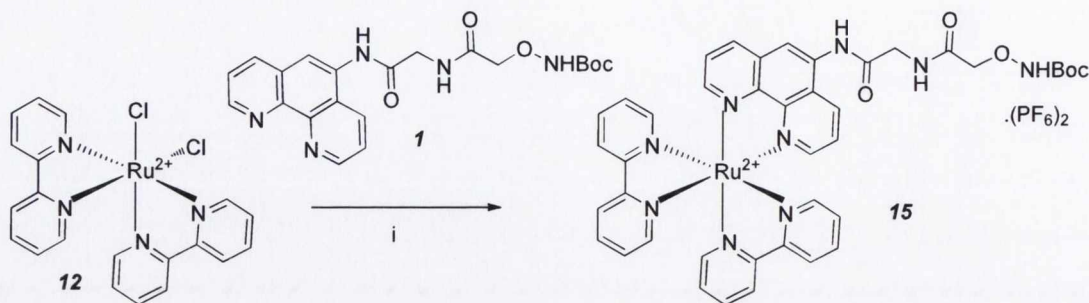
Scheme 2.3 Synthesis of $\text{Ru}(\text{bpy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (**12**) and attempted synthesis of **14** via an acetone intermediate (**13**). (i) 2,2'-bipyridyl, LiCl, DMF, 9 h (ii) Acetone, AgClO_4 ⁶⁹

$\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$ (**11**) was converted to the popular precursor for the synthesis of ruthenium complexes $\text{Ru}(\text{bpy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$ (**12**) following the reported method.⁶⁹ **11** was refluxed in DMF, with 2,2'-bipyridyl in the presence of LiCl and after cooling to room temperature, the product was obtained by precipitation with acetone. The product **12** was filtered and

washed with copious amounts of water to remove the by-product $\text{Ru}(\text{bpy})_3\text{Cl}_2$. A dark green crystalline product was obtained in reasonable yield. This was then further reacted with acetone in the presence of silver perchlorate to give the acetone intermediate **13**. Following filtration of the solution, an excess of ligand was added, and the reaction heated at reflux to afford the appropriate ruthenium complex. A test reaction using 5-nitro-1,10-phenanthroline was used initially with some success, but on application to the required ligand **1** and subsequent trials, the required complex **14** was not obtained. Reference to the fact that the bis(acetone) complex is moisture sensitive was made in the paper and may account for the lack of success in using this reaction procedure. It was found in the majority of instances that the red/brown solution, complex **13**, turned green. Initial thoughts were that the ruthenium complex was being oxidised to Ru^{3+} by the silver perchlorate, but even by reducing the amount used, problems were encountered. The reaction was repeated in HPLC grade acetone, and acetone dried over molecular sieves, but to no avail. All reactions were carried out under nitrogen, using schlenk techniques for the filtration. It is thought that perhaps the scale of the reaction has a role to play, or that some kind of dimer product is being formed, but due to the valuable nature of the ligand, I was not at liberty to try a large scale reaction.

A common approach for the synthesis of ruthenium complexes is to simply reflux the appropriate ligand with $\text{Ru}(\text{bpy})_2\text{Cl}_2$ in methanol and H_2O . A slight variation on this was reported by Ramiro *et al.* where a modified Boc-protected phenanthroline ligand was refluxed in methanol with $\text{Ru}(\text{phen})_2\text{Cl}_2$.⁷⁰

This approach was applied to **1** (Scheme 2.4) and simply refluxing the ligand (1.2 equivalents) with $\text{Ru}(\text{bpy})_2\text{Cl}_2$ (**12**) in HPLC grade methanol, resulted in the required complex **15**, in 77% yield.



Scheme 2.4 Synthesis of Ru(bpy)₂ [2-Boc-aminoxy-N- (1,10) phenanthroline-5-ylcarbamoylmethyl)-acetamide] .(PF₆)₂ **15**. (i) MeOH, reflux.

Mass spectrometry analysis confirmed no loss of the Boc group and the complex was isolated as a PF₆ salt to aid purification. Analysis of the complex **15** by NMR and HPLC confirmed its purity and no further purification was necessary before its use in the ODN conjugation reactions. The absorbance and emission spectra (Figure 2.2) were obtained. The characteristic bands in the absorbance at 283 nm and 450 nm are the LC π - π^* and MLCT d- π^* bands respectively. Exciting through either band, gives an emission centered at 600 nm characteristic of polypyridine ruthenium complexes.

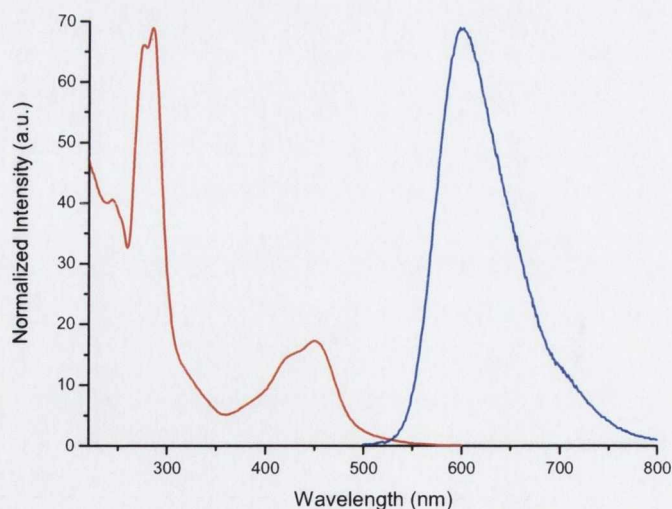


Figure 2.2 Normalised absorbance and emission spectra ($\lambda_{\text{ex}} = 450 \text{ nm}$) of Ru(bpy)₂ [2-Boc-aminoxy-N- (1,10) phenanthroline-5-ylcarbamoylmethyl)-acetamide].(PF₆)₂ **15** ($1 \times 10^{-4} \text{ M}$) in MeOH at room temperature.

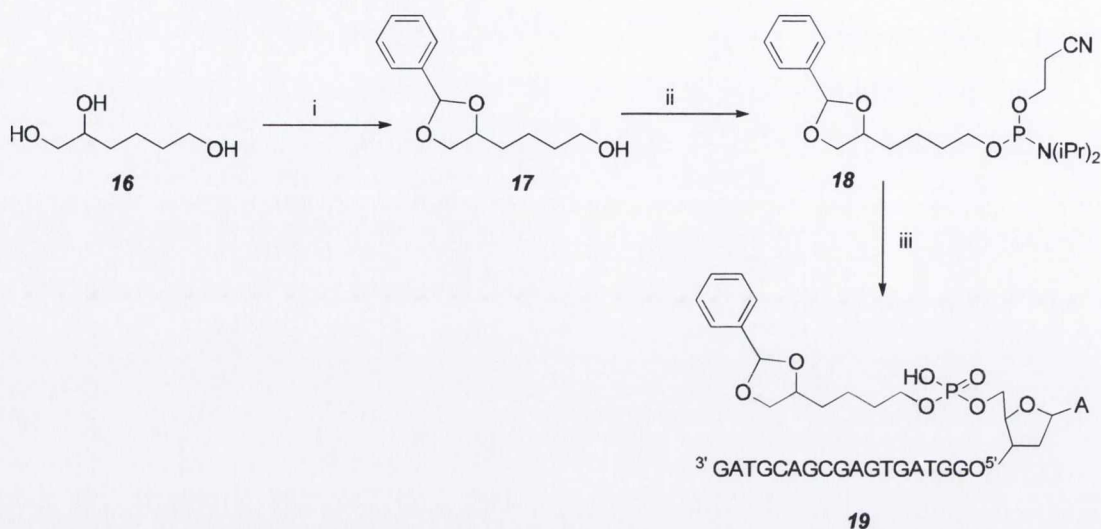
2.12 Synthesis of Aldehyde Modified Oligonucleotides

With the Boc protected oxyamine modified Ru complex **15** successfully prepared, we needed to prepare ODNs with an aldehyde modification for subsequent conjugation reactions. Initial experiments were carried out in Dublin and 5'-aldehyde modified ODNs were prepared to link with the 5'-nanoparticle conjugate work being carried out by Dr. Damian Aherne in the group at the time.

2.13 Synthesis of a Phosphoramidite for the 5' Introduction of an Aldehyde

The synthesis of a phosphoramidite (Scheme 2.5) introducing a protected 1,2 – diol to the 5' extremity of an ODN, in preparation for oxidation to an aldehyde, was previously reported in the literature.^{53,71}

Starting from 1,2,6-hexanetriol (**16**), the 1,2-diol was protected as a benzylidene acetal, using benzaldehyde dimethyl acetal in the presence of pyridinium paratoluene sulfonate to afford the protected diol **17**. The pure product was obtained in moderate yield (58%) after purification by silica gel column chromatography, using dichloromethane and methanol, increasing from 2 to 10% methanol. **17** was then phosphitylated, using 2-cyanoethyl diisopropylchlorophosphoramidite in dry dichloromethane, to afford the phosphoramidite **18**. The phosphoramidite was purified by column chromatography (EtOAc/cyclohexane/Et₃N, 90:10:2).



Scheme 2.5 Synthesis of phosphoramidite **18** for the 5' introduction of an aldehyde to an ODN and subsequent DNA synthesis to give the modified ODN **19**.^{53,71} (i) PPTS, benzaldehyde dimethyl acetal, DMF, 55 °C, 24 h, (ii) DIPEA, 2-cyanoethyl-diisopropylchlorophosphoramidite, dry DCM (iii) Automated DNA synthesis, 28% ammonia for 2 h, 16 h at 55 °C

The ^{31}P NMR showed a single peak downfield at 148 ppm representative of the P(III) product (Figure 2.3). P(V) compounds appear upfield ~ 12 ppm, and as such, this is a great tool for checking the purity of phosphoramidite compounds. It is known that phosphoramidite reagents are not very stable and must be used shortly after synthesis or oxidation to the unreactive P(V) compound is observed.

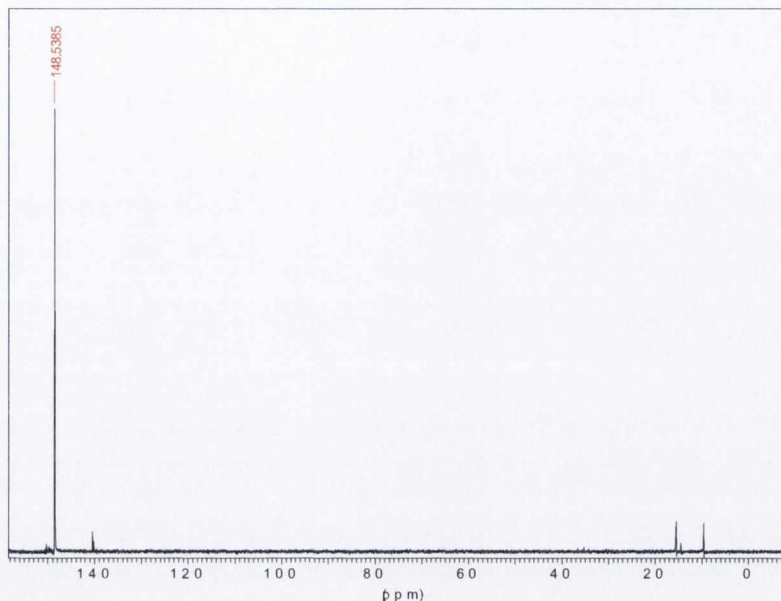


Figure 2.3 ^{31}P NMR of phosphoramidite **18**

2.14 Automated DNA Synthesis of 5'-Diol Oligonucleotide

DNA synthesis (200 nmol scale) was carried out on the TCD Beckmann Oligo 1000M synthesizer, incorporating the prepared phosphoramidite **18** at the 5'-terminus of the ODN. Treatment with ammonium hydroxide solution (55 °C for 16 h) removed the protecting groups from the bases and cleaved the strand from the controlled pore glass (CPG) support, to give **19**. The ammonia was removed by speed-vac. The synthesised ODN was desalted by precipitation with butanol. The solvent was then removed and the pellet dissolved in TEAA (0.1 M)/acetonitrile (9:1) in preparation for HPLC purification. The acetal group is similar to the DMT group in its ability to aid HPLC purification due to its hydrophobicity. The sample was observed on an analytical HPLC system, but unfortunately the yield, as determined by UV-Vis quantification, indicated that the coupling efficiency was very poor, and on inspection, the Beckmann synthesizer was found to be broken and beyond repair. Treatment of **19** with acetic acid would have yielded the corresponding diol and oxidation of this, with sodium periodate, would then afford the required aldehyde modification on the 5' end for conjugation with the prepared ruthenium complex.

All subsequent ODNs prepared by automated DNA synthesis in this thesis were prepared on an ABI 3400 DNA synthesizer (Applied Biosystems) in the lab of Prof. Eric Defrancq, Université J. Fourier, Grenoble, France on a 1 μ mole scale.

2.15 Conjugation of 3'-Aldehyde-ODNs with an Oxyamine Modified Ruthenium Complex

2.15.1 Automated DNA Synthesis of 3'-Diol Modified Oligonucleotides

ODN sequences **21A**, **21B**, **21C** and **21D** (Figure 2.4) were prepared (1 μ mol scale) by Prof. Eric Defrancq on an ABI 3400 DNA synthesizer. The diol moiety was introduced using the commercially available 3'-glyceryl support (**20**) (3-[4,4'-dimethoxytrityl) glyceryl-1-succinyl]) purchased from Eurogentec.⁵⁵ The strands were deprotected and cleaved from the solid support with a 28% ammonia solution for 16 hours at 55 °C. The strands were then purified by reverse phase HPLC. The ODNs were quantified using UV-Vis spectroscopy. The ODN structures were confirmed by MALDI-TOF mass spectrometry.

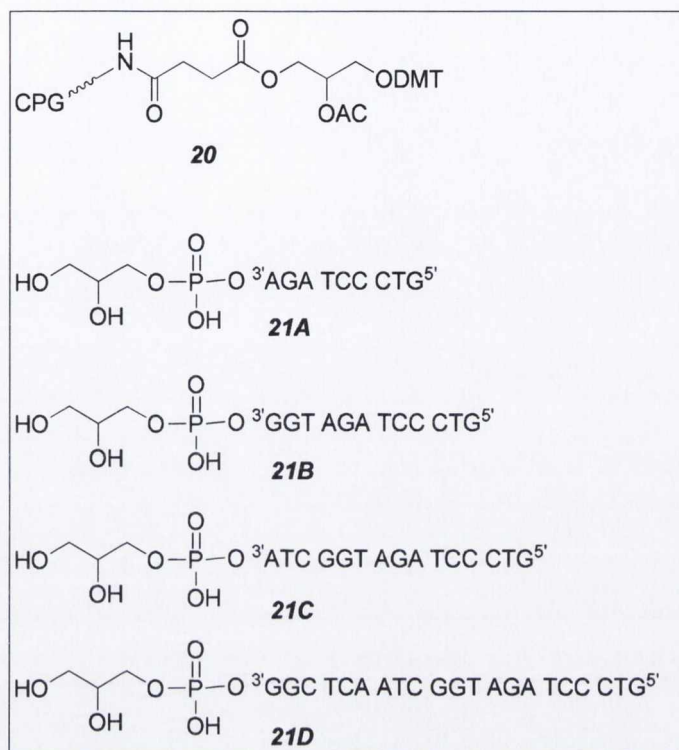
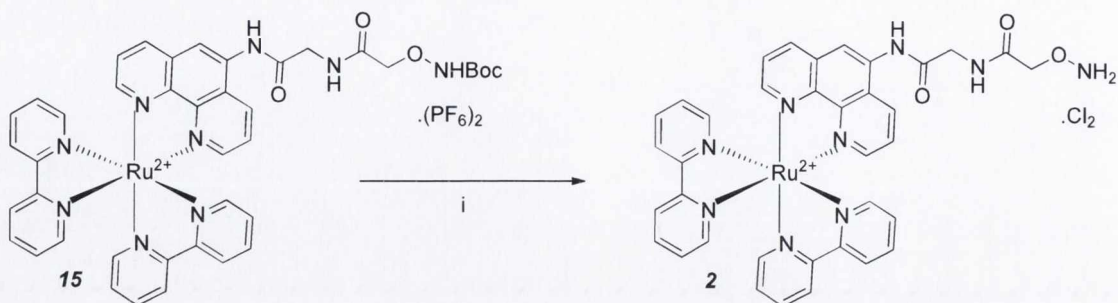


Figure 2.4 Commercially available CPG solid support **20** and the four 3' diol modified ODN sequences prepared, **21A**, **21B**, **21C** and **21D**

2.15.2 Deprotection of the Reactive Oxyamine on the Ruthenium Complex **15**

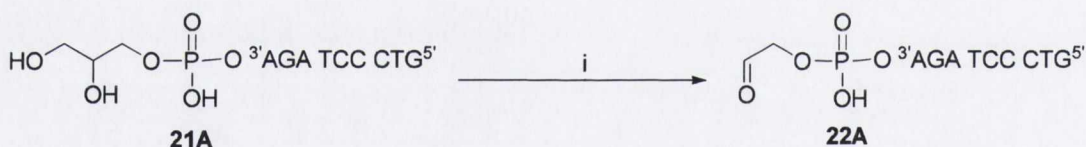
One of the key factors necessary for oxime conjugation is that both components must be aqueous soluble, as the reaction is carried out in aqueous buffer. The ruthenium complex **15** was isolated as a PF_6 salt to aid purification, which is not aqueous soluble. The Boc group, which is labile in acidic conditions, was successfully removed using hydrochloric acid (1 M) instead of the usual TFA (Scheme 2.6). This had the advantage that not only the Boc group was removed, but the PF_6 ions were exchanged for Cl^- ions, imparting solubility to the complex. The deprotection was monitored by analytical reverse-phase HPLC and was observed to be complete within 2.5 hours. The yield was taken to be quantitative.



Scheme 2.6 Removal of Boc group (i) HCl (1 M), 2.5 h

2.15.3 Oxidation of 3'-Diol to 3'-Aldehyde

Oxidation of the diol to the corresponding aldehyde was carried out in H_2O using an excess of sodium periodate. **21A**, **21B**, **21C** and **21D** were converted to the corresponding aldehydes **22A**, **22B**, **22C** and **22D** (Scheme 2.7). The excess NaIO_4 was removed by C18 reverse phase column chromatography, using H_2O and acetonitrile as eluent. Fractions were analysed using UV-Vis spectroscopy and the appropriate fractions combined. The yield was taken to be quantitative.

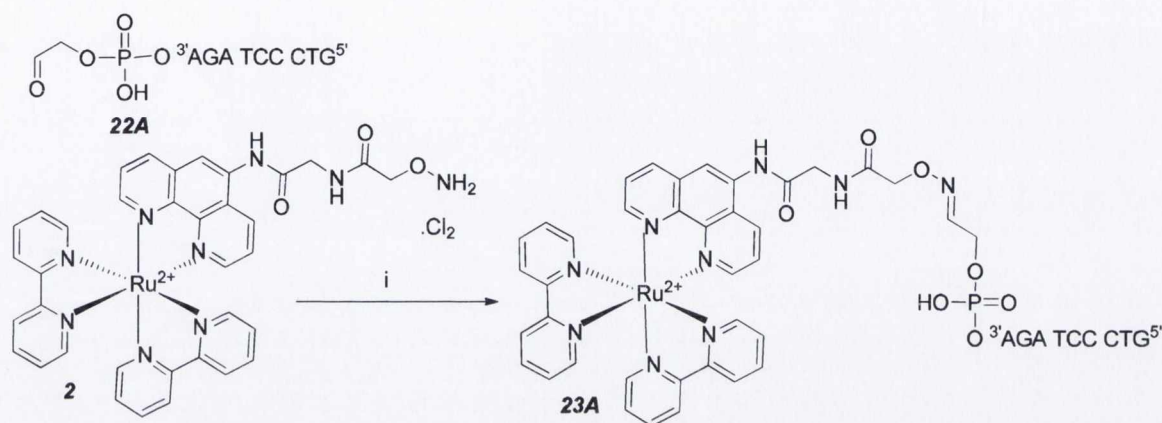


Scheme 2.7 Example of oxidation of **21A** (i) H_2O , NaIO_4 (20 equivalents)

2.15.4 Conjugation of the 3'-Aldehyde ODN with the Ru Complex

The coupling of the ODNs **22A**, **22B**, **22C** and **22D**, with the ruthenium complex **2** was carried out successfully by simply stirring the two components in ammonium acetate buffer (0.4 M, pH 4.5) to give the corresponding conjugates **23A**, **23B**, **23C** and **23D** (Scheme 2.8). Two equivalents of the oxyamine derivative were used because formation of a side product was reported if only one equivalent was used.⁵⁶ This side product was hypothesised to be from the addition of two ODNs to the ruthenium complex and a similar observation where two aldehydes add to an oxyamine derivative has been previously described.⁷² It had also been reported that a low volume (<500 μl) was

optimal for a successful conjugation. Reactions were monitored by reverse-phase HPLC. Reaction times were found to be ~5 hours.



Scheme 2.8 Coupling of ruthenium complex **2** with aldehyde modified ODN **22A** to give conjugate **23A**. (i) Ammonium acetate buffer (0.4 M, pH 4.5)

2.15.5 Purification and Analysis of 3' Ru-ODN Conjugates

The reverse-phase HPLC traces of the reaction mixtures showed evidence of free ODN in all cases (Figure 2.5). Unfortunately, the retention time of the starting materials **22A-D**, and products **23A-D**, were very close together, which excluded purification by HPLC. In the case of the shorter strands, there was a slight separation of the peaks, so HPLC purification might prove viable and should result in a better yield of recovery if the reaction was repeated.

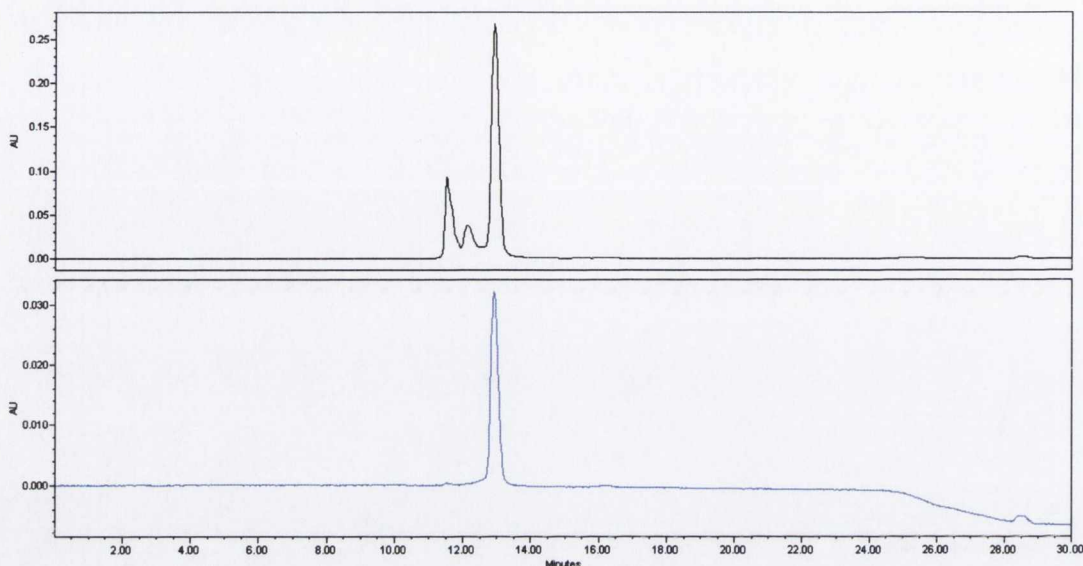


Figure 2.5 RP-HPLC profile of crude reaction mixture of **23A** – Detection at 260 nm (top) and at 420 nm (bottom).

All conjugates were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). Individual orange bands visible by eye were obtained in all cases with conjugates exhibiting slower migration than the free ODNs. The bands were visualised using UV shadowing, excised from the gel, crushed and extracted with water. The conjugates were desalted using C18 reverse phase silica gel chromatography. The pure product was recovered and analysed by HPLC to confirm purification. Individual peaks (Figure 2.6) were obtained for **23A** (9mer), **23C** (15mer) and **23D** (18mer) but two peaks (Figure 2.7) were obtained for **23B** (12mer). All ODNs were confirmed by MALDI-TOF mass spectrometry in negative ionisation mode (Table 2.4). The experimentally determined molecular weights were in excellent agreement with the calculated values. Figure 2.8 shows the actual, predicted and raw mass spectra for **23A**. Only one molecular weight species was observed on analysis of **23B**. The appearance of two peaks in the HPLC can be attributed to the E and Z isomers of the oxime bond and this has since been observed and reported by the Defrancq group in another instance.⁷³

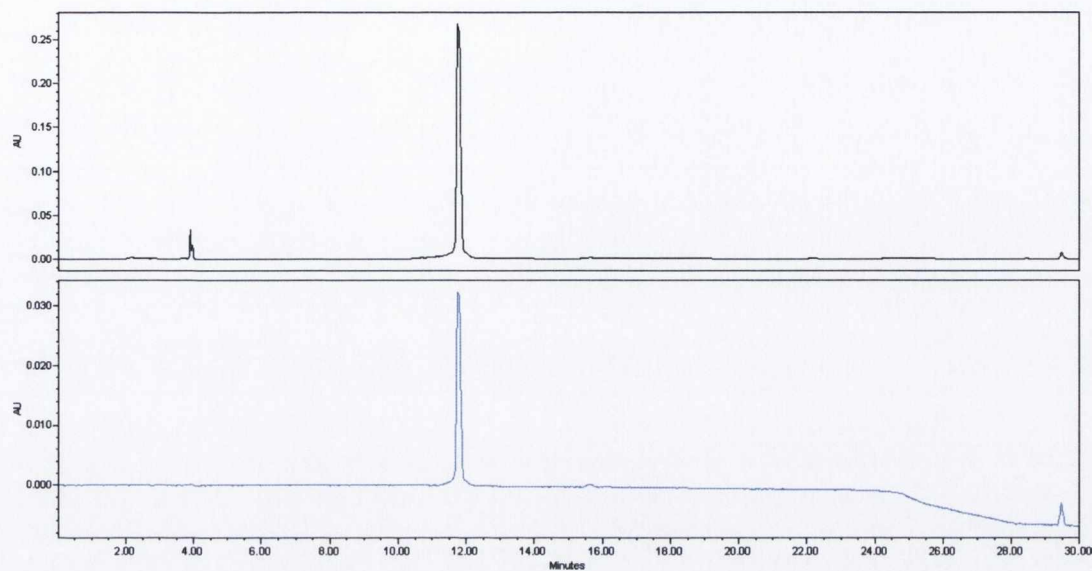


Figure 2.6 RP-HPLC profile after purification of **23A**, also characteristic of **23C** and **23D** – Detection at 260 nm (top) and at 420 nm (bottom).

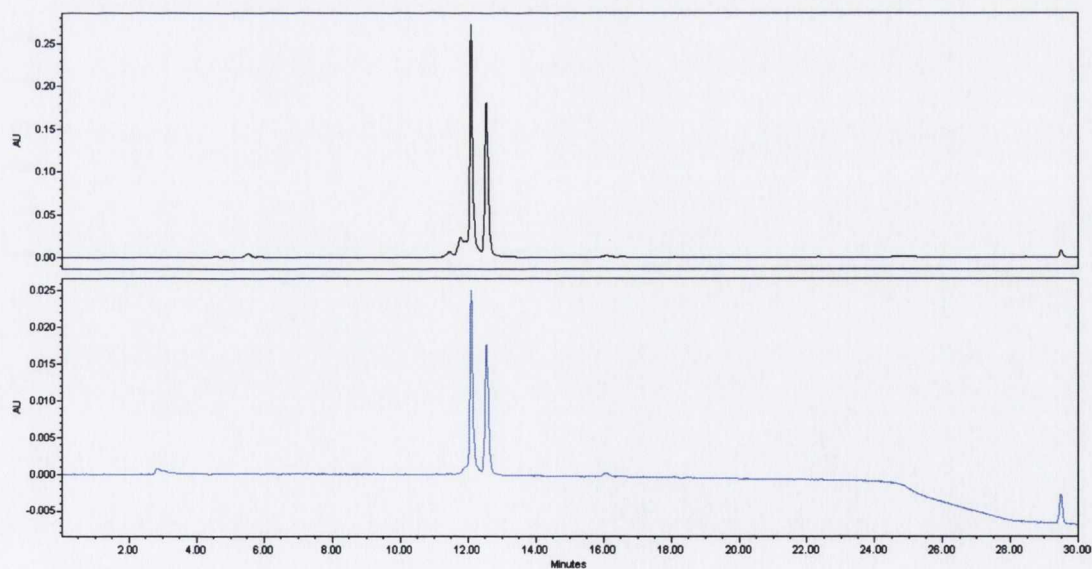
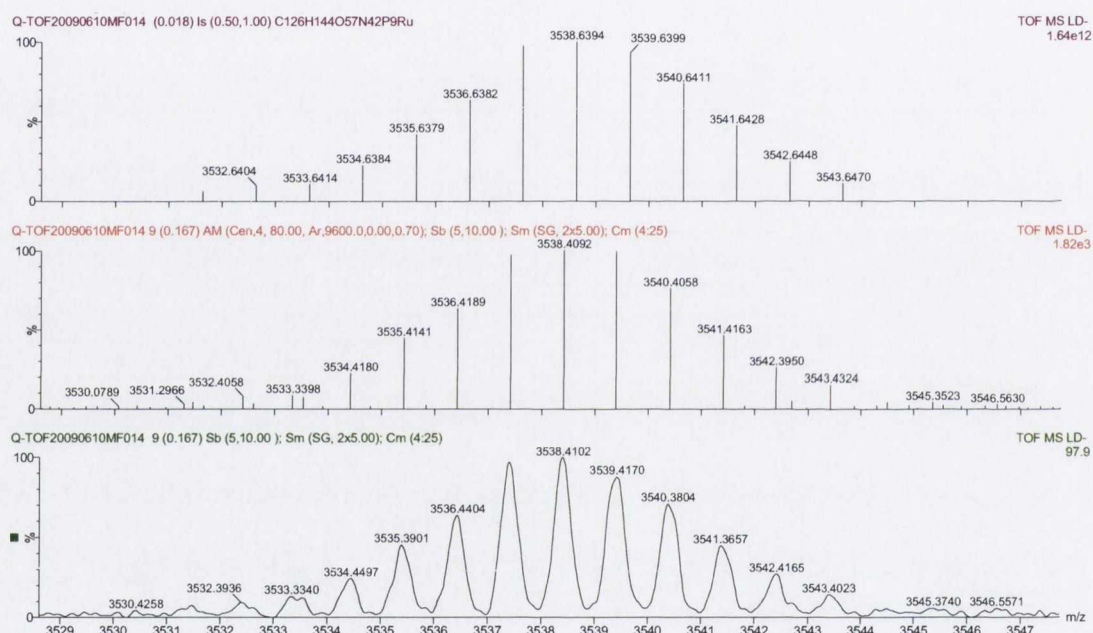


Figure 2.7 RP-HPLC profile after purification of **23B** – Detection at 260 nm (top) and at 420 nm (bottom).

Table 2.4 MALDI-TOF MS analysis of Ru-ODN conjugates in negative mode

ODN Sample Name	m/z Calculated	m/z Found
23A	3538.6	3538.4
23B	4502	4501.26
23C	5429	5429.3
23D	7263.2	7262.5

**Figure 2.8** MALDI-TOF mass spectrum for **23A** using 3-hydroxypicolinic acid as matrix. Calculated for $C_{126}H_{144}O_{57}N_{42}P_9Ru$ = 3538.6394, Found 3538.4092 [M-H]⁻

Yields were quantified by UV, using the extinction coefficient of $Ru(bpy)_2(phen-NH_2).(PF_6)_2$ ⁷⁴ to convert the spectrum into OD units. Yields of **23A** (0.18 mg, 15%), **23B** (0.252 mg, 16%), **23C** (0.2546 mg, 16%) and **23D** (0.15 mg, 7%) were obtained.

No degradation of the ruthenium complex was observed during the coupling reaction or the subsequent purification. The UV-Vis spectrum (Figure 2.9a) illustrated the successful coupling with both a band for the ODN (260 nm) and the MLCT band of the Ru complex (450 nm) observed. The Ru complex still showed its characteristic

emission on conjugation, exciting through the MLCT band at 450 nm, gave an emission centred at 600 nm (Figure 2.9b).

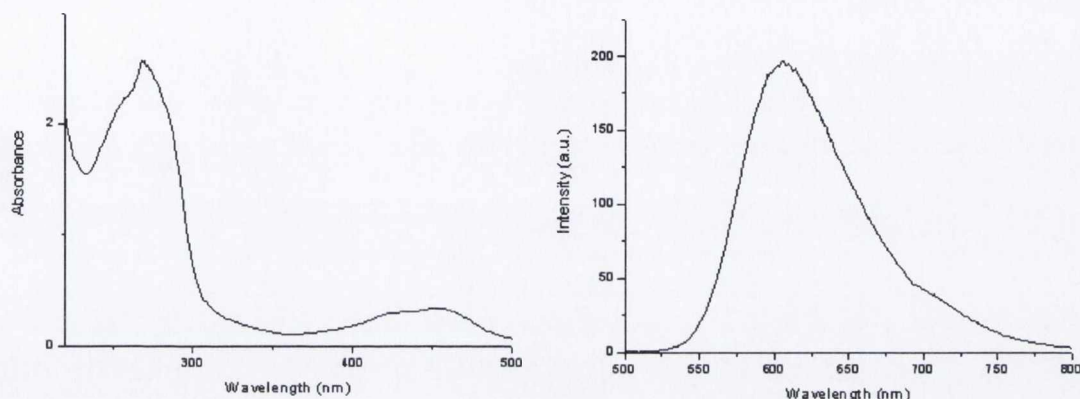


Figure 2.9 a) UV-Vis spectrum of **23A** and b) Emission spectrum ($\lambda_{\text{ex}} = 450$ nm) of **23A** in H_2O at room temperature (4.2×10^{-5} M).

2.16 The use of Aromatic Aldehyde Modified ODNs

The approach outlined in the above section involves the introduction of an aldehyde to the 3'-terminus of an ODN, by oxidation of the corresponding diol. The relatively strong oxidising conditions necessary limit the incorporation of other functionalities. It was noted that a formylindole phosphoramidite **24** and a 5'-aldehyde-modifier C2 phosphoramidite **25** were commercially available from Glen Research (Figure 2.10).⁷⁵⁻⁷⁶

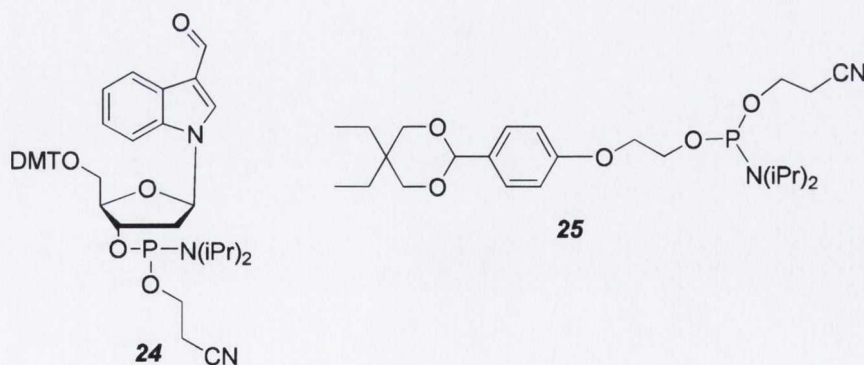


Figure 2.10 Commercial phosphoramidites for incorporation of aromatic aldehydes

It was proposed that they may be reactive enough to successfully form a conjugate via oximation removing the need for the oxidation step. Following discussion with Prof. Defrancq, his group used the 5'-aldehyde-modifier C2 phosphoramidite **25**, to prove that the aromatic aldehyde was indeed reactive enough to form an oxime linkage with an oxyamine derivative.⁷³ The formed linkage was also shown to be stable over a wider pH range than that formed with an aliphatic aldehyde. We used this approach to prepare 5'-Ru-ODN conjugates and then extended this to the preparation of bifunctionalised strands that could be used with nanoparticles (Chapter 4).

2.17 Conjugation of 5'-Aldehyde-ODNs with Oxyamine Modified Ruthenium Complex

2.17.1 Automated DNA Synthesis of 5'-Aldehyde Oligonucleotides

ODN sequences **26A** and **26B** (Figure 2.11) were prepared on a 1 μ mol scale, on an ABI 3400 DNA synthesizer, using standard β -cyanoethyl nucleoside phosphoramidite chemistry. The ODNs were 5' modified by incorporating phosphoramidite **25** during the last step of the automated synthesis. The strands were cleaved from the CPG solid support by treatment with a 28% ammonia solution and deprotected by placing the solution at 55 °C for 16 hours. The ODNs were purified by RP-HPLC assisted by the hydrophobic acetal protecting group.

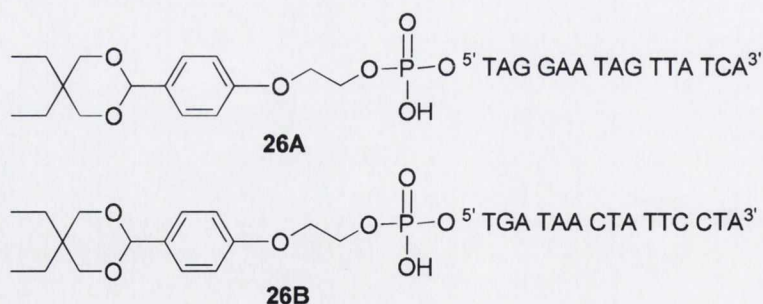


Figure 2.11 ODNs with 5' aromatic aldehyde modification

2.17.2 Removal of the Acetal Protecting Group

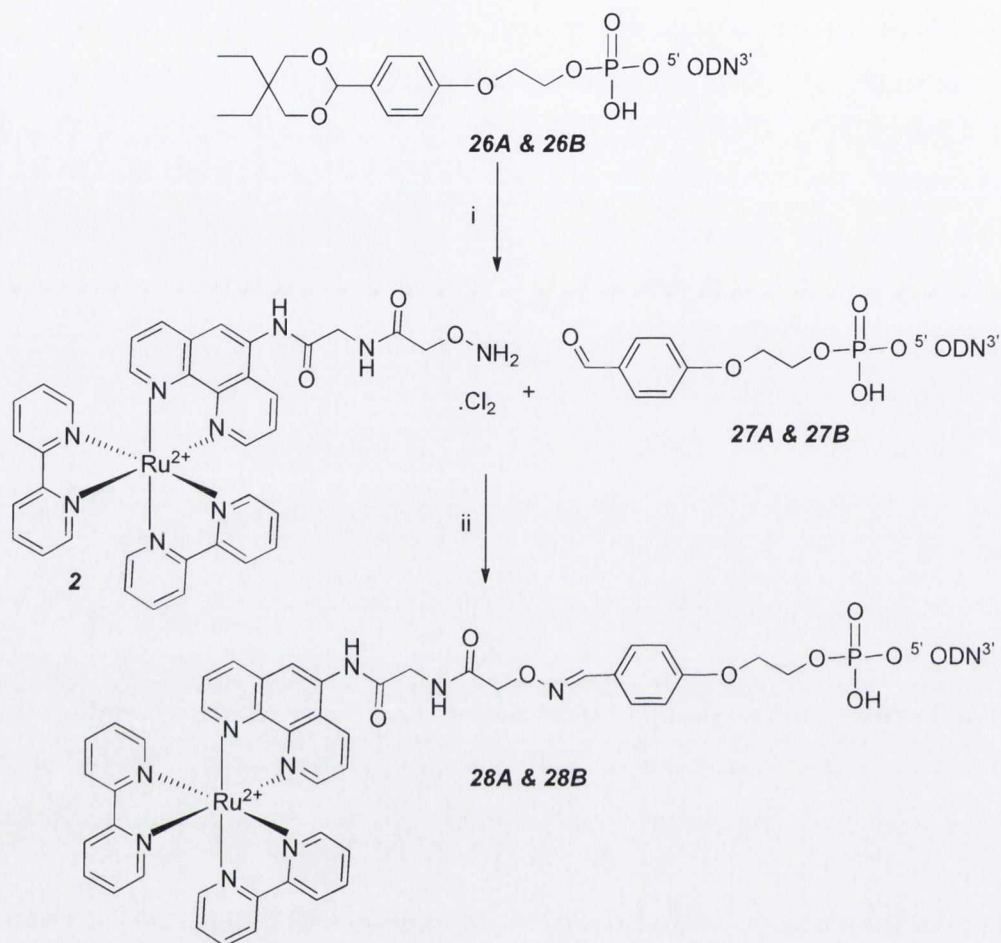
The acetal protecting group on the ODNs **26A** and **26B** was removed by treatment of the strands with 80% acetic acid, for 1 hour, at room temperature, to give **27A** and **27B** (Scheme 2.9). The sample was dried and quantitated using UV-Vis, measuring the OD at 260 nm.

2.17.3 Conjugation of the 5'-Benzaldehyde ODNs with Ru Complex

The oximation reaction was carried out as detailed previously for the 3'-aldehyde. The 5'-aldehyde modified ODN **27A** and **27B** and the ruthenium complex **2** (2 equivalents) were stirred in ammonium acetate buffer (0.4 M, pH 4.5) to give conjugate **28A** and **28B** (Scheme 2.9). The reaction was monitored by reverse phase HPLC (Figure 2.12) with the formation of the conjugate apparent after 3 hours.

2.17.4 Purification and Analysis of 5'-Ruthenium Oligonucleotide Conjugates

The conjugates were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). Individual orange bands, visible by eye, were obtained in all cases with conjugates exhibiting slower migration than the free ODNs. The bands were visualised using UV shadowing, excised from the gel, crushed and extracted with water. The conjugates were desalted using C18 reverse phase silica gel chromatography. Yields were quantified by UV-Vis, using the extinction coefficient of $\text{Ru}(\text{bpy})_2(\text{phen-NH}_2)(\text{PF}_6)_2^{74}$ to convert the spectrum into OD units. Yields of **28A** (0.278 mg, 25%) and **28B** (0.927 mg, 20%) were obtained. No degradation of the ruthenium complex was observed during the coupling reaction or the subsequent purification. Both ODNs were confirmed by MALDI-TOF mass spectrometry in negative ionisation mode (Table 2.5). The UV-Vis spectrum (Figure 2.13a) illustrated the successful coupling with both a band for the ODN (260 nm) and the MLCT band of the Ru complex (450 nm) observed, similar to what was found for the 3' case. The Ru complex still showed its characteristic emission on conjugation, exciting through the MLCT band at 450 nm gave an emission centred at 600 nm. The aromatic linkage can be concluded to have no affect (Figure 2.13b).



Scheme 2.9 Deprotection of acetal protecting group and coupling of aldehyde modified ODN **27A & 27B** with an oxyamine modified ruthenium complex **2** to give conjugate **28A** and **28B**. (i) 80% acetic acid, 1 h, (ii) Ammonium acetate buffer (0.4 M, pH 4.5)

Table 2.5 MALDI-TOF MS analysis of Ru-ODN conjugates in negative mode

ODN Sample Name	<i>m/z</i> Calculated	<i>m/z</i> Found
28A	5563.9	5562.7
28B	5474.9	5473.5

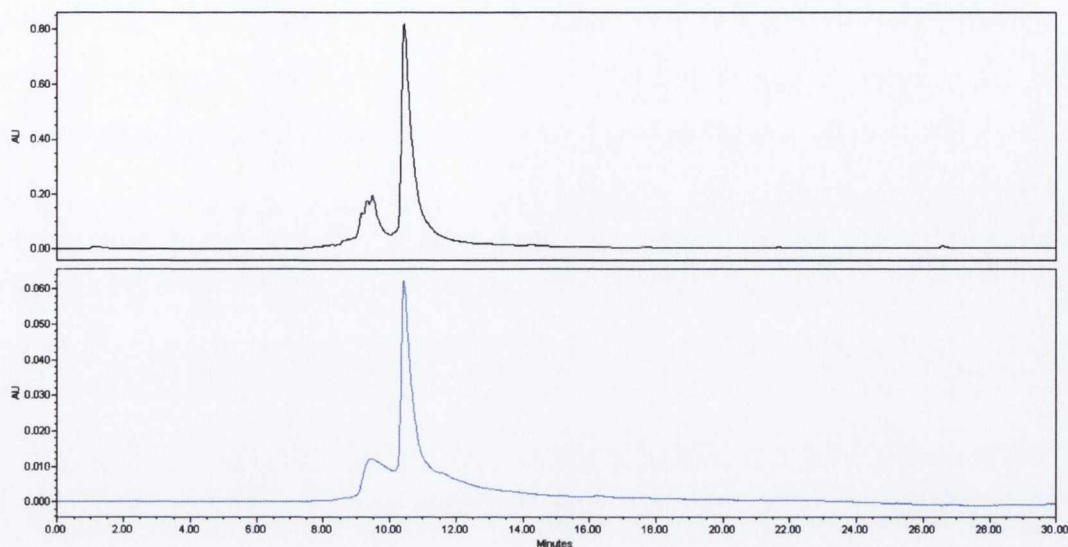


Figure 2.12 RP-HPLC profile of crude reaction mixture for Ru-ODN conjugate **28A** after 3 hours – Detection at 260 nm (top) and 420 nm (bottom).

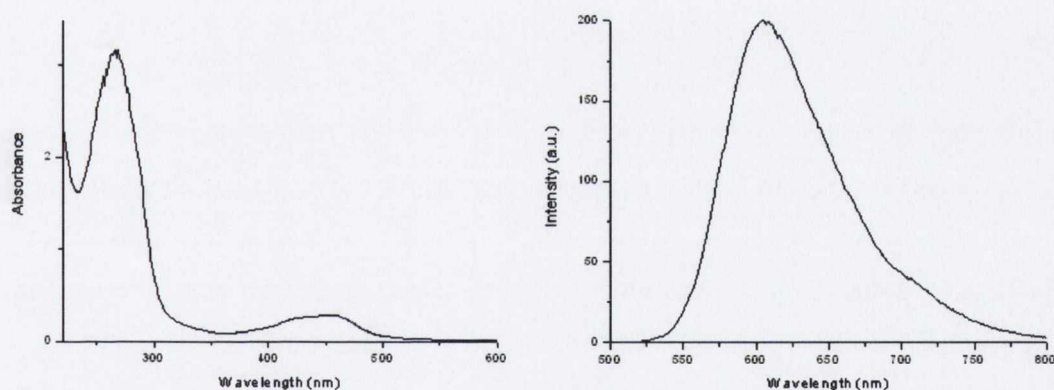


Figure 2.13 a) UV-Vis spectrum of **28A** and b) Emission spectrum ($\lambda_{\text{ex}} = 450 \text{ nm}$) of **28A** in H_2O at room temperature ($3.5 \times 10^{-5} \text{ M}$).

2.18 Conclusions

The oxyamine modified phenanthroline derivative **1** was successfully synthesised, isolated and characterised. It was effectively incorporated into a ruthenium complex **2** and the corresponding complex isolated and characterised. The solubility issue of the complex was addressed and the Boc protecting group successfully removed, for use of the reactive oxyamine functionality in further conjugation reactions.

A phosphoramidite **18** for the introduction of a diol to the 5' end of an ODN was prepared and characterised. It was incorporated into an ODN **19**, but unfortunately due to the DNA synthesizer malfunctioning, the yield obtained was very poor and we were unable to continue with this effort.

Diol moieties were successfully incorporated into the 3'-termini of a series of ODNs (**21A-D**). The corresponding aldehydes (**22A-D**) were successfully obtained by oxidation. Oxime chemistry was efficiently utilised to conjugate the prepared ruthenium complex **2** with the terminal aldehyde ODNs. The conjugates (**23A-D**) were successfully purified by polyacrylamide gel electrophoresis and analysed by HPLC. The prepared conjugates showed no evidence of degradation and their luminescent properties were unaffected. Their structure was confirmed by MALDI-TOF MS in negative ionisation mode.

The ease and efficiency of oxime ligation, as a means of attaching a compound bearing an oxyamine functionality (in particular a ruthenium complex), to an ODN was clearly demonstrated. The key success was the mild conditions necessary for the coupling reaction, improving on reported 'on-column' procedures, whereby the complex must be stable against acid and base. The ease of purification relative to other 'off-column' reports, including within the Kelly group,¹⁶ is another crucial point in favour of this method of conjugation. The viability of this approach for the attachment of many different compounds, both metal and organic, to ODNs is promising.

An aromatic aldehyde was successfully used to prepare 5'-ruthenium ODN conjugates. Benzaldehyde modified ODNs (**26A** and **26B**) were prepared by automated DNA synthesis incorporating a commercially available phosphoramidite. The strands were deprotected and subsequently coupled with the prepared ruthenium complex. The conjugates (**28A** and **28B**) were purified by polyacrylamide gel electrophoresis and analysed by HPLC. The prepared conjugates showed no evidence of degradation and their luminescent properties were unaffected. Their structure was confirmed by MALDI-TOF MS in negative ionisation mode.

The aromatic aldehyde was shown to be as efficient for the preparation of Ru-ODN conjugates as the aliphatic aldehyde. The elimination of the oxidation step expands the potential of the oximation approach, allowing for the possibility of preparing bifunctionalised strands, where previously the oxidation would have been detrimental to certain introduced functionalities. We have expanded this approach to prepare Ru-ODN conjugates, with a thiol moiety on the 3' end, for conjugation to nanoparticles and this is discussed in detail in Chapter 4.

2.19 References

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Chapter 3

Preparation, Stability and Functionalisation of Triangular Silver Nanoplates

3.1 Introduction

As outlined in the introductory chapter of this thesis, we have selected triangular silver nanoplates as our nanoparticle system of choice. The use of nanoparticles, in future applications, is reliant on the properties of the particle being maintained over an extended time period, and thus the long-term stability of the particle is key. Where it is necessary to further functionalise these particles, it is also essential to maintain the particle stability and useful properties of these systems during the functionalisation process. The work discussed in this chapter involves the preparation of such stable triangular silver nanoplates.

The main aim of this work was to prepare stable silver nanoplate oligonucleotide (ODN) conjugates and thus an analysis of the stability of the particles under the required conditions for ODN conjugation is outlined. The particles are shown to present a challenge for direct ODN conjugation, and methods of increasing their stability and more importantly the introduction of functionality for subsequent reaction are discussed. Successful approaches using thiol capping and titania coating are reported. We begin with some background information to the stability and functionalisation of nanoparticles with an emphasis on triangular silver nanoplates (Section 3.3-3.6).

3.2 Aims

- To prepare and investigate the stability of triangular silver nanoplates prepared by the Aherne *et al.* method.
- To stabilise the particles against etching by anions.
- To introduce functionality to the surface of the nanoplates to allow conjugation of ODNs.

3.3 Stabilisation of Metal Nanoparticles

Metal nanoparticles are generally stabilised by two approaches, electrostatically or sterically, or indeed by a combined electrosteric approach.¹⁻² These repulsive forces are required to overcome the attractive van der Waals forces between particles, that would otherwise result in particle aggregation. The stability of nanoparticle solutions are governed by Deryaguin, Landau, Verwey and Overbeek (DLVO) theory, which takes

into account the combination of the van der Waal attractive forces, with the electrical double-layer repulsion. An energy-distance curve can be used to describe the stability or instability of the nanoparticle system. The electric double-layer results from the adsorbed ions on the particle surface, attracting counterions to maintain electrical neutrality (Figure 3.1). Citrate stabilised gold and silver metal nanoparticles are a common example of electrostatically stabilised nanoparticles resulting from the repulsive columbic forces that are generated.³⁻⁴

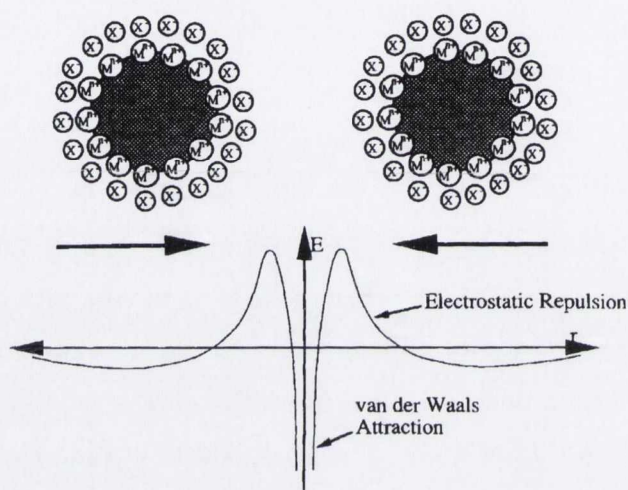


Figure 3.1 Electrostatic stabilisation of nanoparticles. Taken from reference²

Steric stabilisation can be achieved by adsorption of molecules such as polymers, surfactants, bulky ligands or biological molecules on the nanoparticle surface forming a protective layer. Steric repulsion results from entropic and osmotic effects (Figure 3.2).

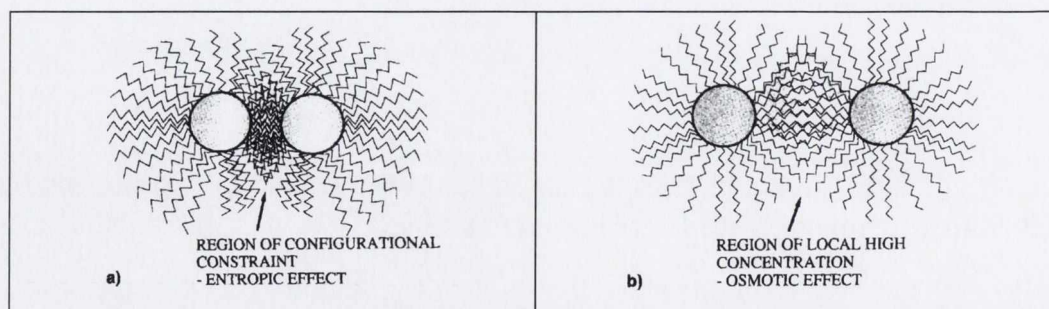


Figure 3.2 Steric stabilisation of nanoparticles. Taken from reference²

3.4 Instability of Silver Nanoparticles

Silver nanoparticles are generally considered to be more sensitive than gold nanoparticles as they are much more susceptible to oxidation. Initial attempts to functionalise spherical silver nanoparticles with ODNs, in an identical manner to spherical gold nanoparticles, were unsuccessful.⁵ The particles were found to undergo irreversible aggregation when heated in a 0.05 M NaCl solution.

The high energy edges of triangular nanoplates are prone to restructuring. Triangular silver nanoplates have been observed to undergo a morphological change after preparation. The large flat face of the triangular particles is known to be {111} and the edges are thought to be comprised of high energy {100} and {110} facets. Triangular nanoplates prepared by lithography have been shown to become thicker and rounded when transferred into solution.⁶ Triangular silver nanoplates prepared using sodium bis(2-ethylhexyl) sulfosuccinate (NaAOT) have been shown to undergo a morphological change to circular discs within 10 hours after preparation.⁷ These nanoplates were reported to maintain a constant thickness. Nanoplates prepared in the Kelly group have also been shown to etch, with PVP shown to accelerate the process.⁸⁻⁹ UV light irradiation, with a wavelength of 365 nm, was used to ‘backwards tune’ the particle LSPR band, with triangular nanoplates undergoing reconstruction to circular particles with an increase in thickness, depending on the exposure time.¹⁰ Heat has also been shown to result in the restructuring of triangular silver nanoplates.¹¹⁻¹² Triangular silver nanoplates have been found to degrade in the presence of certain anions, in particular halides, and this is discussed in detail below.

3.5 Etching of Silver Nanoparticles by Halide Ions

Silver nanoplates are highly sensitive to a variety of anions including the halides; chloride, bromide and iodide. This is of great significance when it comes to the use, and further functionalisation of these silver nanoplates, in particular with DNA. A common approach, which will be detailed in Chapter 4, is to use a salt aging step, to increase DNA loading on the nanoparticle surface. This can result in the restructuring of the triangular nanoplate structure. The salt, which can result in the degradation of the particle, is used to screen the electrostatic repulsion between the negatively charged

DNA backbones and for the hybridisation of complementary ODNs, and thus stability of the systems in the presence of halides is vital.

The dissolution of *spherical* silver nanoparticles in the presence of a variety of nucleophiles including CN^- , Cl^- , I^- and SH^- was reported by Mulvaney *et al.* in the early 1990s.¹³⁻¹⁴ It was shown that the interaction of a nucleophile (N) with a silver nanoparticle surface resulted in the particle acquiring a slight positive charge. In turn, the interior of the particle acquires a slight negative charge, resulting in the Fermi level being raised. This makes it energetically favourable for an oxidising agent, such as oxygen, to oxidise the elemental silver, $\text{Ag}(0)$, to silver ion, Ag^+ . This results in the formation of $\delta^+\text{Ag}-\text{N}\delta^-$ on the particle surface, which eventually leaves as AgN . A schematic representation for this is shown in the upper schematic below (Figure 3.3).

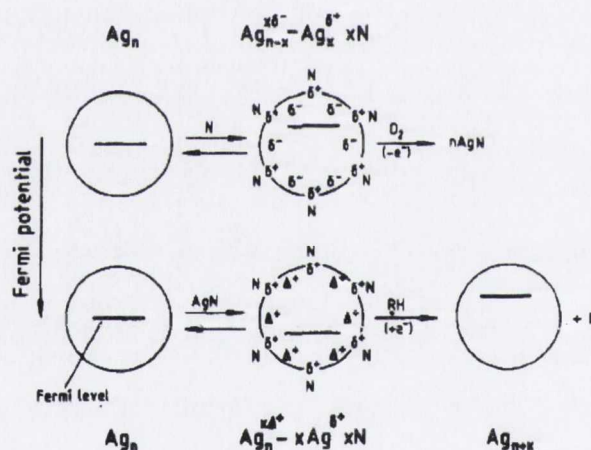


Figure 3.3 Upper schematic shows the complexation of a nucleophile (N) on the nanoparticle surface followed by the oxidation of the particle by O_2 . Taken from reference¹³

The etching phenomenon of triangular silver nanoplates was first observed within our group, by Mesclaux and Ledwith, whilst trying to functionalise an earlier generation of triangular silver nanoparticles, using the layer-by-layer (LBL) approach.⁹ It was found that the chloride counter-ion of poly(allylamine hydrochloride) (PAH) resulted in the rapid degradation of the particles from triangular material to spherical material. This was visualised by TEM. A variety of anions were investigated and in particular, the

halide ions were found to show an order of reactivity of $\text{Cl}^- > \text{Br}^- > \text{I}^-$. Gara subsequently investigated the chloride etching effect on the Aherne *et al.* particles and he found that as little, as 1 mM NaCl, was sufficient to fully convert the triangular nanoplates to spherical material.

Halide ions have been shown to have important shape and size directing effects in the growth stage of silver nanoparticle synthesis.¹⁵⁻¹⁶ During the course of this PhD, a few literature reports on the etching process of triangular silver nanoplates by halide anions post-synthesis have emerged. A variability in the reactivity of the triangular silver nanoplates to the various halide anions has been reported, and it can be proposed, that this is due to the varied synthetic methods used for the preparation of the particles. A full understanding of the etching process is not yet known. A brief summary of the investigations and proposed mechanisms is provided below.

A report by Jiang and Yu showed the sensitivity of triangular silver nanoplates to a variety of inorganic anions.¹⁷ A significant blue shift was observed in the position of the LSPR band in the case of Br^- , Cl^- , and I^- with an order based on the degree of shifting given as $\text{Br}^- > \text{I}^- > \text{Cl}^-$. They attributed the effect of the blue shifting in the LSPR band to particle surface charging and the effect of the various anions was linked with the solubility constant (K_{sp}) of the silver compounds in each case (Table 3.1).

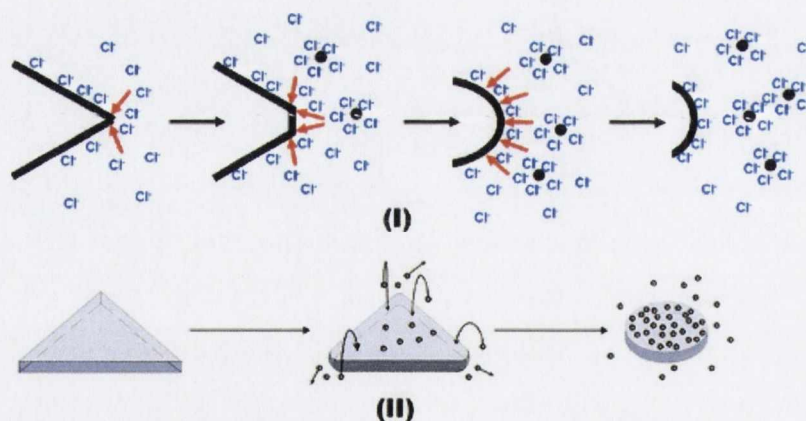
Table 3.1 The solubility constants for silver halide compounds in water¹⁸

Silver compound	K_{sp} (conditions)
AgF	182g/100 ml water (15.5 °C)
AgCl	1.56×10^{-10} (25 °C)
AgBr	7.7×10^{-13} (25 °C)
AgI	1.5×10^{-16} (25 °C)

TEM analysis showed no significant change in particle morphology. This could be due to the low concentrations of halide ions added, although, it should be noted that an earlier report by this group, prepared by an identical method using sodium bis(2-ethylhexyl)sulfosuccinate (NaAOT), showed the evolution of the particles to spherical

material over time.⁷ It is unlikely that such a pronounced shift ~200-250 nm cannot be attributed to a morphological change. F^- , NO_3^- , ClO_4^- , CH_3COO^- , SO_4^{2-} and the cations Zn^{2+} , Cd^{2+} , Cu^{2+} were shown to have no effect. The use of dodecanethiol, or cysteine, was shown to prevent the anion sensing capability of the silver nanoplates.

Citrate stabilised triangular silver nanoplates, prepared by a photoinduced method, were shown to be sensitive to chloride ions, undergoing a shape change to discal material with a slight increase in thickness.¹⁹ The exclusion of oxygen was shown to have no significant effect on the process. The process was attributed to the higher surface energy of the $\{110\}$ side facets relative to the $\{111\}$ facet and also to the fact that citrate preferentially binds to the $\{111\}$ facet. The presence of some small silver clusters ~3 nm was observed by TEM. A proposed mechanism is shown in Scheme 3.1(I). The chloride ion is shown to react at the vertices of the nanoplate, exposing more active areas until a disc is obtained. The dissociated silver atoms could aggregate forming small clusters, stabilised by the chloride ions. The possible redeposition of the silver atoms onto the $\{111\}$ plane is shown in Scheme 3.1(II).



Scheme 3.1 Schematic representation (I) of the proposed mechanism for chloride etching (II) of the redeposition of silver atoms. Taken from reference¹⁹

An increased SERS enhancement of triangular silver nanoplates in the presence of bromide ions was reported by Ciou *et al.*²⁰ It was proposed that the enhancement could derive from the presence of small atomic silver clusters that formed during the sculpturing of the nanoplates. The facet selectivity of halide etching was subsequently

shown by comparing the stability of a variety of silver nanostructures. Silver nanotetrahedrons consisting solely of {111} facets were shown to be stable against etching by halide ions.²¹

The etching process takes place rapidly. Time-resolved extinction spectroscopy was used to monitor the etching effect and it further clarified the mechanism, with evidence for the coexistence of nanoplates and nanodisks observed.²² It was suggested that the halide ions selectively etch some nanoplates (active) and then react with others (less active). The etching rate was determined by monitoring the change in extinction as a function of time. The reaction rate was determined to be concentration dependent with a certain threshold concentration of halide ion required for the etching process to start. An increase in reaction rate was observed with increasing ion concentrations up to 100 mM. The reaction was observed to be pseudo-first order. The activation energies were calculated and the order of reactivity was found to be $\text{Br}^- > \text{I}^- > \text{Cl}^-$. A similar etching effect was observed for thiocyanate (SCN^-), but it was observed to take much longer.

A study of the etching by halide ions of triangular silver nanoplates was carried out by the group of Huang.²³ They found that once a certain threshold concentration of halide anion was added that the reaction would proceed until a yellow solution with an LSPR band centred ~ 400 nm was obtained. It was found that a significantly higher concentration of chloride (3×10^{-4} M) was required compared with bromide (1×10^{-6} M) and iodide (1.5×10^{-6} M), a reactivity order of $\text{Br}^- > \text{I}^- > \text{Cl}^-$. This was correlated with the solubility product, whereby significantly more chloride is required to form a complex with the free silver ion in solution. The amount of free silver ion in the nanoplate sample was determined to be $\sim 10^{-6}$ M from the threshold concentration and the silver halide K_{sp} . Monitoring the process as a function of time, it was observed that a significant blue shift occurred rapidly, followed by a more gradual shifting. The data was fitted to a biexponential decay function, and the fast and slow components were assigned to the truncating and redeposition processes, respectively. The silver ion was in excess, so it was proposed that the halide ions play a catalytic role in the migration of the silver ion.

A further study by the group of Huang involved the termination of the etching process by the addition of 16-mercaptohexadecanoic acid.²⁴ Initial efforts found the reaction rate to be too fast to obtain silver structures with a LSPR band above 580 nm. The addition of PVP was found to slow the etching process substantially. The addition of 16-mercaptohexadecanoic acid, at various times, stopped the etching at various stages, yielding particles with a variety of LSPR bands. The ‘backwards tuning’ of the nanoplates gave stable nanoparticle solutions across a range of wavelengths. Yu *et al.* found an etching reactivity of $\text{Br}^- > \text{Cl}^- > \text{I}^-$ for their aqueous based nanoplate preparation which used PVP in the synthesis. They attributed the difference in reactivity, compared with other reports, to the presence of the PVP.²⁵ Cathcart *et al.*²⁶ investigated the effect of halide ions during and after the synthesis of silver nanoparticles. They proposed that bromide stabilises the {100} facet leading to the formation of 3D structures.

3.6 Methods of Stabilisation of Silver Nanoparticles

A wide range of methods have been used to stabilise metal nanoparticles of a variety of shapes and sizes. A discussion on some of the successful approaches is given below.

3.6.1 Thiol Capping of Silver Nanoparticles

Although the silver thiol bond is weaker than the gold thiol bond, there are a number of reports for the successful stabilisation and functionalisation of spherical silver nanoparticles using thiol ligands.²⁷⁻³¹ The capping of silver particles with neutral ligands, such as the thioalkylated poly(ethylene glycol) described by Doty *et al.*, have been shown to offer excellent stability, but unfortunately are only applicable to particles with diameters less than 20 nm.³⁰ As outlined above, triangular silver nanoplates are highly susceptible to etching by anions, and thus are an added challenge to successfully stabilise. The addition of alkyl thiols (1-hexanethiol, 1-octanethiol, 1-dodecanethiol and 1-hexadecanethiol) to prepared triangular silver nanoplates was shown to ‘freeze’ the morphology of the particles.³² The nanoplates were only stable if the sodium bis(2-ethylhexyl) sulfosuccinate (NaAOT) was not removed from the solution. N,N,N-trimethyl(11-mercaptoundecyl) ammonium bromide has been used to stabilise CTAB stabilised nanoplates.³³ Multi-dentate thiols have been shown to offer an enhanced stability over monothiols, but this approach is only applicable to smaller, spherical

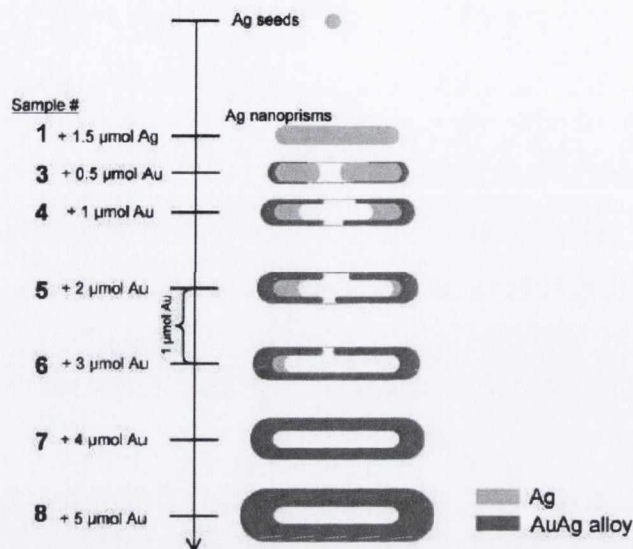
particles. The monothiols pack more readily on the larger particles due to their reduced curvature.³⁴

3.6.2 Silica Coating

The silica coating of triangular silver nanoplates and their subsequent ODN functionalisation was reported by the Mirkin group.³⁵ Dr. Deirdre Ledwith adopted a silica coating strategy as part of her PhD with some success.⁹ The problem with these approaches was that thin coatings were not achievable and there were some problems with reproducibility.

3.6.3 Gold Coating

Ag@Au core shell spherical nanoparticles were used to prepare ODN conjugates because of the extra stability imparted by the gold coating.⁵ The coating of Aherne *et al.*³⁶ triangular silver nanoplates with a thin layer of gold, was shown to stabilise them from etching by chloride.³⁷ The gold was selectively deposited on the edge of the particles and it could be concluded that etching by chloride is face selective. The refractive index sensitivity of these particles was shown to be maintained. This approach was further extended, taking advantage of the galvanic replacement reaction, to prepare triangular hollow AuAg nanoboxes.³⁸ Scheme 3.2 shows the formation of the AuAg nanoboxes from the Ag nanoplates by varying the amount of gold added. The ODN functionalisation of these AuAg nanoboxes is detailed in the results section of Chapter 4.



Scheme 3.2 Diagram showing the growth of triangular AuAg nanoboxes from triangular Ag nanoplates. A cross-sectional illustration of the growth process with different amounts of gold added is shown. Taken from reference³⁸

3.6.4 Titania Coating

3.6.4.1 Why TiO_2 Coating?

Titania coating offers a viable approach to stabilise triangular silver nanoplates and to allow further functionalisation of them. The attractiveness to the use of titania comes from the fact that thin (1-2 nm) shells have been reported in the literature. Previous efforts utilising silica coating, as detailed above, had not only the disadvantage that the method was not reproducible, but also that thin layers were not achievable.⁹ Xue *et al.* reported that the thinnest complete layer possible was 15 nm, when coating triangular silver nanoplates by a modified Stöber method.³⁵ As outlined in Chapter 1, if we want to take advantage of phenomena such as metal enhanced fluorescence that have a distance dependence from the nanoparticle surface, then we need to have the control to place luminophores at a sub 10 nm range as the sensitivity of these phenomena has been shown to drop off with distance from the nanoparticle surface. Coating of the nanoparticles is not only used to impart stability over a wider range of conditions, such as higher ionic strength, but also to allow modification and the tailoring of the nanoparticle properties. Titania coatings are attractive as the shells formed are stable

and chemically inert. The functionalisation of titania particles has been widely developed. Enediols such as catechols have been shown to bind strongly to metal oxides such as titania and thus offer us an approach for the preparation of ODN conjugates.³⁹ Indeed, catechol modified ODNs have been used to successfully prepare ODN conjugates with titania particles.⁴⁰ The preparation of a catechol modified ODN by the efficient method of oximation will be discussed in Chapter 5.

3.6.4.2 Titania Coating of Nanoparticles

The driving force for the development of titania coated nanoparticles has been for their potential use in catalysis, where a high surface area to volume is necessary, and thus many methods have been employed on high surface area colloidal supports including silica, polystyrene and both gold and silver metal nanoparticles. The focus of my attention will be on the titania coating of the noble metal nanoparticles, gold and silver. The key to coating metal nanoparticles with titania is to control the rate of the hydrolysis and condensation of the oxide layer. This can be done by limiting the amount of water in the reaction mixture, but the problem here is that in many instances, metal nanoparticles are not stable in organic media. Efforts to improve the stability of the particles in organic or organic/aqueous mixed solvents have been made, such as capping or priming the surface. Control of the reaction is also achieved through the choice of the titanium precursor and by the addition of complexing agents to slow the condensation process. A variety of approaches have been utilised, for a variety of purposes and an overview of them is given below (Table 3.2).

Table 3.2 Previous reports of the preparation of Au@TiO₂ and Ag@TiO₂ nanoparticles

Group ^{ref}	Metal	Method	Comment
Mayya <i>et al.</i> ⁴¹	Gold	Particles coated with polyelectrolytes. Complexed with titanium (IV) bis(ammonium lactato) dihydroxide (TALH)	30 nm Au, TiO ₂ Shell 10 nm, crystalline
Yu & Mulvaney ⁴²	Gold	Particles treated with MUA and dispersed in ethanol. TOAA and water varied.	12 nm Au, TiO ₂ shell 1 nm, amorphous

Group ^{ref}	Metal	Method	Comment
Buso <i>et al.</i>	Gold	MUA capped particles in MeOH/water treated with titanium butoxide and acetylacetone	10 nm Au, Film formed
Hayakawa <i>et al.</i> ⁴³	Gold	Tiron prepared particles treated with titanium butoxide	8 nm Au
Kwon <i>et al.</i> ⁴⁴	Gold	Triethanolamine was used to slow the hydrolysis of titanium (IV) isopropoxide. MUA capped Au. Coating at 80 °C	15 nm Au, TiO ₂ shell 10 nm, amorphous
McDowell <i>et al.</i> ⁴⁵	Gold	Titanium butoxide and water added to Au in anhydrous ethanol.	Au nanoplates, TiO ₂ shell 5-100 nm, crystalline on heating
Wu <i>et al.</i> ⁴⁶	Gold	TiF ₄ added citrate stabilised Au, reaction carried out at 180 °C	Wedge shaped TiO ₂ shell obtained
Chen <i>et al.</i> ⁴⁷	Gold	PVP stabilised particles treated with titanium butoxide	10-15 nm Au, TiO ₂ shell 1-3 nm, crystalline on heating
Pastoriza-Santos <i>et al.</i> ⁴⁸	Silver	One pot preparation. Reduction of Ag ⁺ by DMF/ethanol in the presence of titanium butoxide and acetylacetone. Refluxed.	Two populations 20 nm and 4 nm, TiO ₂ shell 1-2 nm, amorphous
Tom <i>et al.</i> ⁴⁹	Silver	One pot synthesis. Titanium tetraisopropoxide and acetylacetone used. Refluxed in DMF	30-60 nm Ag, TiO ₂ shell 3 nm, crystalline on heating.
Hirakawa & Kamat ⁵⁰	Silver	One pot synthesis. Titanium tetraisopropoxide used. Increasing addition did not increase coating	2-4 nm particle

Group ^{ref}	Metal	Method	Comment
Sakai <i>et al.</i> ⁵¹	Silver	Sol gel approach with CTAB stabilised particles treated with an ethanolic solution of titanium tetraisopropoxide.	10 nm Ag, TiO ₂ shell 5-10 nm
Zhang <i>et al.</i> ⁵²	Silver	One pot synthesis. Nanobubbles prepared by dissolution of Ag with addition of NaCl	20-40 nm Ag, TiO ₂ shell 2 nm
Wang <i>et al.</i> ⁵³	Silver	Reduction of silver acetate with 1-dodecylamine at 120 °C and subsequent titania coating using titanium tetraisopropoxide with oleic acid at 150 °C.	6 nm particles, anatase shell
Wang <i>et al.</i> ⁵⁴	Silver	Titanium tetraisopropoxide added to aqueous solution of particles	15 nm Ag, TiO ₂ shell 10 nm
Demirörs <i>et al.</i> ⁵⁵	Silver	Non-ionic surfactant Lutensol ON50 and titanium butoxide with particles placed in ethanol. Tunable shell by varying amount of Ti precursor	250 nm Ag, TiO ₂ shell 220 nm

3.6.4.2.1 Characterisation of Titania Coated Nanoparticles

UV-Vis spectroscopy has been used extensively to indicate successful coating of the metal particles. A significant red shift is generally seen, due to the increase in refractive index for TiO₂ (2.08) compared with water (1.33). The point of zero charge for titania is ~pH 6 and the stability of the particles in solution has been used as an indication of successful coating. While TEM has been used, in cases where thin layers are obtained, it may be difficult to see a distinct coating. Figure 3.4 shows a typical TEM image of titania coated silver nanoparticles. EDX and XRD have been used to assess the crystallinity of the shell. The slowed dissolution of coated silver particles by nitric acid has also been used as a test for successful coating.

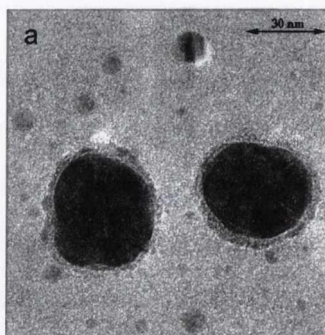


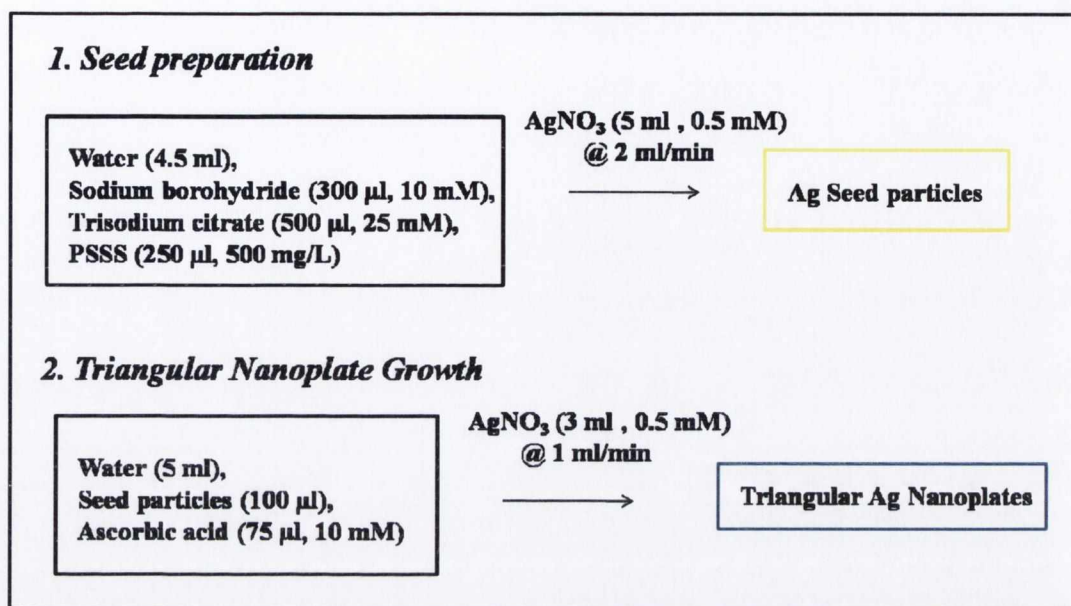
Figure 3.4 HRTEM image of Ag@TiO₂ prepared via a one-pot method. Image taken from reference⁴⁸

To the best of our knowledge, there have been no reports on the preparation of titania coated triangular silver nanoplates. A novel approach building on the literature outlined above will be detailed in the results section below.

3.7 Results

3.8 Preparation of Triangular Silver Nanoplates

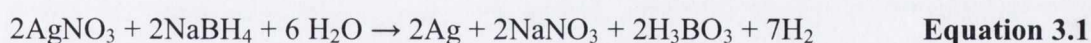
The triangular silver nanoplates used throughout this work were developed by a colleague, Dr. Damian Aherne, during the course of my PhD studies.³⁶ The method improves on previous reports^{8,56} within our group with a high selectivity ($\geq 95\%$) for triangular particles. The particle preparation is a room temperature, two-step, seed-mediated method using simple reagents and mild conditions. An overall schematic for a typical preparation is given in Scheme 3.3.



Scheme 3.3 Schematic for the typical preparation of a blue triangular silver nanoplate sol with a λ_{max} of 750 nm.

3.8.1 Seed Preparation

Silver seed particles were prepared by reducing silver nitrate with sodium borohydride in the presence of poly(sodium 4-styrenesulfonate) (PSSS) (Equation 3.1). Sodium borohydride, a strong reducing agent, was used to rapidly reduce the silver ion, resulting in the formation of a large number of relatively monodisperse silver seed particles.



Citrate is used to stabilise the small silver particles. The role of the PSSS has not yet been fully determined. It has been shown that if the PSSS, is omitted, or simply added, to the seeds after preparation that a wide variety of nanoparticle shapes and sizes are obtained. It has been proposed that the PSSS has a strong influence on the defect structure of the silver seed particles, but as yet it is unclear as to how the PSSS achieves this. It can be assumed that the PSSS has a face selectivity and thus plays a shape directing role in the growth of the seed particle.

The seed particles were characterised using Ultraviolet-visible (UV-Vis) spectroscopy, zeta potential and dynamic light scattering (DLS) measurements. The UV-Vis spectrum

(Figure 3.5) shows the typical single LSPR band that is observed at 396 nm for the prepared silver seed particles. The citrate stabilised seeds were shown to have a zeta potential of -63.8 mV (Figure 3.6). DLS measurements showed the particles to have a hydrodynamic diameter of 7 nm (Figure 3.7).

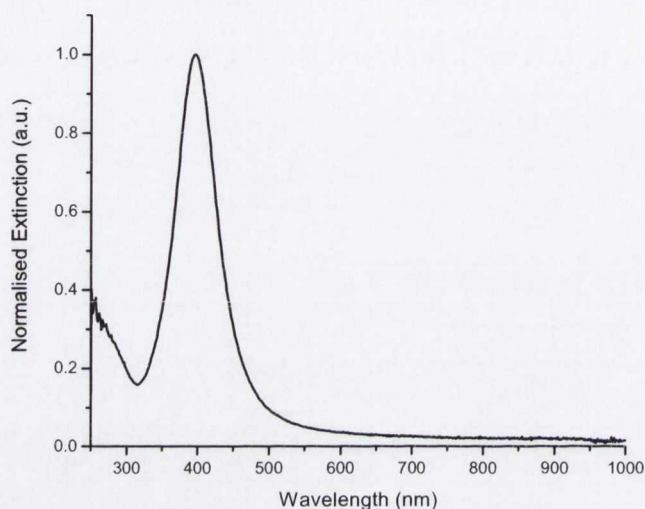


Figure 3.5 UV-Vis spectrum of silver seed particles

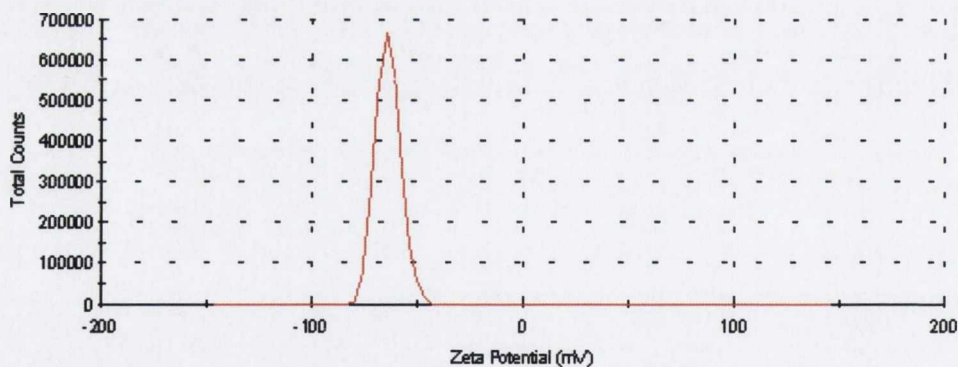


Figure 3.6 Zeta potential measurement of citrate stabilised silver seed particles

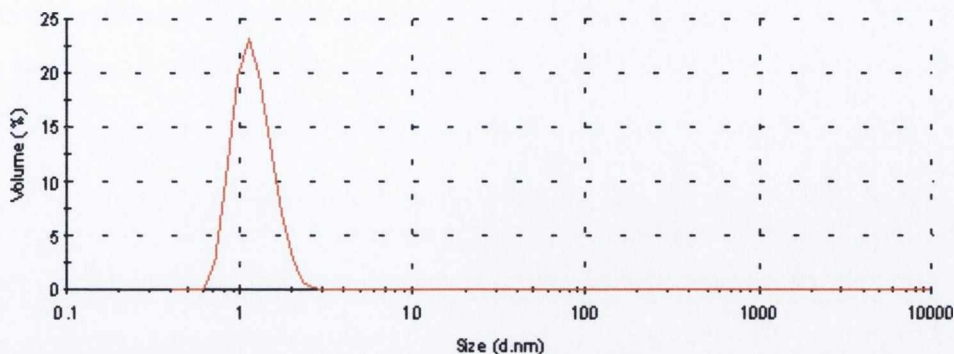
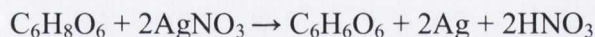


Figure 3.7 Size distribution by volume DLS measurement for citrate stabilised silver seed particles

3.8.2 Triangular Nanoplate Growth

The triangular nanoplates were grown by using a milder reducing agent, ascorbic acid, to reduce the silver ion in the presence of the seed particles. The ascorbic acid is not strong enough to reduce the metal salt without the presence of the seed particles.⁵⁷⁻⁵⁸ The seed particles hence facilitate the reduction of the silver ion and the silver atoms formed are deposited on the seed particles. Nucleation is minimised and even particle growth is achieved. This results in excellent control over the particle size and shape. The ascorbic acid is oxidised to dehydroascorbic acid during the process (Equation 3.2).



Equation 3.2

The beauty of this preparation, as discussed in Chapter 1, is that by simply varying the amount of seed particles used in the growth step, particles of various sizes can be grown, tuning the position of the LSPR band. The particles used throughout this thesis were generally prepared using 100 μl of seed with a resulting maximum LSPR band $\sim$ 750 nm. The particles were found by TEM, to typically have an edge length of 40 nm and a thickness of 8 nm.

The triangular nanoplates were characterised by UV-Vis spectroscopy with a characteristic spectrum depicted in Figure 3.8. The in-plane dipole (λ_{max}) was observed at 747 nm. The other sharp resonance at 337 nm could be assigned to the out-of-plane

quadrupole with the broader and weaker resonance at 460 nm corresponding to the in-plane quadrupole.⁵⁹

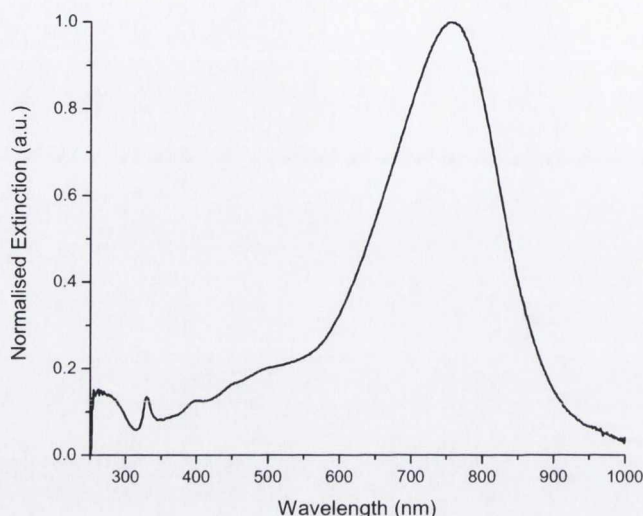


Figure 3.8 UV-Vis spectrum of a typical triangular silver nanoplate sol prepared using 100 μ l of seed particles.

Dynamic light scattering (DLS) was used to determine the size of the particles (Figure 3.9a). Two peaks were observed in the spectrum, the larger peak gave a hydrodynamic diameter of 44 nm and the smaller peak gave a diameter which would correspond to a particle size of 2 nm.

DLS is only effective in describing spherical particles. The observation of two peaks in the DLS has been investigated by scientists at Malvern using these particles, and has been shown to be due to the rotational diffusion of the particle, because of its lack of spherical symmetry. Rotational diffusion is much faster than translational diffusion and hence appears as a smaller particle size. TEM analysis has confirmed that no small material is present that could be responsible for this smaller peak.

A negative zeta potential of -43.8 mV was typically obtained for the ‘as prepared’ citrate stabilised sol (Figure 3.9b).

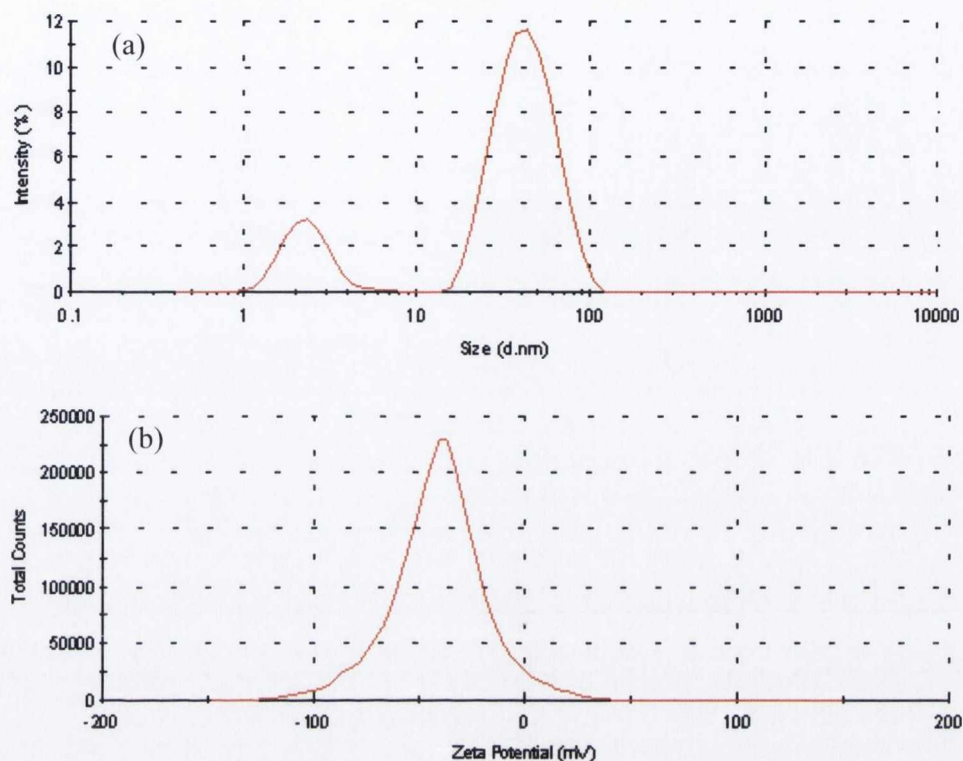


Figure 3.9 (a) Size distribution by intensity DLS measurement for a typical triangular silver nanoplate sol prepared with 100 μL of seed particles. (b) Zeta potential measurement for a typical citrate stabilised triangular silver nanoplate sol

3.9 Concentration of a Typical Triangular Ag Nanoplate Preparation

The concentration of nanoplates in a typical sol was estimated, by presuming that all of the silver nitrate used in the preparation, was converted to nanoplates of exactly the same size, with an edge length of 40 nm and a thickness of 8 nm. The diameter of a silver atom was taken as 0.288 nm and the edge length of a unit cell as 0.4086 nm. It was estimated that a typical sol had a concentration of 2.7×10^{14} particles/L.

Analysis of the supernatant of the triangular nanoplate sol was carried out to determine if it contained any silver, so as to support our assumption that all of the added silver ion is converted into triangular silver nanoplates.

A Volhard titration was attempted but unfortunately the silver concentration was found to be too low for analysis ($<1.7 \times 10^{-4}$ M). The silver nanoplate solution was treated

with nitric acid to yield Ag^+ ions and it was then titrated against ammonium thiocyanate using ammonium iron sulphate as indicator. The silver ions react with the thiocyanate to give silver thiocyanate (a white precipitate) until all the Ag^+ present has reacted (Equation 3.3). The thiocyanate then reacts with the iron of the indicator to give a red colour which can be taken as the end point (Equation 3.4). In our case a red colour was observed immediately.



Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the silver ion content of the supernatant. Analysis showed that a negligible amount of silver ion ($<10^{-7}$ M) was present in the supernatant after passing the particles through a centrifuge filter. The measurement was carried out in triplicate.

3.10 Stability of ‘As-Prepared’ Triangular Ag Nanoplates

The stability of the particles is very important, both in the short-term and the long-term, as the properties of the particle correspond with the particle shape and corresponding spectrum. Trisodium citrate (0.5 ml, 25 mM) is added to the nanoparticle preparation after synthesis to give a final citrate concentration of 1.4 mM. The particles have been observed to be somewhat stabilised from the residual citrate present in the seeds, but on analysis a significant amount of degradation is observed overnight with a blue shift, 27 nm, in the LSPR band observed if no citrate is added after preparation (Figure 3.10a). A more dramatic blue shifting, 47 nm, was observed over a 7 day period (Figure 3.10b). This blue shifting has been attributed to the rounding of the high energy corners of the triangular particles.⁵⁹

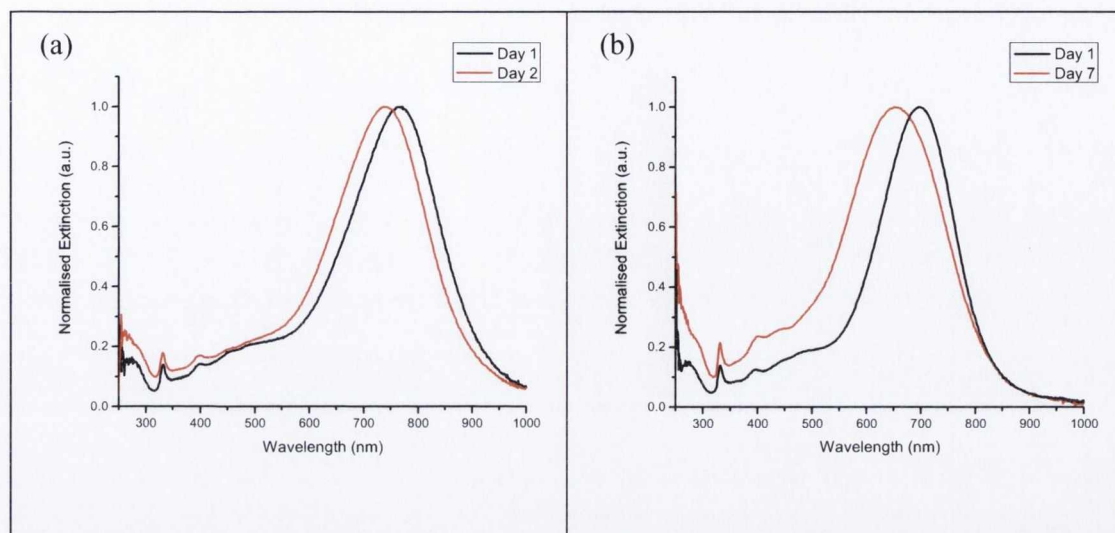


Figure 3.10 UV-Vis spectra of triangular Ag nanoplates with no added citrate (a) after 1 day and (b) after 7 days.

The addition of citrate to electrostatically stabilise the sample, the storage of the samples in the dark and at 4 °C significantly reduced the degradation of the nanoplates (Figure 3.11a).

Centrifugation at 6000 rpm for 30 mins was required to fully pellet the triangular silver nanoplates. This resulted in a blue shift of 15 nm for the redispersed pellet (Figure 3.11b). This can be attributed to the truncation or snipping of the corners of the particles.

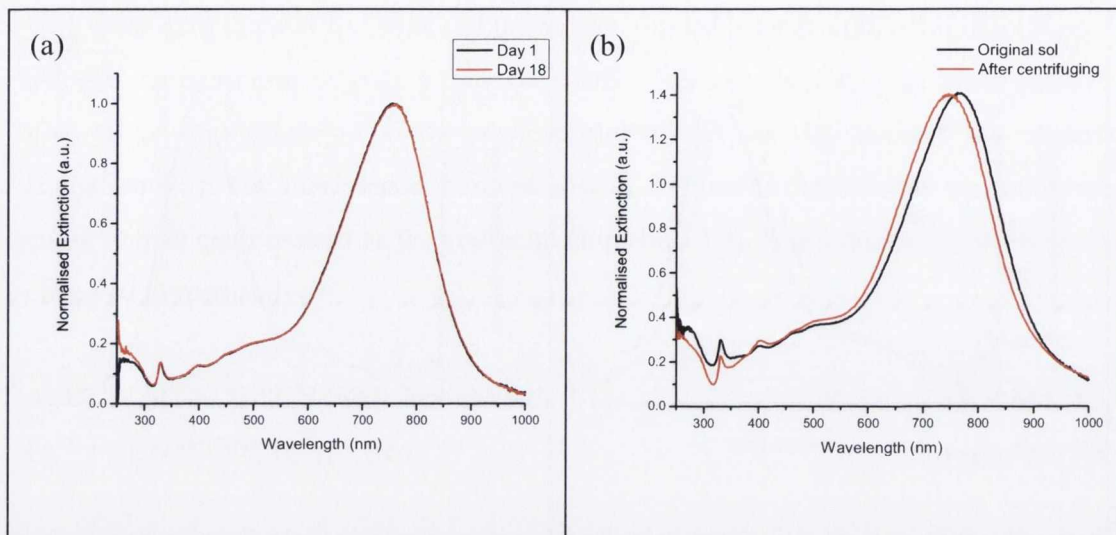


Figure 3.11 UV-Vis spectra of triangular Ag nanoplate sol (a) with citrate added monitored for 18 days. The sample was stored in the dark at 4 °C. (b) after centrifuging at 6000 rpm for 30 mins.

3.10.1 Stability of ‘As-Prepared’ Triangular Nanoplates to Halide Anions

As detailed in the introduction of this chapter silver nanoplates are highly sensitive to halide anions. This is particularly relevant to this work, as a common approach for the preparation of nanoparticles with a dense loading of oligonucleotides is to use a salt-aging step. Sodium chloride is also usually used for the hybridisation of the complementary ODNs. The etching of a variety of anions on an earlier generation of particles (prepared by a seed mediated method using citrate and PVP in the growth step) showed them to have an order of reactivity of $\text{Cl}^- > \text{Br}^- > \text{I}^-$.⁹ A detailed study of the effect of chloride on the Aherne *et al.* particles was carried out by Matthew Gara (unpublished results). It was noted that some literature reports showed a different order of reactivity to that previously observed for the earlier generation of particles, and so I undertook a brief study to see if our new generation (Aherne *et al.*) particles showed any different behaviour and if any conclusions could be drawn.

Stock aqueous potassium halide solutions (0.25 M) were prepared. The silver nanoplate sol (1 ml) was placed in a cuvette and the potassium halide solution (4 μl) added to give a final salt concentration of 10 mM. The sample was shaken and the UV-Vis spectrum

obtained immediately (Figure 3.12a). Taking the extent of blue shifting as a measure of the reactivity of the halide ions, an order of $\text{Br}^- > \text{Cl}^- > \text{I}^-$ was found. The samples were left overnight at room temperature and analysed (Figure 3.12b). A yellow solution was obtained in the case of samples treated with KBr and KCl. The sample treated with KI had turned cloudy and this could be attributed with the formation of AgI nanoparticles.

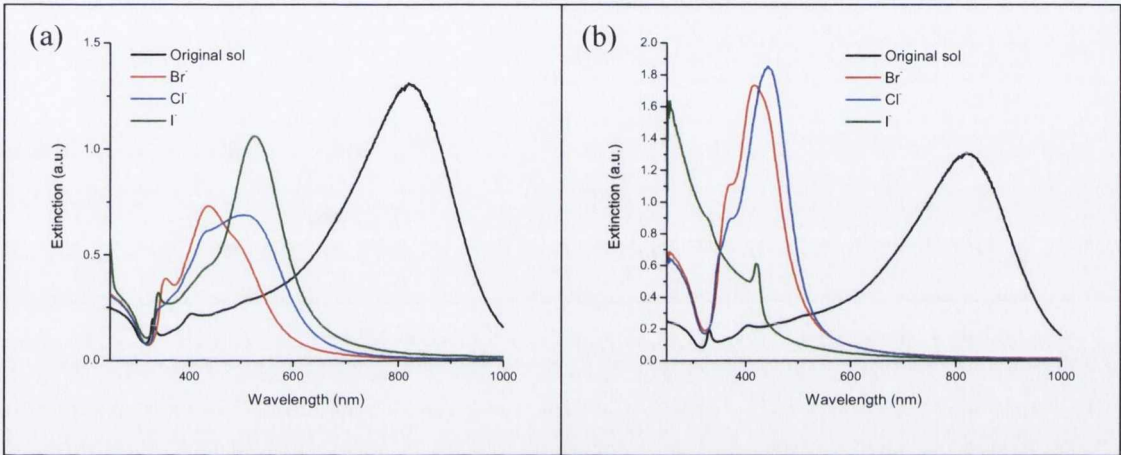


Figure 3.12 (a) UV-Vis spectra of triangular silver nanoplates taken immediately after addition of 10 mM KBr, KCl and KI. (b) Solutions after reacting overnight.

This order of reactivity $\text{Br}^- > \text{Cl}^- > \text{I}^-$ was different to that previously observed for the earlier generation silver nanoplates⁸ which was $\text{Cl}^- > \text{Br}^- > \text{I}^-$ and is also different to that reported in the literature $\text{Br}^- > \text{I}^- > \text{Cl}^-$ (Table 3.3).²³ There is no doubt that further investigation and an understanding of the etching mechanism is needed.

Table 3.3 Comparison of the halide etching order of various nanoplate preparations

Triangular Ag Nanoplate preparation	Order of Reactivity
Aherne <i>et al.</i> ³⁶	$\text{Br}^- > \text{Cl}^- > \text{I}^-$
Ledwith <i>et al.</i> ⁸⁻⁹	$\text{Cl}^- > \text{Br}^- > \text{I}^-$
Hsu <i>et al.</i> ²³	$\text{Br}^- > \text{I}^- > \text{Cl}^-$

Comparing the results obtained it can be proposed that the surface chemistry of the particles has a role to play. The earlier generation particles were prepared by a similar aqueous, two step seed mediated method, differing in that PVP is used in the growth step. The addition of PVP to a nanoplate preparation has been shown by Lee *et al.*²⁴ to

slow the etching of triangular silver nanoplates sufficiently, so that addition of a thiol can stop the etching process. PVP has been shown recently to preferentially bind to the {100} facet of triangular silver nanoplates.⁶⁰

3.11 Thiols for Stabilising Triangular Silver Nanoplates

3.11.1 16-Mercaptohexadecanoic Capping

16-mercaptohexadecanoic acid (MHA) was shown by Mirkin *et al.* to prevent the degradation of triangular silver nanoplates by dimethylamine during the silica coating of the particles using the Stöber method.³⁵ We investigated the potential of using this compound to stabilise our triangular silver nanoplates, in particular to etching by chloride.

Addition of an aliquot of a 1 mM ethanolic solution of 16-mercaptohexadecanoic acid to the prepared triangular nanoplate sol to give a final concentration of 20 μ M, resulted in a 33 nm red shift in the main LSPR band of the particle from 739 nm to 772 nm. (Figure 3.13a) The red shift was apparent after 5 minutes and further monitoring of the sample did not result in any further red shifting. This shift in the position of the LSPR band is indicative of high surface coverage on the particle surface. The LSPR band of triangular silver nanoplates are very sensitive to refractive index changes.⁶¹ The centrifugation of these particles at 6000 rpm for 30 minutes resulted in no change in the position of the LSPR band of the particles (Figure 3.13b). Previous reports have shown the freezing of the shape of triangular silver particles by sufficient thiol capping and no change in the LSPR spectrum was observed on monitoring a MHA capped sol for a 4 week period.³²

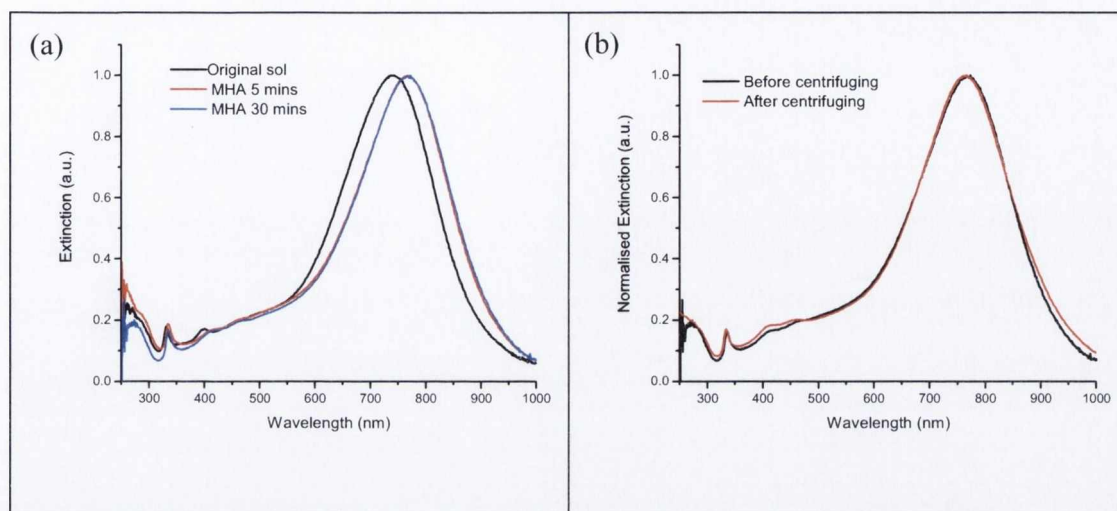


Figure 3.13 UV-Vis spectra of triangular Ag nanoplate sol (a) before and after capping with MHA. (b) MHA capped sol before and after centrifugation at 6000 rpm for 30 mins.

The effect of MHA capping on the size and charge of the triangular silver nanoplates was analysed. The hydrodynamic diameter of the particles was observed to increase, the larger peak increased by 13 nm, from 44 nm to 57 nm, and the smaller peak increased by 2 nm, from 2 nm to 4 nm (Figure 3.14). A zeta potential of -43.8 mV was obtained for the capped particles (Figure 3.15). This was indicative that the carboxylic acid groups are ionised and thus provide electrostatic stabilisation to the nanoplate sol.

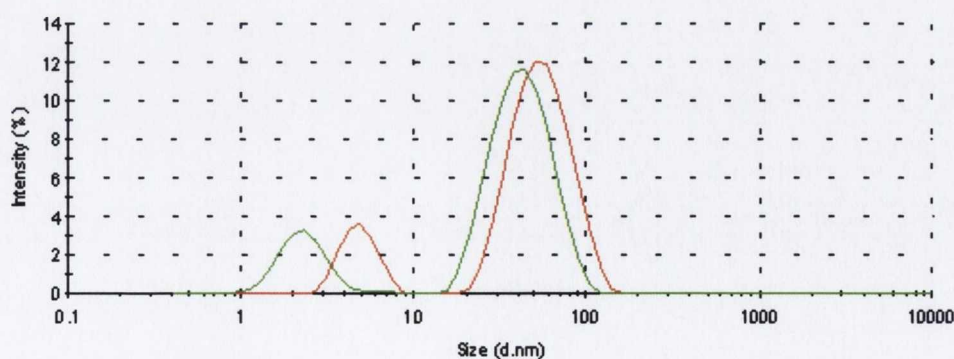


Figure 3.14 Size distribution by intensity DLS measurement of triangular Ag nanoplate before and after capping with MHA.

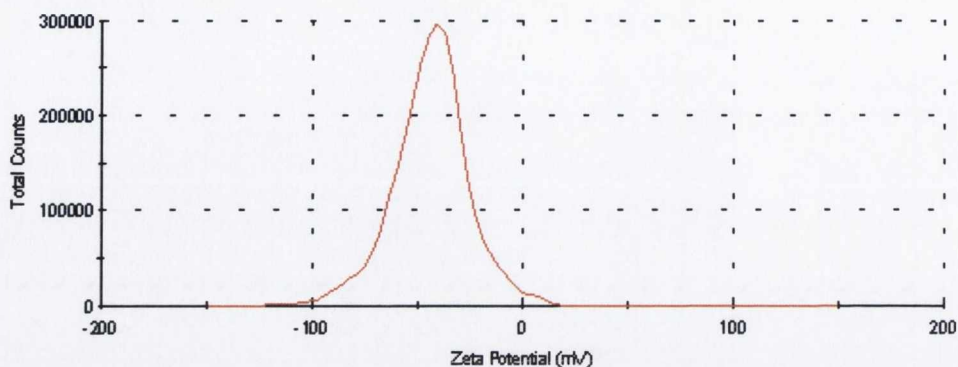


Figure 3.15 Zeta potential of MHA capped triangular Ag nanoplates.

The capping of the particles with MHA was further investigated and confirmed by Raman and infrared spectroscopy. Infrared measurements were obtained by combining a number of sols capped with MHA, centrifuging to remove any excess MHA and then concentrating and drying a sample by lyophilisation. The sample was then analysed by FTIR. The presence of the MHA on the silver surface was evident from the spectrum obtained (Figure 3.16). The strong peaks at 2914 cm^{-1} , 2849 cm^{-1} and 1472 cm^{-1} can be assigned to the CH_2 stretching and CH_2 bending respectively. The band at 1692 cm^{-1} corresponds to the $\text{C}=\text{O}$ stretch, 3105 cm^{-1} the OH stretch and the band at 2560 cm^{-1} corresponds to the SH stretch. This 2560 cm^{-1} peak disappears in the nanoplate spectrum as the MHA binds via Ag-S bond.

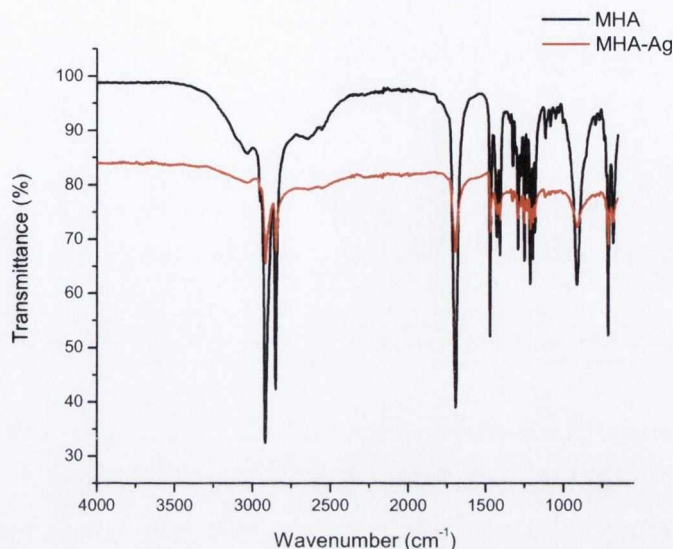


Figure 3.16 IR spectrum of 16-mercaptohexadecanoic acid (MHA) and MHA capped triangular silver nanoplate sol

Raman measurements were carried out on an Avalon Instruments Raman station with an excitation wavelength of 785 nm. The background fluorescence of the particles was extremely high and no signals could be determined for the citrate stabilised sol or the MHA capped sol. A metal liquid-like film (MELLF) was prepared of each sample by adding dichloromethane and a stabiliser. The sample was shaken. The raman spectra of the MELLFs were taken and in this case it was possible to observe the citrate on the surface of the particles, for the citrate stabilised sol (Figure 3.17).⁶²⁻⁶³ The corresponding MHA capped sol, did not give any signals corresponding to either citrate or MHA, and thus it was inferred that the citrate had been displaced from the particle surface or it was in such a low concentration that it could not be observed.

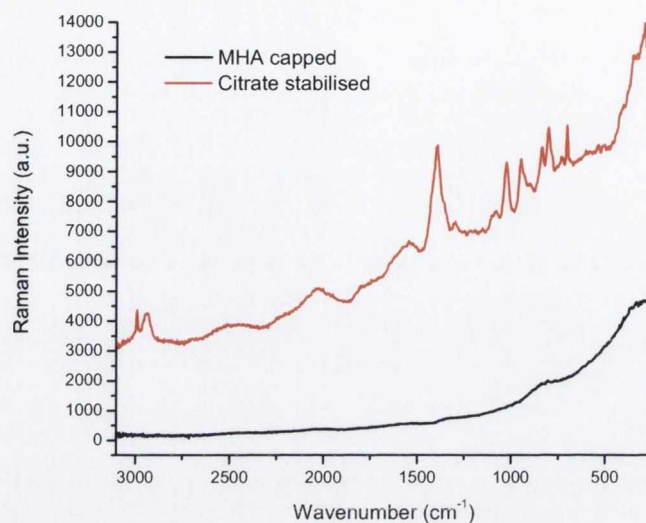


Figure 3.17 Raman spectra of the MELLFs prepared of citrate stabilised and MHA capped triangular silver nanoplates.

Significantly the MHA was shown to protect the particles sufficiently from etching by halide ions. The particles were shown to be stable even in 100 mM sodium or potassium halide salt solutions. Figure 3.18 shows the UV-Vis spectrum of the MHA capped triangular silver nanoplates in the presence of 100 mM NaCl. The capped particles were also found to be stable in 100 mM KBr and KI solutions for an extended period of time (> 1 week).

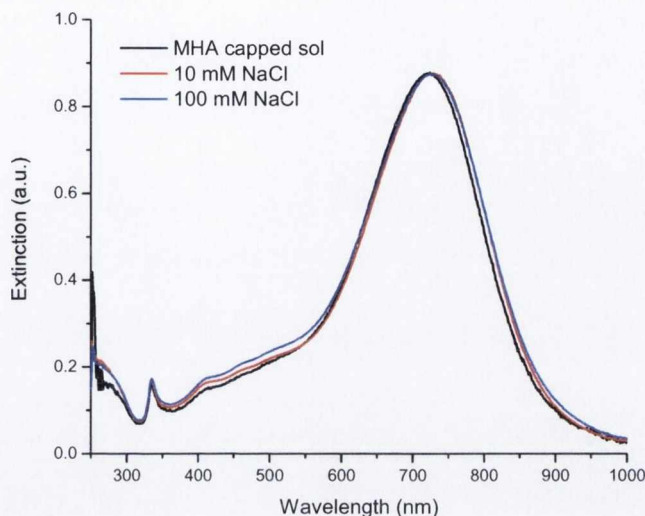


Figure 3.18 UV-Vis spectra of MHA capped triangular silver nanoplates in the presence of 10 and 100 mM NaCl.

It should be noted that the stabilising effect of 16-mercaptohexadecanoic acid on triangular silver nanoplates was published by Lee *et al.* during the course of this work.²⁴ They showed that their triangular silver nanoplates were stable in the presence of 100 mM KBr.

An investigation into the required concentration of MHA needed to protect the particles was carried out. A triangular silver nanoplate sol was divided in four and MHA (1 mM) added to give final concentrations of 20 μM , 10 μM , 5 μM and 2.5 μM . The original sol had a λ_{max} at 724 nm, and it shifted by varying degrees, depending on what concentration of MHA was added (Figure 3.19a). The 20 μM sample underwent a 33 nm shift to 757 nm, the 10 μM sample underwent a 25 nm shift to 749 nm, the 5 μM sample underwent a 13 nm shift to 737 nm and the 2.5 μM sample underwent a 11 nm shift to 735 nm. The samples were treated with sodium chloride (10 mM final concentration) and their spectra monitored for 15 minutes. It was evident from the UV-Vis spectra (Figure 3.19b) that the 10 μM sample was sufficiently capped to offer stability against etching. This was further investigated with a series of samples with MHA concentrations of 10, 9, 8, 7 and 6 μM respectively. It was found that a 9 μM final concentration of MHA was needed to provide stability.

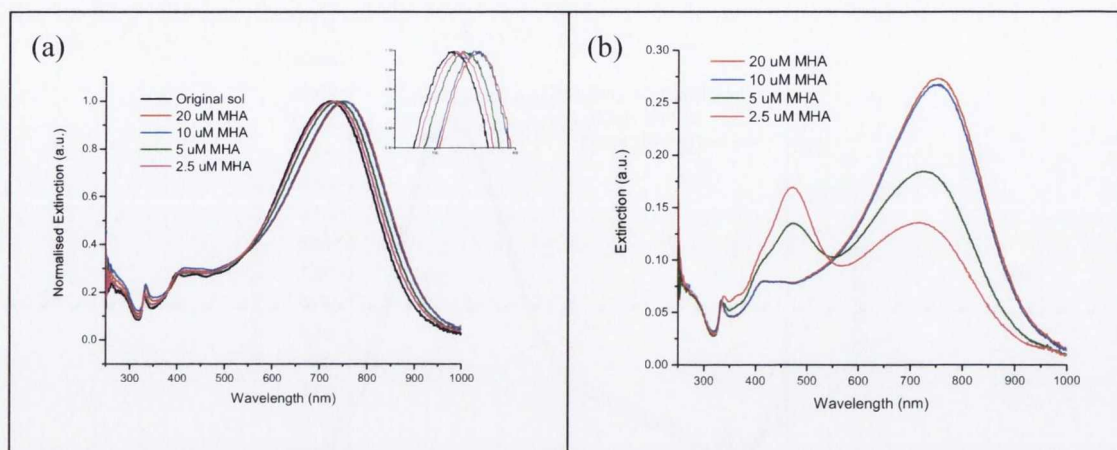


Figure 3.19 (a) UV-Vis spectra of triangular silver nanoplates before and after addition of MHA of various final concentrations (20, 10, 5 and 2.5 μM) (b) UV-Vis spectra of the various MHA capped particles in the presence of 10 mM NaCl.

This is an interesting result as the variance in spectral shifts from the 10 μM and 20 μM samples would suggest that the 10 μM sample is not fully capped with the MHA but yet it offers protection to the sample from chloride etching. Indeed Aherne *et al.* has shown that epitaxial deposition of gold on these triangular silver nanoplates is sufficient to protect the particles from etching in 10 mM NaCl.³⁷ Mirkin has shown that the thiol oligonucleotide conjugation of triangular gold nanoplates takes place initially on the side faces before functionalising the flat $\{111\}$ face.⁶⁴

The stability of the MHA capped particles was analysed by the addition of dithiothreitol (DTT) (Figure 3.20). The DTT displaced the MHA from the surface of the particles and resulted in the particles aggregating because of the overriding charge neutralisation.

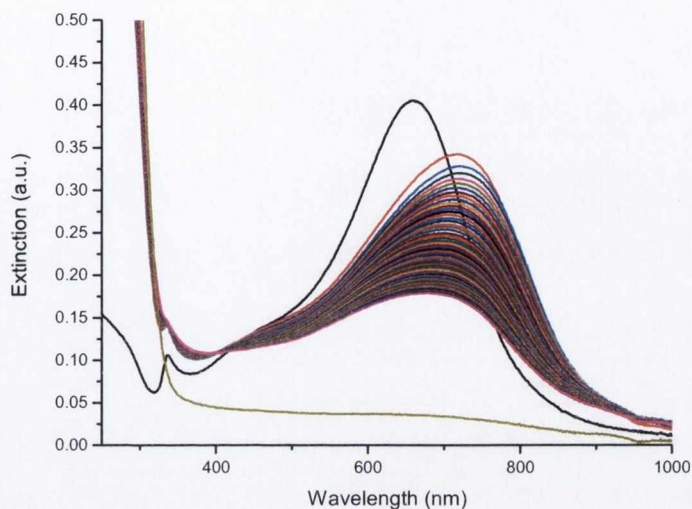


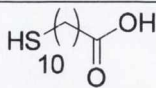
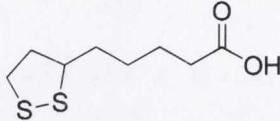
Figure 3.20 UV-Vis spectrum of MHA capped triangular silver nanoplate sol after addition of DTT (1 M). Scanned every 2 mins for 60 cycles. Final scan after 13 hours

3.11.2 Capping with Other Thiol Molecules

Some other thiol compounds were analysed for comparison and effectiveness with that of 16-mercaptohexadecanoic acid (**29**) (Table 3.4).

Table 3.4 Structures of thiolated compounds used for nanoparticle capping

Name	Structure
16-mercaptohexadecanoic acid	$\text{HS}-(\text{CH}_2)_{15}-\text{COOH}$ 29
Tiopronin	$\text{H}_3\text{C}-\text{CH}(\text{SH})-\text{CO}-\text{NH}-\text{CH}_2-\text{COOH}$ 30
Thiolated PEG	$\text{HS}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{O}-(\text{CH}_2-\text{CH}_2)_n-\text{OCH}_3$ 31

Name	Structure
11-mercaptoundecanoic acid	 32
Thioctic acid	 33

Tiopronin (**30**) has been used readily with spherical silver particles.⁶⁵⁻⁶⁶ It was proposed that the possible hydrogen bonding between the molecules would give it an added advantage, and increased packing, over some other shorter thiols, but although it slowed the degradation of the nanoplates by anion etching, the effect was still very pronounced and rapid. Thiolated PEG (**31**) has been used, to impart a solvent stability to gold particles particularly in the coating of the particles with silica⁶⁷ and we were keen to take advantage of this particularly with regard to the titania coating of the nanoplates. Treatment of the nanoplates with PEG-SH again offered some slowed resistance to etching by chloride but offered little protection to the particle. It was interesting to note that if higher concentrations of the PEG-SH were used, the particles appeared to etch. It is proposed that this was due to an impurity in the compound, but due to the small amount of material (250 mg) we were not at liberty to investigate this. Thioctic acid (**33**) and 11-mercaptoundecanoic acid (MUA) (**32**) were also utilised but again, although the etching process was slowed relative to the untreated particles, the compounds did not offer any long term protection.

3.11.3 Discussion on Thiol Stabilisation of Triangular Silver Nanoplates

The contrasting behaviour of the various thiols may be correlated with the packing of the compounds on the surface of the particles. It has been shown that larger shaped particles are more like flat planar structures and the packing is not as high as that of smaller spherical particles.³⁴ This packing can be correlated with the coverage of the particle. It can be assumed that the advantage and significance of the MHA, is that the long alkyl chain imparts stability to the particles, with the lateral van der Waal interactions creating a well defined and structured self assembled monolayer (SAM).

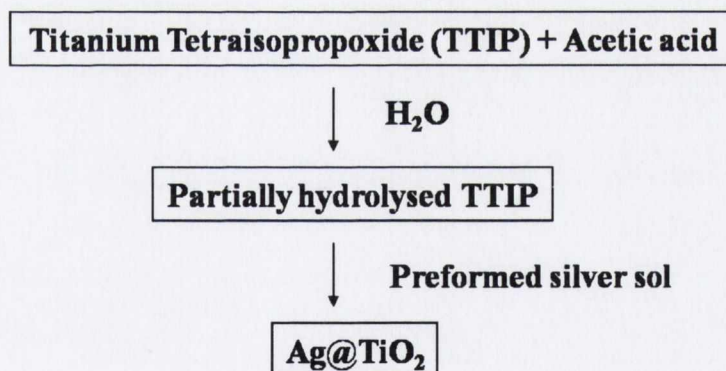
The resulting dense packing creates a hydrophobic area around the particle surface imparting a stability to the particles. The significant red shift in the LSPR band is indicative of formation of a SAM.

The beauty of the MHA capping is that not only does it stabilise the particles efficiently against etching, but the introduction of the carboxylic acid group offers us a route to further functionalise these particles. We have utilised this in Chapter 5 to prepare ODNs modified with a long sulfhydryl chain.

3.12 TiO₂ Coating of Triangular Silver Nanoplates

The method for titania coating the particles was based on a preparation developed by Dr. Siby Padmanabhan within our laboratory (Scheme 3.4).⁶⁸ It involved the complexation of titanium tetraisopropoxide (TTIP) with acetic acid, the addition of water to partially hydrolyse the TTIP, followed by addition of the preformed silver nanoplates.

Sol gel approach for preparation of Ag@TiO₂ particles



Scheme 3.4 General procedure for the preparation of Ag@TiO₂ using a sol gel approach

3.12.1 Coating of Citrate Stabilised Silver Nanoplate Sol

Preliminary experiments were carried out with the ‘as prepared’ citrate stabilised nanoplates with a particle concentration of 2.7×10^{14} particles/L. Titanium tetraisopropoxide (0.25 ml, 0.84 mmol) was placed stirring in a beaker. Acetic acid

(0.24 ml, 4.19 mmol, 5 equivalents) was added. A white precipitate was obtained for the complex. Water (1 ml) was added to start the hydrolysis, the solution turned clear. The preformed nanoplate sol was added. The beaker was covered with parafilm and the sample stirred at room temperature overnight. A cloudy blue suspension was obtained. Efforts to separate the free titania from the possibly coated triangular silver nanoplates by centrifugation resulted in the formation of a gel like substance that we can propose was the result of forming a titania network. It was not possible to determine if the particles were coated or if they were simply embedded in a titania network.

3.12.2 Development of a Concentrated Triangular Ag Nanoplate Preparation

There were two options to try and optimise the method for titania coating, to decrease the concentration of the titanium precursor or increase the concentration of nanoparticles in the reaction mixture. It was decided that the addition of more particles to the reaction mixture was the best approach. If the coating reaction is a surface limited process, then the more particles that are in the reaction mixture, the more coated particles achieved, with the amount of free titania particles obtained reduced. It has been shown in Section 3.10, Figure 3.11 that the centrifugation of the nanoplates can lead to the degradation of the particles. The blue shift in the UV-Vis spectrum is presumably due to the removal of the sharp corners of the triangular nanoplates. The concentration of the particles by centrifugation can also be a laborious and time consuming process. A concentrated nanoplate preparation was needed. The aim was to develop a more concentrated preparation of nanoplates, whilst maintaining the quality and the monodispersity of the original nanoparticle preparation, i.e., a high number of triangular particles.

3.12.2.1 Preparation of a Ten Times Concentrated Triangular Silver Nanoplate Sol

Initially the preparation of a ten times more concentrated sample (3 ml of 5 mM AgNO₃) was tried but similar particles to the original preparation were not obtained. Attempts varying the rate of addition of the silver nitrate and the ascorbic acid were made, but with little success. A lot of spherical material was present, evidenced by a peak ~ 400 nm in the UV-Vis spectrum and aggregation was also observed in most instances.

3.12.2.2 Preparation of a Five Times Concentrated Triangular Silver Nanoplate Sol

A five times more concentrated sol was prepared with much more success. The preparation built on the original preparation. Water (5 ml), seed (varied 100-500 μ l) and ascorbic acid (375 μ l, 10 mM) were combined, followed by the controlled addition of AgNO_3 (3 ml 2.5 mM) at a rate of 1 ml/min. Citrate (2.5 ml, 25 mM) was added immediately after preparation, or the particles were observed to aggregate. Although the quantity of seed used was varied, a similar position of the LSPR band was obtained regardless of the amount of seed used. In all subsequent preparations 500 μ l of seed particles were used (Figure 3.21).

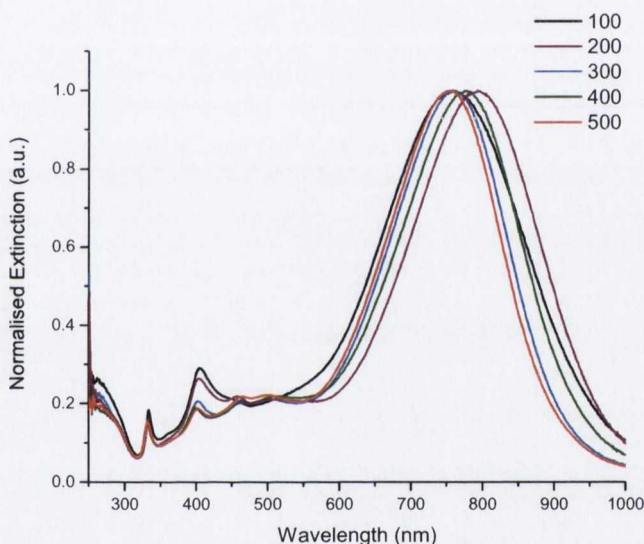


Figure 3.21 UV-Vis spectra of a five times concentrated preparation of triangular silver nanoplates prepared using various seed quantities, 100 μ l - 500 μ l.

The size of the particles was determined using DLS. A similar size profile was obtained for the concentrated preparation, as compared with the standard triangular silver nanoplate preparation. Hydrodynamic diameters of 40 nm and 1 nm were obtained for the large and small peaks respectively (Figure 3.22). TEM analysis showed that the particles were triangular in morphology though the size distribution was greater than achieved for the standard preparation.

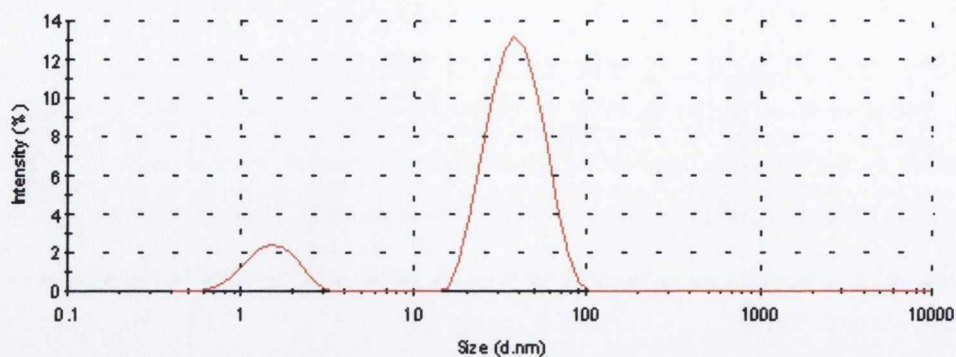


Figure 3.22 Size distribution by intensity measured by DLS for a five times concentrated preparation of triangular silver nanoplates.

3.12.3 Titania Coating of Concentrated Citrate Stabilised Ag Nanoplate Sol

Experiments were carried out using the five times concentrated citrate stabilised particles without using any priming step. A particle solution containing $\sim 10^{13}$ particles was prepared, by combining three five times concentrated sols (24 ml), pelleting the samples by centrifugation and redispersing in 12 ml of water. Titanium tetraisopropoxide (0.25 ml, 0.84 mmol) was placed stirring in a beaker. Acetic acid (0.24 ml, 4.19 mmol, 5 equivalents) was added. A white precipitate was obtained for the complex. Water (1 ml) was added to start the hydrolysis, the solution turned clear. The preformed nanoparticle solution (12 ml) was added. The beaker was covered in parafilm and stirred in the dark. A blue shift of the main LSPR band was evident in the UV-Vis spectrum relative to the starting nanoplate sol. A band for titania could also be observed (Figure 3.23). The original sol was centred at 722 nm, a 12 nm shift to 710 nm and a further shift to 699 nm was observed with time. The sample could be centrifuged and redispersed.

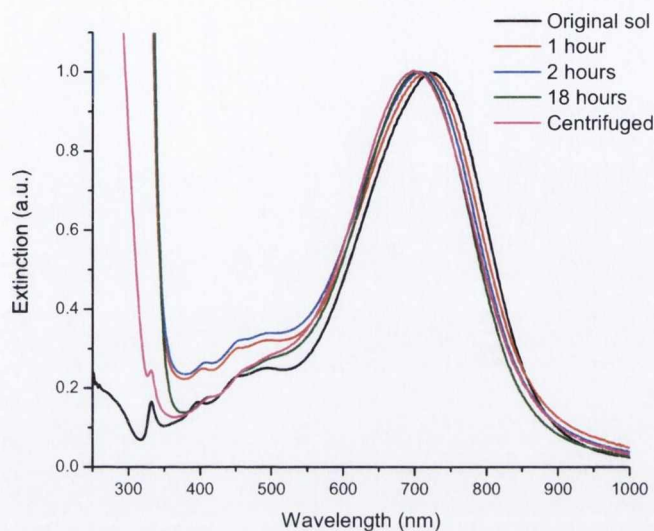


Figure 3.23 UV-Vis spectra of citrate stabilised concentrated triangular silver nanoplates before and after treating with titania coating mixture.

The size of the particles was measured by DLS and was shown to increase on treatment with the titania mixture (Figure 3.24). The large peak increased by 21 nm, from 36 nm to 57 nm. An increase in the smaller peak of 2 nm was also observed, from 1 nm to 3 nm. The zeta potential changed from -40.9 mV for the citrate stabilised sol to +30.6 mV for the titania treated sol (Figure 3.25).

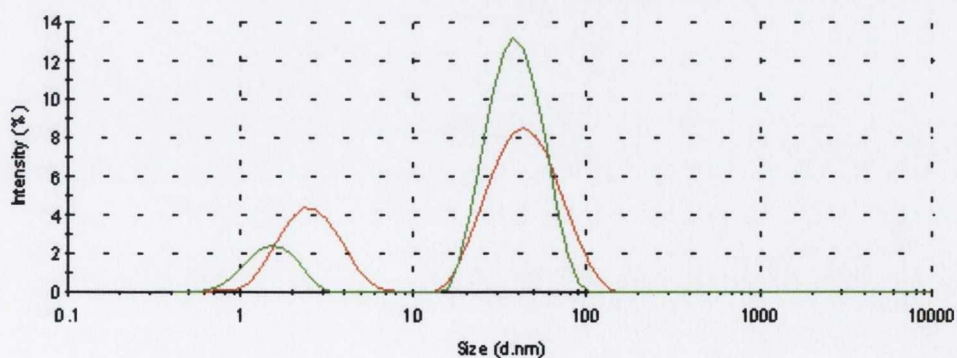


Figure 3.24 Size measurement by intensity measured by DLS for citrate stabilised concentrated triangular silver nanoplates before and after coating

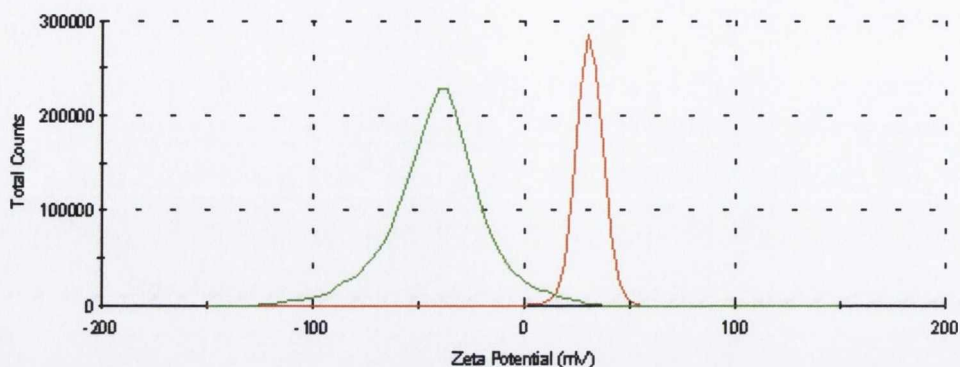


Figure 3.25 Zeta potential measurements for citrate stabilised concentrated triangular silver nanoplates before and after coating.

TEM analysis (Figure 3.26) showed that the morphology of the particles had been disrupted by the coating process. No evidence of a distinct titania coating was observed. The presence of free titania was not visualised.

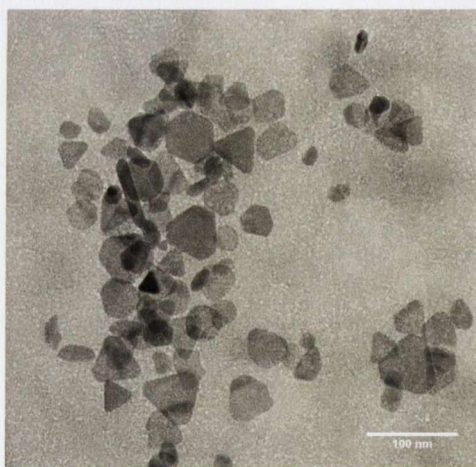


Figure 3.26 TEM of Ag@TiO₂ sample prepared with concentrated citrate stabilised triangular Ag nanoplates.

The particles were shown to have some increased stability to etching by chloride as shown in Figure 3.27, when compared with the ‘as prepared’ triangular silver nanoplate sol. The untreated particles have been shown to etch immediately with the addition of 10 mM NaCl with a peak ~400 nm obtained (Figure 3.12). The treated sample took ~ 12 hours for the sample to turn yellow. The limited stability may be due to a thin titania

shell. Titania shells have been shown to be porous, so complete stability to etching is not expected.

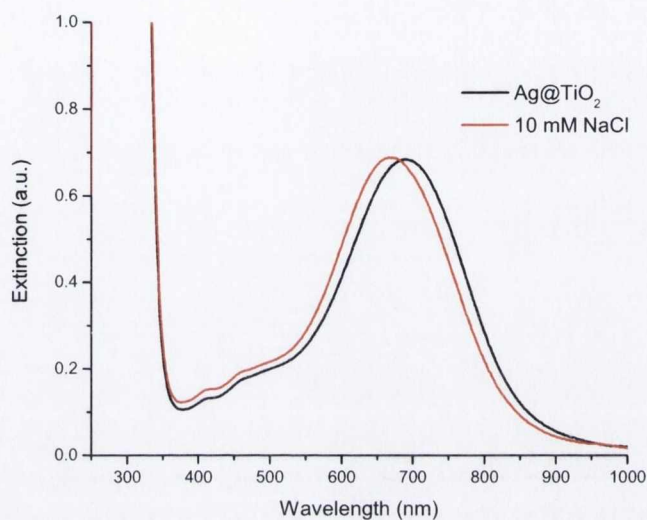


Figure 3.27 UV-Vis spectra of Ag@TiO₂ sample prepared with citrate stabilised concentrated triangular silver nanoplates after addition of 10 mM NaCl after 30 minutes.

Silver nanoparticles are readily dissolved by nitric acid. Addition of a dilute solution of nitric acid to the ‘as prepared’ particles showed an immediate shift in the spectrum and had substantially changed after 8 hours (Figure 5.28a). The addition of nitric acid to the titania treated particles resulted in little change to the particles over the same time period (Figure 5.28b).

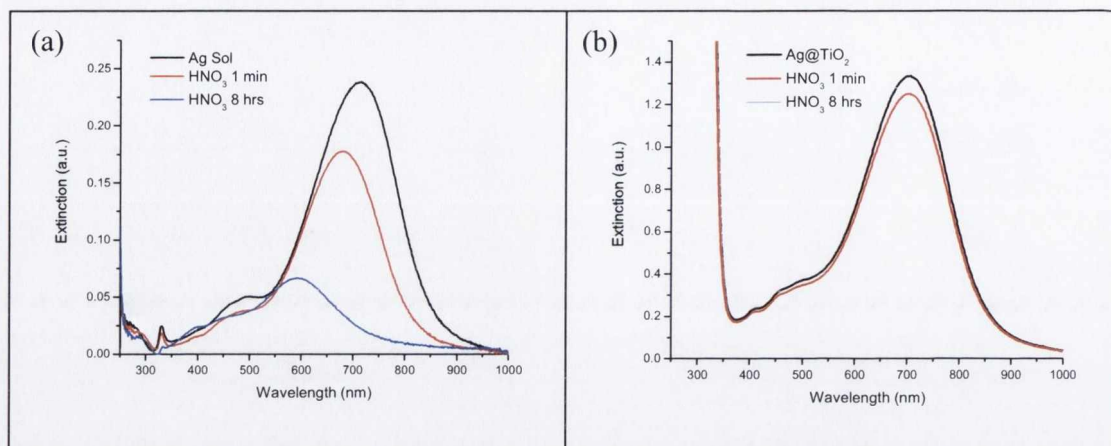


Figure 5.28 (a) UV-Vis spectra of ‘as-prepared’ citrate stabilised triangular silver nanoplates before and after addition of nitric acid. The sample was monitored for 8 hours. (b) UV-Vis spectra of Ag@TiO₂ particles prepared with citrate stabilised concentrated triangular silver nanoplates before and after addition of nitric acid.

The long term stability of the potentially coated sample was analysed. The UV-Vis spectrum after 25 days showed a broadening in the in-plane LSPR band (Figure 3.29).

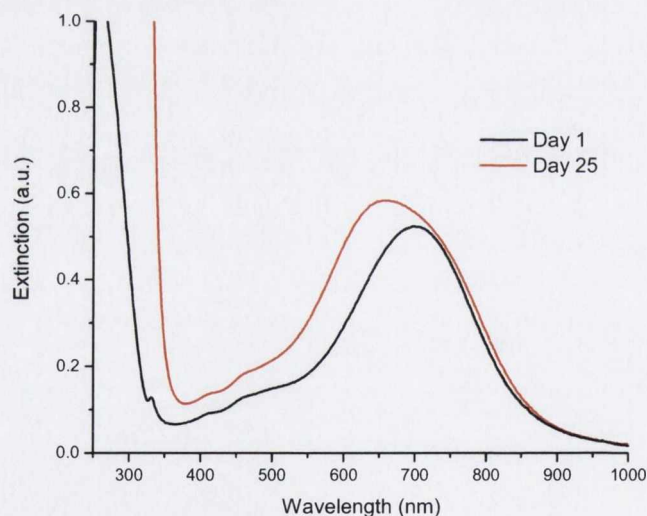


Figure 3.29 UV-Vis spectra of Ag@TiO₂ sample on day 1 and day 25.

Analysis of the particles in the presence of the reagents used, namely acetic acid and titanium tetraisopropoxide (TTIP) showed that the acetic acid resulted in the degradation of the particles within 1 hour of addition, with a more profound effect

observed with time (Figure 3.30a). The LSPR band shifted from 727 nm to 712 nm and then to 671 nm. A growth of a peak at 400 nm was also observed. The TTIP was shown to have no effect on the particle spectrum (Figure 3.30b).

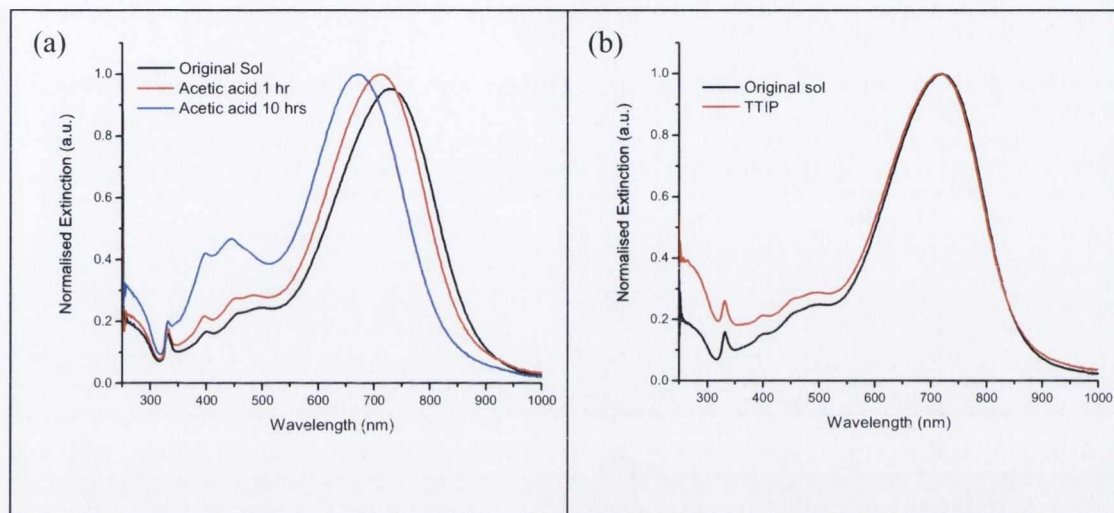


Figure 3.30 UV-Vis spectra of triangular Ag nanoplates in the presence of (a) 0.35 M acetic acid (b) 0.07 M titanium tetraisopropoxide (TTIP)

3.12.3.1 Summary and Discussion of Titania Coating of Citrate Stabilised Ag Nanoplates

In the above section we have presented results on our efforts to titania coat citrate stabilised triangular nanoplates. Initial studies were carried out with the ‘as-prepared’ particles, but these were unsuccessful with a gel obtained. A five times concentrated preparation was successfully developed. Titania coating of this particle preparation showed some promising results. Analysis of the particles by UV-Vis after treating with titanium tetraisopropoxide and acetic acid showed a blue shift in the in-plane LSPR band. The refractive index of titania (2.5 for anatase) is substantially higher than water (1.33) and thus a red shift would be expected. Such a red shift has been reported in the literature for the titania coating of silver and gold nanoparticles. An increase in size was observed when the particles were analysed by DLS. Significantly, a zeta potential change from negative to positive was observed. The point of zero charge for titania is ~ 5 . The pH for this reaction solution was determined to be 3.4 and thus positively charged titania should be obtained due to OH_2^+ species on the surface. TEM analysis

did not show any obvious coating, perhaps it is thin as required. TEM significantly though, showed a change in the particle morphology that would help account for the observed blue shift in the UV-Vis. Further evidence that the particles are coated, although without maintaining the original particle structure, was obtained from the enhanced stability that these particles showed in sodium chloride and nitric acid solutions. The reaction conditions were mimicked to decipher how the degradation of the triangular structure was occurring. It was clear that the acetic acid was responsible, with a blue shift evident in the UV-Vis spectrum. It is proposed that the acetic acid is responsible for disrupting the nanoparticle structure during the early stage of the coating process, and that as the process proceeds, and the particles become coated they have an increased stability. It was decided to adapt the method, incorporating a priming step that would offer protection at the early stage of the coating procedure.

3.12.4 Titania Coating of Concentrated MHA Capped Ag Nanoplate Sol

MHA has been shown to sufficiently cap the particles and prevent the degradation by anions. It has also been observed by Banks *et al.*⁶⁹ that a titania layer can be grown on an acid capped surface. It was thought that the carboxylate groups could help to template the titania growth on the particle surface, whilst protecting the particle from degradation by acetic acid. We were also keen to prevent the degradation of the particles by anions and we have shown already that MHA can prevent the halide etching of the nanoplates (Section 3.11). We have shown in the previous section that titania coating slows the etching process, but we believe it is unlikely to inhibit it. Titania coatings have been shown to be porous, with them being used to form titania nanoshells.⁷⁰ The possibility here is that we can take advantage of the MHA on several levels. We can use the MHA to protect the particle during the coating process. It may help direct the coating on the particle surface, and indeed, it could leave the particles stable to anions after coating, whilst the titania coating will impart a functional surface that we can use for conjugation.

An identical concentration of particles ($\sim 10^{13}$) was used here to that outlined in the previous section (Section 3.12.3). The particles were fully capped with MHA, left overnight to ensure complete capping, centrifuged to remove any excess MHA and

redispersed to yield a concentrated nanoplate solution. The addition of the MHA to the particle sol resulted in a red shift of 33 nm, from 718 nm to 751 nm, as previously outlined. A further red shift of 16 nm was observed on treatment of the sample with the titanium mixture, from 751 nm to 767 nm (Figure 3.31).

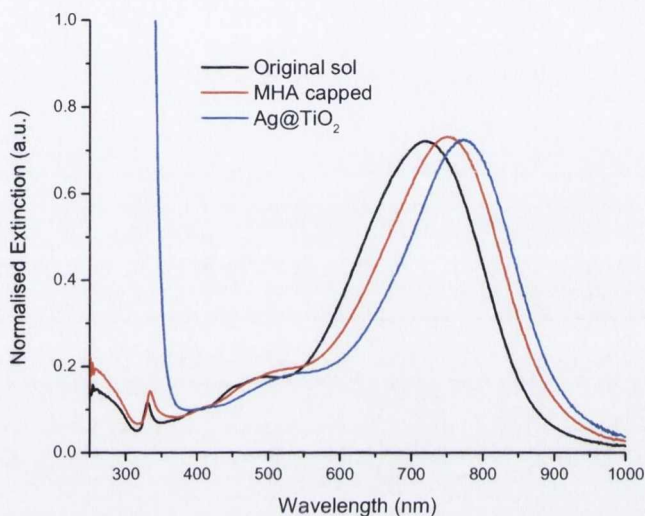


Figure 3.31 UV-Vis spectra for the original sol (black), MHA capped sol (red) and titania coated sol (blue)

This is suggestive of successful coating of the particles because the refractive index of titania (2.5) is much higher than water (1.33). A similar trend to this has been observed before by Mirkin for the silica coating of MHA capped triangular silver nanoplates.³⁵

3.12.4.1 Analysis of Titania Coated MHA Capped Silver Nanoplates

The size of the particles at each stage was monitored by DLS (Figure 3.32a). An increase in both the larger and the smaller peaks was evident. It can be imagined that a peak due to rotation will appear larger on coating, as the rotation of the particle will be substantially slowed. An increase on capping with MHA was observed of 13 nm in the larger peak, from 40 nm to 53 nm, and of 2 nm in the smaller peak, from 1 nm to 3 nm. A further increase was observed on treating the sample with the titania coating mixture. The larger peak increased by 16 nm, from 53 nm to 69 nm, and the smaller peak by 2 nm, from 3 nm to 5 nm. The zeta potential changed dramatically on coating from -

50.5 mV for the MHA capped particles to +36.2 mV for the coated preparation (Figure 3.32b).

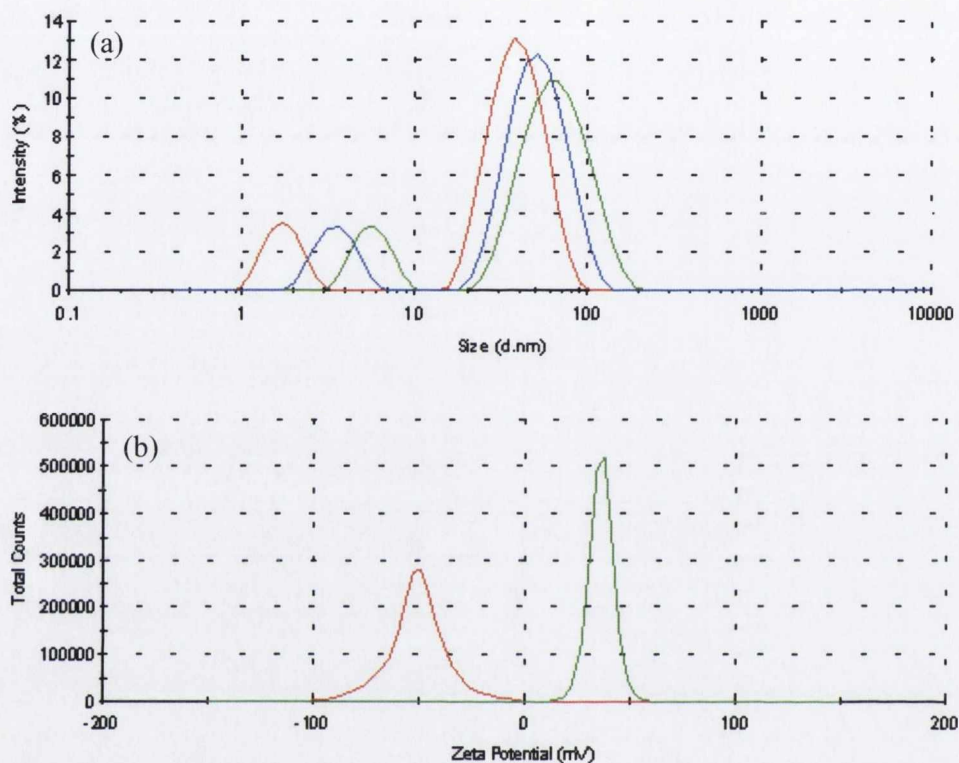


Figure 3.32 (a) Size distribution by intensity measured by DLS for the citrate stabilised (red), MHA capped (blue) titania coated (green) triangular silver nanoplate samples. (b) Zeta potential measurement of MHA capped (red) and titania coated (green) triangular silver nanoplates.

TEM analysis of the particles was performed (Figure 3.33). No distinct titania coating was observed but importantly the particles were mainly triangular and their morphology was intact. It should be noted that the resolution of titania coatings in TEM can be quite poor, and in the case where a very thin coating is obtained, particularly with these thin nanoplates, it could be very difficult to observe.

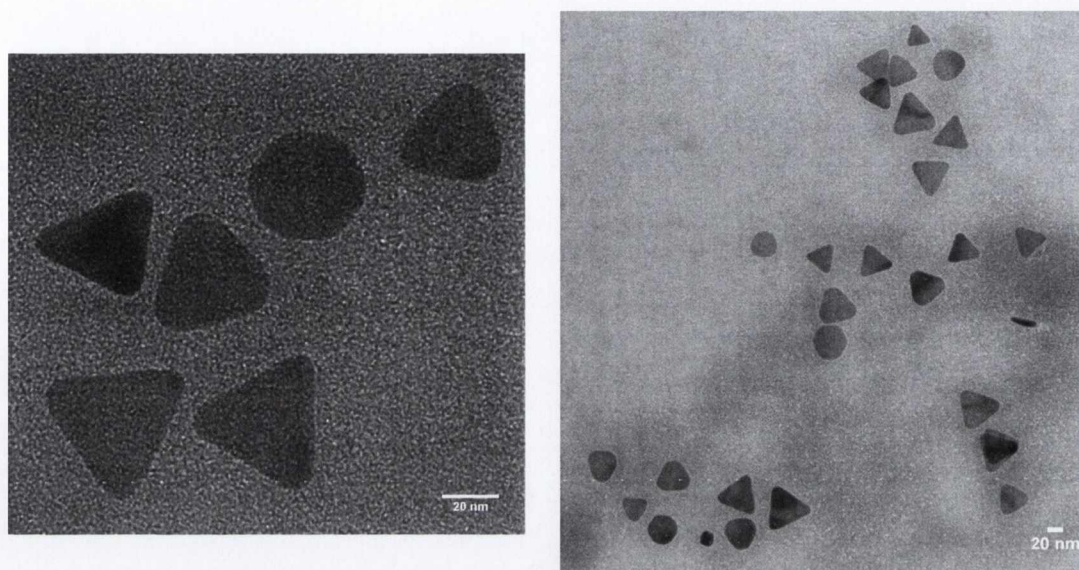


Figure 3.33 TEM images of Ag@TiO₂ prepared with concentrated MHA capped triangular silver nanoplate preparation

The pH of the reaction solution was analysed. The starting sols had a pH 7 but on treatment with the coating mixture, the pH was found to drop to 3.5. The pK_a of the acid group of the MHA may be slightly lower on the surface of the particle, but even at pH 3.5, the acid group should be protonated and the particles would precipitate from solution. The stability of the MHA capped sol in 0.34 M acetic acid with a solution pH of 3.5 was investigated for comparison. The MHA capped particles were found to aggregate almost immediately, at this low pH, with a black precipitate evident. The UV-Vis spectrum shows the scatter band obtained on flocculation of the particles (Figure 3.34). We obtained a dark blue sol for our coated preparation. The stability of the dispersion at pH 3.5 suggests that the surface is modified with titania. The pH of the final solution is lower than the point of zero charge (PZC) of titania (pH 5.5-6.8) and thus a positive zeta potential reading is obtained due to the protonation of the surface hydroxyl groups forming $Ti-OH_2^+$ species. This combined with the red shift in the UV-Vis and an increase in size by DLS would all indicate that the particles are indeed coated with titania.

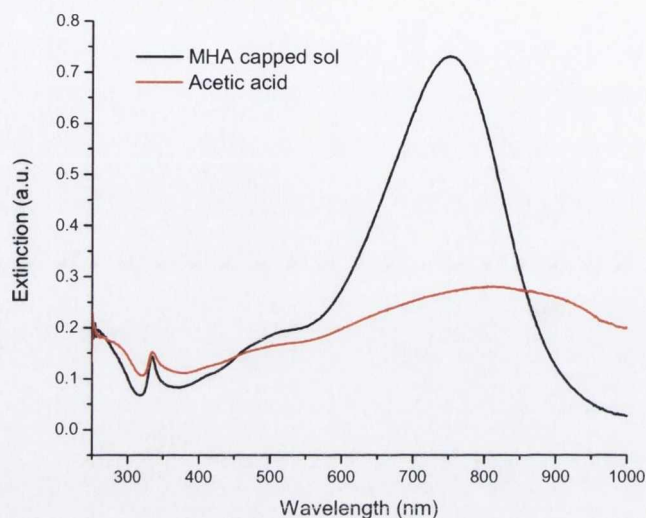


Figure 3.34 UV- Vis spectra of MHA capped particles before and after addition of acetic acid (0.35 M, pH of 3.5).

Importantly the particles could be pelleted by centrifugation and redispersed. The stability of the sample in nitric acid was analysed. The UV-Vis showed the particles to be stable in nitric acid for greater than an 8 hour period (Figure 3.35). (Bare silver particles dissolve readily in nitric acid.)

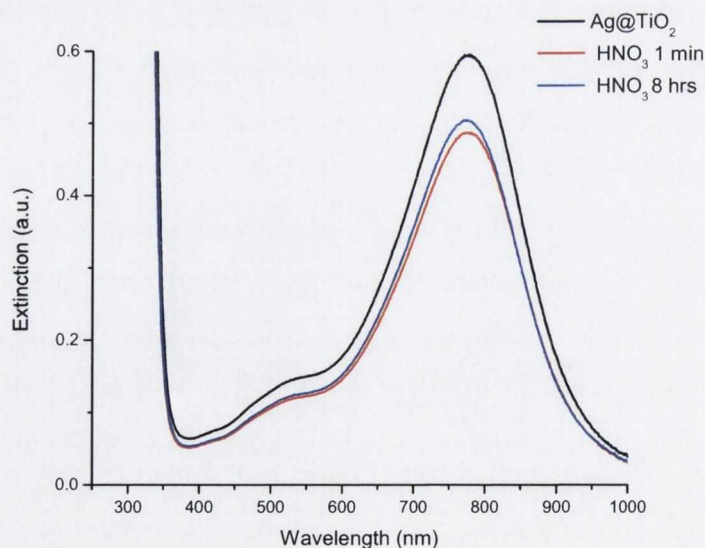


Figure 3.35 UV-Vis spectra of Ag@TiO₂ particles prepared from concentrated MHA capped triangular silver nanoplate preparation after addition of nitric acid.

The salt stability of the particles was assessed. The particles were shown to be stable in 10 mM NaCl for an extended time period (Figure 3.36a). At the higher salt concentration of 100 mM NaCl the sample was observed to gel (Figure 3.36b). (Note however that the plasmon band position is unaffected.)

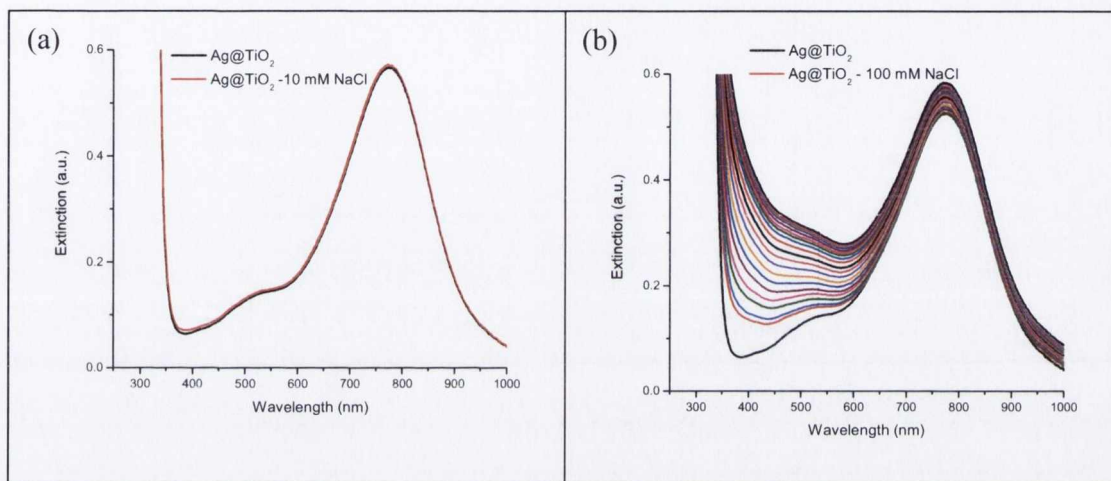


Figure 3.36 UV-Vis spectrum (a) of Ag@TiO₂ particles prepared from concentrated MHA capped triangular silver nanoplates in the presence of 10 mM NaCl (b) in the presence of 100 mM NaCl

We have shown that MHA capped particles aggregate after addition of DTT due to the displacement of the acid groups from the surface (Figure 3.20). Addition of DTT to the Ag@TiO₂ sample prepared with MHA capped particles did not have any effect in the UV-Vis spectrum (Figure 3.37). The particles remained dispersed in solution.

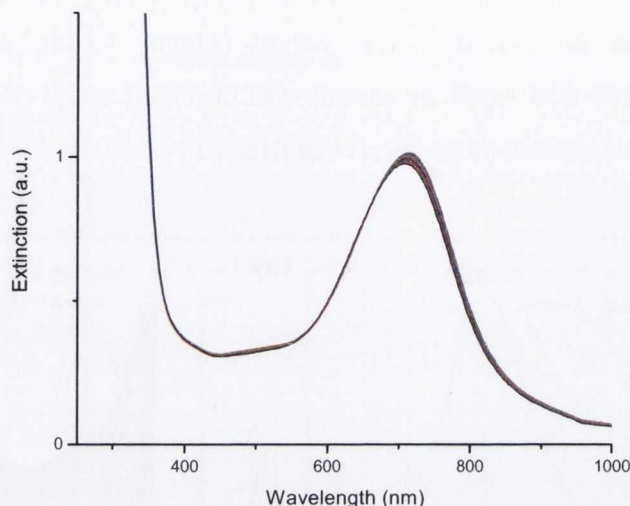


Figure 3.37 UV-Vis of Ag@TiO₂ prepared with concentrated MHA capped triangular silver nanoplate sol after addition of DTT (1M). The sample was scanned every 5 minutes for 100 minutes.

The titania coating formed at room temperature can be presumed to be amorphous from literature reports. Efforts to crystallise the titania coating were made, so as to aid in the characterisation and confirmation of the coating. The sample was heated at 80 degrees for 7 days, unfortunately the sample irreversibly aggregated.

A large peak in the UV-Vis at 350 nm could be attributed to titania including any free titania particles. Dopamine has been shown to readily form a charge transfer band on binding with titania. We used this concept to help visualise the small titania particles that were present in our reaction mixture. The large triangular nanoplates were selectively pelleted by centrifugation leaving a clear supernatant. Addition of dopamine hydrochloride to this resulted in the observation of an orange yellow colour, which was clear evidence for the presence of free titania. This was visualised by UV-Vis spectroscopy with the appearance of a band at 390 nm (Figure 3.38).

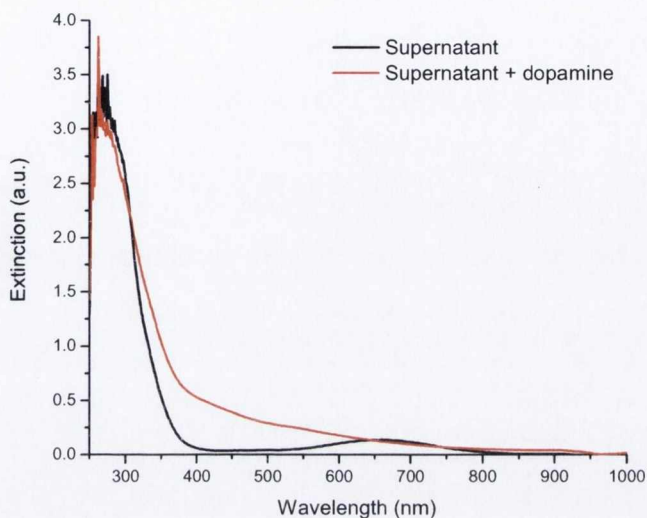


Figure 3.38 UV-Vis spectrum of reaction supernatant after centrifuging the Ag@TiO₂ sample before and after addition of dopamine

Efforts to use size exclusion chromatography to separate the two distinct particle population sizes were unsuccessful, with the pores of the NAP column becoming blocked. We proposed that this was due to the titania gelling or forming networks which could be a result of the pH of the eluent. More success may be achieved if the pH of the solution was more carefully controlled.

3.12.4.2 Discussion on Titania Coating of MHA Capped Silver Nanoplates

The key detail that suggests that these particles are indeed coated is their stability in the acidic solution with a pH of 3.5, where the MHA capped particles would precipitate. This observation, along with the change in zeta potential, red shift in the UV-Vis and size increase by DLS are all suggestive of successful coating. No distinct layer is observed in the TEM and thus it can be assumed that the titania shell is very thin.

The hydrolysis of titania is a very fast process, much faster than the corresponding silica system and thus it is not surprising that a large amount of free titania is obtained. Some literature reports show the controlled growth of titania shells of various thicknesses on metal nanoparticles by varying the amount of titanium precursor in the reaction mixture.^{45,55} These reported experiments are carried out in anhydrous or organic

solution, with a titanium precursor with an efficient complexing agent to slow the hydrolysis, whereby on reaction and attachment of the first layer a second layer of titania is grown by further reaction with the titania precursor. In our case the particles are being coated in aqueous media. It is energetically favourable to coat the particles as opposed to the titanium precursor self condensing,⁷¹ but because of the speed of the hydrolysis and condensation, no further growth occurs as all the material has already undergone reaction. This is possibly the key and reason why there are many reports for very thin titania shells, as compared with the analogous silica system.

Efforts to reduce the amount of titania self nucleation within our system and to grow thicker shells were tried by switching to a solvent system of ethanol, or an ethanol/water mix. The MHA capped particles were found to precipitate readily in these solutions and it is suggested that this is due to the lower dielectric constant of ethanol as compared with water. If the coated nanoplates could be readily removed from the free titania, then they could be dissolved in ethanol and used as templates for the growth of thicker shells.

3.13 Conclusions

The stability of the prepared triangular silver nanoplates is quite good with little shifting in the UV-Vis spectrum observed if sufficient citrate is added after preparation, light is excluded and the samples are kept at 4 °C

The triangular silver nanoplates have been shown to be very sensitive to halide ions, the reactivity appears to be different to that observed in the literature and for an earlier generation of nanoplates prepared within our group, with an order of reactivity of $\text{Br}^- > \text{Cl}^- > \text{I}^-$ found. It can be concluded that the surface chemistry of the particles has a significant role to play with the particle preparation and additives having a role. Further investigation and an understanding of the etching mechanism is required.

16-mercaptohexadecanoic acid (MHA) has been shown to form a sufficiently dense surface assembled monolayer on the triangular nanoplates preventing them from degradation by a variety of anions. The MHA also helps preserve the particle structure when centrifuged and was shown to prevent the degradation of the particle morphology

during the coating process. Interestingly it would appear that particles do not have to be fully capped by the MHA for them to be stable to halide ions. There is some literature precedent for the face selective functionalisation of anisotropic nanoparticles. The introduction of the carboxylic acid functionality offers possibilities for the further functionalisation of the nanoplate particles. This will be dealt with in Chapter 5.

Concentrated preparations of triangular silver nanoplate sols can be prepared by scaling up the Aherne *et al.* procedure, but there is a limit to this. A tens time concentrated preparation proved unsuccessful, but a five times preparation was achieved. Importantly a high selectivity for triangular particles is maintained, but the size distribution was increased.

Titania coating has been carried out using both concentrated preparations of citrate stabilised and MHA capped particles. It can be concluded that a thin layer of TiO_2 can be achieved using the combined evidence in each case.

- (1) Coated citrate stabilised particles were shown to have an increased stability to chloride ions and nitric acid, compared with the untreated particles. An increase in the size by DLS and a change in the zeta potential were both indicative of successful coating. However TEM showed the particles to have undergone a morphological change. This was shown to be as a result of the acetic acid and could account for the unexpected blue shift in the UV-Vis.
- (2) The use of MHA capped particles prevented the degradation of the particle morphology due to acetic acid. A significant red shift in the UV-Vis, an increase in the size of the particles by DLS and a change in the zeta potential all suggested successful coating. This combined with the stability of the particles at pH 3.5 confirmed the successful coating of the particles.

TEM showed that a very thin layer was obtained, as required. The use of MHA capping also resulted in the long term stability of the particles in sodium chloride. Significantly, the titania layer introduces a functionality to the particles, which should provide a route for the conjugation of ODNs. The preparation of a catechol modified ODN is detailed in Chapter 5. To the best of our knowledge, this is the first report for the preparation of Ag@TiO_2 core shell particles with triangular silver nanoplates.

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Chapter 4

Nanoparticle Oligonucleotide Conjugates

4.1 Introduction

Nanoparticle oligonucleotide (ODN) conjugates offer many possibilities for the advancement of technologies, not only in the creation of assemblies and the building of new materials, as envisaged by Seeman,¹ but they also show great promise in the field of detection.² A review of the work that has been carried out with metal nanoparticle ODN conjugates will be described below. The results presented in this chapter are focused on the functionalisation of gold nanoparticles; spherical gold nanoparticles and AuAg triangular nanoboxes, with thiolated ODNs.

4.2 General Strategies for the Preparation of Nanoparticle ODN Conjugates

There are various strategies for the preparation of nanoparticle ODN conjugates.³ These include: (1) The introduction of ODNs by direct adsorption of a functional group onto the nanoparticle surface, e.g., alkyl thiol modified ODNs onto gold or silver.⁴ (2) Reaction with a pre-activated surface that already has a recognition site in place, e.g., an avidin modified surface with a biotin labelled ODN.⁵ (3) Surface coupling of functionally capped particles, e.g., NHS ester activated carboxylic acid capped particles with amino modified ODNs.⁶

A wide range of different nanoparticles have been conjugated with ODNs including gold,⁴ silver,⁷ quantum dots,⁸ magnetite,⁹ silica¹⁰ and titania.¹¹ The salt concentration is a major consideration when preparing conjugates with nanoparticles. Relatively high salt concentrations are necessary for DNA hybridisation, but the high salt concentration can be detrimental to the nanoparticle stability, causing irreversible aggregation. The focus of this thesis is on the work involving gold and silver metal nanoparticles. Much more work has been carried out with gold nanoparticles, than with silver, and the reasons for this will be addressed below.

4.3 Gold Nanoparticle ODN Conjugates

The preparation of gold nanoparticle ODN (Au-ODN) conjugates has been the study of numerous reports over the past 14 years.¹² The first reports in 1996, by the groups of Mirkin and Alivisatos, paved the way for what is an ongoing study and area of

development.^{4,13} The Mirkin group reported the preparation of particles densely functionalised with ODNs, whilst Alivisatos *et al.* reported the functionalisation of particles with a discrete number of ODNs. Densely functionalised particles have an increased stability and have been used in detection.^{2,14} Discretely functionalised particles have potential in the creation of arrays and assemblies.¹⁵⁻¹⁶

The initial study by the Mirkin group used alkyl thiol modified ODNs to conjugate 13 nm citrate stabilised gold nanoparticles.⁴ The particles were functionalised with non-complementary ODN sequences. On mixing, no colour change was observed, the solution remained red, but on addition of a complementary linking segment, an immediate colour change to blue/purple and a precipitation reaction ensued (Figure 4.1). The process was shown to be reversible upon heating and a sharp melting curve was observed when it was monitored at 260 nm or 700 nm. The sharpness and the selectivity of these systems, has been shown to be excellent for DNA detection, with the ability to distinguish a target strand with a single base mismatch.¹⁷

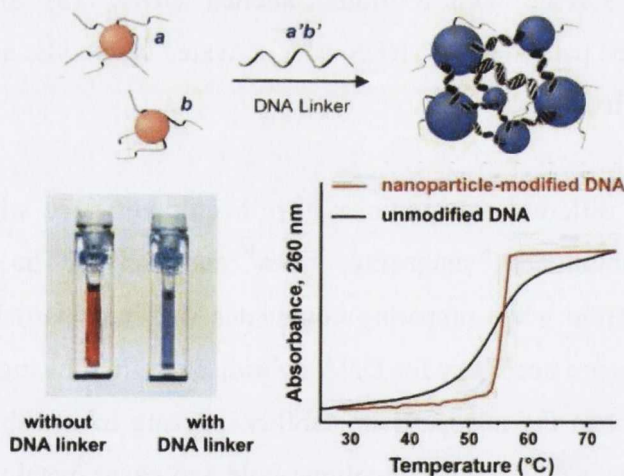
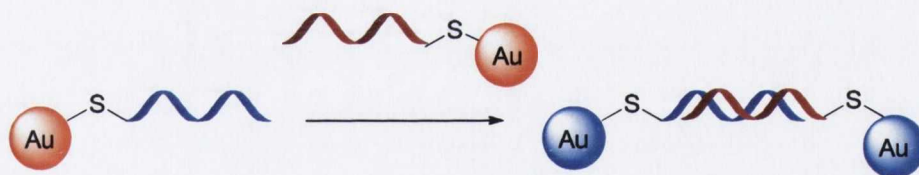


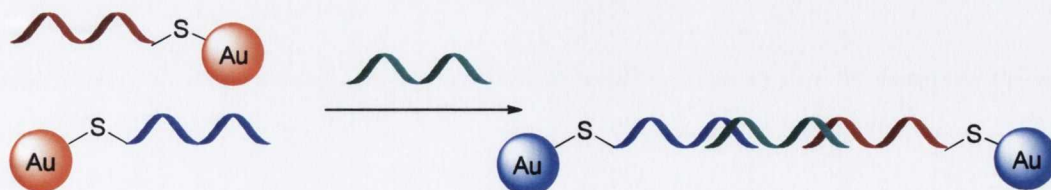
Figure 4.1 Schematic representation (top) of the colour change induced on addition of a complementary target to DNA functionalised gold nanoparticles. Photograph (bottom left) of the sample before and after addition of a DNA target strand. Melting curve (bottom right) for the free duplex compared with the nanoparticle duplex. Adapted from references^{2,18}

In contrast, Alivisatos *et al.* prepared phosphine passivated 1.4 nm gold clusters with one N-propylmaleimide group.¹³ These particles were subsequently reacted with thiolated ODNs, to give discretely functionalised particles that could form assemblies and could be analysed by gel electrophoresis.¹⁹⁻²⁰ The use of these discretely functionalised particle systems has been limited by the methods for their purification. The separation of conjugates with a specific number of functionalised strands has been documented. Agarose gel electrophoresis has only been shown to be useful for the separation of small particles functionalised with long ODN strands.^{15,21} Anion-exchange HPLC offered an approach for the separation of larger nanoparticles with shorter ODNs, but particles any larger than 15 nm were reported to aggregate, due to their instability in the buffer solution.¹⁶ However AE-HPLC showed a greater improvement for the separation of small nanoparticles with long ODNs in comparison with the agarose gel.

The focus of the work in this thesis is on the preparation of nanoparticles densely functionalised with ODNs. Both two-strand²²⁻²³ and three-strand systems^{4,22} (sometimes referred to as ‘sandwich’ systems) have been utilised. Schematics for the different systems are shown below (Scheme 4.1 and 4.2).

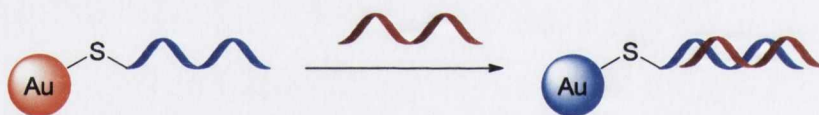


Scheme 4.1 Simplified schematic of a two-strand system. The mixing of two complementary ODN conjugates results in the formation of an aggregated assembly.



Scheme 4.2 Simplified schematic of a three-strand system. A complementary crosslinking segment is used to induce the assembly of two distinct nanoparticle ODN conjugates.

At higher salt concentrations (>0.5 M NaCl), the addition of complementary ODNs to gold nanoparticle ODN conjugates, has resulted in the aggregation of the systems without the crosslinking of the nanoparticles (Scheme 4.3).²⁴⁻²⁶ These systems are referred to as non-crosslinking systems. Monitoring the thermal denaturation of these systems has shown them to show a sharp melting transition.⁷ They have also been shown to have selectivity for terminal mismatches, at the end of the ODN sequence opposite the conjugation site. It is proposed that the aggregation results from the conformational change, on hybridisation at the nanoparticle surface, which results in a tighter conformation of the ODN. This has the effect of decreasing the electrostatic repulsion, because duplexes have a higher binding constant with cations than single strands, and the steric repulsion is also reduced, as the stiffening of the system lowers the entropy. An investigation of the interaction of DNA modified gold nanoparticles and a DNA modified gold plate suggested that a helix stacking interaction is responsible for the aggregation in these systems.²⁷ The ODN density on the nanoparticle surface has recently been shown to be important as to whether this aggregation phenomenon is observed.²⁸



Scheme 4.3 Simplified schematic of a non-crosslinking system. Addition of the complementary ODN to a nanoparticle ODN conjugate induces aggregation under high salt conditions.

4.4 Properties of Conjugates

4.4.1 Melting Temperature of DNA

The denaturation/melting of a double-stranded helix of DNA, results in an increase in the observed absorbance signal normally measured at 260 nm.²⁹ This is because the extinction of a double-stranded helix is lower, due to the base stacking, than the extinction of the single-stranded systems. This is known as the hypochromic effect. The melting temperature (T_m), the temperature at which 50% of the ODN and its complement are in duplex form, is generally determined by measuring the absorbance at 260 nm as a function of temperature. A sigmoidal curve is obtained. The T_m for a

particular ODN duplex is affected by a number of factors including the length and sequence of the ODN, and, the ionic strength and pH of the solution.

4.4.2 Melting Temperature of Nanoparticle-ODN Conjugate Assemblies

A sharp melting transition is characteristically observed for the dehybridisation of nanoparticle-ODN conjugates.¹⁸ The T_m occurs at a higher temperature than that for the free ODN duplex. In the case of the nanoparticle systems, the DNA concentration is very low, and thus, the observed melting behaviour results from monitoring the nanoparticle rather than the DNA. This has been shown for gold nanoparticles by monitoring the signal at longer wavelengths, 525 nm or 700 nm.⁴ The term melting is used loosely and it is better to term it a phase transition.

There are many contributing factors to the observed T_m in these systems. Along with those outlined for the free DNA duplex above; the length and sequence of the ODN, and, the ionic strength and pH of the solution, there are other factors specific to the nanoparticle systems, which include the size of the nanoparticles, the surface density of the ODNs on the nanoparticle surface, the distance of the nanoparticles from each other and their positioning within the aggregate and the spacer length. As is observed for DNA duplexes not attached to nanoparticles, as the salt concentration is increased, the T_m is observed to linearly increase. The longer the ODN and the higher the GC content the more stable the system that is achieved and a higher T_m is observed.

A similar behaviour is observed for all nanoparticle ODN systems, but different theories account for the sharpness for the transition. It is proposed that the behaviour arises from the number of ODN interactions that take place on a nanoparticle surface. One theory that is proposed for the observed melting transition is that it is as a result of a cooperative melting effect, with the localised salt concentration being reduced as some strands denature, resulting in the lowering of the T_m of subsequent duplexes.^{18,30} The other theory attributes the observed transition to entropic cooperativity, comparing the nanoparticle aggregate to a gel undergoing a phase transition to a liquid.³¹⁻³² The localised salt concentration has been shown to be less important. This has been further

exemplified recently using experimental and computational analysis of a simple system.³³

The number of base pairs needed to induce the assembly of nanoparticle ODN conjugates has been shown to be much less than for the free duplex.³⁴ Base pair ‘slipping’ has been observed on particles with a high degree of curvature whereby non-Watson-Crick base pairing interactions can take place along with regular Watson-Crick base pairing, resulting in increased T_{ms} .³⁵

4.4.3 Preparation and Adjustments in the Preparation of Au-ODN Conjugates

The thiol gold bond is relatively strong (45 kcal/mol), but it is not the only process involved in the binding of ODNs to gold surfaces. Non-specific adsorption of the nucleobases has been observed, and is attributed to the amines in the pyrimidine or purine rings of the nucleotides, with a reactivity sequence of $T < A < C < G$.³⁶⁻³⁸ Approaches have been utilised to try and overcome this non-specific binding including the addition of mercaptohexanol³⁹⁻⁴⁰ and addition of short thiolated ODN segments.⁴¹ The use of spacer segments on the end of ODNs has also been used to increase the surface loading of ODNs and to ensure that the ODNs are free for hybridisation. T_{10-20} or A_{10-20} ODN segments^{36,41} and PEG segments have been used.⁴² The conformation of ODNs on the surface of nanoparticles has been shown to be important for hybridisation. The conformation of the ODN on the nanoparticle surface is dependent on the number of ODN strands on the surface (Figure 4.2).⁴³ Too many ODNs on the surface will inhibit efficient hybridisation, as the ODNs will not be accessible for hybridisation.

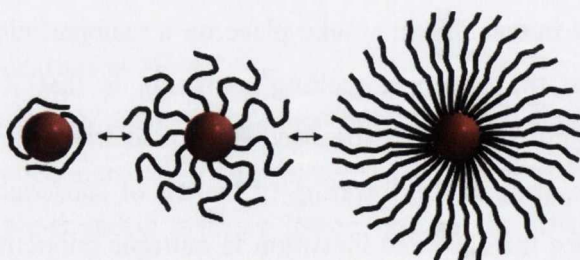


Figure 4.2 Representation of the different conformation of ODNs on a gold nanoparticle surface dependent on the number of ODNs conjugated. Taken from reference⁴³

Earlier preparations involved a gradual salt-aging step, whereby the addition of NaCl screens the negatively charged phosphate backbone, reducing the repulsion between the strands and allowing them to pack tighter together. Hurst *et al.* showed that the loading increased with increasing salt concentration, plateauing between 0.7 M and 1 M NaCl.⁴² The use of sonication and the addition of surfactants, has been shown to be advantageous in reducing preparation time and increasing ODN loading. More recent reports have introduced adaptations to reduce the time required for conjugate preparation, including the use of a non-ionic fluorosurfactant (Zonyl FSN)⁴⁴ or the addition of mononucleotides during the preparation.⁴⁵ The rate of target hybridisation has been increased by using short internal complement DNA.⁴⁶

4.4.4 Measurement of Surface Loading and Hybridisation

It is important to be able to quantify the number of ODNs attached to the nanoparticle surface. A wide range of methods have been utilised, to measure the surface loading and hybridisation of ODNs on nanoparticle surfaces. The most common method of measuring ODN concentration is by measuring the absorbance at 260 nm, but this is a relatively insensitive method. Herne and Tarlov used ³²P radiolabelling to determine the surface coverage and hybridisation on gold plates.³⁹ Radiolabelling has also been used to monitor the denaturation of double-stranded DNA on a gold nanoparticle surface.⁴⁷ Fluorescently labelled ODNs were used to monitor coverage and hybridisation on gold nanoparticles.⁴¹ Electrochemistry,⁴⁸ surface plasmon resonance spectroscopy⁴⁹ and real-time PCR have also been used.⁵⁰ Oligreen[®] is a commercially available fluorescent stain for quantifying ODNs and single-stranded DNA.⁵¹ It is 10,000 times more sensitive than analysis by absorbance. Oligreen is an asymmetrical cyanine dye, its actual structure is proprietary. It offers advantages over the other methods, as it requires no special ODNs or labelling steps. The reaction is very fast and the results have been shown to be reproducible, as long as the standard curve is prepared with an ODN with a similar base composition to that used. Oligreen has been shown to have a significant base selectivity. It has been used successfully to quantify the number of ODN strands on nanoparticle surfaces.^{23-24,52}

4.4.5 Stability of Nanoparticle-ODN Conjugates

The stability of these nanoparticle conjugates is of utmost importance if they are to form the basis of a medical detection system. Biological fluids and buffers contain many additives, which could actively compete for the binding sites. An improved conjugate stability was observed with the use of multiple thiol or disulfide tethers.⁵³⁻⁵⁵ The preparation of 3'-thioctic acid labelled ODN nanoparticle conjugates showed an enhanced stability, when compared with the corresponding alkyl thiol ODN conjugates.⁷ Interestingly, the stability was not found to correlate with the surface coverage. Dithiocarbamate modified ODNs have also been used to prepare stable gold nanoparticle ODN conjugates.⁵⁶

4.4.6 Size and Shape of Particles

Spherical particles, as large as 250 nm, have been successfully conjugated with ODNs and their stability in high salt solutions demonstrated.⁴² A more recent concept has been the role of curvature in the loading and stability of the various particle sizes. Larger particles (>60 nm) can be considered to be more like a planar plate surface, and the packing density is greatly reduced.⁵⁷⁻⁵⁸ The use of multithiol linkers has been shown to be more useful when functionalising smaller particles.⁵⁹

There has been very little work carried out on the functionalisation of anisotropic gold nanoparticles. The earliest reports involved the functionalisation of gold nanorods, but contradictory results were observed.⁶⁰⁻⁶¹ In one case, the rods assembled end-to-end, whereas in the other case they assembled along their long axis. This eluded to the possibility that the faces of anisotropic particles could be selectively functionalised. Mirkin used alkyl thiol modified ODNs to prepare conjugates with CTAB stabilised triangular gold nanoprisms.²³ Analysis using EELS, monitoring the phosphorus signal intensity, allowed the monitoring of the functionalisation over time. A facet selectivity was observed, with the alkyl thiol modified ODNs going on the high energy side facets, before the flat {111} plane. This was further shown by monitoring the assembly of complementary spherical gold nanoparticle ODN conjugates. A longer reaction time of over 12 hours was found to be needed for maximum ODN loading, compared with 6 hours for the spherical nanoparticles. A higher surface loading was also found than

predicted and this was attributed to the curvature at the edges of the particles. The melting temperature for this system was found to be higher and this was attributed to the greater surface contact resulting in a higher number of ODN interactions involving full complementary base pairing.³⁵ A recent report details the formation of superlattices from a variety of different shaped gold nanoparticles functionalised with ODNs.⁶² Gold nanorods, triangular nanoprisms, rhombic dodecahedra and octahedra were all successfully functionalised with ODNs. The face-to-face assembly of triangular gold nanoprisms was observed, which was consistent with the fact that particles associate in a manner to maximise the ODN hybridisation interactions.

4.5 Silver nanoparticle ODN conjugates

The preparation of silver nanoparticle ODN (Ag-ODN) conjugates has proven much more challenging than the analogous gold nanoparticle system. Silver nanoparticles are more susceptible to oxidation and the silver thiol interaction is weaker than with gold. These are both contributing factors to the difficulty of conjugating silver nanoparticles with ODNs. Mirkin initially used a strategy of gold coating spherical silver nanoparticles, prior to ODN conjugation, because it was found that, the use of alkyl thiol modified ODNs directly with silver nanoparticles, resulted in the irreversible aggregation of the nanoparticles under the necessary conditions for hybridisation, i.e., heating in the presence of 0.05 M NaCl.⁶³

The first report for the direct preparation of Ag-ODN conjugates, involved the use of homo-ODNs, adenine and thymine sequences, with a disulfide modification.⁶⁴ EDTA stabilised silver nanoparticles (30 nm) were functionalised by a very slow salt-aging process. The use of an adenine sequence was found to be more successful, than using a thymine sequence, but all Ag-ODN conjugates were only found to be stable for a number of hours at the required salt concentration. A significant difference in the behaviour of the silver hybridised conjugates was observed, in comparison with the Au-ODN conjugates with a large dampening in the LSPR and a slight red shift being observed. The slope of the melting curve in the silver nanoparticle case was found to be broader than the gold case, and with all other parameters the same, it was concluded that the surface density of DNA was lower on the silver particles. Vidal *et al.*⁶⁵ had more

success in preparing Ag-ODN conjugates with alkyl thiol modified ODNs. They used a similar procedure to that of the original Mirkin gold strategy, but with the addition of a pH adjustment step before the salt-aging. FAM labelled ODNs showed this to result in a greater loading of DNA strands.

The use of cyclic disulfide modified ODNs for the successful preparation of Ag-ODN conjugates was carried out by the Mirkin group.⁵⁵ This method improved greatly on previous reports, which were limited to the introduction of poly-nucleotide sequences, and which showed a relatively short and low stability under hybridisation conditions. The reports by Tokareva *et al.*⁶⁴ and Vidal *et al.*⁶⁵ had lengthy procedures requiring days for preparation whereas the Ag-ODN conjugates prepared with cyclic disulfides could be prepared within 30 minutes.

As outlined above for gold, the use of 3'-thioctic acid labelled ODNs was shown to stabilise particles more than the corresponding alkyl thiol labelled ODNs.⁷ This approach was successfully applied to spherical silver nanoparticles, and again, it was observed that this enhanced stability was not due to an increased surface coverage. The disulfide did not stabilise silver to the same extent as gold, presumably because of the weaker thiol-silver interaction, although the disulfide modified silver nanoparticles were observed to be more stable than the alkyl thiol modified gold particles. A sharp melting curve was observed on hybridisation of the Ag-ODN with a complementary ODN sequence. This was similar to that reported by Sato *et al.* with gold.²⁴

The detection of a DNA target sequence using silver nanoparticle ODN conjugates in a 'sandwich' configuration was carried out by Thompson *et al.*⁶⁶ The conjugates were prepared using alkyl thiols, analogous to the gold strategy, with a gradual salt-aging process. The system was shown to be fifty times more sensitive, than the corresponding gold system, because of the larger molar extinction coefficient of silver. A colour change from yellow to pale red was observed in the presence of a complementary target ODN (Figure 4.3). Interestingly, a stability difference between the 3' and 5' modified Ag-ODN conjugates was observed. The difference in the modifications was in the length of the carbon spacer, with a C₆ spacer at the 5' end, with only a C₃ spacer at the

3' end. The longer alkyl chain was seen to impart a greater stability, and it was proposed, that this was due to a hydrophobic effect, resulting in the screening of the nanoparticle surface from detrimental ions in the troublesome buffer solution.

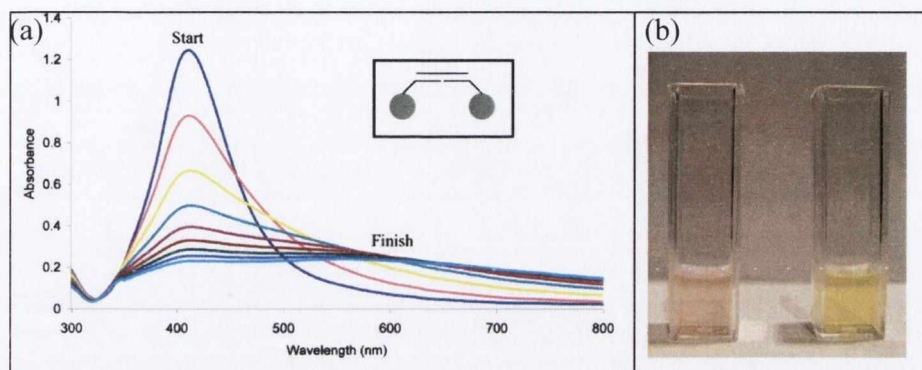


Figure 4.3 (a) UV-Vis spectrum of silver nanoparticle ODN conjugate monitored after addition of complementary target ODN. (b) Photograph of silver nanoparticle ODN conjugate in the presence of a complementary ODN target (pale red) and a non-complementary ODN target (yellow). Taken from reference⁶⁶

Amino modified ODNs have been used for the preparation of gold and silver nanoparticle conjugates,⁶⁷ while Pal *et al.* have used ODNs with a phosphorthioate segment to prepare silver nanoparticle ODN conjugates.⁶⁸⁻⁶⁹ The use of a greater number of phosphorthioates was shown to increase the conjugate stability.

A sole report emerged during the writing of this thesis for the direct preparation of shaped silver nanoparticle ODN conjugates.⁷⁰ Prior to this Mirkin reported the preparation of ODN conjugates with silica coated silver nanoprisms.¹⁰ Kim and Lee prepared ODN conjugates using thiolated ODNs and triangular silver nanoplates prepared by the Aherne *et al.*⁷¹ method.⁷⁰ As has been shown in Chapter 3, the Aherne *et al.* nanoplates are highly sensitive to etching by halide anions. Kim and Lee reported the aggregation of the bare silver nanoprisms in the presence of 0.15 M NaCl, but it is clear from the TEM image in the supporting document that this degradation is primarily due to the etching of the particles to spherical material. A blue shift in the main LSPR is observed on conjugation of the particles with ODNs, and this again, can be attributed to

the change in the particle morphology, due to etching by chloride ions, which is apparent in the TEM images (Figure 4.4).

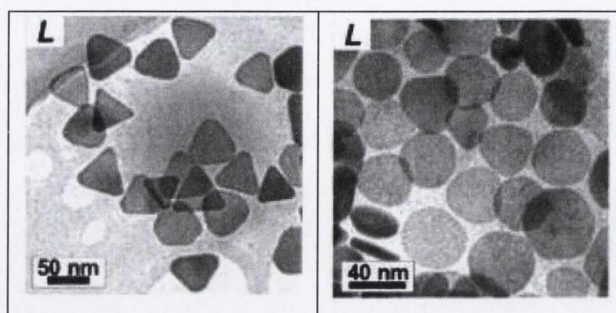


Figure 4.4 TEM image of triangular silver nanoplates before (left) and after (right) conjugation with thiolated ODNs. Taken from reference⁷⁰

AFM was used to confirm ODN conjugation and it was found that conjugation was effective within 4 hours. Two complementary conjugates were mixed and examined by thermal denaturation. The assembly was shown to be reversible, but interestingly for the larger particles, a two step phase transition was observed. This was attributed to the formation of two different assemblies within the system, edge-to-edge/face or face to face. The formation of the different assemblies was controlled by hybridising the system at different temperatures (Figure 4.5).

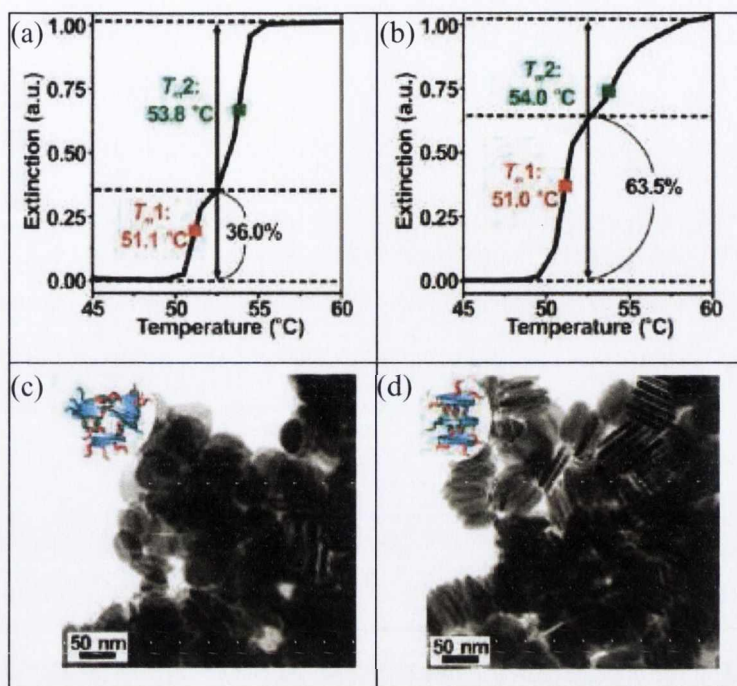


Figure 4.5 Thermal denaturation curves for silver nanoprism-ODN conjugate aggregates assembled at (a) room temperature and (b) -20 °C and then at 4 °C and the corresponding TEM images showing particles assembled (c) edge-to-edge/face and (d) face-to face. Taken from reference⁷⁰

The detection capabilities of the nanoplate ODN conjugates was also investigated. An unoptimised limit of detection of 1 nM was obtained. The potential of triangular silver nanoplates for colorimetric detection of DNA was clearly shown (Figure 4.6).

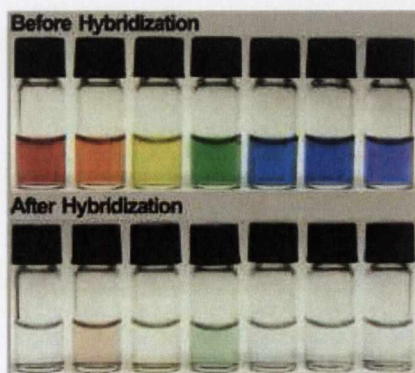


Figure 4.6 Photograph of the silver nanoprism-ODN conjugates before and after hybridisation with a complementary target strand. Taken from reference⁷⁰

4.6 Assemblies of Nanoparticle-ODN Conjugates

Many reports have focused on the assembly of gold nanoparticle-ODN conjugates into defined structures with distinct properties. Particles of various sizes have been assembled together by varying the ratio of the particle populations (Figure 4.7).^{68,72-73} Mixed nanoparticle systems have been used to take advantage of the high extinction coefficient of silver.⁷⁴⁻⁷⁵ Spherical gold-ODN conjugates have been assembled into crystal lattices⁷⁶⁻⁷⁸ and this has been extended to include shaped gold nanoparticles.⁶² Silver nanoparticles have been assembled on pre-engineered triangular DNA origami structures.⁶⁹

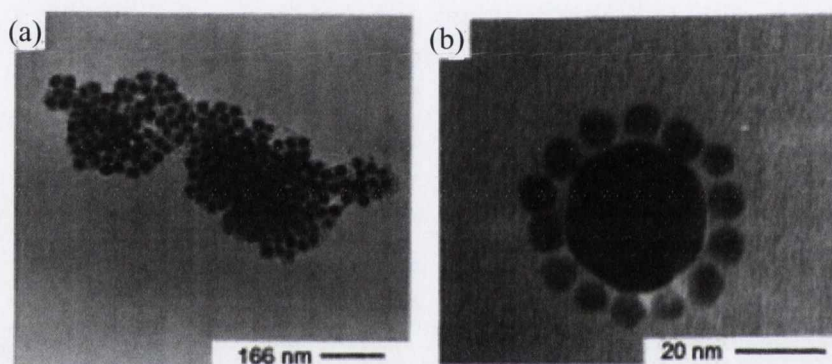


Figure 4.7 TEM images of (a) 8 nm gold and 31 nm gold particle assembly and (b) a satellite structure obtained from mixing the 8 nm and 31 nm particles in a ratio of 120:1. A linking ODN is added in each case. Taken from reference⁷²

4.7 Use of Nanoparticle-ODN Conjugates for Detection and Medicine

Nanoparticle-ODN conjugates have been used extensively in the field of detection and this is an area of continued research. Detection systems involving molecular beacons,⁷⁹⁻⁸⁰ based on the assembly of two nanoparticles in the presence of complementary targets,^{14,66} based on the aggregation of nanoparticles in the presence of a complementary target²⁶ and systems that decipher between single and double-stranded DNA have all been utilised.⁸¹ Nanoparticle-ODN conjugates offer much promise in medicine and have been shown to be readily taken up by a number of cells.⁸² The potential of these nanoparticle-ODN systems is huge, and the prospects in the coming years across a range of applications, is extensive.

4.8 Overview of Results

The results in this chapter involve the preparation of nanoparticle oligonucleotide (NP-ODN) conjugates. The main aim of this work was to functionalise triangular silver nanoplates with ODNs, but due to the instability of these particles to various anions as detailed in Chapter 3, this was found to be quite challenging. The triangular nanoplates have been shown to etch to spherical nanoparticles, in the presence of small amounts of some anions, including chloride. Chapter 5 details further efforts towards the preparation of ODN conjugates with triangular silver nanoplates. The focus of this chapter is on the preparation of ODN conjugates with spherical gold nanoparticles or triangular AuAg nanoboxes derived from triangular silver nanoplates.

Aims and Objectives:

- To functionalise spherical gold nanoparticles with sulfhydryl modified ODNs following reported procedures and to hybridise two complementary ODN conjugates to prove successful conjugation. (Section 4.9)
- To prepare a bifunctionalised ODN for fluorescently labelling metal nanoparticles using efficient oxime ligation. (Section 4.10)
- To investigate the effect of the proximity of 13 nm spherical gold nanoparticles on the emission of ruthenium tris(bipyridine). (Section 4.10.5)
- To functionalise and characterise spherical gold nanoparticles with a ruthenium modified ODN. (Section 4.10.6 - 4.11)
- To prepare triangular AuAg nanoboxes and subsequently functionalise the particles with ODNs. (Section 4.11 – 4.11.4)
- To hybridise complementary AuAg nanobox ODN conjugates and to investigate the properties of the formed aggregates. (Section 4.11.4 -4.12)
- To investigate the assembly of gold nanoparticle ODN conjugates with complementary AuAg nanobox ODN conjugates. (Section 4.13)

4.9 Spherical Gold Nanoparticle ODN Conjugates

4.9.1 Preparation of Spherical Gold Nanoparticles

Citrate stabilised spherical gold nanoparticles were prepared by citrate reduction of HAuCl_4 .⁸³⁻⁸⁴ An aqueous solution of hydrogen tetrachloroaurate (III) trihydrate (1mM) was brought to the boil and trisodium citrate (38.8 mM) was added. The solution turned from yellow to clear, to black, to purple and finally to deep red. The particles were characterised by UV-Vis spectroscopy (Figure 4.8) with a characteristic surface plasmon band observed at 519 nm.

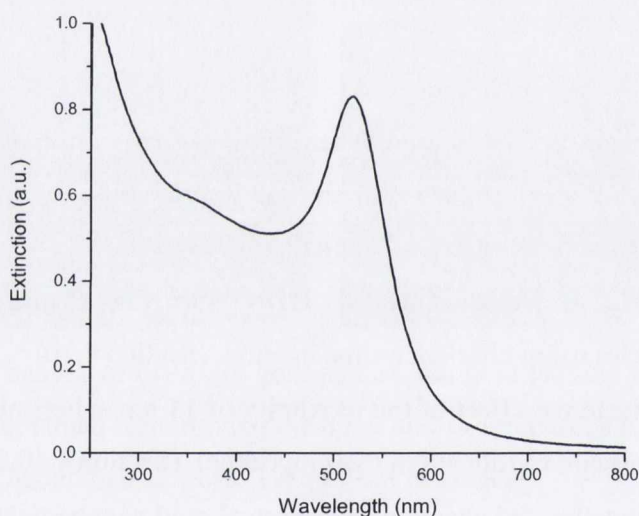


Figure 4.8 UV-Vis spectrum of citrate stabilised spherical gold nanoparticles with a λ_{max} at 519 nm.

Transmission electron microscopy (TEM) showed the average particle size to be 13 ± 1 nm (200 particles sampled). The TEM image (Figure 4.9) showed that a relatively monodisperse sol was obtained.

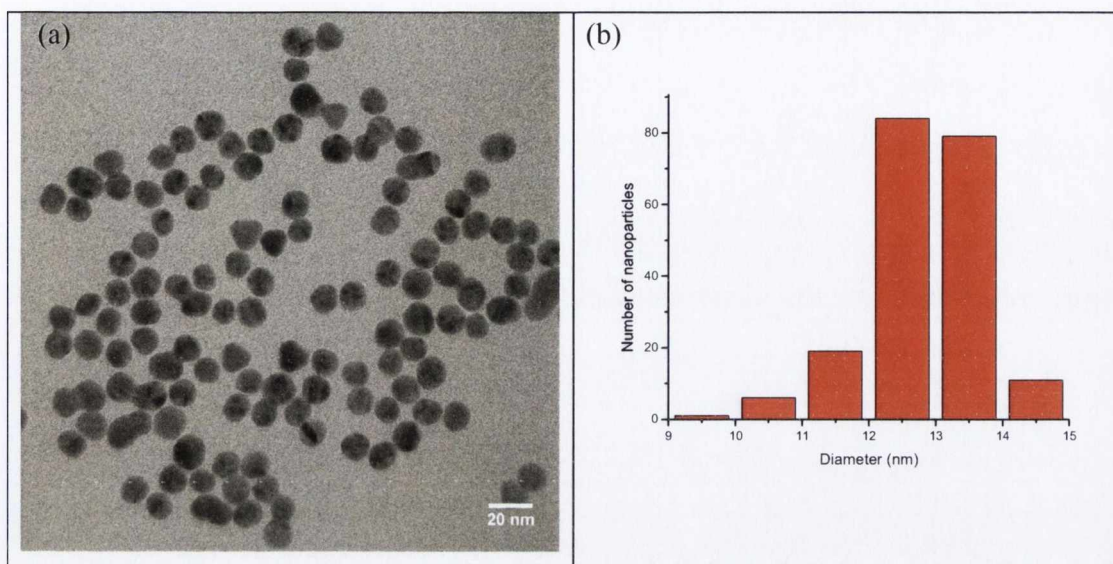


Figure 4.9 (a) TEM image of citrate stabilised spherical gold nanoparticles and (b) Histogram showing the particle size distribution for a population of 200 nanoparticles. Particle size of $13 \text{ nm} \pm 1 \text{ nm}$.

The particle concentration was determined to be 12.3 nM , using a reported extinction coefficient for 13 nm particles, of $2.4 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$,²² assuming Beer-Lambert behaviour. Dynamic light scattering (DLS) measurements gave particles with a hydrodynamic diameter of 20 nm and the zeta potential of the citrate stabilised gold particles was determined to be -31.4 mV (Figure 4.10).

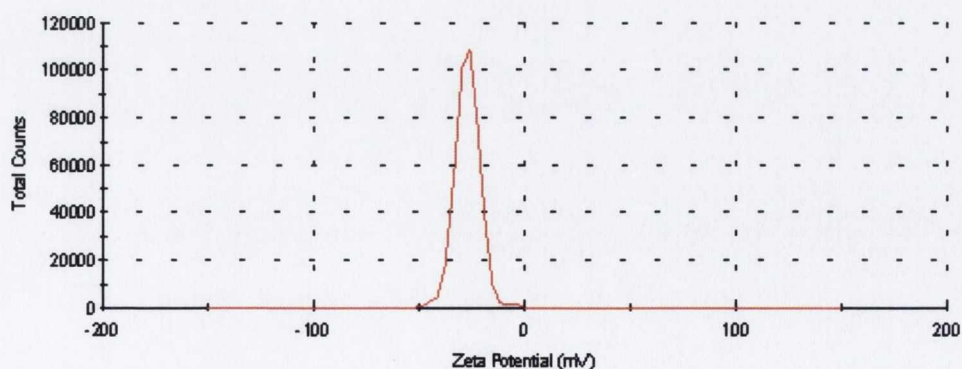


Figure 4.10 Zeta potential measurement for the 'as-prepared' citrate stabilised spherical gold nanoparticles.

4.9.2 Choice of ODN Sequences

The sequences **35A** (5' Tr-S-A₁₀ TCT AGG GAC 3') and **35B** (5' Tr-S-A₁₀ GTC CCT AGA 3') were selected to be complementary to each other, without the formation of any secondary structures. A stretch of ten adenines was included, to help maximise loading on the particle surface,⁴² ensuring that the orientation of the ODN would be such as to allow hybridisation. **35A** is also complementary to the Ru-ODN (9-mer, **23A**, 5' GTC CCT AGA-Ru 3') sequence prepared in Chapter 2.

4.9.3 Synthesis of 5'-Sulfhydryl Modified ODNs

The ODNs **35A** and **35B** were prepared on an ABI 3400 DNA synthesizer (Applied Biosystems), using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 1 μ mol scale. The ODNs were 5'-modified with a trityl protected sulfhydryl group by incorporating the commercially available S-Trityl-6-mercaptohexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite **34** (Glen Research) during the last step of the automated synthesis (Figure 4.11).

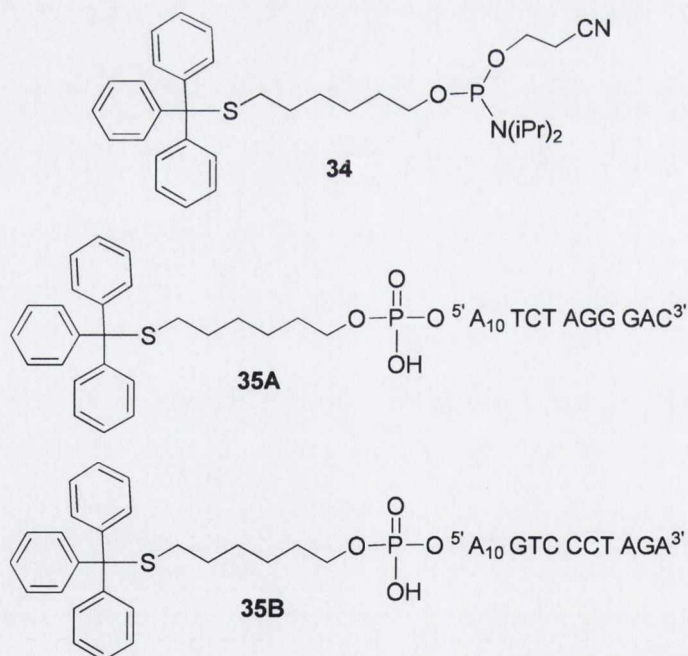


Figure 4.11 Commercial phosphoramidite for the 5' modification of ODNs with an alkyl thiol **34** and the prepared ODN sequences **35A** and **35B**

The ODN was cleaved from the solid support and released into solution by treatment with 28% aqueous ammonia for 16 h at 55 °C. The ODNs were analysed by reverse phase HPLC with a retention time of 19 minutes observed for the required ODN (Figure 4.12). The trityl group renders the ODN hydrophobic and thus it has a longer retention time than the failure sequences. This aids in its purification.

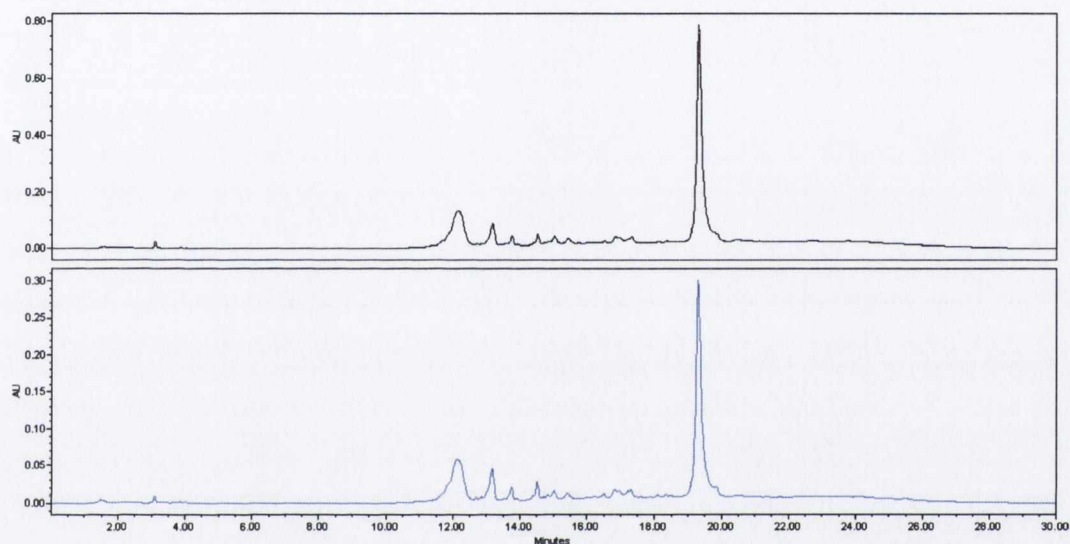


Figure 4.12 Reverse phase HPLC analysis of ODN **35A** before purification. Top at 260 nm, Bottom at 280 nm.

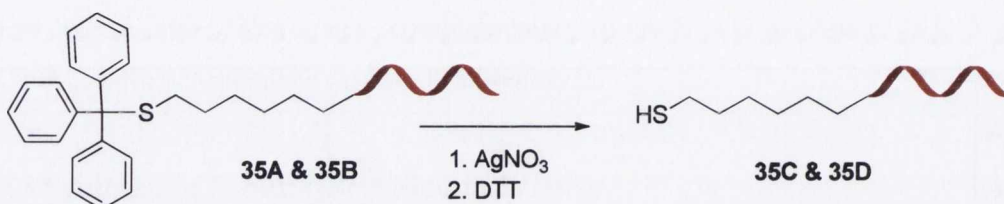
The ODNs were purified by semi-preparative reverse phase HPLC and quantified using UV-Vis spectroscopy. 195 nmoles of **35A** and 215 nmoles of **35B** were obtained. The sequences were confirmed by MALDI-TOF mass spectrometry (Table 4.1).

Table 4.1 MALDI-TOF MS analysis of 5'-sulfhydryl modified ODNs in negative mode

Sample Name	m/z Calculated	m/z Found
35A	6308.9	6307.7
35B	6268.9	6269.9

4.9.4 Cleavage of Trityl Protecting Group

The trityl group on **35A** and **35B** was removed by oxidative cleavage with a silver nitrate solution for 30 minutes to give **35C** and **35D** (Scheme 4.4). A cloudy white suspension was observed.



Scheme 4.4 Cleavage of trityl group of **35A** and **35B** to give sulfhydryl **35C** and **35D**

The silver nitrate was removed by complexation with dithiothreitol (DTT). The sample was treated with DTT and a yellow precipitate (the silver-DTT complex) was observed to form instantly. The precipitate was pelleted by centrifugation and the supernatant containing the ODN collected. The precipitate was washed a number of times to maximise recovery of the ODN. The fractions collected were analysed by UV-Vis for ODN content, by monitoring the absorbance at 260 nm. The recovered ODN samples were combined, and the excess DTT was removed using a NAP column, with water as eluent. The ODNs were quantified by UV-Vis and used immediately for nanoparticle conjugation.

4.9.5 Calculation of Required Amount of ODN for Functionalisation

The amount of ODN needed to densely functionalise the spherical gold nanoparticles was determined as outlined in the current protocols in nucleic acid chemistry series.⁸⁵

$$\begin{aligned} \text{moles of conjugated ODN} &= A_n \times c_n \times D \times V \\ &= 4\pi r^2 \times c_n \times 35 \text{ pmol/cm}^2 \times V \end{aligned} \quad \text{Equation 4.1}$$

where A_n is the surface area of the nanoparticle, C_n is the nanoparticle concentration in number of nanoparticles per litre, D is the ODN density per nanoparticle and V is the volume of the nanoparticle solution (in L). Taking the radius (r) as 6.5 nm and the highest reported density value (D) from the literature as 35 pmol/cm²,⁴¹ it was

determined that 4 nmoles of ODN were needed to functionalise 3 ml of 12.3 nM gold nanoparticles.

4.9.6 Preparation of ODN Nanoparticle Conjugates

The gold nanoparticle solution was placed in a glass sample vial. The 5'-sulfhydryl ODN **35C** or **35D** was added and the sample agitated overnight on an orbital shaker. A slow salt-aging process was adopted. Sodium phosphate buffer (100 mM, pH 7.4) and SDS (1%, w/v) were added to give final concentrations of 10 mM and 0.01% respectively. The particles were shaken for 30 minutes. Aliquots of NaCl (2 M) were added, increasing the salt concentration by 0.05 M increments over 48 hours, until a final concentration of 0.3 M was achieved.

This was the first evidence for successful conjugation, as the 'as-prepared' citrate stabilised particles are observed to aggregate and turn dark even at a concentration of 0.1 M NaCl (Figure 4.13), due to the screening of the repulsive electrostatic interactions between the particles.

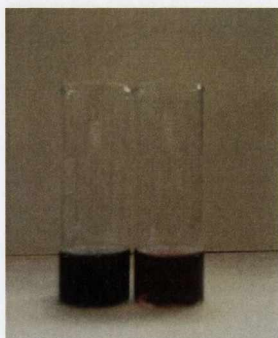


Figure 4.13 Citrate stabilised 'as-prepared' particles (left) and ODN functionalised particles **Au-35C** (right), after addition of 0.1 M NaCl, 10 mM sodium phosphate and 0.01% SDS. The 'as-prepared' particles are observed to aggregate.

Once the final salt concentration had been reached, the particles were left to equilibrate overnight. The excess ODN was removed by multiple centrifugation steps. The particles were pelleted, the supernatant was removed, and the pellet redispersed in 0.3 M PBS. This process was repeated 3 times. The particles were finally redispersed in 0.3 M NaCl, 10 mM sodium phosphate, 0.01% SDS and characterised and quantified by UV-Vis. A

slight red shift in the surface plasmon band of 5 nm, from 519 nm to 524 nm, was observed, which was consistent with previous reports (Figure 4.14).^{4,17}

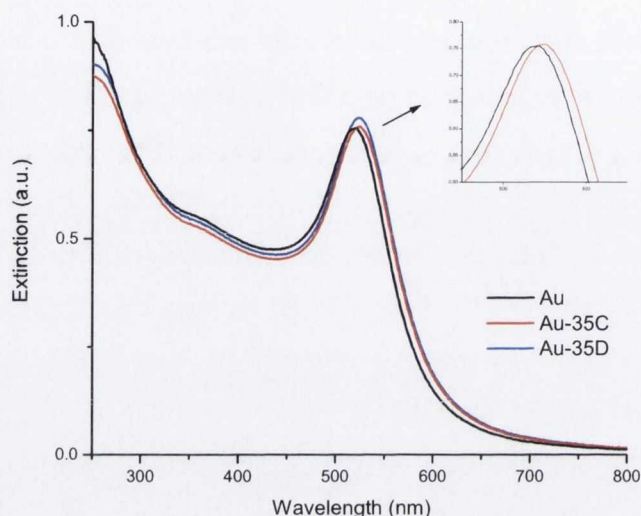
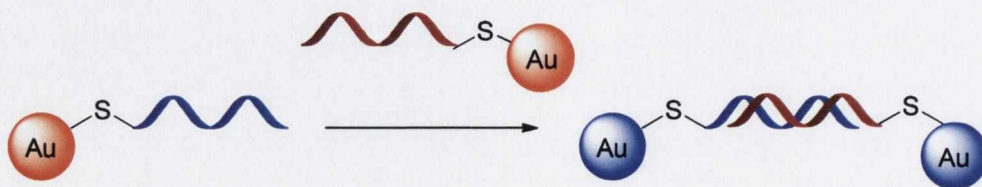


Figure 4.14 UV-Vis spectra of Au and AuNP-ODN conjugates, **Au-35C** and **Au-35D**. The inset shows the 5 nm red shift, from 519 nm to 524 nm, observed on conjugation with the ODN.

4.9.7 Hybridisation of Complementary Conjugates

Having successfully prepared and purified the Au-ODN conjugates, we wanted to investigate the aggregate assembly, from the hybridisation of two particles with complementary ODN sequences (Scheme 4.5). Here we are using two 9-mer complementary strands with an adenine spacer. Hybridisation will only occur if the ODN strands are orientated away from the nanoparticle surface and thus a high loading of ODN is desirable. Likewise, if the ODN density on the nanoparticle surface is too large then hybridisation will not occur because there will not be enough space.



Scheme 4.5 Simplified schematic showing the assembly of two complementary gold nanoparticle-ODN conjugates

Equimolar Au-ODN conjugates (**Au-35C** and **Au-35D**), in 0.3 M PBS, were mixed to give a final ODN concentration of 1.4 μM . A colour change, from red to purple, was observed within 5 minutes (Figure 4.15).

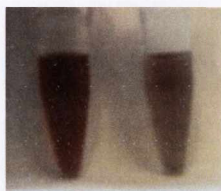


Figure 4.15 Photograph of **Au-35C** conjugate (left) and hybridised **Au-35C** and **Au-35D** after mixing for 5 mins

4.9.8 Analysis of the Hybridisation by UV-Vis Spectroscopy

Analysis by UV-Vis showed a red shift in the main surface plasmon resonance band and a dampening. The sample was left overnight at room temperature and a precipitate was observed to settle in the bottom of the tube. The sample was agitated, to resuspend the aggregate and the UV-Vis spectrum obtained (Figure 4.16). A band at 595 nm was observed for the aggregate.

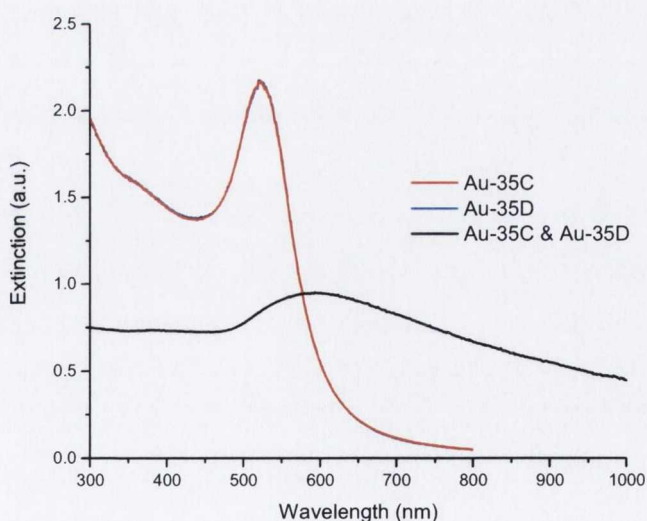


Figure 4.16 UV-Vis spectrum of the resuspended aggregate ($\lambda_{\text{max}} = 595 \text{ nm}$) formed from hybridisation of **Au-35C** and **Au-35D** in 0.3 M NaCl, 10 mM sodium phosphate buffer, 0.01% SDS compared with the individual conjugate spectra.

The surface plasmon band of the gold nanoparticles has been shown to be very sensitive to interparticle distance, and thus a red shift and surface plasmon dampening are observed on hybridisation, due to the assembly of the particles in close proximity relative to each other.

4.9.9 Analysis of the Hybridisation by Dynamic Light Scattering

Dynamic light scattering was used to analyse the individual conjugates, **Au-35C** and **Au-35D** (Figure 4.17). An increase in the hydrodynamic diameter, of 4 to 6 nm, relative to the ‘as-prepared’ citrate stabilised particles was observed.

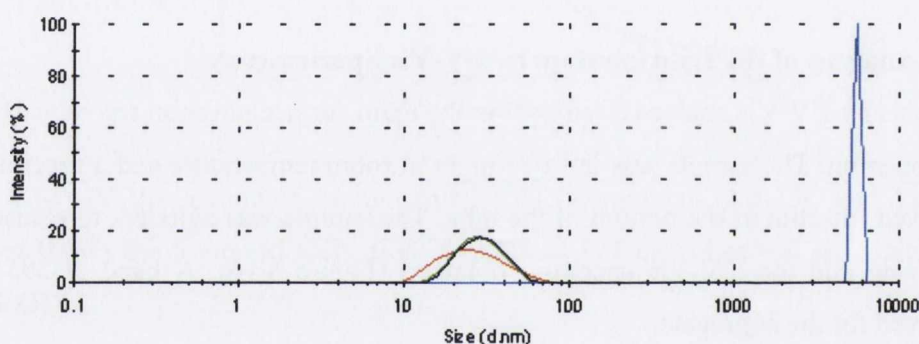


Figure 4.17 DLS measurements of ‘as-prepared’ gold nanoparticles **Au** (red), **Au-35C** (green), **Au-35D** (black), hybridised conjugates **Au-35C** and **Au-35D** (blue). A size increase of 4 to 6 nm was observed for **Au-35C** and **Au-35D** relative to the **Au** particles. A large aggregate with a size of 5.5 μm was observed on mixing **Au-35C** and **Au-35D**.

This correlates well with the ODN length. The ODN is a 19-mer (9-mer of a recognition sequence and a 10-mer adenine spacer) therefore it has a length of 6.46 nm, if fully outstretched in its B DNA form. The flexibility of the single stranded ODN chain, the conformation of the ODN depending on the number of strands, and the interaction of the A_{10} segment with the particle surface would account for a lower value than the persistence length of the ODN being observed. Analysis of the hybridised conjugates showed evidence for formation of a large aggregate with a particle size of 5.5 μm obtained.

The denaturation of the hybridised sample was also analysed by DLS as a function of temperature (Figure 4.18). The aggregate size was shown to decrease, from an average value of 1590 ± 580 nm (20 to 35 °C) to 52 ± 20 nm (50 to 65 °C), with increasing temperature. Unfunctionalised gold nanoparticles were monitored as a control. No change in particle size was observed with increasing temperature.

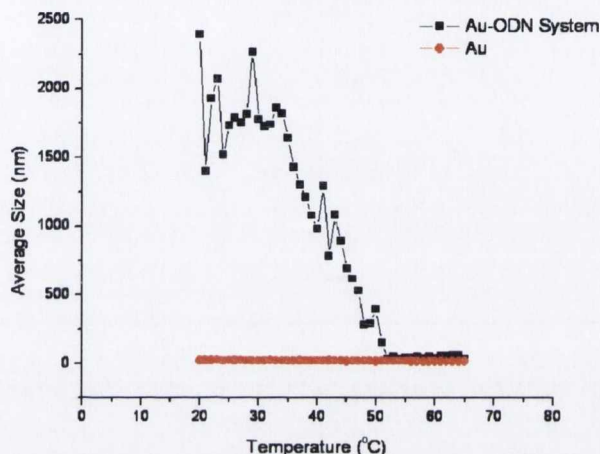


Figure 4.18 Dynamic light scattering of Au-ODN hybridised conjugates as a function of temperature. An unfunctionalised gold nanoparticle sample was monitored as a control (red).

4.9.10 Analysis of the Hybridised Conjugates by TEM

The hybridised gold nanoparticle-ODN conjugates were analysed by TEM (Figure 4.19). Analysis of the nanoparticles at the edge of the assembly, showed that the particles were identical to the unfunctionalised particles, with an average diameter of 13 nm. The particles within the assembly could be observed as discrete particles and were not irreversibly melted together. Unfunctionalised gold nanoparticles have been observed to undergo particle growth reactions resulting in irreversible aggregation.⁸⁶

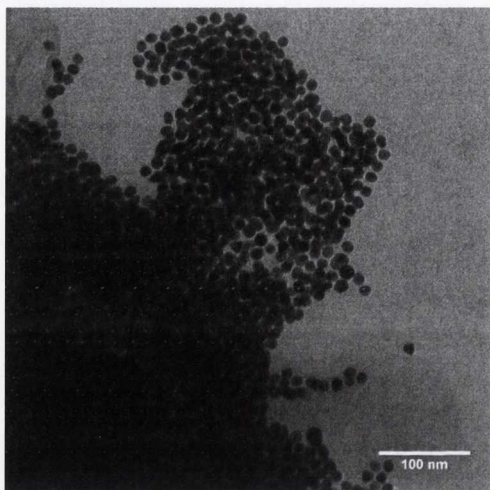


Figure 4.19 Transmission Electron Microscopy image showing a 3D assembly formed from the hybridisation of equimolar **Au-35C** and **Au-35D** in 0.3 M PBS.

4.9.11 Analysis of the Hybridised Conjugates by Thermal Denaturation Experiments

The absorbance of the hybridised conjugates was monitored as a function of temperature (Figure 4.20) at 260 nm (where DNA absorbs) and at 525 nm (the maximum LSPR band of the nanoparticles). The melting temperature (T_m) was determined to be 49 °C for the gold nanoparticle aggregate.

This was higher than the T_m for the 9-mer free duplex, which was observed to be 39 °C. A sharp denaturation profile relative to the free duplex was obtained, which was consistent with previous literature reports.¹⁸ A similar profile was obtained when the sample was monitored at 260 nm and at 525 nm.

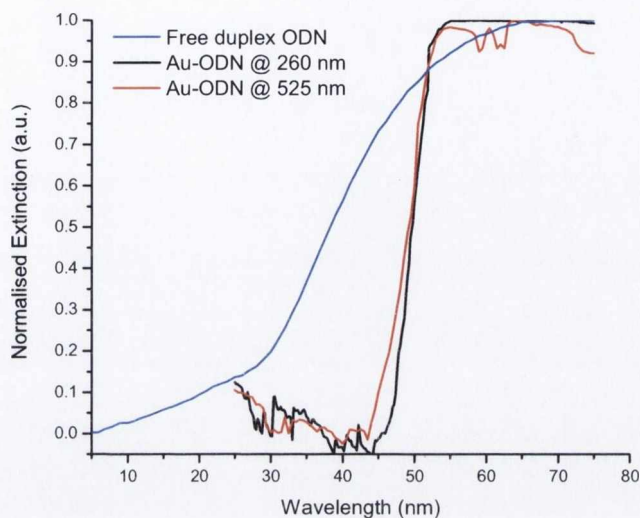


Figure 4.20 Thermal melting curve for the aggregate formed from hybridised conjugates **Au-35C** and **Au-35D** monitored at 260 nm (where DNA absorbs) and at 525 nm (the maximum LSPR band of the gold nanoparticles) and the free duplex DNA (8 μ M, monitored at 260 nm). The denaturation curves are normalised for clarity.

4.9.12 Quantification of the Number of ODNs on Gold Nanoparticles

The surface coverage of nanoparticles with ODNs varies in the literature, with reported values ranging from ~ 7 pmol/cm² to 35 pmol/cm².^{37,41} The ODN coverage has been shown to depend on a number of factors including the sequence of the ODN and the conditions used in the conjugation process, as discussed in Section 4.4.3.

Quant-IT[™] Oligreen[®], a fluorescent stain (Section 4.4.4) for the quantification of ODNs and single-stranded DNA, was used to determine the amount of ODN on the surface of the nanoparticles by carrying out an assay in a 96 well plate, using a fluorescence microplate reader. A standard curve for each ODN sequence was prepared as the reagent has been shown to have a significant base selectivity (Figure 4.21).

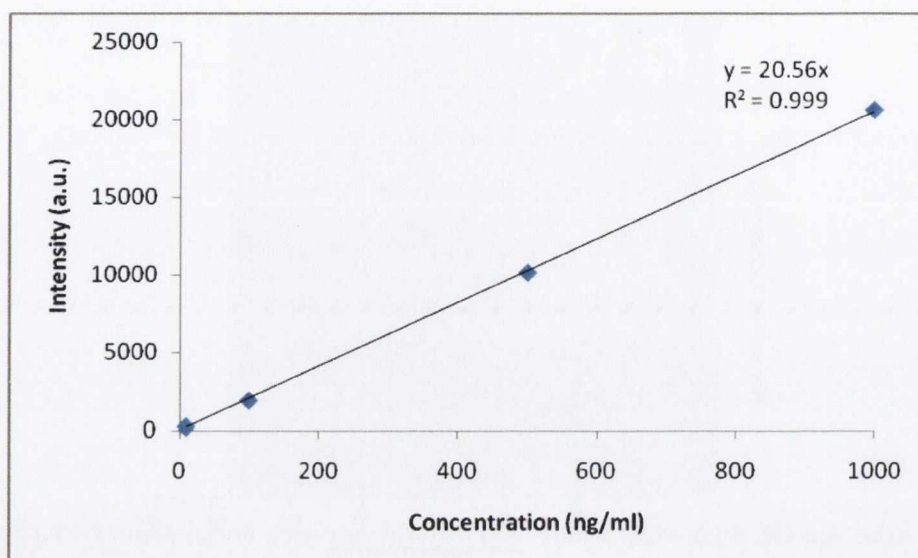


Figure 4.21 Example standard curve obtained for ODN sequence **Au-35C** with Oligreen reagent.

An aliquot of each gold nanoparticle ODN conjugate was taken and treated with dithiothreitol (DTT) to displace the surface bound ODNs. The samples were centrifuged to pellet the particle aggregate and a portion of the supernatant removed for analysis. Oligreen reagent was added to the samples and the fluorescence measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The ODN concentration in the nanoparticle solutions was determined from the standard curves to be 867 nM for **Au-35C** and 1434 nM for **Au-35D**.

The samples were found to have loading values comparable with the literature reported value of 35 pmol/cm² (112 strands for a 13 nm particle). **Au-35C** was found to have a lower value of 71 strands/particle and **Au-35D** had a slightly higher value of 117 strands/particle.

This is indicative that the composition of the sequence has an effect on the observed loading on the particles. Both strands contain an identical stretch of adenines but based on previous studies one can imagine that the lower loading for the **35C** sequence could be accounted for because of the attraction of the G nucleobase for the nanoparticle surface.³⁶

4.9.13 Summary and Discussion of Gold Nanoparticle-ODN Conjugates

13 nm spherical gold nanoparticles were functionalised with thiolated ODNs. A 5 nm red shift in the LSPR band and an increase in the hydrodynamic diameter, as determined by DLS, were indicative of successful conjugation. Complementary conjugates were mixed to form hybridised assemblies. The reversible nature of the assemblies was monitored by thermal denaturation. The melting temperature for the nanoparticle conjugate system was found to be 10 °C higher than that for the free duplex. The curve was also observed to be significantly sharper than for the free duplex. These observations are in agreement with previous literature reports.⁴ TEM analysis showed the particles to be intact within the assembly. Interestingly, DLS analysis of the aggregate as a function of temperature showed a similar phase transition for the ‘disassembly’ of the hybridised aggregate. The ODN surface coverage on the nanoparticles was determined using Oligreen and found to correlate with literature reports.⁴¹ Significantly a difference was found for the individual sequences albeit the samples were prepared under identical conditions.

4.10 Bifunctionalised ODN for Nanoparticle Labelling

The efficiency of oximation as a coupling strategy has been clearly illustrated in Chapter 2, where it has been used to successfully prepare Ru-ODN conjugates. Initial experiments involved incorporation of a diol on the ODN, necessitating oxidation with sodium periodate to the corresponding aldehyde before coupling. This relatively harsh oxidation step would deem this approach incompatible with the use of certain functionalities, particularly sulfur derivatives, in the case where we want to have a bifunctionalised strand. One of the visions of this project was to create sensitive molecular beacon systems, incorporating a luminescent molecule by oxime chemistry on one end of the ODN, whilst introducing functionality to attach the other end to a metal nanoparticle. The sufficient reactivity of the benzaldehyde for oximation and its use in the successful preparation of Ru-ODN conjugates was shown in Chapter 2. Here we show how a disulfide can be introduced on the 3' end of this strand and used for subsequent functionalisation of gold nanoparticles (Figure 4.22).



Figure 4.22 Simplified diagram illustrating the aim of this section, to functionalise gold nanoparticles with a Ru labelled ODN.

4.10.1 Preparation of a Bifunctional ODN with a 3'-Disulfide and 5'-Benzaldehyde

The ODN **37** was prepared on an ABI 3400 DNA synthesizer (Applied Biosystems), by using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 1 μ mol scale. The commercially available 1-O-Dimethoxytrityl-propyl-disulfide, 1'-succinyl-lcaa-CPG (**36**) was used to introduce the disulfide at the 3' end. The ODN was 5'-modified by incorporating the commercially available 2-[4-(5,5-Diethyl-1,3-dioxan-2-yl)phenoxy]ethan-1-yl-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (**25**) during the last step of the automated synthesis. The ODN was cleaved from the solid support and released into solution by treatment with 28% aqueous ammonia for 16 hours at 55°C (Figure 4.23). The ODN was purified by RP-HPLC.

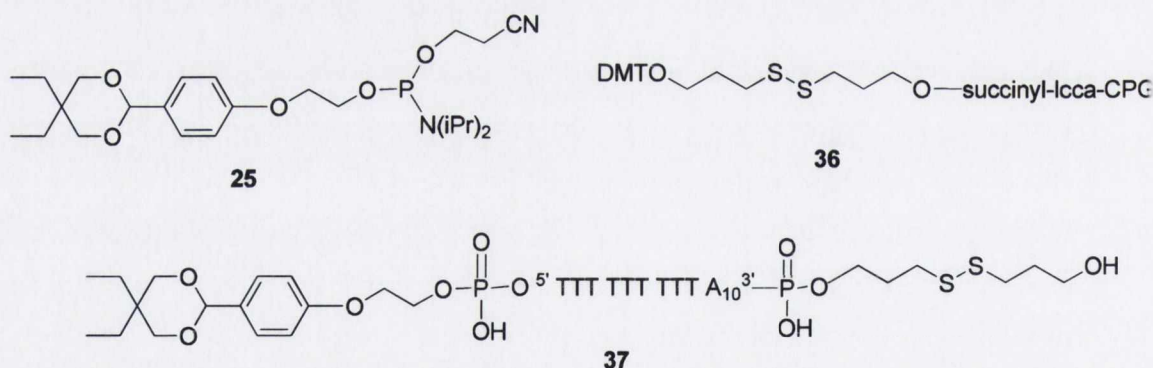
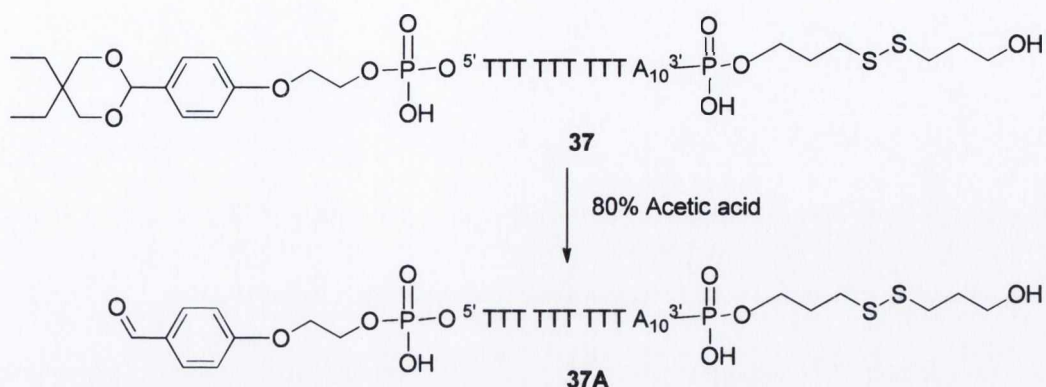


Figure 4.23 Commercially available phosphoramidite **25** and CPG support **36** for ODN modification and the sequence of the prepared ODN **37**

4.10.2 Removal of the Acetal Protecting Group

The acetal protecting group on **37** was cleaved by treatment of the strands with 80% aqueous acetic acid for 1 hour at room temperature (Scheme 4.6). The sample **37A** was dried and quantified using UV-Vis, measuring the OD at 260 nm.



Scheme 4.6 Removal of acetal protecting group on ODN **37** to give **37A**

4.10.3 Conjugation of the 5'-Benzaldehyde of **37A** with a Ru Complex

The oximation reaction was carried out as detailed previously in Chapter 2. The 5' aldehyde modified ODN **37A** and the ruthenium complex **2** (2 equivalents) were stirred in ammonium acetate buffer (0.4 M, pH 4.5) to give conjugate **37B** (Scheme 4.7). The reaction was monitored by reverse phase HPLC (Figure 4.24) with the formation of the conjugate apparent after 3 hours. The ruthenium conjugate could be affirmed by monitoring the reaction at 420 nm.

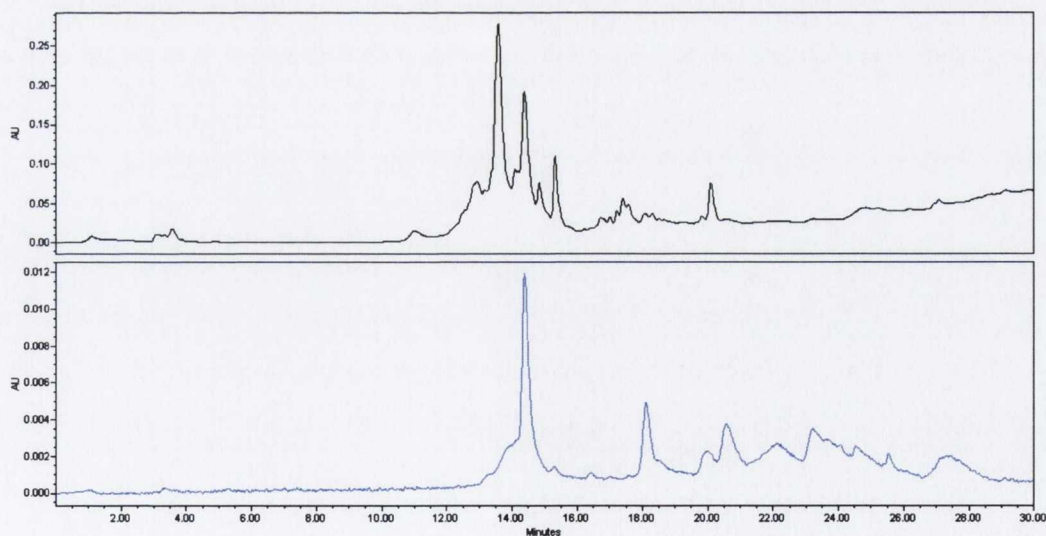
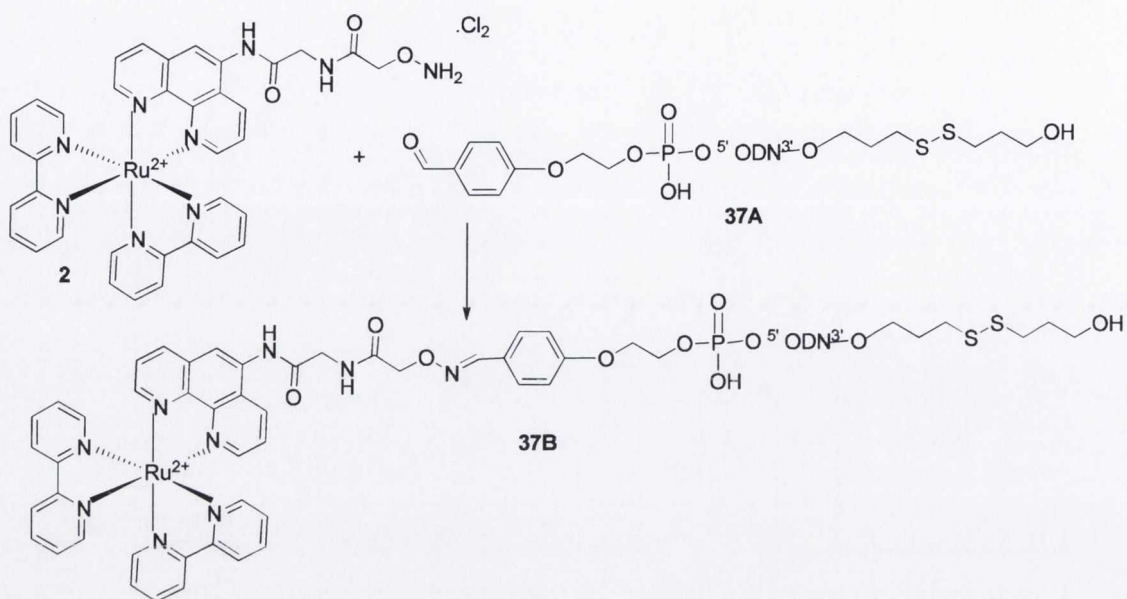


Figure 4.24 HPLC trace of crude coupling of **37B**. Top at 260 nm, Bottom at 420 nm.



Scheme 4.7 Coupling of ODN **37A** with an oxyamine modified ruthenium complex **2** to give conjugate **37B**

4.10.4 Purification and Analysis of Ru-ODN-SS

The ODN was purified using denaturing polyacrylamide gel electrophoresis. A single orange band was observed. UV shadowing was used to visualise the ODN. The ODN was excised from the gel, crushed and collected by elution in water. A yield of 14% was determined using UV-Vis. The sequence was confirmed by MALDI-TOF mass spectrometry: Calculated 7000.1 Da, Found 6999.3 Da.

The UV-Vis spectrum (Figure 4.25a) illustrated the successful coupling with both a band for the ODN (260 nm) and the MLCT band of the Ru complex (450 nm) observed. The Ru complex still showed its characteristic emission on conjugation, exciting through the MLCT band at 450 nm, gave an emission centred at 600 nm (Figure 4.25b). No degradation of the ruthenium complex was observed during the coupling reaction or the subsequent purification.

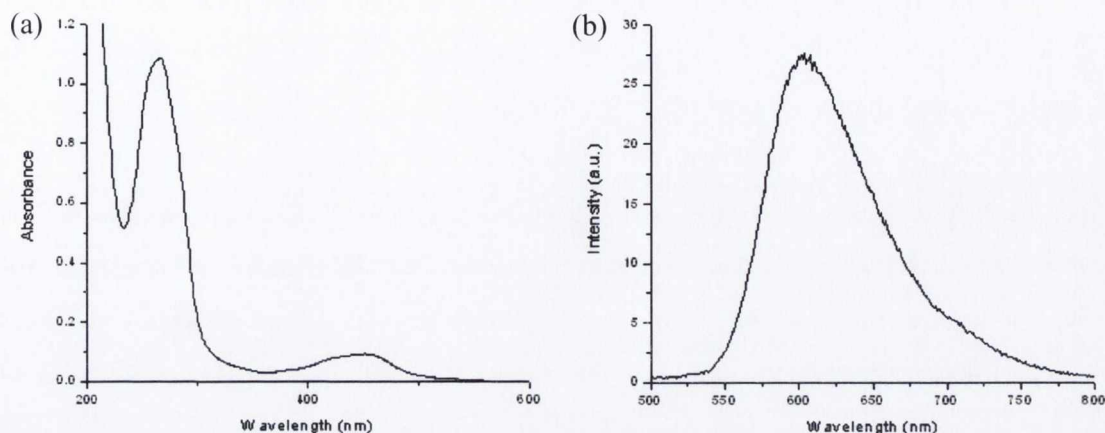


Figure 4.25 a) UV-Vis spectrum of **37B** (5.8 μM) and b) Emission spectrum ($\lambda_{\text{ex}} = 450$ nm) of **37B** (2.9 μM) in H_2O at room temperature.

4.10.5 Investigation of Effect of Gold Nanoparticles on the Emission of $\text{Ru}(\text{bpy})_3$

Before functionalising the spherical gold nanoparticles with the Ru-ODN-SS **37B**, it was decided to investigate the quenching effect of the 13 nm spherical gold nanoparticles on the luminescence of ruthenium tris(bipyridine) (Figure 4.26).



Figure 4.26 Diagram depicting the adsorption of ruthenium complexes on the gold nanoparticle surface.

Reports in the literature have shown that the emission of ruthenium tris(bipyridine) can be quenched effectively by gold nanoparticles. The earlier reports looked at the interaction of small mercapto capped gold nanoparticles (1-2 nm).⁸⁷ In a report by Glomm *et al.*⁸⁸ on the quenching of both mercaptooctanoic acid capped particles and citrate stabilised gold nanoparticles (10 nm), the citrate stabilised particles were observed to flocculate in the presence of relatively low concentrations of the ruthenium complex.

An experiment analysing the quenching effect of the 13 nm citrate stabilised gold nanoparticles was devised. Flocculation of the particles was evidenced at very low concentrations of the ruthenium complex. Measurements were carried out to assess the quenching ability of the particles. Due to the flocculation, we were limited by the number of measurements that could be achieved. Ruthenium tris(bipyridine) with a final concentration of 1×10^{-7} M was added to solutions of gold nanoparticles with concentrations of 4×10^{-9} M (25:1, Ru:particle) and 2×10^{-9} M (50:1). The samples were equilibrated for an hour. UV-Vis (Figure 4.27) showed no sign of flocculation. The emission spectrum (Figure 4.28) was obtained, exciting at 450 nm. Significant quenching was observed with less quenching observed on the addition of less gold.

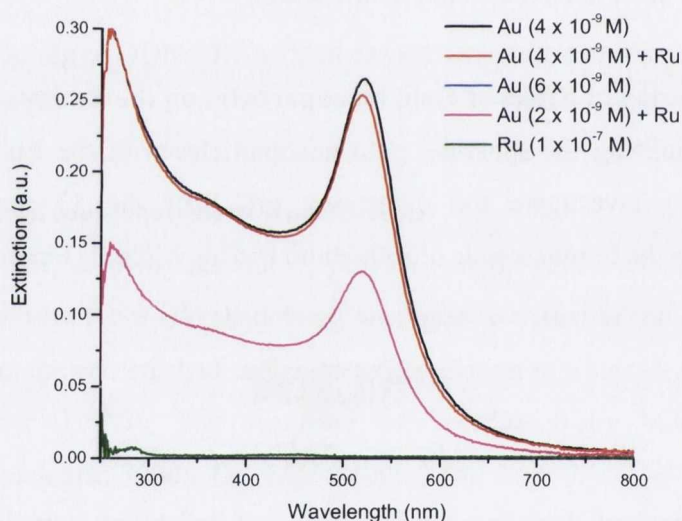


Figure 4.27 UV-Vis of 1×10^{-7} M ruthenium tris(bipyridine) in the presence of gold nanoparticles (13 nm) with final nanoparticle concentrations of 4×10^{-9} M and 2×10^{-9} M. No flocculation was observed.

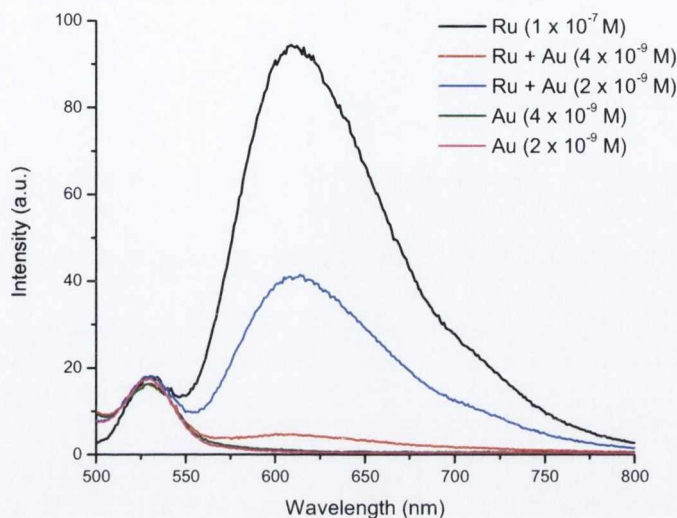
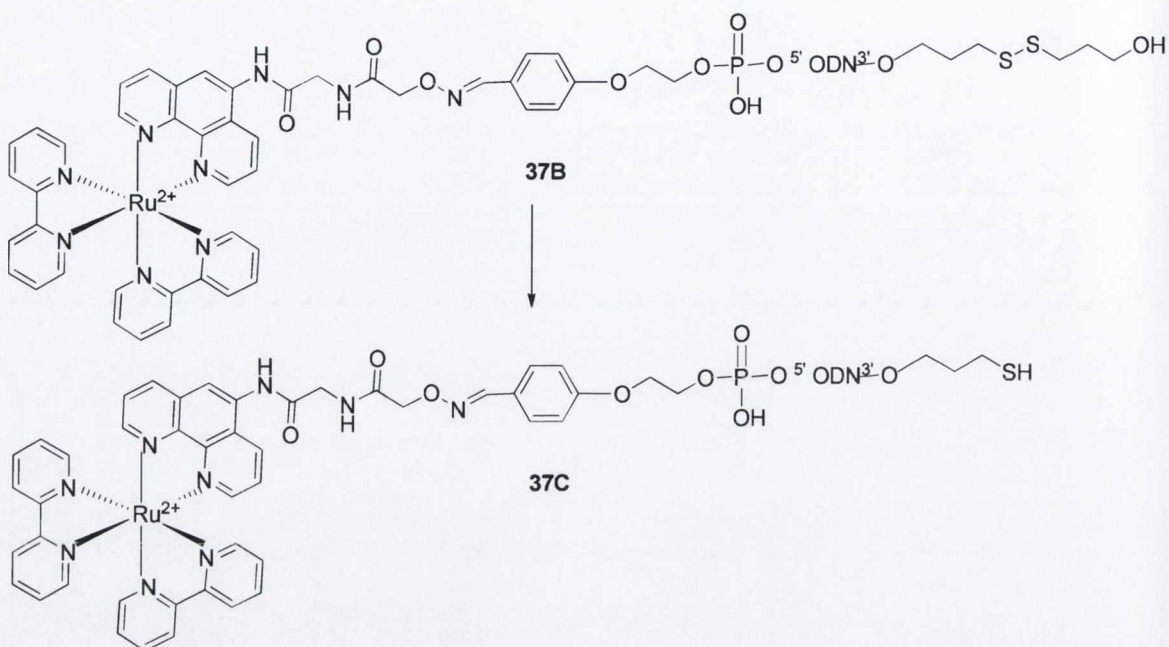


Figure 4.28 Emission spectrum of 1×10^{-7} M ruthenium tris(bipyridine) ($\lambda_{\text{ex}} = 450$ nm) in the presence of gold nanoparticles with final nanoparticle concentrations of 4×10^{-9} M and 2×10^{-9} M. The band at 530 nm is the Raman band of water. Slits of 20 nm.

4.10.6 Conjugation of Ru-ODN with Spherical Gold Nanoparticles

The amount of ODN **37B** needed was determined as before in Section 4.9.5 using the reported value of 35 pmol/cm^2 .⁴¹ The disulfide was reduced using dithiothreitol (0.1 M) to the corresponding thiol **37C** (Scheme 4.8).



Scheme 4.8 Reduction of disulfide of **37B** to the corresponding thiol **37C** using dithiothreitol

The DTT was removed by NAP column with water as eluent and the purified ODN added directly to the citrate stabilised gold nanoparticles (0.5 ml, 12.4 nM). The sample was agitated overnight on an orbital shaker. Sodium phosphate (100 mM, pH 7.4) and SDS (1%, w/v) were added to give final concentrations of 10 mM and 0.01% respectively. Aliquots of NaCl (2 M) were added, increasing the salt concentration by 0.05 M increments over 48 hours, until a final concentration of 0.3 M was achieved.

This was the first evidence for successful conjugation as the ‘as-prepared’ citrate stabilised particles are observed to aggregate as illustrated earlier in the Chapter (Figure 4.13).

Once the required salt concentration was reached, the particles were left to equilibrate overnight. The excess ODN was removed by multiple centrifugation steps. Three successive washings were used. The particles were finally redispersed in 0.3 M NaCl, 10 mM sodium phosphate, 0.01% SDS and characterised and quantified by UV-Vis. A slight broadening and a red shift of 5 nm, from 519 nm to 524 nm, were observed

(Figure 4.29a). Transmission electron microscopy (Figure 4.29b) showed that the nanoparticle morphology was unaffected by conjugation and that no aggregation had occurred.

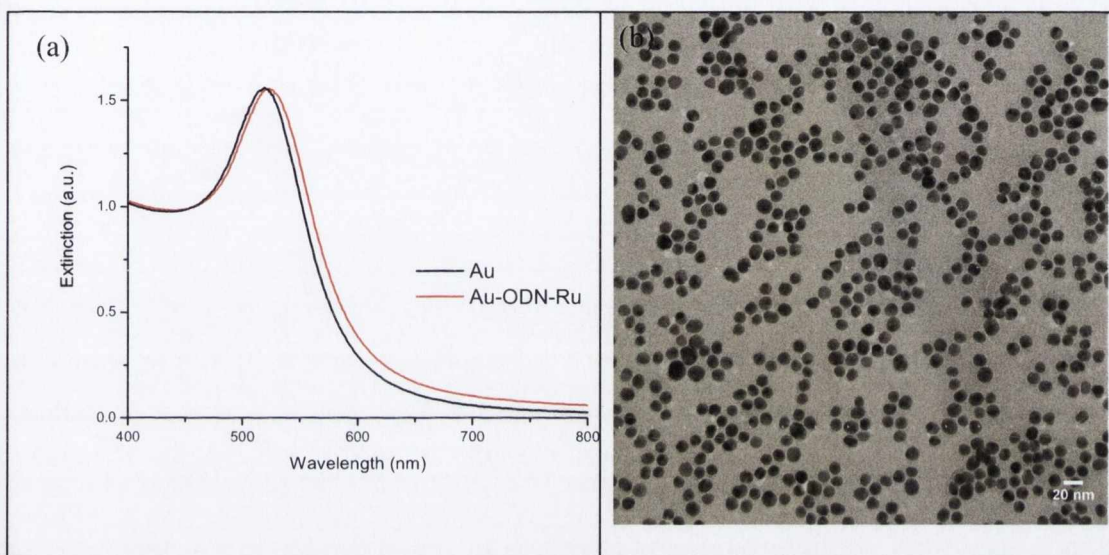
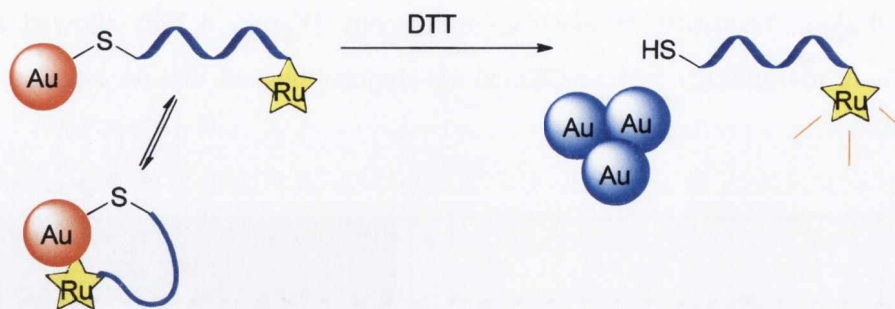


Figure 4.29 (a) Normalised UV-Vis of gold nanoparticles before conjugation (black line) and after conjugation (red line). A red shift of 5 nm was observed, from 519 nm to 524 nm. (b) TEM image of the functionalised particles. Average particle size of 13 nm.

4.10.7 Analysis of the Ru-ODN-Au Conjugate

The sample was analysed by photoluminescence (Figure 4.30) but no definitive signal for the Ru emission was observed. It was considered that the Ru complex could be sufficiently close to the nanoparticle surface to be quenched. The persistence length of the fully outstretched ODN including the A_{10} spacer segment is 6.46 nm. The flexibility of the single stranded ODN and the attraction of the Ru complex for the nanoparticle surface could bring the Ru close to the nanoparticle surface (Scheme 4.9).



Scheme 4.9 Simplified schematic depicting the release of the ODN from the nanoparticle surface on addition of dithiothreitol.

Dithiothreitol (DTT) was added to the sample to displace the ODN from the nanoparticle surface and the emission was monitored with time. A defined emission band at ~ 610 nm was observed to appear immediately and was observed to plateau within 30 minutes (Figure 4.30).

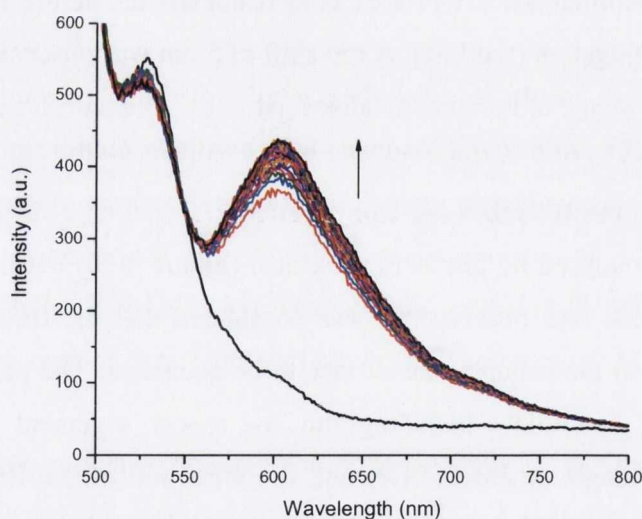


Figure 4.30 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450$ nm) of **37C** Ru-ODN-Au conjugate (black line) and after addition of DTT (coloured lines). An increase in the luminescence was observed over 30 mins. The band at 530 nm is the raman band of water. Slits of 20 nm and a high PMT voltage were used.

This is direct evidence for the successful conjugation of the ODN on the nanoparticle surface, with luminescence of the ruthenium complex being quenched by the close proximity to the nanoparticle surface.

Single photon counting (SPC) lifetime analysis was also carried out on the Ru ODN nanoparticle conjugate before and after addition of DTT (Figure 4.31). No detectable decay profile was observed for the conjugate. It could be concluded that the ruthenium emission was being quenched on the nanoparticle surface. The sample was analysed in aerated solution after addition of DTT and a decay profile obtained. The decay profile was fitted to monoexponential kinetics. A lifetime of 499 ns was obtained.

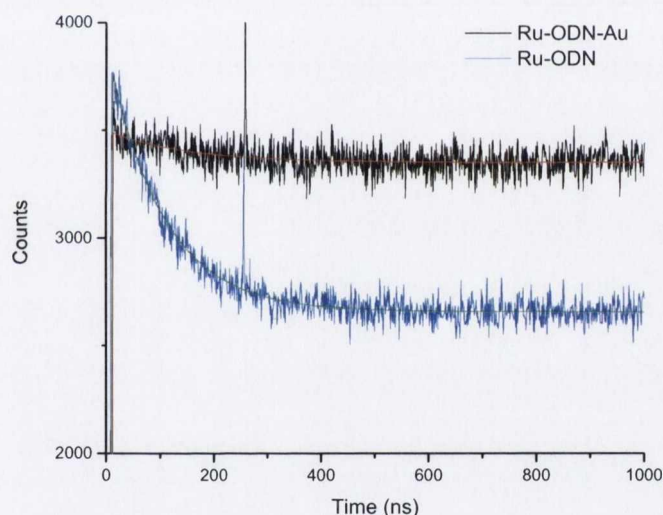
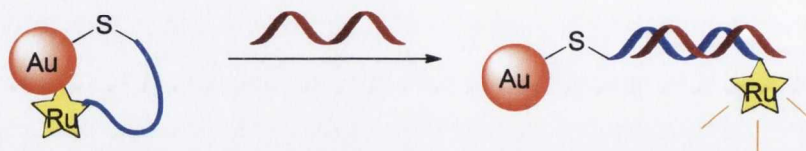


Figure 4.31 Single photo counting lifetime analysis of the Ru-ODN-Au conjugate before (black trace) and after (blue trace) addition of DTT. The straight lines represent the data fitted to a single exponential decay. (Note the background is due to the long (18 hour) acquisition time)

The conformation of the ODN on the nanoparticle surface could have a role to play in the observed quenching as both the ruthenium complex and the nucleobases have an affinity for the nanoparticle surface and the single-stranded DNA segment is relatively flexible. It has been shown that nanoparticle-ODN-dye conjugates can act as molecular beacons without the need for a hairpin segment.⁸⁰

A complementary ODN sequence was added to a Ru-ODN-Au sample to see if an increase in the emission could be observed upon hybridisation (Scheme 4.10).



Scheme 4.10 Simplified schematic showing the switching on of the Ru luminescence on hybridisation and formation of the DNA duplex

The emission was observed to switch on upon hybridisation with the complementary ODN (Figure 4.32). While this might suggest the success of this reaction, further investigation showed this not to be the case.

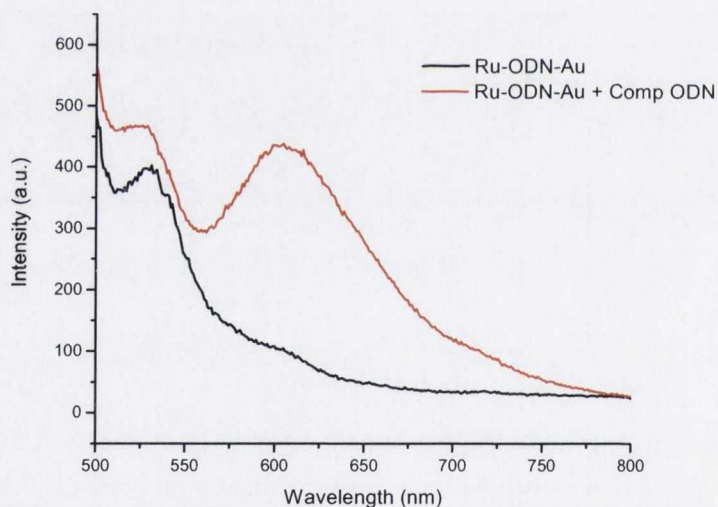


Figure 4.32 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450 \text{ nm}$) of **37C** gold nanoparticle conjugate before (black line) and after (red line) addition of the complementary ODN.

The sample was observed to have altered colour. The emission from the sample was further investigated. It was found that the ODN had been displaced from the nanoparticle surface. The sample was centrifuged and the emission from the supernatant and the redispersed pellet analysed (Figure 4.33).

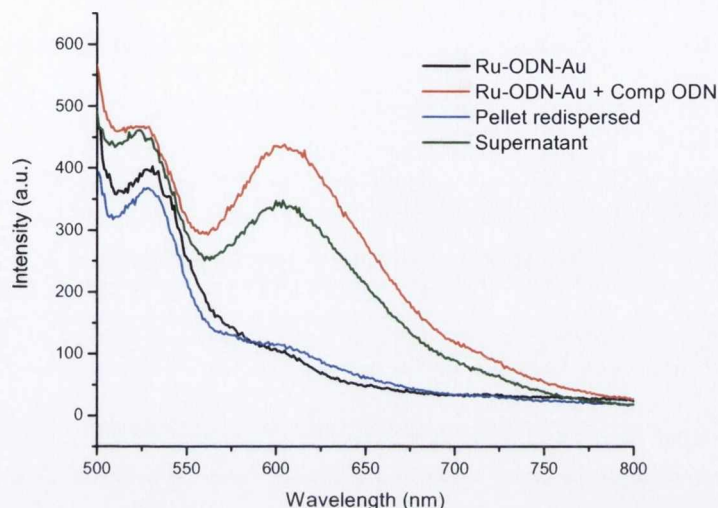


Figure 4.33 Photoluminescence spectrum ($\lambda_{\text{ex}} = 450 \text{ nm}$) of **37C** gold nanoparticle conjugate before (black line) and after (red line) addition of the complementary ODN. The sample was centrifuged and the supernatant (green line) and pellet (blue line) analysed.

4.10.8 Discussion of the Ru-ODN-Au Conjugate Section

A bifunctionalised ODN was successfully prepared, modified with a 5'-Ru complex and a 3'-disulfide using oximation. The ODN was purified and used to functionalise spherical gold nanoparticles. Analysis of the photoluminescence of the sample, showed no signal for the Ru emission, but after removing the ODN from the surface of the nanoparticle using DTT, an emission at 610 nm was observed. This is not surprising, the flexibility of the single-stranded ODN could leave the Ru in close proximity to the gold surface, resulting in quenching. Lifetime analysis of the sample before and after addition of DTT was also indicative that the Ru emission was quenched by the gold nanoparticle. A separate conjugate sample was treated with a complementary ODN. Double-stranded DNA is rigid, in comparison with single-stranded DNA, and it was thought that on hybridising with a complementary sequence, that a signal for the emission would be observed. A switching on of the emission was observed after addition of the complementary ODN, but on further analysis the Ru-ODN was found to have been displaced from the surface. A colour change in the nanoparticle sample, to a pale purple, was indicative that the sample had irreversibly aggregated after becoming

unstable. The ODN sequence Ru-T₉A₁₀ has the ability to hairpin. Hairpinned DNA segments do not pack as readily and tightly on nanoparticle surfaces and on hybridising can leave the nanoparticle surface exposed to the high salt concentration of the buffer.⁸⁹ Further analysis using a non-hairpinning ODN would be necessary to confirm this. The use of short ODN helper segments or MCH could also prove useful in optimising this conjugate system, to ensure a stable working system is achieved.

4.11 AuAg Nanobox ODN Conjugates

There have been very few reports in the literature on the functionalisation of anisotropic nanoparticles with ODNs. Mirkin has reported the preparation of triangular gold nanoplate ODN conjugates,²³ and during the course of writing this thesis, a publication documenting the functionalisation of an array of different shaped gold nanoparticles was published.⁶² Another recent publication, reports the preparation of ODN conjugates with triangular silver nanoplates.⁷⁰ It is important to note that etching of the triangular particles was observed, but the paper illustrated some interesting results due to the assembly of the anisotropic particles and showed the potential use of these shaped nanoparticle-ODN conjugates in the area of detection.

Anisotropic particles are more challenging to functionalise because of the reactivity of their different faces and the reduced surface packing. Many recent reports have looked at the role that curvature has to play in the surface packing on the different shaped particles.⁵⁷ Larger, nanoplate structures are more like planar plates and the packing density as such is significantly less. These shaped particles are more susceptible to irreversible aggregation.

4.11.1 Preparation of Hollow AuAg Nanoboxes

The instability of triangular silver nanoplates to halide ions was detailed in Chapter 3. A colleague, Dr. Damian Aherne, gold coated the triangular silver nanoplates and showed them to be stable to etching.⁹⁰ Leading from this work triangular AuAg nanoboxes subsequently were prepared.⁹¹ Some years ago, Mirkin⁶³ used the enhanced stability of gold coated spherical silver nanoparticles to prepare ODN conjugates with unique

optical properties and we decided to apply this approach to prepare ODN conjugates with triangular AuAg nanoboxes, taking advantage of the stability of these particles.

The AuAg nanoboxes were prepared according to the reported procedure.⁹¹ Triangular silver nanoprisms (8.175 ml) were prepared as outlined in Chapter 3, excluding the addition of trisodium citrate after preparation. An excess of ascorbic acid (825 μ l, 10 mM) was added to the silver sol and gold (III) chloride trihydrate (10 ml, 0.5 mM) was added at a rate of 1 ml/min. The particles were observed to red shift, and then to gradually blue shift, with the surface plasmon band of the final sol at a shorter wavelength than the original sol (Figure 4.34). The particles were used immediately without the addition of any stabilisers.

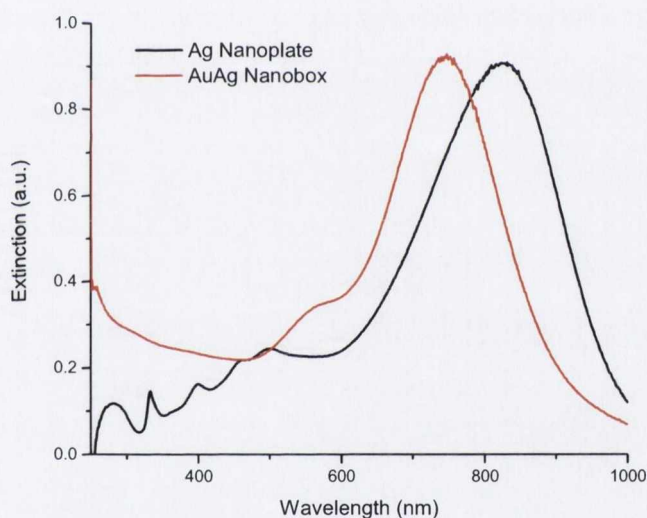


Figure 4.34 UV-Vis of triangular silver nanoplates (black line) and the corresponding AuAg nanoboxes (red line) prepared from addition of ascorbic acid (825 μ l, 10 mM) and HAuCl_4 (10 ml, 0.5 mM).

Transmission electron microscopy analysis was carried out to characterise and determine the size of the prepared nanoboxes (Figure 4.35). Triangular nanoboxes with an edge length of 90 nm were obtained.

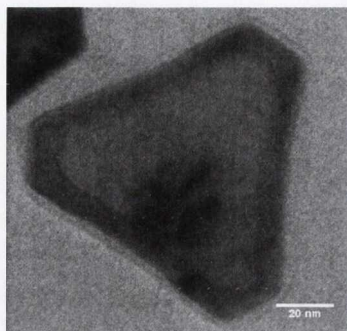


Figure 4.35 TEM image of triangular silver nanobox

4.11.2 Amount of ODN Needed

An estimate of the amount of ODN required was made based on the assumption that a monodisperse nanoparticle sol of triangular silver nanoplates are obtained and that these are then converted to nanoboxes with dimensions of 90 nm. It has been observed by Mirkin that a higher than expected loading of ODNs was obtained on triangular gold nanoplates due to the curvature at the sides.²³ An ODN surface footprint of 15 nm² was used for the calculation.

4.11.3 Functionalisation of AuAg Nanoboxes with 5'-Thiol Modified ODNs

The trityl group of ODNs **35A** and **35B** were cleaved using silver nitrate to give **35C** and **35D** as previously detailed (Section 4.9.4). The DTT was removed using a NAP-5 column and the ODN (excess) was added to the AuAg Nanobox sol (3 ml). The samples were agitated overnight on an orbital shaker. Sodium phosphate (100 mM) and SDS (1%) were added to give final concentrations of 10 mM and 0.01% respectively. The sample was agitated for 30 minutes and NaCl (2 M) was added gradually over time, incrementally increasing the salt concentration to a final concentration of 0.1 M. Some aggregation was observed during the salt-aging of the **35C**-nanobox conjugate. This was overcome by adopting a more gradual salt-aging process. Analysis of the surface coverage of the spherical gold nanoparticle conjugates showed that a lower loading was achieved with the **35C** sequence so this helps account for the difficulty in functionalisation with this particular sequence over the other. The excess unconjugated ODN was removed by centrifugation. The particles were pelleted by centrifuging at 5000 rpm for 5 minutes. The supernatant was removed and the pellet redispersed in 0.1 M NaCl, 10 mM sodium phosphate and 0.01% SDS. This was repeated three times and

the pellet was finally redispersed in a lower volume to yield a concentrated sample. The conjugates **35C-Nanobox** and **35D-Nanobox** were analysed by UV-Vis, a red shift of 18 nm, from 744 nm to 762 nm, relative to the original sol being observed (Figure 4.36).

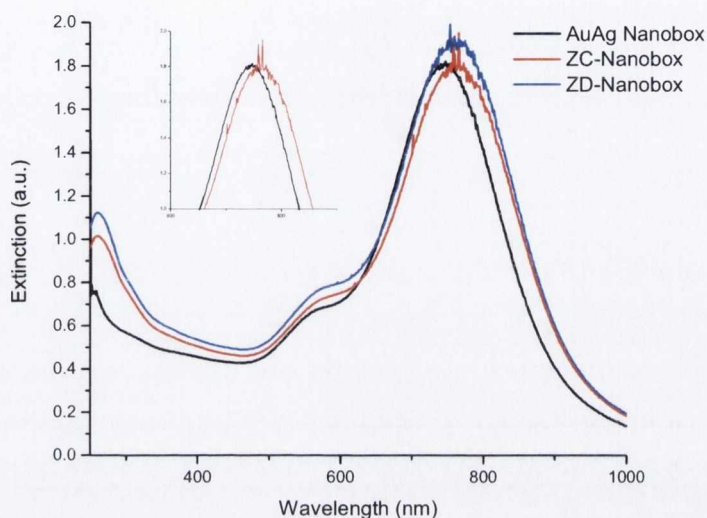


Figure 4.36 UV-Vis of the nanoboxes before and after conjugation with the ODNs. A red shift of 18 nm, from 744 nm to 762 nm, was observed on functionalisation

The nanobox ODN conjugates were analysed by dynamic light scattering (Figure 4.37). An increase in the size was observed. The larger peak changed from 67 nm to 87 nm and the smaller peak from 7 nm to 15 nm. An increase in the size of the particles was observed on conjugation. Two peaks were observed because of the shape of the particles. This was investigated previously by a scientist in Malvern Instruments with our triangular silver nanoplates and the smaller peak was attributed to the rotational diffusion of the non-spherical particle.

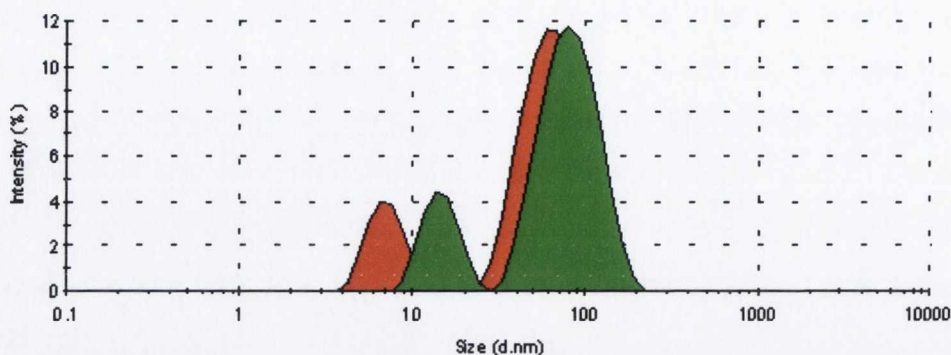
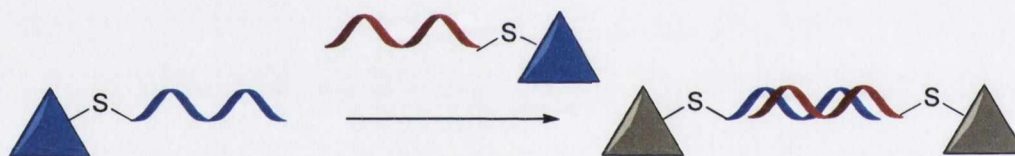


Figure 4.37 DLS measurement of AuAg nanobox before (red) and after (green) functionalisation with ODN 35C.

4.11.4 Hybridisation of Two Complementary Nanobox Conjugates

The samples were quantified by UV-Vis and equimolar amounts of conjugates **35C-Nanobox** and **35D-Nanobox**, based on the OD, were mixed (Scheme 4.11).



Scheme 4.11 Schematic representation for the assembly formed from the mixing of two complementary ODN conjugates.

A colour change from dark blue/grey to light grey was observed on hybridisation of the complementary conjugates (Figure 4.38a). The aggregate was allowed to settle overnight. The UV-Vis/NIR spectrum showed a dampening and a red shift on hybridisation (Figure 4.38b).

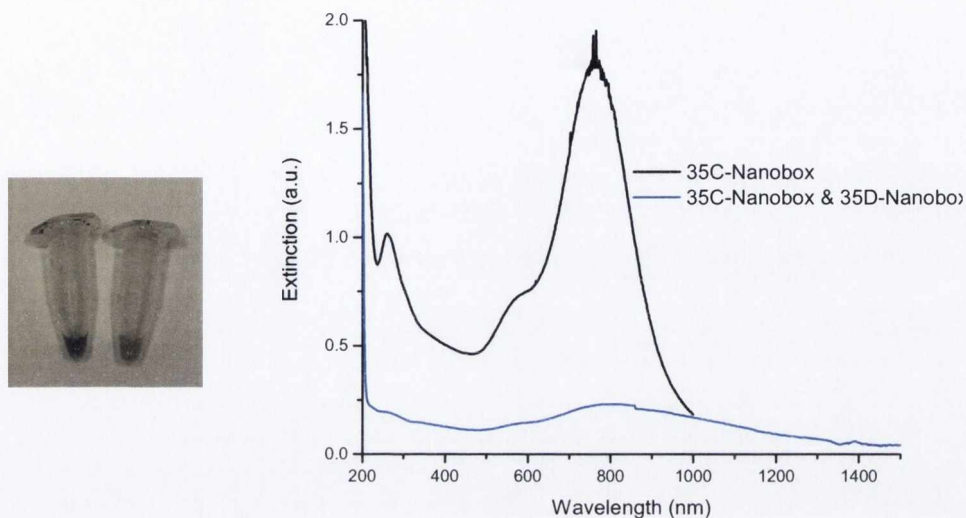


Figure 4.38 (a) Photograph of the nanoboxes before (left) and after (right) hybridisation. (b) UV-Vis/NIR spectrum of nanobox-ODN conjugate and the hybridised nanobox conjugates.

4.11.5 Analysis of the Thermal Denaturation of the Nanobox ODN Assembly

The sample was diluted in 0.3 M PBS buffer, the pellet was redispersed by agitation and the absorbance at 760 nm of the hybridised conjugate was measured as a function of temperature (Figure 4.39). The melting temperature (T_m) was determined to be 37 °C. A control sample of citrate stabilised AuAg nanoboxes were monitored with temperature. No phase transition was observed. The increase in extinction, observed at the LSPR band of the particles, indicates that the transition is the result of a dehybridisation with the recovery of the initial spectrum of the particles.

It is worth mentioning that an experiment in 0.1 M PBS was initially attempted, but no hybridisation was evidenced. This is not surprising as one can assume that the T_m would be lower and closer to room temperature than that depicted in Figure 4.40.

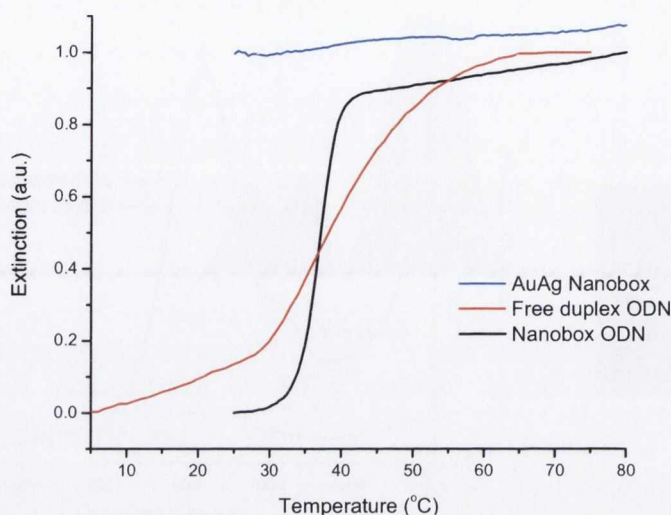


Figure 4.39 Thermal melting curve for the hybridised conjugates **35C-Nanobox** and **35D-Nanobox** (0.3 M PBS, monitored at 760 nm) and the free duplex ODN (8 μ M, monitored at 260 nm). The citrate stabilised particles (blue line) show no behaviour.

The reproducibility of the hybridisation process was tested by repeatedly performing melting analysis on the same sample. A similar profile was obtained with each cycle for the repeated hybridisation/dehybridisation events.

The dehybridisation was also monitored by DLS as a function of temperature (Figure 4.39). The particle size was seen to decrease with temperature. A citrate stabilised nanobox system was used as a control.

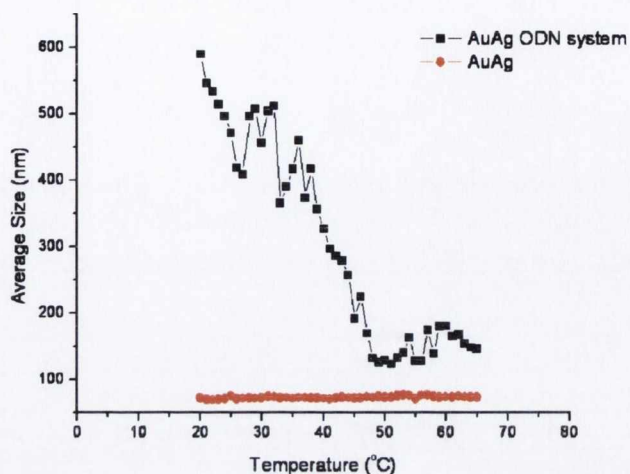


Figure 4.40 Graph showing the particle size of the nanobox assembly as a function of temperature. Data shown is an average of three measurements.

4.11.6 TEM Analysis of the Nanobox ODN Assembly

TEM analysis of a hybridised sample of **35C-Nanobox** and **35D-Nanobox** showed the assembled aggregation of the nanoboxes (Figure 4.41a). A control of the **35C-Nanobox** sample in the same concentration was dropped and analysed. The particles were observed to be well separated and this excluded the possibility that the aggregation was due to the TEM sample preparation (Figure 4.41b)

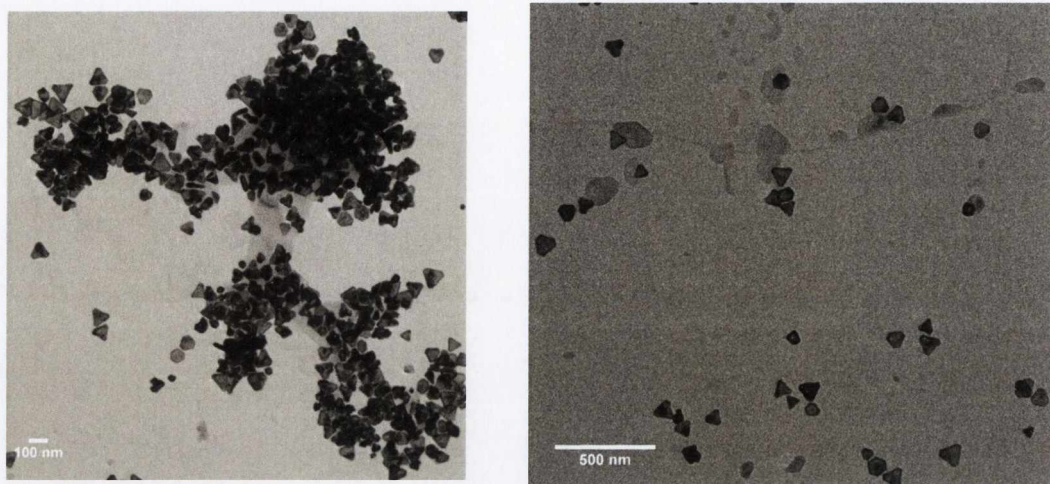


Figure 4.41 (a) TEM image of the assembly formed on hybridisation of **35C-Nanobox** and **35D-Nanobox** in 0.3 M PBS (b) TEM image of **35C-Nanobox**, no large aggregates were observed.

4.11.7 Effect of Increasing Salt Concentration on the Nanobox ODN Assembly

An increase in the salt concentration resulted in an increase in the observed melting temperature for the aggregate system, as previously observed for gold nanoparticle ODN systems (Figure 4.42 and 4.43).

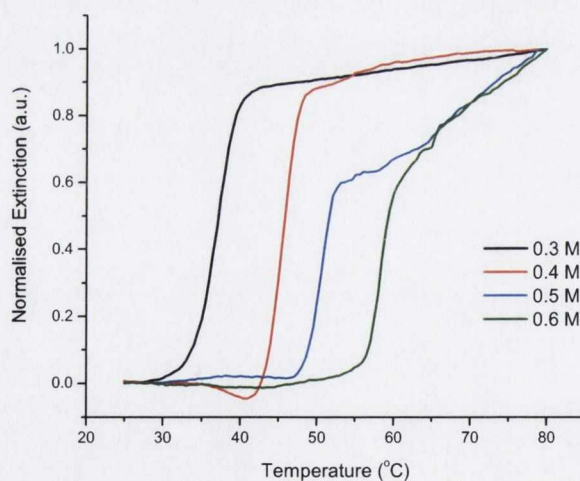


Figure 4.42 The effect of salt concentration on the melting of nanobox aggregates. Sample in different concentrations of PBS monitored at 760 nm.

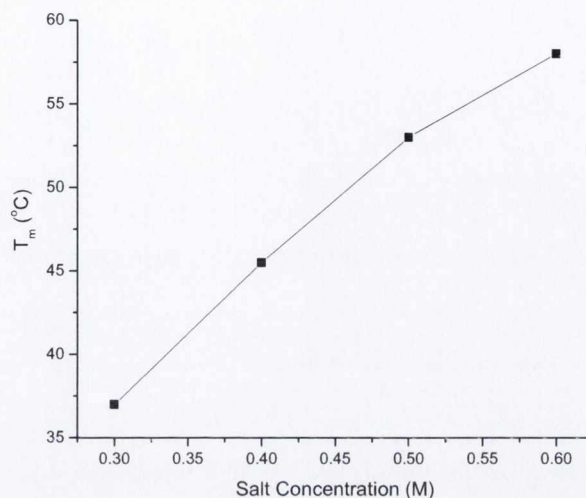


Figure 4.43 Plot of T_m for hybridised nanoboxes as a function of salt concentration

During the course of these experiments, it was observed in the majority of instances, that rather than plateauing after the sharp melting transition that an increase in the extinction was observed as the sample was heated. We wanted to further investigate this to try and decide if the phenomenon was due to the shape and structure of the particles or if it was just an artefact from the sample. A full spectrum was obtained at 1 °C increments. The spectrum was observed to become very noisy (Figure 4.49 and 4.50, Appendix Section 4.16). The reason for this behaviour is not known.

4.11.8 Summary and Discussion of Nanobox-ODN Conjugate Assemblies

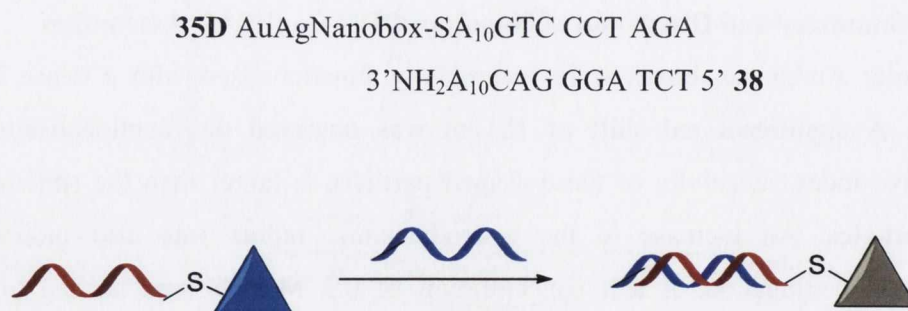
Triangular AuAg nanoboxes were successfully functionalised with a dense layer of ODNs. A significant red shift of 18 nm was observed on functionalisation. The refractive index sensitivity of these shaped particles is larger than the spherical gold nanoparticles. An increase in the hydrodynamic radius was also indicative of successful conjugation. A salt concentration of 0.3 M PBS was needed to induce hybridisation. The melting temperature of the system was seen to be similar to the free duplex (unlike the spherical gold system) but significantly sharper (similar to the spherical gold system). This is different to that previously observed with triangular gold nanoplates, where a significantly higher melting temperature was obtained compared to spherical particles. DLS and TEM showed the formation of smaller aggregates, for the

hybridised nanoboxes, even though the nanoboxes are substantially bigger than the spherical particles. A few possibilities could account for this observation. The ODN packing on the different facets of the nanoboxes could be different. Mirkin has previously shown the ODN to have a facet selectivity.²³ If the basal plane is not densely functionalised then the ODN conformation could be such that the ODNs are not readily available for hybridisation. The side facets have a higher degree of curvature and are considered to be more reactive, and thus are more likely to be densely functionalised and the hybridisation is more likely to occur.

4.12 Non-Crosslinking Melting of AuAg Nanobox-ODN Conjugate

Reports by Sato *et al.* have shown that if the salt concentration is greater than 0.5 M, addition of the complementary sequence to the gold nanoparticle ODN conjugate results in aggregation and this aggregation can be visualised as a sharp melting transition and has been shown to be useful in the detection of terminal mismatches.²⁴⁻²⁵ Graham has also carried out a non-crosslinking experiment with spherical silver nanoparticle ODN conjugates.⁷

An aliquot of **35D-nanobox** in 0.6 M PBS was treated with ODN **38** (Scheme 4.12). The sample was observed to aggregate (Figure 4.44)



Scheme 4.12 Diagram depicting the non-crosslinking experiment on **35D-nanobox**

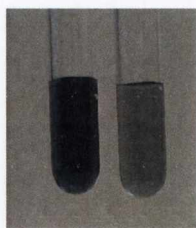


Figure 4.44 Photograph of **35D-Nanobox** with (right) and without (left) **ODN 38**

The sample was allowed to aggregate completely. The aggregate was resuspended in 0.6 M PBS buffer. The sample was monitored at 760 nm as a function of temperature (Figure 4.45). A sharp melting transition was observed with a T_m of 41 °C.

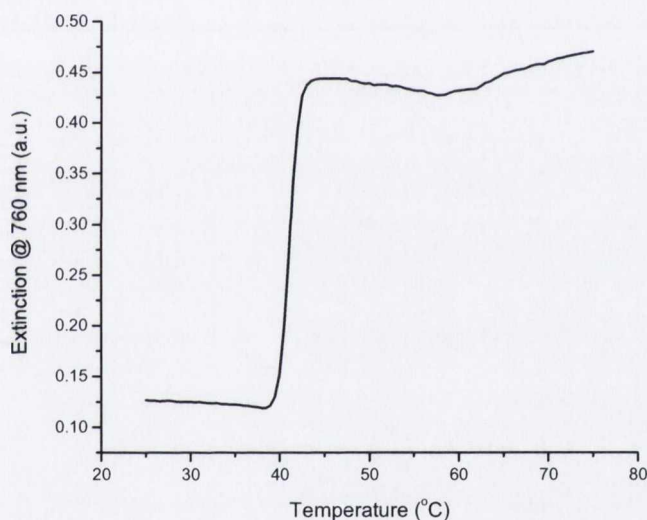
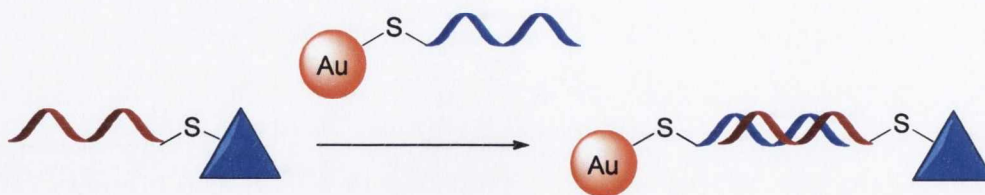


Figure 4.45 Melting curve obtained for **35D-nanobox** and **ODN 38** in 0.6 M PBS. The absorbance was monitored at 760 nm.

4.13 Assembly of Spherical Gold Nanoparticles with AuAg Nanoboxes

The use of ODN modified nanoparticles in the area of assembly has been alluded to in the introductory chapter of this thesis. Here we investigated the assembly of large AuAg nanoboxes functionalised with ODNs (Section 4.11.3) with smaller spherical gold nanoparticles functionalised with the complementary ODN sequence (Section 4.9.6) as depicted in Scheme 4.13.



Scheme 4.13 Simplified schematic for the assembly of **35C-Nanobox** and **35D-Au**

4.13.1 Preparation and Analysis of Nanobox-ODN and Au-ODN Assembly

35C-Nanobox and **35D-Au** (in excess) were combined in 0.3 M PBS and allowed to hybridise. The sample was diluted with 0.3 M buffer to a volume of 1 ml for UV-Vis analysis. A dampening and a red shift were apparent for both plasmon bands, from (Figure 4.46). A control sample was prepared by mixing two non-complementary strands, **35C-Nanobox** and **35C-Au**. No dampening or red shift was apparent for this system. The plasmon bands of both the spherical gold nanoparticles and the AuAg nanoboxes remained unchanged. Addition of the ‘as-prepared’ citrate stabilised gold to **35C-Nanobox** in 0.3 M PBS unsurprisingly resulted in the observation of an aggregate band, due to the aggregation of the spherical gold nanoparticles, but the surface plasmon band could still be observed in its original position, with a λ_{max} of 760 nm, for the AuAg nanoboxes.

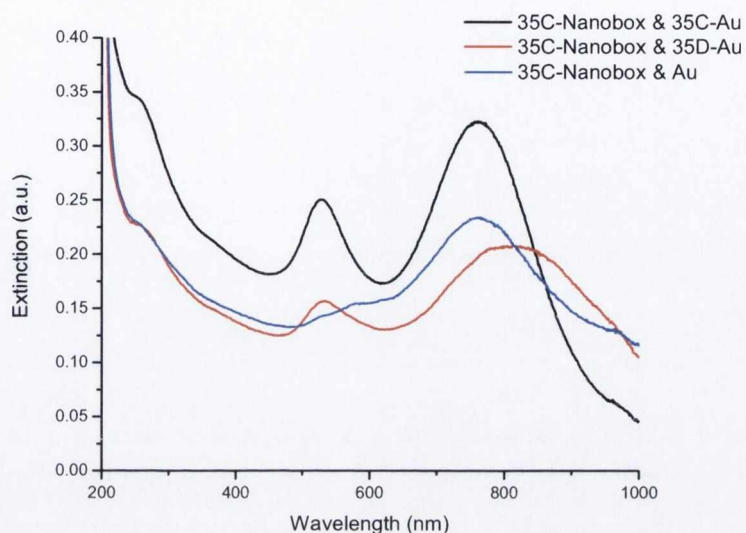


Figure 4.46 UV-Vis spectra of the assembly of **35C-Nanobox** and **35D-Au** in 0.3 M PBS (red) and control samples, **35C-Nanobox** and **35C-Au** (black) and **35C-Nanobox** and citrate stabilised gold (blue).

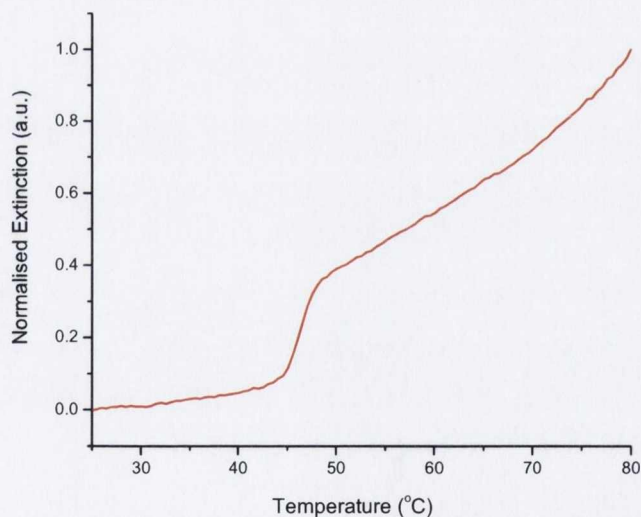


Figure 4.47 Normalised melting curve obtained for the assembly of **35C-nanobox** and **35D-Au** in 0.3 M PBS, monitored at 760 nm.

4.13.2 TEM Analysis

The sample was prepared as detailed above in Section 4.13.1 and a 10 μl aliquot was dropped on a TEM grid after 3 hours. A variety of assemblies were observed (Figure 4.48).

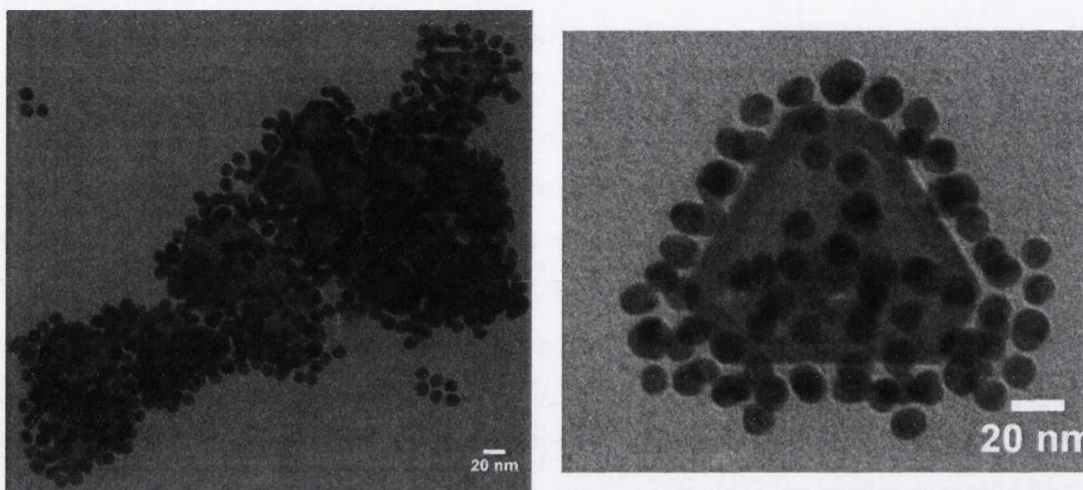


Figure 4.48 TEM images of the assembly of **35C-Nanobox** and **35D-Au** in 0.3 M PBS.

4.13.3 Discussion of Assembly of Nanobox-ODN and Au-ODN

From the TEM images it would appear that hybridisation occurs more readily on the side faces. There is a possibility that these faces are more densely functionalised due to the higher reactivity of these faces over the $\{111\}$ face. Curvature has been shown to have an affect on the particle loading so this could also account for this. It would appear that there is less ODN free for hybridisation on the top flat face of the particles. The loading on planar surfaces has been shown to be reduced because of the repulsion of the negative backbones of the ODNs. The ODN on the $\{111\}$ face may be orientated in a way that is not accessible for hybridisation. These observations could also help account and support the lower T_m obtained for the nanobox nanobox assembly system.

4.14 Overall Summary and Conclusions

Au-ODN Conjugates

Spherical gold nanoparticles were successfully loaded with a dense packing of thiol modified ODNs. The particles were shown to be stable in salt solutions and the mixing of two complementary nanoparticle conjugates resulted in a red to purple colour change due to the assembly of the nanoparticles. A sharp melting transition was observed for these particles monitoring at both 260 nm and at 525 nm. The T_m was found to be higher than that for the free duplex. This result confirmed the successful conjugation and supported previous reports in the literature. The hybridisation of the nanoparticle conjugates was further affirmed by DLS measurements. The denaturation was monitored by light scattering, the particle size was shown to decrease with increasing temperature. The number of ODN strands on each nanoparticle was determined by an Oligreen fluorescent assay. A difference was observed for the individual sequences.

Quenching of Ru Emission by Au and Preparation of Ru-ODN-Au Conjugate

Citrate stabilised gold nanoparticles were shown to effectively quench the emission of ruthenium (trisbipyridine). A bifunctionalised ODN for the labelling of metal nanoparticles with a ruthenium complex was successfully prepared. The compatibility of oxime chemistry (via the benzaldehyde approach) with sulfur chemistry was shown. The strand was effectively conjugated with spherical gold nanoparticles. Significantly the ruthenium complex was shown to withstand the reduction of the disulfide with DTT. The emission of the ruthenium was shown to be quenched. The successful conjugation was confirmed by monitoring the emission as a function of time after displacing the ODNs with dithiothreitol. Unfortunately the addition of the complementary ODN resulted in the destabilisation of the nanoparticles and the ODN was observed to detach from the nanoparticle surface. The conjugation strategy needs optimisation but the preparation of a bifunctionalised strand is an exciting result that could be used to prepare a wide variety of labelled conjugates with metal nanoparticles. By varying the length of the ODN, control over the distance from the nanoparticle surface is possible.

Triangular AuAg Nanobox- ODN Conjugates

AuAg nanoboxes were prepared from triangular silver nanoplates following a reported procedure. The nanoboxes were successfully functionalised with ODNs. A slower salt-aging process had to be adopted than for the spherical particles in particular for sequence **35C** which correlated with the reduced loading observed for this sequence determined by the Oligreen assay. The salt stability of the particles was the first evidence for successful functionalisation. A red shift of 18 nm was observed in the main surface plasmon band. Interestingly no hybridisation was observed at 0.1 M NaCl but on increasing the salt concentration to 0.3 M a colour change from blue to grey was observed. A sharp melting transition was observed when the absorbance at 760 nm was monitored as a function of temperature. The melting temperature for this system was shown to be very close to that for the free duplex. This is different to that previously reported for triangular gold nanoplates and may have something to do with the degree of ODN packing on the different facets. There have been very few reports in the literature on the functionalisation of anisotropic particles and to date there are no reports of ODN functionalised nanoboxes.

The non-crosslinked aggregation of these nanobox particles was observed on addition of the complementary strand under high salt conditions. A colour change from blue to grey occurred and a sharp melting transition was observed by monitoring the absorbance at 760 nm as a function of temperature. To the best of our knowledge this is the first report of this non-crosslinking aggregation on an anisotropic particle with previous reports pertaining to spherical gold and silver nanoparticles.

Assembly of Au-ODN and Triangular AuAg Nanobox-ODN Conjugates

On combining complementary ODN functionalised nanoboxes and spherical gold nanoparticles, aggregated assemblies were observed by TEM. Interestingly, the spherical nanoparticles appeared to assemble more readily on the side facets of the AuAg nanoboxes. This would suggest that the DNA loading is greater on these faces and more accessible for hybridisation and could help account for the lower melting temperature observed for the nanobox nanobox assemblies.

A number of nanoparticle ODN conjugate systems were successfully prepared and the expansion of the results outlined in this chapter should lead to some exciting discoveries with potential in the area of detection.

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4.16 Appendix

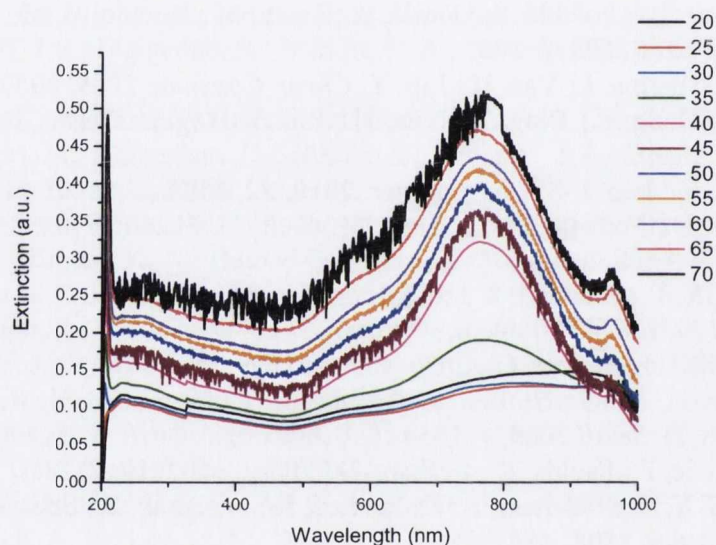


Figure 4.49 UV-Vis spectrum of nanobox-ODN dehybridisation monitored every 5 °C from 20 – 70 °C

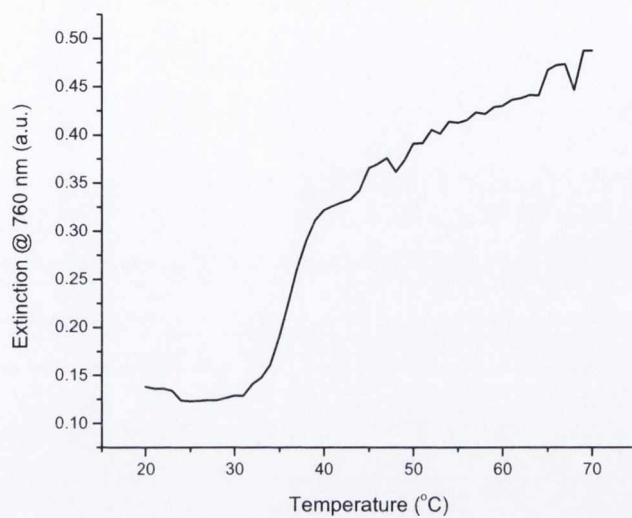


Figure 4.50 Plot of temperature vs extinction monitored at 760 nm for nanobox-ODN

Chapter 5

Modified Oligonucleotides for Nanoparticle Conjugation

5.1 Introduction

This Chapter details efforts that were made to prepare ODN conjugates with triangular silver nanoplates, taking advantage of the stability imparted by using 16-mercaptohexadecanoic acid capping and titania coating of the triangular silver nanoplates, as discussed in Chapter 3.

The aim was to prepare ODNs modified with a long sulfhydryl alkyl chain for direct conjugation with triangular silver nanoplates and catechol modified ODNs for conjugation with the Ag@TiO₂ particles described in Chapter 3.

The results detailed here include:

- An ‘on-column’ approach to the preparation of ODNs with a long sulfhydryl alkyl chain (Section 5.2)
- Preparation of catechol modified ODNs by an efficient oximation approach (Section 5.3)

As will become apparent, we were unable to bring these approaches to completion due to time constraints, although very satisfactory progress was made.

5.2 Synthesis of ODNs Modified with a Long Sulfhydryl Alkyl Chain

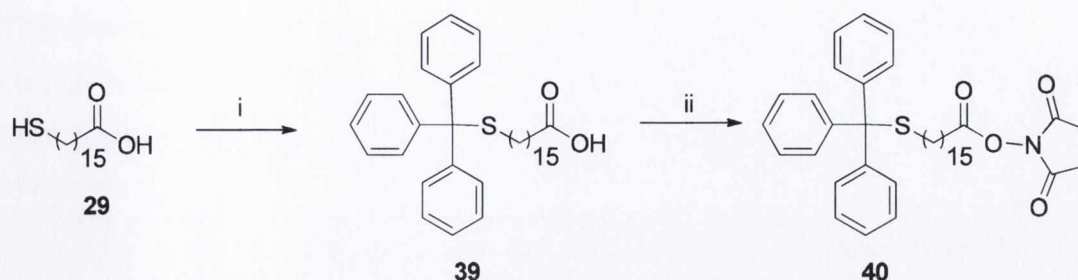
In Chapter 3, 16-mercaptohexadecanoic acid (MHA) was shown to stabilise the triangular silver nanoplates. The key success here was that the particles, were not only stabilised against etching by anions, but the introduction of a carboxylic acid functionality opened up the possibility to allow further conjugation. We wanted to take advantage of this to prepare modified ODNs with a long alkyl chain terminated with a thiol. A number of approaches were considered including both ‘on-column’ and ‘off-column’ methods. In this chapter we discuss the ‘on-column’ preparation of an ODN with a long-chain alkyl thiol modification.

5.2.1 Incorporation of a C₁₆ Sulfhydryl Unit on the 5' End of ODNs by an 'On-Column' Approach

An 'on-column' approach, coupling the NHS activated ester of a protected 16-mercaptohexadecanoic acid derivative, with 5'-amino modified ODNs was adopted. Thiols are very reactive functional groups. They undergo reaction or oxidation readily and thus need to be protected. A variety of protecting groups are reported for the protection of sulfhydryl groups.¹ Here we needed a protection strategy that was compatible with the cleavage of the ODN from the solid support, therefore stable to cleavage by base, and that could be removed easily when needed, without any damage to the ODN. A trityl group is commonly used for the protection of sulfhydryl containing phosphoramidites such as that used for the 5' modification of ODNs with thiols (Chapter 4, Section 4.9.3) and thus was the protecting group selected.

5.2.2 Protection and Activation of 16-mercaptohexadecanoic acid²⁻³

The sulfhydryl of 16-mercaptohexadecanoic acid (MHA) (**29**) was trityl protected by reacting it with trityl chloride in anhydrous DMF under nitrogen (Scheme 5.1). Chloroform was added and the organic layer washed with water. The organic layers were combined and concentrated under reduced pressure. The residue was dissolved in a minimum amount of ether, a small amount of hexane was added and the sample placed in the freezer overnight. The crystallised trityl alcohol was removed by filtration. The filtrate was evaporated under reduced pressure. A white precipitate was obtained on addition of methanol. The solid was collected by filtration, washed with cold methanol and dried. 16-tritylmercaptohexadecanoic acid (**39**) was obtained in good yield (62%). The purity was confirmed by NMR and mass spectrometry.

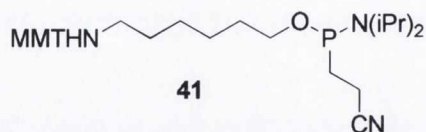


Scheme 5.1 Synthesis of 16-tritylmercaptohexadecanoic acid (**39**) and succinimidyl 16-tritylmercaptohexadecanoate (**40**). (i) Trityl chloride, DMF, N_2 , room temperature, 48 hours (ii) NHS, EDC, DMAP, DCM, room temperature, 2 hours

The acid group of **39** was converted to the corresponding NHS ester **40** by reacting it with N-hydroxysuccinimide using the coupling reagent ethyldiisopropylcarbodiimide in the presence of N,N-dimethylaminopyridine to give succinimidyl 16-tritylmercaptohexadecanoate (**40**) as a white solid in excellent yield (86%). It was characterised by NMR and mass spectrometry.

5.2.3 Preparation of 5'-Amino Modified Oligonucleotides and Choice of Sequences

A series of ODNs (**41A–41C**, Figure 5.1) were prepared by Prof. Defrancq, on an ABI 3400 DNA synthesizer (Applied Biosystems), by using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 1 μmol scale. The ODNs were 5'-modified with a monomethoxytrityl (MMT) protected amino group, by incorporating the commercially available 6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (**41**) (Glen Research) during the last step of the automated synthesis. The oligonucleotides were left attached to the CPG solid support for 'on-column' reaction.



5' MMTNH-TTT TTT-CPG 3' **41A**

5' MMTNH-A₁₀ TAG GAA TAG TTA TCA-CPG 3' **41B**

5 MMTNH-A₁₀ TGA TAA CTA TTC CTA- CPG 3' **41C**

Figure 5.1 Commercial phosphoramidite for 5'-amino incorporation 6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite **41** and ODN sequences prepared (**41A**, **41B** and **41C**)

The reason and advantage of using **41A**, the T₆ sequence, asides from being a simpler sequence, was that analysis of the reaction could be made much more quickly. The thymidine phosphoramidite does not have any protecting groups, and thus simply reaction with an ammonia solution for 1 hour cleaves the ODN from the solid support, allowing analysis of the reaction. The other bases, adenine, cytosine and guanine, require treatment for 16 hours at 55 °C to remove the acyl protecting groups on the exocyclic amines. A benzoyl group is used to protect the amine of adenine and cytosine, and an isobutyryl group is used to protect the amine of guanine (Figure 5.2).

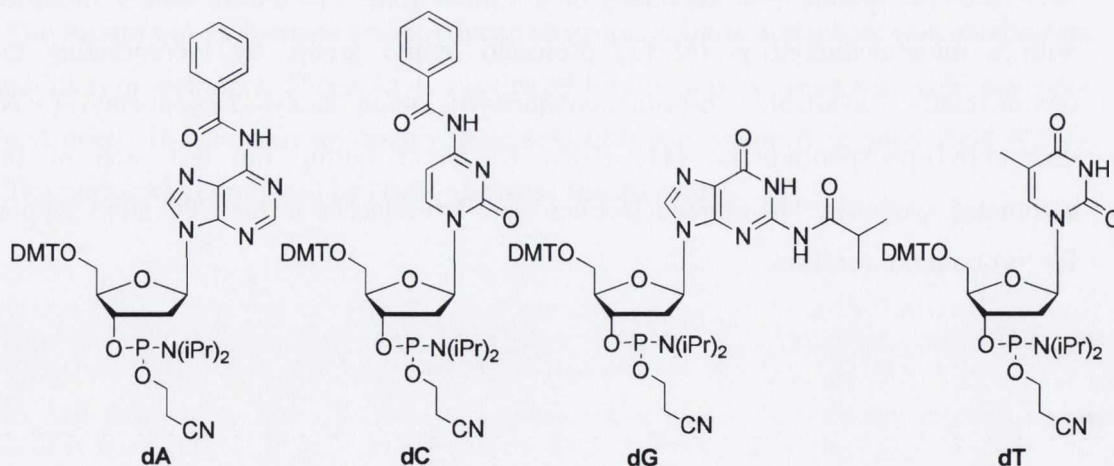
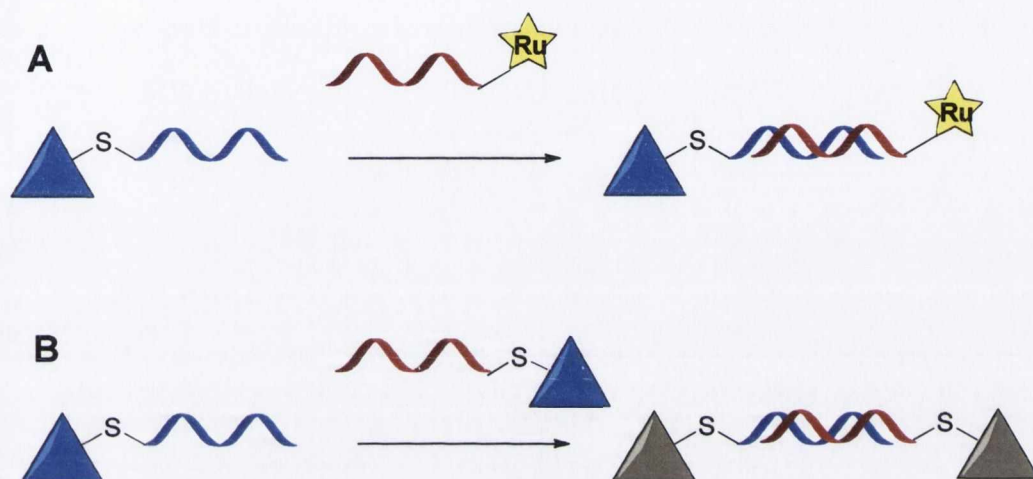


Figure 5.2 Structure of phosphoramidites **dA**, **dC**, **dG** and **dT**. The exocyclic amines of **dA** and **dC** are protected with a benzoyl group and the amine of **dG** with an isobutyryl group.

The sequences **41B** and **41C** were chosen to be complementary to the Ru-ODN conjugates (**28A** and **28B**) as described in Section 2.17.3, Chapter 2 and complementary to each other for use in a nanoparticle cross-linking ‘sandwich experiment’ as depicted in Scheme 5.2B.



Scheme 5.2 Simplified representation of the possible systems A and B that could be created with the selected ODN conjugates **41B** and **41C**.

5.2.4 Confirmation of Successful 5'-Amino Modification

A small aliquot of the CPG resin of **41A** was treated with ammonia for 1 hour and analysed by HPLC (Figure 5.3). A peak, with a retention time of 20 minutes, indicated successful incorporation of the MMT amino modification at the 5' end of the ODN. The MMT group results in the ODN being hydrophobic and thus it has a longer retention time relative to the free ODN (~12 minutes).

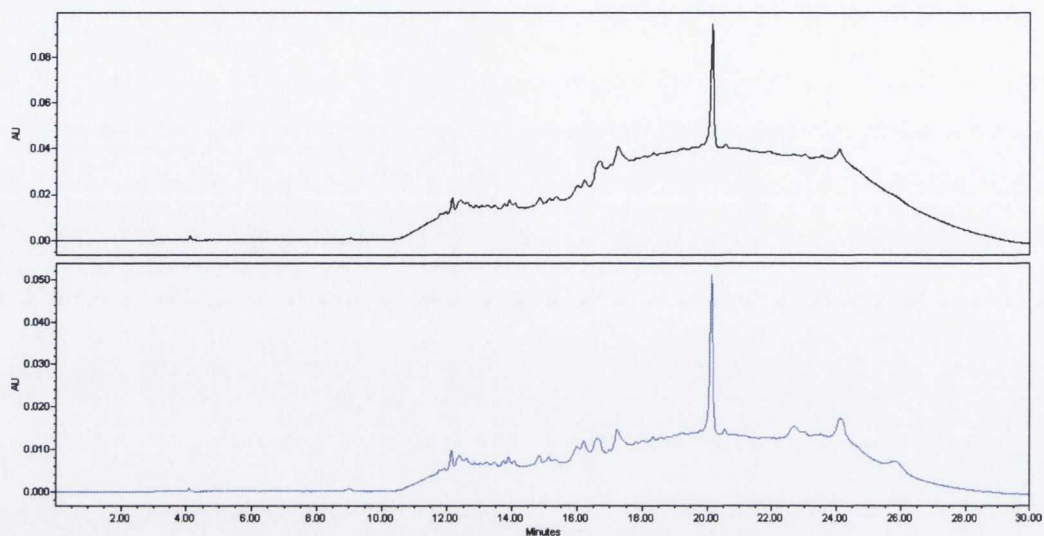
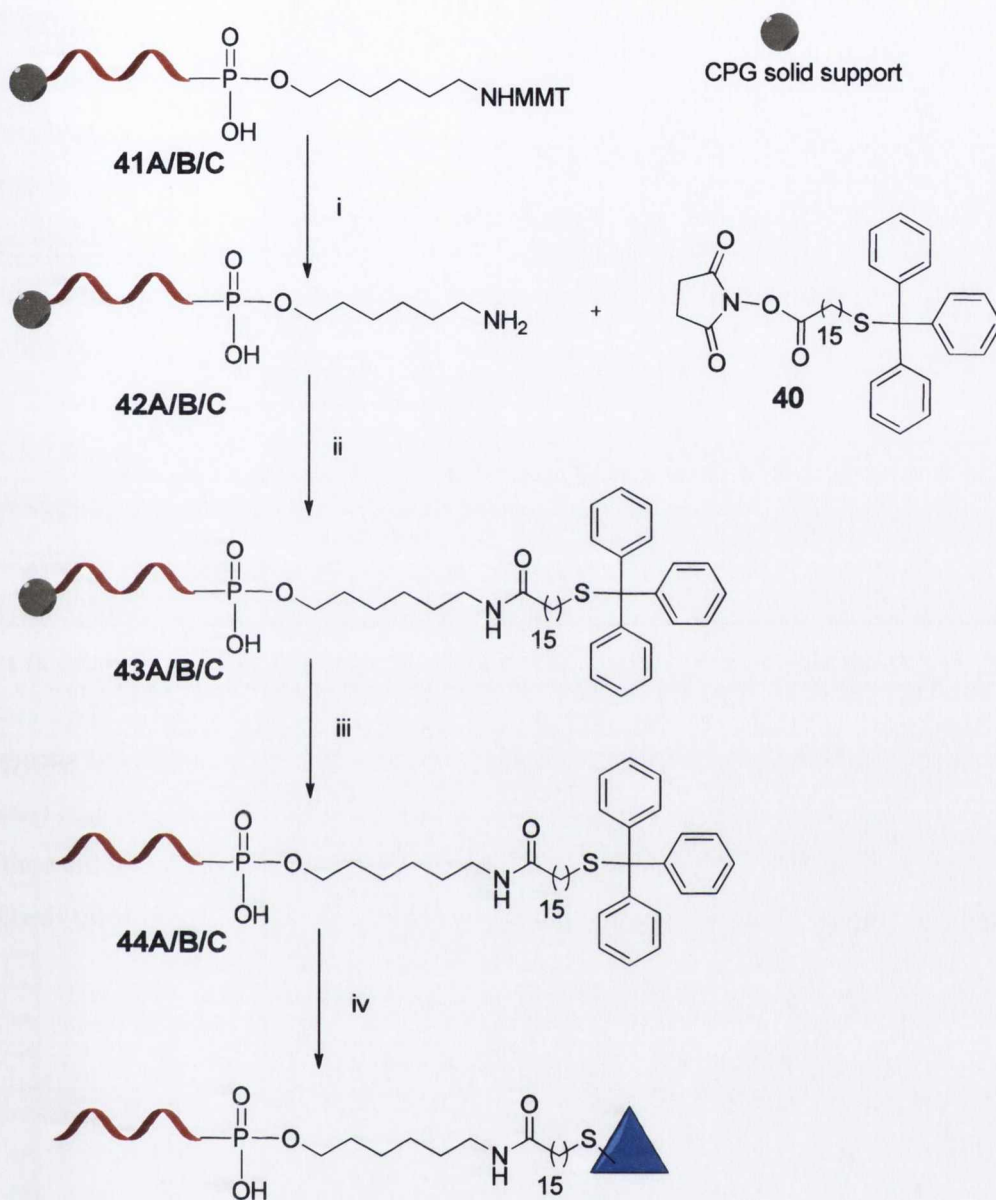


Figure 5.3 HPLC trace of the CPG resin of **41A** treated with ammonia. Top at 260 nm, Bottom at 280 nm. (Note background due to low concentration of ODN injected)

5.2.5 Deprotection of the MMT Protecting Group

The CPG resin of **41A** was treated with 80% acetic acid for 1 hour to remove the MMT group (Scheme 5.3, Step 1), using two syringes, to give **42A**. The solution was agitated, every 10 minutes, over the 1 hour period. The resin was washed with acetonitrile and dried with argon. An aliquot of the resin was taken and treated with ammonia, and analysed by HPLC to confirm successful deprotection (Figure 5.4).



Scheme 5.3 Preparation of MHA modified ODN via ‘on-column’ coupling and subsequent use to prepare nanoparticle conjugate. (i) 80% AcOH, (ii) Anhydrous DCM, DIPEA, (iii) 28% ammonia, (iv) (a) DTT, (b) triangular Ag nanoplates

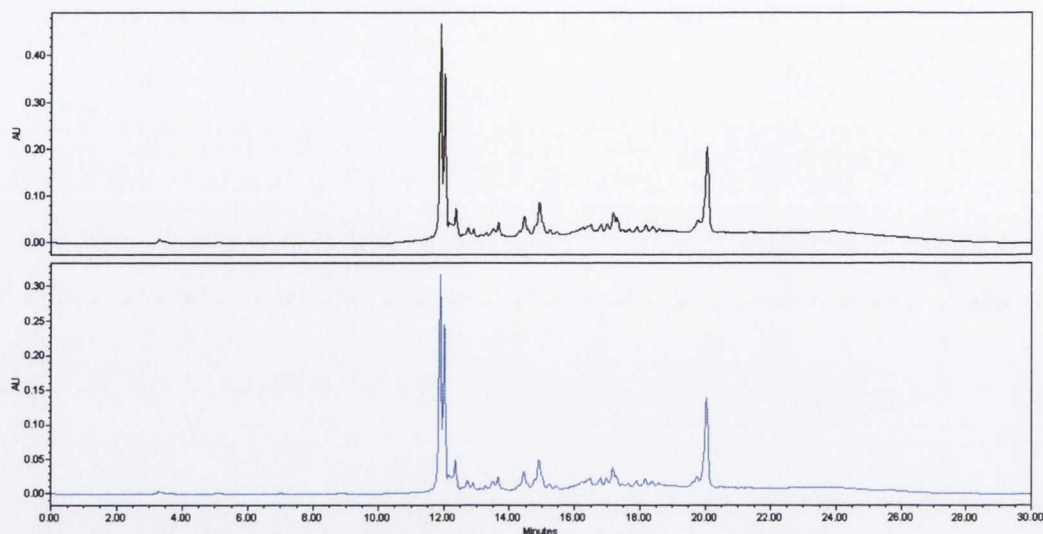


Figure 5.4 HPLC trace of **42A** after deprotection of **41A** with 80% acetic acid. Top at 260 nm, Bottom at 280 nm.

The appearance of a peak with a retention time of 11.9 minutes indicated successful removal of the MMT group, although some protected ODN **41A** still remained (peak at 20 minutes). The MMT deprotection step is an equilibrium reaction and on the confines of the solid support it is difficult to completely remove the MMT group from the ODN because it reattaches (Scheme 5.4).



Scheme 5.4 Reattachment of the MMT protection group on the confines of the CPG solid support

Subsequent deprotection reactions were carried out using 3% trichloroacetic acid in dichloromethane for 10 minutes via syringe, but again a peak for the protected ODN was apparent. When repeating this I would advise that detritylation should be performed directly on the DNA synthesizer by using twice the DCM/TCA procedure. It is expected that the MMT cleavage should be increased by flowing the cleavage reagent through the cartridge.

5.2.6 Coupling

This work was carried out on two separate visits to the lab of Prof. Defrancq in Grenoble. On the first occasion anhydrous acetonitrile was used as the solvent. On analysis, by mass spectrometry, no evidence for successful coupling was observed. It was decided to alter the conditions, changing the solvent, increasing the number of equivalents of the NHS activated ester and reducing the reaction volume to create forcing conditions to help reaction on the confines of the solid support.

5.2.6.1 Coupling with Test Sequence 42A

The deprotected CPG resin **42A** (0.5 μ moles) was transferred to a small centrifuge tube. Anhydrous dichloromethane (125 μ l), DIPEA (10 μ l) and succinimidyl 16-tritylmercaptohexadecanoate (**40**) (6.27 mg, 10 μ moles, 20 equivalents) were added. The mixture was agitated on an orbital shaker. An aliquot of **43A** was removed after 3.5 hours and treated with ammonia for 1 hour to cleave it from the solid support and analysed by HPLC **44A** (Figure 5.5).

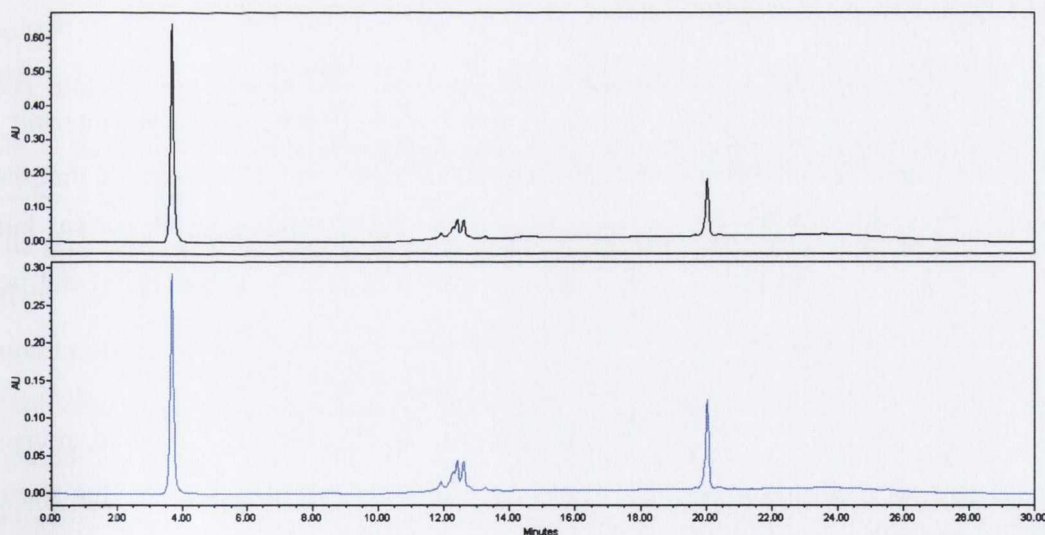


Figure 5.5 HPLC trace of **44A** coupling reaction after 3.5 hours. Top at 260 nm, Bottom at 280 nm

No evidence of an ODN with a longer retention time was observed. The peak $\sim$ 4 minutes corresponded to the activated ester **40**. The mixture was allowed to react

overnight. The remaining resin was washed numerous times with dichloromethane and dried. The resin was treated with ammonia for 1 hour and analysed by HPLC (Figure 5.6).

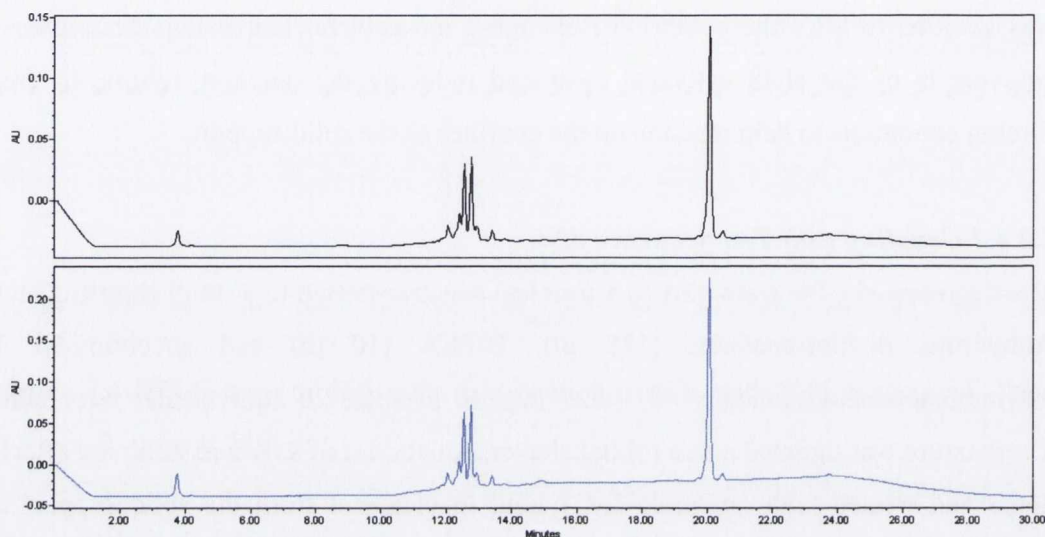


Figure 5.6 HPLC trace of **44A** coupling reaction after 24 hours. Top at 260 nm, Bottom at 280 nm.

There was no appearance of a new peak in the HPLC but a reduction in the intensity of the peak eluting at 12 minutes was suggestive of reaction. It was considered that there was a possibility that the peak at 20 minutes could be the required product **44A**, but it was also possible that this was the remaining MMT-NH-ODN **41A** that had not been successfully cleaved on the confines of the solid support.

A co-injection of the cleaved starting sample and the final reaction mixture, revealed only one peak at 20 minutes which would suggest that this peak was in fact the starting ODN **41A**. The crude reaction mixture was analysed by ESI-MS (Figure 5.7).

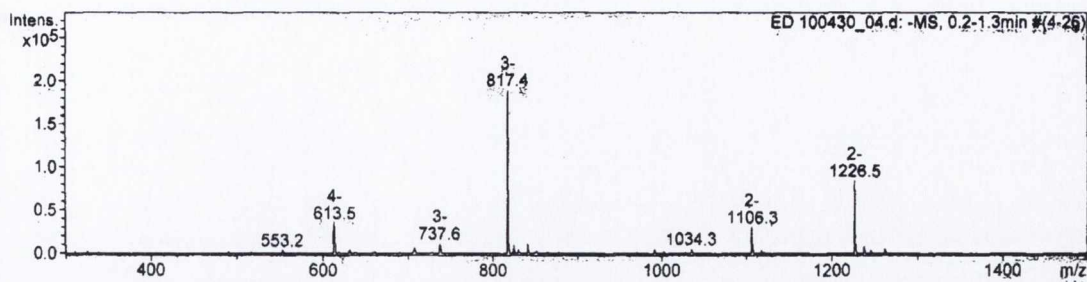


Figure 5.7 ESI-MS of **44A** coupling reaction. 1226.5 [M^2], 1106.3 [MMT-NH-ODN $^{2-}$], 817.4 [M^3], 737.6 [MMT-NH-ODN $^{3-}$], 613.5 [M^4]. M = Required ODN, Tr-S-C $_{16}$ -ODN

The ESI-MS confirmed successful preparation of the ODN conjugate **44A**, ($M_{\text{calcd}} = 2455$ Da). The presence of some MMT-NH-ODN **41A** ($M_{\text{calcd}} = 2214$ Da) was also observed, which again supported the theory that the peak at 20 minutes in the HPLC trace was simply this, and that our product was too hydrophobic to elute. However, for ESI-MS analysis, no signal corresponding to T $_6$ -NH $_2$ **42A** is observed ($M = 1943$ Da) as well as the T $_6$ ODN without the linker ($M = 1763$ Da). The crude sample was purified by HPLC and the peak at 20 minutes collected and submitted for ESI-MS analysis, the only mass observed was that of the MMT protected ODN **41A**.

5.2.6.2 Coupling of **41B** and **41C**

The process was repeated for the sequences **41B** and **41C**. It was hoped that the longer ODN sequence would override the extreme hydrophobicity imparted by the combination of the long alkyl chain with the trityl protecting group. As already mentioned, the deprotection of the MMT group of **41A** was not quantitative, so the method was adapted to try and improve the yield of amino modified ODN. DNA synthesizer deblock reagent (3% trichloroacetic acid in DCM) was used via syringe for 10 minutes. An aliquot of the resin was cleaved with ammonia solution at 55 °C for 16 h and analysed by HPLC (Figure 5.8). The peak at 12 minutes was the required ODN **42B/C** and a peak at 17 minutes could be attributed to the protected ODN as a result of the incomplete cleavage on the confines of the solid support **41B/C**.

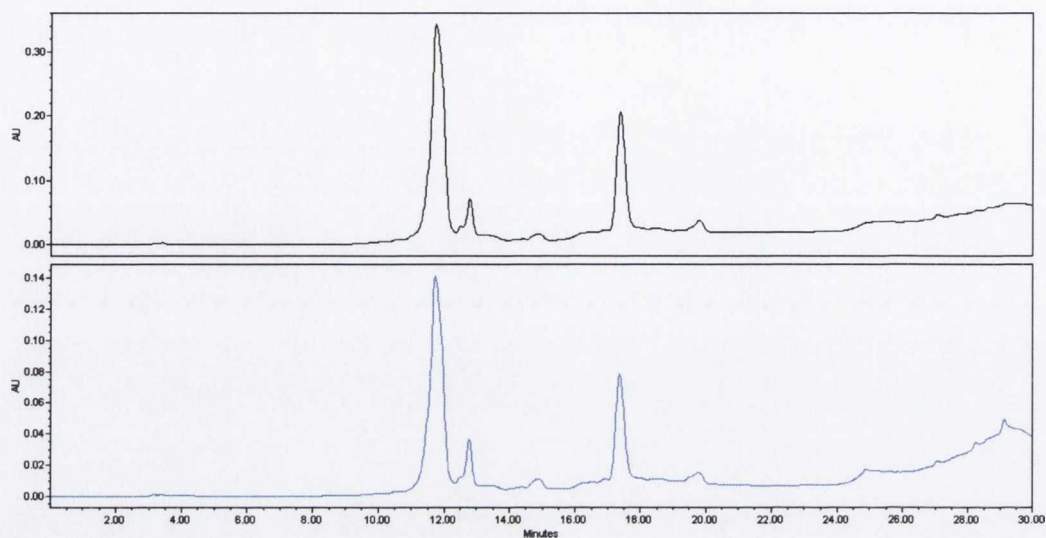


Figure 5.8 HPLC trace of **42B** after deprotection of **41B** with 3% TCA in DCM. Top at 260 nm, Bottom at 280 nm

The deprotected CPG resin **42B/C** (1 μ mole) was transferred to a small centrifuge tube. Anhydrous dichloromethane (250 μ l), DIPEA (20 μ l) and succinimidyl 16-tritylmercaptohexadecanoate **40** (12.54 mg, 20 μ moles, 20 equivalents) were added. The mixture was agitated on an orbital shaker overnight. The beads **43B/C** were washed vigorously with dichloromethane and dried. Ammonia solution was added and the samples placed at 55 $^{\circ}$ C for 16 h. The samples were analysed by HPLC **44B/C** (Figure 5.9).

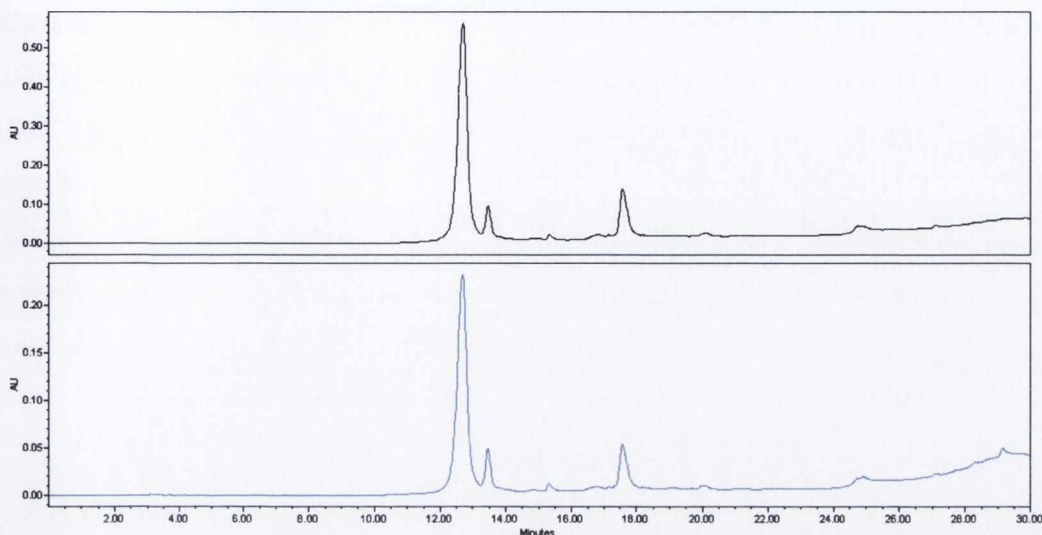


Figure 5.9 HPLC trace of **44B** conjugate. Top at 260 nm, Bottom at 280 nm.

No evidence for the product was seen in the HPLC trace. The combination of the long alkyl chain and the trityl group render the ODN so hydrophobic that it does not elute in the 30 minute timeframe of the HPLC analysis. Attempts to analyse the sample by MALDI-TOF MS did not give any conclusive evidence for successful incorporation of the MHA unit.

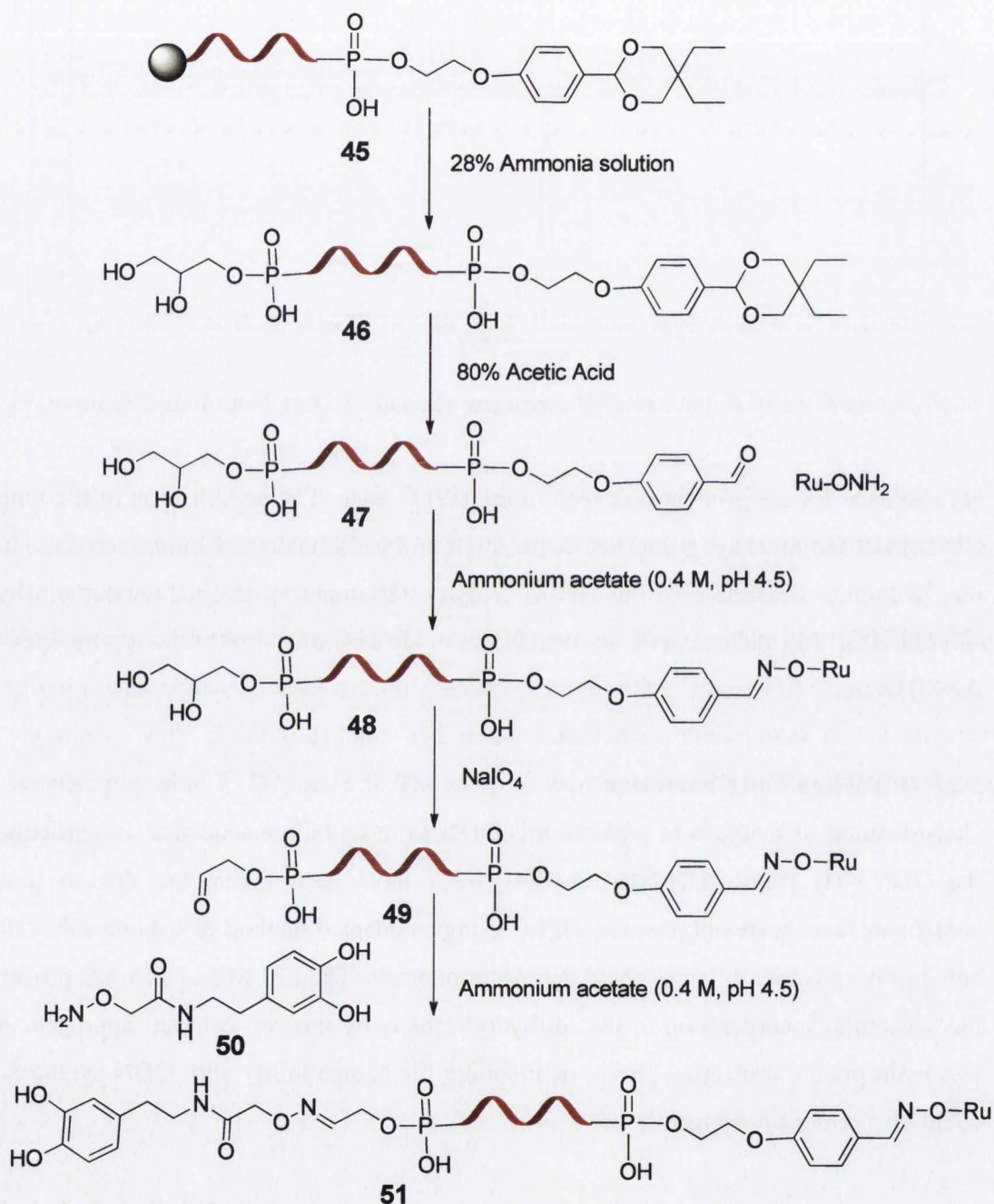
5.2.7 Discussion and Conclusion

Consideration of methods to separate this ODN from its failure sequence, in particular the ODN-NH₂ from ODN-NH-CO-(CH₂)₁₅-STr have been given, but due to time constraints these were not pursued. HPLC using an adapted method or column substrate could prove advantageous, as could gel electrophoresis. The ESI-MS of **44A** has proven the successful incorporation of the sulfhydryl moiety by this ‘on-column’ approach. A less hydrophobic protecting group, maintaining the compatibility with ODN synthesis, could also prove advantageous.

5.3 Catechol Modified ODN

Catechols have been shown to bind efficiently to various oxides, including TiO₂⁴ and Fe₃O₄,⁵ and thus this offers another approach for the conjugation of ODNs to nanoparticles. The Ag@TiO₂ particles outlined in Chapter 3, Section 3.12 could be

conjugated with ODNs, and by using the success of the benzaldehyde modified ODNs for successful oximation (Chapter 2, Section 2.17), a bifunctionalised luminescent ODN nanoparticle system could be created by a bis-oximation strategy (Scheme 5.5)



Scheme 5.5 Schematic showing the possible preparation of a bifunctionalised ODN, with a 3'-catechol and a 5'-Ru complex using two oximation steps.

There are only a few reports in the literature for catechol modified ODNs and they involve the activated ester coupling of a catechol derivative with an amino modified ODN.⁴ We wanted to apply the efficient oxime strategy to improve on this, showing the versatility of this system and further applying the approach to prepare a bifunctionalised ODN combining two oximation steps. The synthesis of an oxyamine derivative of a catechol moiety and the subsequent preparation of a catechol labelled ODN **52** is reported (Figure 5.10).

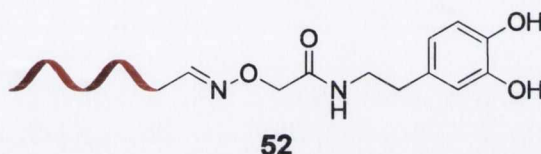


Figure 5.10 Catechol modified ODN

5.3.1 Initial Coupling Reaction

A Boc protected oxyamine derivative of dopamine **53** was prepared by Dr. Gerard Doorley as part of his Ph.D. thesis (Figure 5.11).⁶

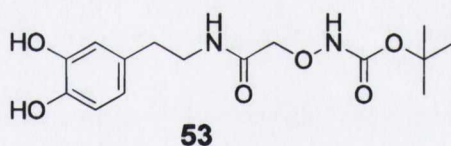


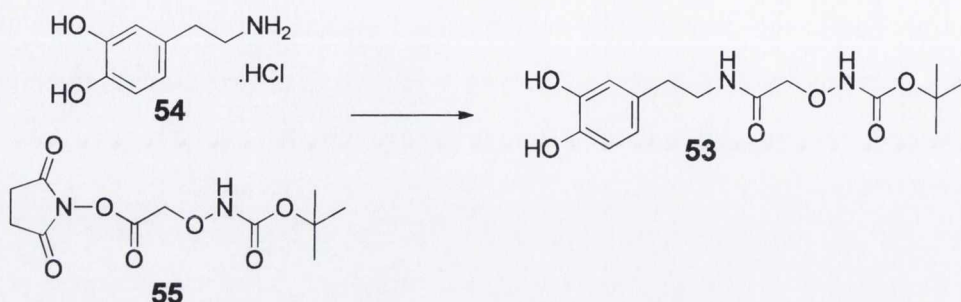
Figure 5.11 Boc protected oxyamine derivative of dopamine

Initial experiments were carried out using the compound prepared by Dr. Doorley (stored at room temperature for a number of months), but on further analysis (mass spectrometry and NMR) it was found to have seriously decomposed, with only a small amount of the required compound present, which was evidenced by mass spectrometry. NMR showed that the Boc group had been lost with no peak evident at 1.4 ppm for the *tert*-butyl and a number of new peaks upfield could be observed.

5.3.2 Synthesis of *tert*-butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate

The resynthesis of compound **53** (*tert*-butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate) was undertaken following the procedure reported by Dr. Doorley

in his thesis with some minor adjustments (Scheme 5.6). It proved to be quite a challenge, particularly to purify it without any detrimental oxidation or rearrangement.



Scheme 5.6 Schematic for synthesis of *tert*-butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate (**53**).

The dopamine hydrochloride salt **54** was placed stirring in anhydrous DMF under nitrogen. DIPEA and the NHS ester of Boc protected O-(carboxymethyl)hydroxylamine (**55**) were added. The reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure. The product was purified by flash chromatography on silica, using chloroform:methanol (9:1) as eluent. The fractions were analysed by TLC. The catechol moieties were stained using ferric chloride giving the characteristic dark black spots. Initial experiments resulted in some problems with decomposition, with the sample turning brown in colour. Any prolonged exposure to air and heat was eliminated from the preparation. The sample was dried for a number of days on a high vacuum line. A sticky white solid was obtained in 58% yield. The purity was confirmed by mass spectrometry and ^1H (Figure 5.12) and ^{13}C NMR.

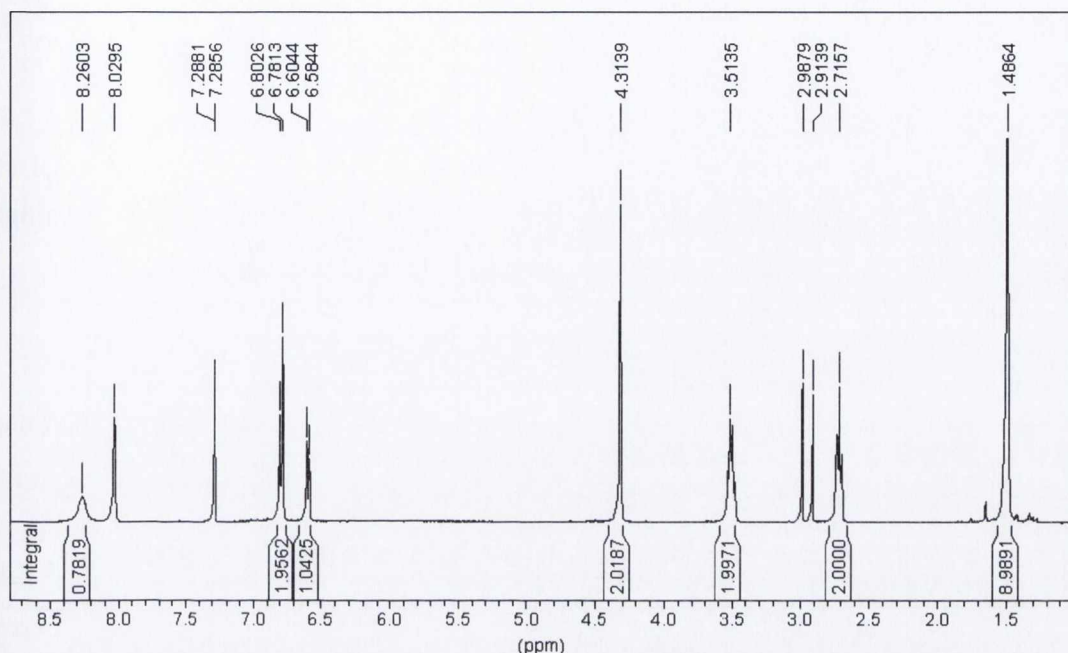
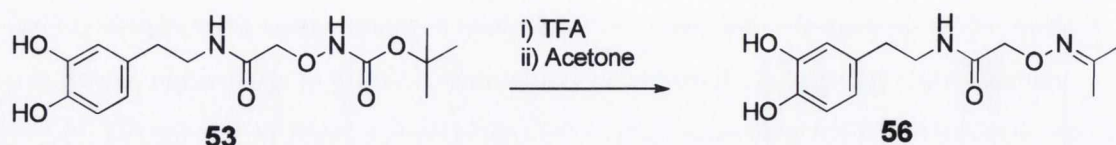


Figure 5.12 ^1H NMR spectrum of *tert*-butyl-2-((3,4-dihydroxyphenethyl)amino)-2-oxoethoxycarbamate (**53**) Note peaks at 8.02 (DMF), 7.28 (CHCl_3), 2.98 (DMF) and 2.91 (DMF) are due to residual solvent.

5.3.3 Removal of the Boc Protecting Group

Due to the sensitivity and reactivity of compound **53**, we wanted to confirm that it was possible to remove the Boc protecting group and further react the oxyamine group without any degradation or side reactions.

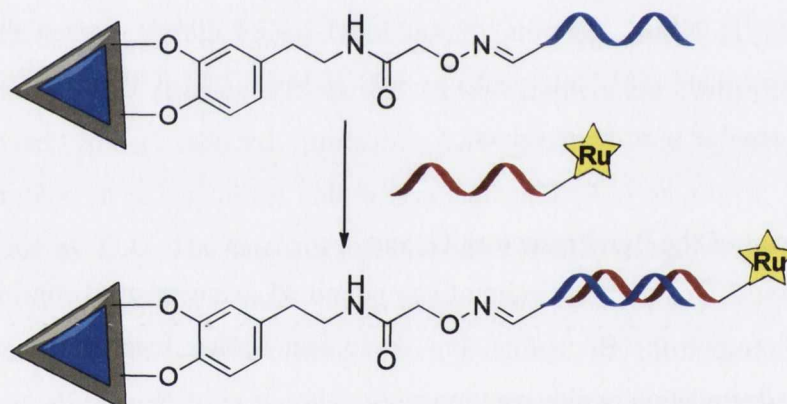
The removal of the Boc protecting group was achieved using DCM/TFA (50/50, v/v) with 5% triisopropylsilane as a carbocation scavenger, at room temperature for 2 hours. Acetone was added to the reaction. The successful deprotection was confirmed by observing the formation of the acetone oxime **56** by mass spectrometry (Scheme 5.7).



Scheme 5.7 Schematic showing the removal of the Boc group on the oxyamine derivative **53** with TFA and subsequent formation of the acetone oxime **56**.

5.3.4 Choice of ODN Sequences

The ODN sequence selected was 5'CCA TCT AGG GAC A₁₀-diol 3' (**57**). The ODN was selected to be complementary to the Ru-ODN conjugate **23B** prepared in Chapter 2, 5'GTC CCT AGA TGG-Ru3'. The potential use of these prepared sequences is illustrated below (Scheme 5.8)



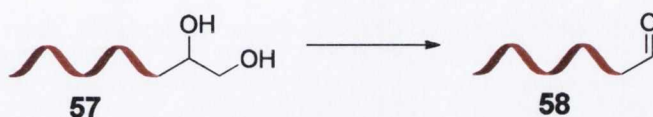
Scheme 5.8 Schematic representation of the potential use of the ODNs **57** and **23B**

5.3.5 Preparation of ODNs

The ODN was prepared by Prof Defrancq on an ABI 3400 DNA synthesizer (Applied Biosystems), by using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 1 μ mol scale. The diol moiety was introduced using the commercially available 3'-glyceryl support, (3-[4,4'-dimethoxytrityl] glyceryl-1-succinyl)] (Glen Research). The ODN was deprotected and cleaved from the solid support by treating it with a 28% ammonia solution for 16 h at 55 °C. The strand was purified by reverse phase HPLC. The ODN concentration was determined by UV-Vis spectroscopy. The ODN structure was confirmed by MALDI-TOF mass spectrometry.

5.3.6 Oxidation of Diol to Aldehyde

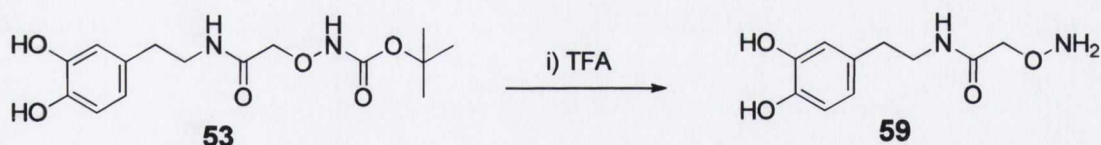
The ODN **57** containing the 3'-diol was oxidised to the corresponding 3'-aliphatic aldehyde **58** using sodium-*m*-periodate (20 equivalents) (Scheme 5.9). The excess NaIO₄ was removed using a NAP-10 column. The yield was taken to be quantitative.



Scheme 5.9 Oxidation of diol **57** with sodium periodate to give the corresponding aldehyde **58**

5.3.7 Deprotection of *tert*-butyl-2-(3,4-dihydroxyphenethylamino)-2-oxoethoxycarbamate

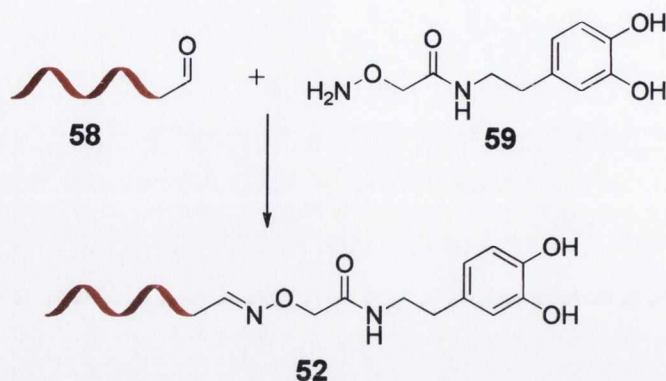
Tert-butyl-2-(3,4-dihydroxyphenethylamino)-2-oxoethoxycarbamate **53** (10 mg, 0.03 mmoles) was placed in a round bottom flask. Dichloromethane/TFA (2 ml, 50:50, v/v) was added with 5 % triisopropylsilane, as a scavenger for the carbocation, and the mixture stirred at room temperature for 2 hours (Scheme 5.10). The solvent was removed by rotary evaporation. The yield of **59** was taken to be quantitative.



Scheme 5.10 Schematic for the removal of the Boc group on the oxyamine derivative **53** to give **59**

5.3.8 Coupling of ODN **58** with **59**

The aldehyde modified oligonucleotide **58** was dissolved in ammonium acetate buffer (0.4 M, pH = 4.6). The oxyamine **59** (2 equivalents) was added (Scheme 5.11). The reaction was stirred at room temperature and monitored by HPLC.



Scheme 5.11 Oxime coupling of ODN **58** and oxyamine **59** to give catechol modified ODN **52**.

5.3.9 Analysis and Purification of Catechol ODN Conjugate **52**

HPLC showed no separation. Denaturing polyacrylamide gel electrophoresis was used to separate the required conjugate **52** from the free ODN and the excess oxyamine **59**. Two very close bands were observed, they were excised from the gel, crushed and collected by elution in water. The samples were desalted using NAP size exclusion chromatography. MALDI-TOF MS analysis confirmed successful conjugation with a mass of 7090.13 observed for the band that migrated more slowly. ($M_{\text{Calculated}} = 7092$ Da). The required conjugate **52** complexed with iron was also observed ($M = 7145$ Da). Some of the starting ODN ($M = 6882.94$ Da, $M_{\text{Calculated}} = 6884$ Da) was observed (Figure 5.13).

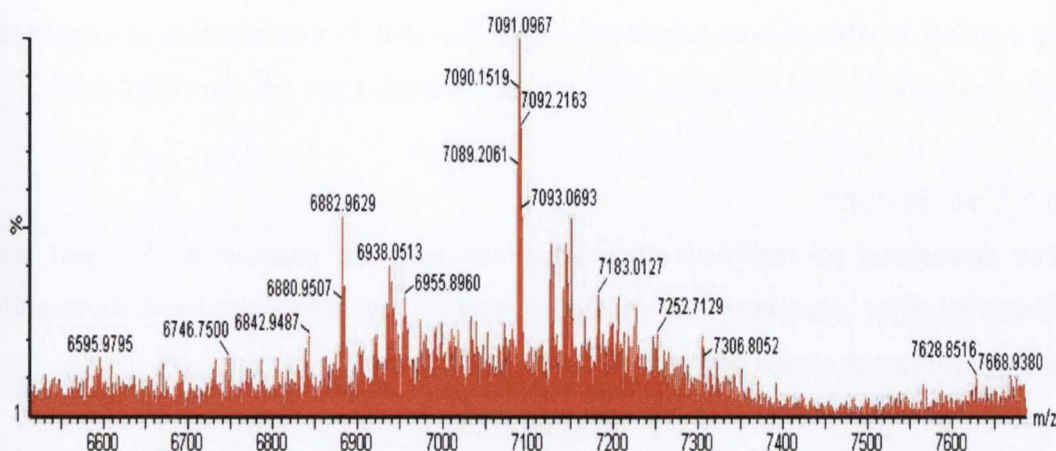


Figure 5.13 MALDI-TOF MS of the more slowly migrating band excised from the gel in negative ionisation mode.

5.3.10 Summary on Catechol Modified ODN

A catechol modified ODN was successfully prepared by oximation. Purification using PAGE resulted in two very close bands being obtained. MALDI-TOF mass spectrometry confirmed the successful preparation of the conjugate, although some of the starting ODN was also observed. The purification of this reaction needs to be optimised. The prepared catechol modified ODN offers a route for the functionalisation of the triangular Ag@TiO₂ particles, TiO₂ nanoparticles^{4,7} and indeed the direct conjugation to silver nanoparticles.⁸

5.4 Future Work

The need to be able to prepare bifunctionalised oligonucleotides with both, a fluorescent label and a group for nanoparticle conjugation, has already been outlined in Chapter 2 and 4, with the benzaldehyde being shown to be reactive enough for oximation. The possibility of using two oxime reactions, taking advantage of the efficiency of this coupling, is an exciting prospect. The preparation of a bifunctionalised strand, using a bis oxime approach was started, but unfortunately due to the initial challenges with the catechol approach this was not completed. An ODN with a 3' diol and a 5' benzaldehyde was prepared **46** (Scheme 5.5), and the 5' end was successfully coupled with a Ru complex to give **49**. A test reaction showed no degradation of the Ru complex

on treating it with sodium periodate, suggesting that further reaction to couple the catechol could be used to give an ODN with a 3'-catechol and a 5'-Ru complex **51**.

5.5 Conclusions

The preparation of modified oligonucleotides with the potential to be used with triangular silver nanoplates and triangular Ag@TiO₂ nanoparticles were successfully prepared although challenges were met with each system.

Long chain alkyl thiol modified ODNs were successfully prepared. However they were found to be extremely hydrophobic, excluding purification by HPLC. ESI-MS confirmed the successful preparation of the ODN.

The oxyamine modified catechol was found to present a synthetic challenge due to the sensitivity of the catechol. This was overcome and a catechol-ODN conjugate successfully prepared. PAGE purification gave the required ODN, which was confirmed by MALDI-TOF MS. Initial work on the preparation of a bifunctionalised strand showed great promise but due to time constraints was not completed.

5.6 References

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Chapter 6

Experimental

6.1 Reagents and Solvents

Chemical reagents were purchased from Sigma Aldrich and Fluka excluding the DNA synthesis reagents, which were obtained from Glen Research and the Quant-iT Oligreen[®] reagent was purchased from Biosciences Ltd. (Invitrogen). All solvents were reagent grade unless otherwise specified within the text. All reagents and solvents were used as received unless otherwise indicated. For the nanoparticle preparations distilled water passed through a Millipore Synergy 185 unit was further distilled before use. All glassware for the nanoparticle work was treated with aqua regia and rinsed thoroughly with distilled water before use.

6.2 Instrumentation

6.2.1 Raman

Raman measurements were carried out in Queens University Belfast using an Avalon Instruments raman station with an excitation wavelength of 785 nm.

6.2.2 UV-Vis Spectroscopy

A Cary Varian 50 Scan spectrophotometer and a Perkin Elmer Lambda 1050 UV/VIS/NIR spectrophotometer were used for all UV-Vis measurements. Thermal denaturation experiments were performed using a PerkinElmer Lambda 35 UV-Vis spectrophotometer equipped with a PTP1 peltier temperature controller.

6.2.3 Photoluminescence

Emission and excitation measurements were performed using a Cary Varian Eclipse spectrophotometer. Lifetime measurements were performed on a Horiba Jobin Yvon Fluorolog TCSPC. The excitation source was a 458 nm NanoLED. A Tecan GENios Microplate reader was used for the Oligreen assay. Measurement mode – Fluorescence top, Excitation wavelength – 480 nm, Emission wavelength – 535 nm, Gain – optimal, Number of flashes – 10, Lag time – 0, Integration time – 20 μ s

6.2.4 Infrared Spectroscopy

Infrared measurements were performed using a Perkin Elmer, Spectrum 100, FT-IR spectrometer fitted with a universal ATR sampling accessory.

6.2.5 Mass Spectrometry

MALDI-TOF measurements were performed using a Waters MALDI Q-TOF Premier system and ESI-MS measurements were performed using a LCT Orthogonal Acceleration TOF Electrospray mass spectrometer. Negative and positive ionisation modes were used where appropriate.

6.2.6 Nuclear Magnetic Resonance Spectroscopy

A Bruker Avance III 400 and a Bruker Avance II 600 were used for all ^1H , ^{13}C and ^{31}P NMR measurements.

6.2.7 DNA Synthesis

A Beckmann Oligo 1000M DNA synthesizer was used in Trinity College Dublin. DNA synthesis performed in Université J. Fourier, Grenoble, France was carried out on an Applied Biosystems ABI 3400 DNA synthesizer. An extended coupling time was used for incorporation of all modified phosphoramidites.

6.2.8 Transmission Electron Microscopy

TEM images were taken on a Jeol 2100 La-B6 TEM operating at an accelerating voltage of 200 keV with a Lanthanum hexaboride emission source and a resolution of 0.2 nm. Samples were prepared on Agar[®] Carbon film grids and images were analysed using IMAGE J software to determine the particle dimensions.

6.2.9 Dynamic Light Scattering

Particle sizing measurements were carried out using a Malvern Instruments Zetasizer Nano ZS. Samples were filtered through 0.45 μm PVDF filters before use. Low volume clear disposable acrylic cuvettes (Sarstedt) were used. Measurements were performed at 25 °C using the 173° backscatter set-up. The number of runs per measurement was set at automatic and the results were averaged over 5 measurements for each sample.

Temperature experiments were carried out in a quartz cuvette using a temperature trend method. The sample was heated from 20 °C to 65 °C at 1 °C intervals.

6.2.10 Zeta Potential

Zeta potential measurements were carried out using a Malvern Instruments Zetasizer Nano ZS. Clear disposable zeta capillary cells (Particular Sciences) were used. The surface tension of the cell was broken with ethanol prior to use. The cell was washed thoroughly with water to avoid any contamination. Measurements were performed at 25 °C using the general purpose mode. The number of runs per measurement was set at automatic and the results were averaged for 5 measurements for each sample.

6.2.11 Speed-vac

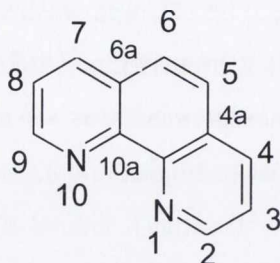
A Savant Speedvac concentrator was used to dry all ODN samples.

6.2.12 HPLC

RP-HPLC analysis was performed on a Waters RP-HPLC system with a dual wavelength detector using a Nucleosil C18 column (Macherey Nagel, 250 x 4.6 mm, 5 µm). A solvent system of; solvent A (50 mM TEAAc buffer containing 5% acetonitrile) and solvent B (acetonitrile containing 5% water) was used. A flow rate of 1 mL min⁻¹ and a linear gradient of 0-30 % B was applied over 20 or 30 minutes. The ODNs were purified on a µ-Bondapak C-18 column (Macherey-Nagel Nucleosil, 250 x 10 mm, 7 µm) with similar gradient at a flow rate of 4 mL min⁻¹ on a Gilson HPLC system with a dual wavelength detector.

6.3 Methods

Numbering of phenanthroline ring



6.3.1 5-amino-1,10-phenanthroline (**6**)¹⁻³

5-nitro-1,10-phenanthroline (**5**) (1.5 g, 6.67 mmol), and Pd/C (10%, 0.428 g, 4 mmol, 0.6 equivalents) were added to a tri-necked round bottom flask and placed under nitrogen. Ethanol (100 ml) was added and the reaction heated. Hydrazine hydrate (1.06 g, 33 mmol, 1.02 ml, 5 equivalents) was added dropwise and the mixture refluxed for 4 hours. The palladium was removed by filtration through celite to leave a golden yellow filtrate. The solvent was removed by rotary evaporation to yield a golden solid. The solid was washed with ether (2 x 15 ml) and dried under vacuum. A yield of 1.2 g (94%) was obtained. ¹H NMR (400 MHz, d₆-DMSO): δ= 9.06 (dd, J=4.1 Hz, 1H, H₂), 8.69 (dd, J=4.1 Hz, 1H, H₉), 8.67 (dd, J=8.2 Hz, 1H, H₄), 8.06 (dd, J=8.2 Hz, 1H, H₇), 7.74 (dd, J=8.2 Hz, J=4.1 Hz, 1H, H₃), 7.51 (dd, J=8.2 Hz, J=4.1 Hz, 1H, H₈), 6.86 (s, 1H, H₆), 6.17 (br s, 2H, NH₂). ¹³C NMR (100 MHz, d₆-DMSO): δ= 149.35 (CH), 146.18 (C_q), 144.81 (CH), 142.67 (C_q), 140.49 (C_q), 132.70 (CH), 130.80 (CH), 130.56 (C_q), 123.20 (CH), 122.06 (CH), 121.80 (C_q), 101.71 (CH). MS-ESI: Calculated for C₁₂H₉N₃ m/z = 195.0796, Found m/z = 196.0880 [M+H]⁺. IR (ATR, cm⁻¹): 3416, 3319, 3228, 1636, 1610, 1594, 1563, 1505, 1488, 1455, 1428, 1406, 1335, 1303, 1272, 1218, 1173, 1127, 1107, 1065, 1035, 961, 938, 885, 841, 826, 812, 791, 730, 712, 661.

6.3.2 N-(tert-butoxycarbonyl)glycine anhydride⁴

To a solution of commercial Boc-Glycine (3.65 g, 20 mmol) in acetonitrile (100 mL), DCC (2.1 g, 10 mmol, 0.5 equivalents) was added under nitrogen. The solution was stirred at room temperature for 1 hour and then filtered under N₂ to remove the DCU

side product. The solution was concentrated under vacuum (~5 ml) and used without purification.

6.3.3 2-Boc-amino-N-[1,10]-phenanthroline-5-yl-acetamide (7)⁴

5-amino-1,10-phenanthroline (**6**) (1.04 g, 5 mmol) and dry acetonitrile (50 ml) were placed in a tri-necked round bottom flask. The Boc-Gly-anhydride (~5 ml, 4 equivalents, above) was added and the reaction mixture stirred under nitrogen at room temperature. The reaction was monitored by TLC. Three more batches (12 equivalents) of anhydride were added until the reaction turned from a milky yellow (insoluble starting material) to a clear orange solution (product soluble in MeCN). The solvent was removed by rotary evaporation to yield a sticky orange solid. The compound was purified by flash chromatography, silica gel 60. A solvent system of DCM:MeOH (10%) + 1% NH₄OH was used. Ferrous chloride was used to stain the TLC plates. The fractions were combined and the solvent removed by rotary evaporation to yield a yellow solid. The compound was dried under high vacuum. Yield (1.23 g, 3.49 mmol, 70%). ¹H NMR (400 MHz, d₆-DMSO): δ= 10.20 (s, 1H, phen-NH), 9.12 (dd, J= 4.5Hz, J= 1.5Hz, 1H, H₂), 9.04 (dd, J=4.5 Hz, J=1.5 Hz, 1H, H₉), 8.64 (dd, J=8 Hz, J=1.5 Hz, 1H, H₄), 8.47 (dd, J=8 Hz, J=1.5 Hz, 1H, H₇), 8.15 (s, 1H, H₆), 7.80 (dd, J=8 Hz, J=4.5 Hz, 1H H₃), 7.74 (dd, J=8 Hz, J=4.5 Hz, 1H, H₈), 7.28 (t, J=6 Hz, 1H, CH₂NH), 3.96 (d, J=6 Hz, 2H, CH₂NH), 1.42 (s, 9H, tBu). ¹³C NMR (100 MHz, d₆-DMSO): δ= 169.67 (phen-NH-CO), 156.12 (NH-CO-O), 149.90 (CH), 149.39 (CH), 145.82 (C_q), 143.85 (C_q), 135.86 (CH), 131.78 (CH), 131.62 (CH), 128.06 (C_q), 124.78 (C_q), 123.59 (CH), 122.77 (CH), 120.26 (CH), 78.18 (C_q), 43.89 (CH₂), 28.21 (CH₃). MS-ESI: Calculated for C₁₉H₂₀N₄O₃ m/z =352.1535, Found 353.1627 [M+H]⁺. IR (ATR, cm⁻¹): 3327, 2980, 2929, 2852, 1704, 1680, 1643, 1622, 1601, 1519, 1481, 1454, 1418, 1390, 1365, 1280, 1253, 1222, 1151, 1089, 1068, 1052, 1031, 994, 962, 941,892, 868, 838, 805, 793, 730, 711.

6.3.4 2-amino-N-[1,10]-phenanthroline-5-yl-acetamide (8)⁴

The protected phenanthroline derivative **7** (0.59 g, 1.68 mmol) was stirred in a DCM/TFA solution (10 ml, 50/50, v/v) at room temperature for 2 h. The solvent was removed under reduced pressure by successive evaporation with toluene and acetonitrile. A yellow solid was obtained in quantitative yield. It was used without further

purification. MS-ESI: Calculated for $C_{14}H_{12}N_4O$ $m/z = 252.1011$, Found $m/z = 253.1100$ $[M+H]^+$ and 275.1122 $[M+Na]^+$

6.3.5 N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine⁵

O-(Carboxymethyl) hydroxylamine hemihydrochloride (**9**) (1 g, 9.14 mmol) and triethylamine (1.156 g, 11.425 mmol, 1.6 ml, 1.25 equivalents) were dissolved in water (15 ml). Di-tert-butylidicarbonate (2.2 g, 10 mmol, 1.09 equivalents) was dissolved in 1,4-dioxane (15 ml) and added to the flask. The reaction was stirred at room temperature for 3 hours. Water (25 ml) was added to the reaction. The aqueous phase was extracted with ethyl acetate (3 x 50 ml). The aqueous phase was cooled to 0 °C and adjusted to pH 2 with 5 M HCL. It was further extracted with ethyl acetate (3 x 20 ml). The combined layers were washed with precooled 5% HCL (3 x 15 ml) and saturated NaCl (3 x 15 ml). The organic phase was dried over Na_2SO_4 and the solvent removed by rotary evaporation. A white solid (1.12 g, 6.4 mmol, 70% yield) was obtained. 1H NMR (400 MHz, d_6 -DMSO): $\delta = 12.82$ (br s, 1H, COOH), 10.12 (br s, 1H, NH), 4.26 (s, 2H, CH_2), 1.40 (s, 9H, tBu). ^{13}C NMR (100 MHz, d_6 -DMSO): $\delta = 170.19$ (C_q), 156.44 (C_q), 80.04 (C_q), 71.98 (CH_2), 27.96 (CH_3). IR (ATR, cm^{-1}): 3374, 2986, 2959, 2573, 1747, 1718, 1437, 1413, 1395, 1368, 1284, 1211, 1161, 1119, 1056, 1023, 975, 895, 853, 776, 758, 693.

6.3.6 NHS ester of N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (**10**)⁵

N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (0.910 g, 5.2 mmol) and NHS (0.66 g, 5.72 mmol, 1.1 equivalents) were dissolved in dry acetonitrile (20 ml) and dicyclohexyldicarbodiimide (1.14 g, 5.72 mmol, 1.1 equivalents) was added. The reaction was stirred overnight under nitrogen. The DCU was removed by filtration. The solvent was removed by rotary evaporation. The crude product was dissolved in hot carbon tetrachloride (60 ml) and filtered. The filtrate was placed in the fridge at 4 °C overnight. A white precipitate was obtained. It was collected and dried by suction filtration. A crystalline white solid was obtained (760 mg, 2.63 mmol, 51% yield). 1H NMR (400 MHz, d_6 -DMSO): $\delta = 10.38$ (br s, 1H, NH), 4.83 (s, 2H, CH_2O), 2.84 (s, 4H, CH_2-CH_2), 1.42 (s, 9H, tBu). ^{13}C NMR (100 MHz, d_6 -DMSO): $\delta = 170.12$ (C_q), 165.23

(C_q), 156.68 (C_q), 80.70 (C_q), 69.95 (CH₂), 27.98 (CH₃), 25.50 (CH₂). IR (ATR, cm⁻¹): 3341, 2982, 1822, 1784, 1754, 1479, 1450, 1425, 1418, 1393, 1367, 1271, 1247, 1202, 1164, 1132, 1094, 1068, 1048, 997, 971, 908, 861, 794, 778, 750.

6.3.7 2-Boc-aminoxy-N-[(1,10)-phenanthroline-5-ylcarbamoylmethyl]-acetamide.(PF₆)₂ (**1**)⁴

To a suspension of the deprotected phenanthroline derivative **8** (1.68 mmol) in anhydrous DMF (10 ml), the activated ester of N-Boc-O-(carboxymethyl)-hydroxylamine (**10**) (0.63 g, 2.184 mmol, 1.3 equivalents) and DIPEA (5.54 mmol, 0.97 ml, 3.3 equivalents) were added. The mixture was stirred at room temperature for 1 h under nitrogen. The solvent was removed under reduced pressure and the product was purified by silica gel column chromatography (eluent from 8 to 10% methanol in dichloromethane with 2% NH₄OH). The protected oxyamine ligand was obtained as a white solid (300 mg, 0.7 mmol, 42% yield). ¹H-NMR (400 MHz, d₆-DMSO): δ = 10.33 (br s, 1H, ONHBoc), 10.23 (br s, 1H, phen-NH), 9.14 (dd, J = 4 Hz, J = 1.5 Hz 1H, H₂), 9.06 (dd, J = 4 Hz, J = 1.5 Hz, 1H, H₉), 8.64 (dd, J = 8 Hz, J = 1.5 Hz, 1H, H₄), 8.48 (m, 2H, H₇ & CH₂NH), 8.16 (s, 1H, H₆), 7.85 (dd, J = 8 Hz, J = 4 Hz, 1H, H₃), 7.77 (dd, J = 8 Hz, J = 4 Hz, 1H, H₈), 4.30 (s, 2H, CH₂O), 4.19 (d, J = 5.5 Hz, 2H, CH₂NH), 1.40 (s, 9H, tBu). ¹³C NMR (100MHz, d₆-DMSO): δ = 168.66 (C_q), 156.69 (C_q), 149.94 (CH), 149.44 (CH), 145.76 (C_q), 143.85 (C_q), 135.94 (CH), 131.84 (CH), 131.48 (C_q), 128.02 (C_q), 124.80 (C_q), 123.63 (CH), 122.90 (CH), 120.47 (CH), 80.55 (C_q), 74.71 (CH₂), 42.3 (CH₂), 27.93 (CH₃). MS-ESI: Calculated for C₂₁H₂₃N₅O₅ m/z = 425.1698, Found m/z = 426.1778 [M+H]⁺. IR (ATR, cm⁻¹): 3283, 3108, 2928, 1711, 1653, 1601, 1535, 1479, 1455, 1415, 1393, 1367, 1318, 1280, 1254, 1228, 1166, 1108, 1016, 998, 968, 882, 826, 801, 740, 658.

6.3.8 Ru(bpy)₂Cl₂·2H₂O (**12**)⁶

RuCl₃·3H₂O **11** (5 g, 19 mmol), 2,2'-bipyridyl (5.95 g, 38.2 mmol, 2 equivalents) and LiCl (5.6 g, 133.7 mmol, 7 equivalents) were placed in a round bottom flask. DMF (75 ml) was added and the reaction refluxed for 8.5 hours. The mixture was cooled to room temperature and acetone (375 ml) added. The flask was placed at 0 °C overnight. The mixture was filtered leaving a dark green solid and a red filtrate. The solid was

washed with excessively with H₂O until the washings were clear and subsequently with ether. A dark green microcrystalline product was obtained (4.027 g, 8.37 mmol, 44% yield). MS-ESI: Calculated for C₂₀H₁₆N₄RuCl₂ m/z = 483.9795 Found m/z = 483.9815 [M]⁺. λ (max) in MeOH = 362nm, 522nm. IR (ATR, cm⁻¹): 3521, 3097, 3067, 1607, 1598, 1480, 1461, 1441, 1416, 1306, 1260, 1155, 1122, 1033, 1016, 901, 888, 800, 767, 747, 730, 723, 702, 656.

6.3.9 Attempted Synthesis of Ru(bpy)₂(acetone)₂. (13)⁶

An acetone solution (40 ml) was deaerated using N₂. Ru(bpy)₂Cl₂·2H₂O (**12**) (70 mg, 0.13 mmol) was added. AgClO₄ (56 mg, 0.27 mmol, 2 equivalents) was added and the reaction stirred under nitrogen for 3 hours. The reaction mixture was filtered. A red brown solution was obtained, but on addition of the ligand (and in a few instances before) a green/blue solution was obtained. Mass spectrometry and UV-Vis were used to attempt analysis, but exactly what formed was not deciphered and varied with each reaction.

6.3.10 Ru(bpy)₂[2-Boc-aminoxy-N-(1,10)phenanthroline-5-ylcarbamoylmethyl]acetamide.(PF₆)₂ (15)⁷

Methanol HPLC grade (30 ml) was placed in a tri-necked round bottom flask. The methanol was deaerated with N₂ for 15 mins. Ru(bpy)₂Cl₂ (**12**) (189 mg, 0.39 mmol) was added. The oxyamine modified phenanthroline ligand **1** (200 mg, 0.47 mmol, 1.2 equivalents) was added and the reaction stirred at reflux under N₂ for 12 hours. The reaction mixture turned a strong orange colour. The solvent was concentrated ~5 ml by rotary evaporation. A saturated solution of ammonium hexafluorophosphate (1 ml) was added. Water was subsequently added to favour precipitation. A flocculent orange precipitate was obtained. The precipitate was collected by Buchner filtration. An orange solid was obtained (250 mg, 0.298 mmol, 77% yield). ¹H NMR (600 MHz, d₆-DMSO): δ = 10.51 (s, 1H, phen-NH), 10.32 (s, 1H, NH-Boc), 8.91 (m, 3H, Ar-H), 8.84 (d, 2H, Ar-H), 8.77 (d, 1H, Ar-H), 8.61 (s, 1H, H₆), 8.48 (t, 1H, CH₂NH), 8.23 (m, 2H, Ar-H), 8.17 (d, 1H, Ar-H), 8.13 (dd, 2H, Ar-H), 8.05 (d, 1H, Ar-H), 7.94 (dd, 1H, Ar-H), 7.85 (m, 3H, Ar-H), 7.60 (m, 4H, Ar-H), 7.38 (m, 2H, Ar-H) 4.30 (s, 2H, CH₂O), 4.23 (d, 2H, CH₂NH), 1.40 (s, 9H, tBu). ¹³C NMR (125 MHz, d₆-DMSO): δ = 168.67 (C_q),

168.68 (C_q), 156.75 (C_q), 156.50 (C_q), 152.33 (CH), 151.42 (CH), 151.34 (CH), 151.24 (CH), 147.17 (C_q), 144.66 (C_q), 137.90 (CH), 137.79 (CH), 136.30 (CH), 133.46 (C_q), 132.62 (CH), 130.11 (C_q), 127.78 (CH), 127.67 (CH), 126.47 (CH), 125.71 (CH), 124.41 (CH), 124.33 (CH), 119.78 (CH), 80.54 (C_q), 74.65 (CH₂), 42.52 (CH₂), 27.87 (CH₃). MS-ESI: Calculated for C₄₁H₃₉N₉O₅Ru m/z = 839.2118, Found M = 839.2084. Observed 419.6042 where $z = 2$. λ_{max} in MeOH = 283nm, 450nm. IR (ATR, cm⁻¹): 3386, 1674, 1603, 1533, 1465, 1446, 1423, 1370, 1314, 1244, 1161, 1113, 859, 761, 725, 660.

6.3.11 4-[2-Phenyl-(1,3)-dioxolan-4-yl]-butanol-1-ol (**17**)⁸⁻⁹

1,2,6-hexanetriol (**16**) (3.68 g, 27.52 mmol) and a catalytic amount of PPTS (0.3 g) were added to a solution of benzaldehyde dimethyl acetal (2.095 g, 13.76 mmol) in dry DMF (30 ml). The reaction mixture was stirred for 24 h at 55 °C under anhydrous conditions. The solvent was removed by rotary evaporation. The oily residue was dissolved in ethyl acetate (50 ml), extracted with NaHSO₃ solution (3 x 20 ml), NaHCO₃ (3 x 20 ml) and brine (3 x 20 ml). The organic layer was dried with Na₂SO₄, filtered and the solvent removed by rotary evaporation. The crude product was purified by silica column chromatography eluent DCM:MeOH increasing from 2 to 10% MeOH. A golden oil (1.75 g, 7.88 mmol, 58%) was obtained. ¹H NMR (400 MHz, CDCl₃): δ = 7.52-7.48 (m, 2H, Ar-H), 7.41-7.37 (m, 3H, Ar-H), 5.93 and 5.81 (2s, 1H, O-CH-O), 4.25-4.11 (m, 2H, CH₂O), 3.69-3.57 (m, 3H, CHO & CH₂CHO), 2.28 (br s, 1H, OH), 1.61-1.56 (m, 6H, 3CH₂). MS-ESI: Calculated for C₁₃H₁₈O₃ m/z = 222.1154, Found m/z = 245.1142 [M+Na]⁺

6.3.12 Phosphoramidite (**18**)⁸⁻⁹

DIPEA (302 mg, 0.4 ml, 2.33 mmol) and 2-cyanoethyldiisopropyl chlorophosphoramidite (359 mg, 0.34 ml, 1.5 mmol) were added under nitrogen to a solution of **17** (260 mg, 1.16 mmol) in anhydrous DCM (3 ml). The reaction mixture was stirred at room temperature for 4 h and DCM (20 ml) was then added. The organic layer was washed with 10% aqueous NaHCO₃ and brine and dried over anhydrous Na₂SO₄. The crude mixture was purified by silica gel column chromatography (EtOAc/cyclohexane/Et₃N) 90:10:2 and a golden oil (95 mg, 0.226 mmol, 19%) was obtained. ¹H NMR (400 MHz, CDCl₃): δ = 7.51-7.47 (m, 2H, Ar-H), 7.39-7.37 (m, 3H,

Ar-H), 5.92 and 5.80 (2s, 1H, O-CH-O), 4.28-4.09 (m, 2H, CH₂O), 3.86-3.76 (m, 2H, CH₂O), 3.71-3.58 (m, 5H, CH₂O & 3CH), 2.62 (t, 2H, CH₂CN), 1.79-1.48 (m, 6H, 3CH₂), 1.29-1.25 (m, 12H, 4CH₃). ³¹P NMR (162 MHz, CDCl₃): δ = 148.53. MS-ESI: Calculated for C₂₂H₃₅N₂O₄NaP m/z = 422.2232, Found m/z = 445.2234 [M+Na]⁺

6.3.13 Automated ODN Synthesis in Dublin (19)⁸⁻⁹

Automated DNA synthesis was carried out on a Beckmann Oligo 1000M DNA synthesizer using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 200 nmole scale. The modified phosphoramidite **18** (0.67 M in dry acetonitrile) was coupled to the support bound oligonucleotide during the last step using an extended coupling time (15 min). The oligonucleotide **19** was treated with a 28% ammonia solution for 2 h and deprotected from the CPG support by keeping the ammonia solution at 55 °C for 16 hours.

6.3.14 Desalting of Oligonucleotide (19) in Preparation for HPLC

The ODN **19** was dissolved in water (100 μ l) and butanol (1 ml) was added. The mixture was agitated vigorously for 30 secs and then the eppendorf was centrifuged for 5 mins at 13,200 rpm. A pellet gathered at the bottom of the tube. The supernatant was removed using a pasteur pipette. The pellet was dissolved in TEAA (0.1 M)/MeCN (9:1) (500 μ l) and the sample agitated for 20 seconds.

6.3.15 Ru(bpy)₂[2-aminoxy-N-(1,10)phenanthroline-5-ylcarbamoyl methyl]acetamide.(PF₆)₂ (2)

The protected Ru complex **15** (10 mg, 11.9 μ mol) was stirred in analytical grade HCL (2 ml) at room temperature for 2.5 hours. The reaction was monitored by HPLC. The retention time shifted from 4.77 mins to 4.32 mins. The solvent was removed by rotary evaporation and the yield taken to be quantitative. MS-ESI: Calculated for C₃₆H₃₁O₃N₉Ru m/z = 738.41, Found 304.5787 [M-129/z]⁺ where z = 2. λ_{max} in MeOH = 285 nm, 450 nm.

6.3.16 General Procedure for Automated ODN Synthesis (Grenoble)

Automated DNA synthesis was carried out on an Applied Biosystems ABI 3400 DNA synthesizer using standard β -cyanoethyl nucleoside phosphoramidite chemistry on a 1 μ mole scale. An extended coupling time was included for the incorporation of any modified phosphoramidites. ODNs were cleaved from the CPG solid support by treatment with 28% aqueous ammonia solution for 1 hour followed by 16 hours at 55 °C.

6.3.17 General Procedure for Oxidation of 3'-diol-ODN to 3'-aldehyde-ODN

The oligonucleotide was dissolved in H₂O (1 ml) and NaIO₄ (20 equivalents) added. The reaction was stirred at room temperature for 2 h. The product was purified by reverse phase C18 column using H₂O as eluent followed by a 50/50 mixture of H₂O and MeCN or using a NAP size exclusion column with water as eluent. UV analysis of the fractions was used to determine the oligonucleotide content. The solvent was removed by rotary evaporation. The product was transferred to an eppendorf with washings and dried by speed-vac. The procedure was repeated for the individual strands. The yield was taken to be quantitative.

6.3.18 General Procedure for Coupling Reaction Between Aldehyde-ODN and Ru Complex (2)

Ammonium acetate buffer (400 μ l, 0.4 M, pH 4.5) was added to the aldehyde-ODN. A solution of the deprotected Ru complex **2** was prepared (~20 μ l, 2 equivalents) and added to the eppendorf. The reaction was stirred at room temperature and monitored by HPLC. Reaction times varied from a few hours (aliphatic aldehyde) to a number of hours (aromatic aldehyde).

6.3.19 General Procedure for Polyacrylamide Gel Electrophoresis (PAGE) Purification of ODNs

The crude ODN conjugate was dissolved in urea (7 M, 400 μ l). The conjugates were purified by denaturing PAGE using acrylamide/bis-acrylamide (19:1, 150 ml), TBE buffer (30 ml), urea (150 g), H₂O (10.5 ml), TEMED (150 μ l) and 10% APS (2.2 ml). The bands containing the conjugates were visualised on the gel by UV shadowing. The band was sliced from the gel, crushed and extracted with water. The conjugates were

desalted using C18 reverse phase chromatography, eluent H₂O (15 x 10 ml) followed by H₂O/MeOH (50/50) or using a NAP size exclusion column with water as eluent. The fractions were combined and the solvent removed by rotary evaporation. The sample was transferred to an eppendorf with washings and dried by speed-vac. Yields were calculated by quantification using An extinction coefficient for Ru(bpy)₂(phen-NH₂).(PF₆)₂, $\epsilon = 15900 \text{ cm}^{-1} \text{ m}^{-1}$ at 455 nm was used for the Ru-ODN conjugates.

6.3.20 General Procedure for MALDI-TOF MS Analysis of Oligonucleotides

A clean sample plate was soaked in ammonium acetate (10 mM) solution for 30 minutes, rinsed with millipore water, followed by ethanol and dried. 3-hydroxypicolinic acid (0.5 M) in 50/50 water/acetonitrile was prepared fresh. Cation exchange beads (Dowex 50 WX8) were regenerated with ammonium acetate (10 mM), filtered and washed with millipore water. An oligonucleotide concentration of 10 μM was used. The beads were placed in the wells of the plate and allowed to dry. The matrix solution (1 μl) and the ODN sample (1 μl) were dropped in each sample well, mixed in the tip and allowed to dry. The beads were carefully removed and the samples were analysed. Measurements were performed in triplicate.

6.3.21 Triangular Silver Nanoplate Preparation¹⁰

6.3.21.1 Preparation of Silver Seed Particles

Water (4.5 ml), poly(sodium styrenesulphonate) (PSSS; 0.25 ml, 500 mg/L; 1000 kDa), trisodium citrate (0.5 ml, 25 mM) and NaBH₄ (0.3 ml, 10 mM, freshly prepared) were placed stirring in a beaker. An aqueous AgNO₃ solution (5 ml, 0.5 mM) was added via syringe pump at a rate of 2 ml/min

6.3.21.2 Preparation of Triangular Silver Nanoplates

Water (5 ml), ascorbic acid (75 μl , 10 mM) and various quantities of seed solution were placed stirring in a beaker. An aqueous AgNO₃ solution (3 ml, 0.5 mM) was added via syringe pump at a rate of 1 ml/min. After synthesis trisodium citrate (0.5 ml, 25 mM) was added to stabilise the particles. The particles were protected from light and stored at 4 °C until use.

6.3.21.3 Five Times Concentrated Preparation of Triangular Silver Nanoplates

Water (5 ml), ascorbic acid (375 μ l, 10 mM) and seed particles (500 μ l) were placed stirring in a beaker. An aqueous AgNO₃ solution (3 ml, 2.5 mM) was added via syringe pump at a rate of 1 ml/min. After synthesis, trisodium citrate (2.5 ml, 25 mM) was added to stabilise the particles. The particles were protected from light and stored at 4 °C until use.

6.3.22 Anion Etching Experiments

Stock aqueous potassium halide solutions (0.25 M) were prepared. The silver nanoplate sol (1 ml) was placed in a cuvette and the potassium halide solution (4 μ l) added to give a final salt concentration of 10 mM. The sample was shaken and the UV-Vis spectrum obtained immediately.

6.3.23 MHA Capping of Triangular Silver Nanoplates

A 1 mM ethanolic solution of 16-mercaptohexadecanoic acid (MHA) was added to the triangular silver nanoplate sol to give a final concentration of 20 μ M. The sample was stirred for 30 minutes at room temperature. The excess MHA was removed by centrifugation.

6.3.24 TiO₂ Coating of Triangular Silver Nanoplates

Titanium tetraisopropoxide (0.25 ml, 0.84 mmol) was placed stirring in a beaker. Acetic acid (0.24 ml, 4.19 mmol, 5 equivalents) was added. A white precipitate was obtained for the complex. Water (1 ml) was added, the solution turned clear. The preformed nanoparticle solution (12 ml) was added. The beaker was covered with parafilm and stirred in the dark.

6.3.25 Preparation of Spherical Gold Nanoparticles¹¹

Hydrogen tetrachloroaurate (III) trihydrate (500 ml, 1 mM) was placed in a round bottom flask (1 L) fitted with a condenser and brought to the boil with vigorous stirring. Trisodium citrate (50 ml, 38.8 mM) was added. The mixture was refluxed for 20 minutes. The solution turned from pale yellow to deep red. The solution was allowed to cool to room temperature and was filtered (0.2 μ m Omnipore filter). The resulting

solution of colloidal particles was characterised by an absorption maximum at 519 nm. Dynamic light scattering gave a diameter of 20 nm and TEM analysis showed the particle size to be 13 nm. The particles were stored in the dark at 4 °C before use.

6.3.26 General Procedure for Cleavage of Trityl Protected Sulfhydryl ODN

A silver nitrate solution (150 μ l, 50 mM) was added to the dry ODN sample and allowed to stand for 30 minutes. A cloudy white suspension was observed. Dithiothreitol (200 μ l, 10mg/ml) was added and allowed to react for 5 minutes. A yellow precipitate formed and was removed by centrifugation. The excess DTT was removed on a NAP column with water as eluent. The ODN was used immediately.

6.3.27 General Procedure for Preparation of Gold-ODN Conjugates

The gold nanoparticle solution was placed in a glass sample vial. The 5'sulfhydryl ODN was added and the sample agitated overnight on an orbital shaker. Sodium phosphate buffer (100 mM, pH 7.4) and SDS (1%, w/v) were added to give final concentrations of 10 mM and 0.01% respectively. The particles were shaken for 30 minutes. Aliquots of NaCl (2 M) were added, increasing the salt concentration by 0.05 M increments over 48 hours, until a final concentration of 0.3 M was achieved. The sample was left to equilibrate overnight. The particles were pelleted by centrifugation. The supernatant was removed and the pellet redispersed. This was repeated three times to remove any unconjugated ODN.

6.3.28 Oligreen Assay

A 250 μ l aliquot of each gold nanoparticle-ODN conjugate was taken and treated with dithiothreitol (DTT) (250 μ l, 0.2M). The sample was placed at 40 °C for 30 minutes. The samples were centrifuged to pellet the particle aggregate and a portion of the supernatant removed for analysis. Oligreen reagent (diluted 200 fold) was added to the samples and the fluorescence measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A standard curve was prepared for each ODN with known ODN concentrations.

6.3.29 Thermal Denaturation of Nanoparticle ODN Assemblies

Equimolar amounts of the complementary nanoparticle ODN conjugates in 0.3 M PBS were mixed and allowed to hybridise and precipitate overnight. The sample was shaken and monitored using a PerkinElmer Lambda 35 UV-Vis spectrophotometer equipped with a peltier temperature controller. Samples were heated at a rate of 0.5 °C/min, from 25 °C to 80 °C, taking a reading every 1 °C. The samples were monitored at 260 nm or at the λ_{max} for the nanoparticle solutions (519 nm for Au and 750 nm for AuAg nanoboxes).

6.3.30 Thermal Denaturation of Free Duplex ODN

A solution of the two complementary sequences [8 μM] was prepared in 0.3 M NaCl, 10 mM sodium phosphate buffer pH 7. The sample was annealed for 5 mins at 95 °C and allowed to cool gradually to room temperature and placed at 4 °C overnight to ensure hybridisation. The change in extinction of the hybridised DNA duplex was monitored at 260 nm by incrementally heating the sample from 5 °C to 80 °C.

6.3.31 Preparation of Hollow Triangular AuAg Nanoboxes¹²

Triangular silver nanoplates were prepared as in Section 6.3.21.2. An excess of ascorbic acid (825 μl , 10 mM) was added to the silver sol and gold (III) chloride trihydrate (10 ml, 0.5 mM) was added at a rate of 1 ml/min via syringe pump. The particles were used immediately without the addition of any stabilisers.

6.3.32 Triangular AuAg Nanobox ODN Conjugates

The AuAg nanobox solution was placed in a glass sample vial. The 5'sulfhydryl ODN was added and the sample agitated overnight on an orbital shaker. Sodium phosphate buffer (100 mM, pH 7.4) and SDS (1%, w/v) were added to give final concentrations of 10 mM and 0.01% respectively. The particles were shaken for 30 minutes. Aliquots of NaCl (2 M) were added, increasing the salt concentration by 0.025 M increments over 48 hours, until a final concentration of 0.1 M was achieved. The sample was left to equilibrate overnight. The particles were pelleted by centrifugation. The supernatant was removed and the pellet redispersed. This was repeated three times to remove any unconjugated ODN.

6.3.33 16-tritylmercaptohexadecanoic acid (39)¹³⁻¹⁴

16-mercaptohexadecanoic acid (**29**) (0.5 g, 1.73 mmol) and trityl chloride (0.723 g, 2.59 mmol, 1.5 equivalents) were placed stirring in anhydrous DMF (5 ml) under nitrogen at room temperature for 48 hours. Chloroform (10 ml) was added and the reaction mixture was extracted with water (3 x 10 ml). The organic layer was dried with sodium sulphate and filtered. The solvent was removed by rotary evaporation. The residue was dissolved in the minimum amount of ether and a small amount of hexane added. The mixture was placed in the freezer overnight. The crystallised, trityl alcohol, was removed by filtration. The filtrate was concentrated by rotary evaporation. Methanol was added and the solid collected by filtration. The solid was washed with methanol and dried. A white solid was obtained with a yield of 62%. ¹H NMR (400 MHz, d₆-DMSO): δ = 1.27 (br s, 24 H, CH₂), 1.64 (m, 4 H, CH₂), 2.13 (t, 2 H, CH₂COOH), 2.35 (t, 2 H, CH₂SH), 7.20-7.32 (br m, 15 H, Trityl CH). ¹³C NMR (100 MHz, d₆-DMSO): δ = MS-ESI: Calculated for C₁₉H₂₀N₄O₃ m/z = 352.1535, Found 353.1627 [M+H]⁺. IR (ATR, cm⁻¹): 2915, 2849, 1692, 1472, 1428, 1409, 1325, 1291, 1273, 1252, 1233, 1214, 1197, 1182, 1117, 1084, 1050, 914, 715, 681.

6.3.34 Succinimidyl 16-tritylmercaptohexadecanoate (40)¹³

16-tritylmercaptohexadecanoic acid (**39**) (100 mg, 0.2 mmol) in dichloromethane (3 mL) was mixed with N,N-dimethylaminopyridine (DMAP) (2.4 mg, 0.02 mmol, 0.1 equivalents), ethyldiisopropylcarbodiimide (57 mg, 0.3 mmol, 1.5 equivalents), and N-hydroxysuccinimide (27.6 mg, 0.24 mmol, 1.2 equivalents). The reaction was stirred at room temperature for 2 h. The solution mixture was extracted with 10% citric acid three times and the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford the white solid product 0.107g (86% yield). ¹H NMR (400 MHz, d₆-DMSO): δ = 1.23 (br s, 24 H, CH₂), 1.58 (m, 4H, CH₂), 2.04 (t, 2 H, CH₂COOH), 2.62 (t, 2H, CH₂SH), 2.81 (s, 4 H, succinimidyl), 7.23-7.33 (m, 15 H, trityl CH). MS-ESI: Calculated for C₃₉H₄₉NO₄S m/z = 627.8884, Found 650.3284 [M+Na]⁺. IR (ATR, cm⁻¹): 3474, 3060, 2919, 2851, 1807, 1782, 1738, 1596, 1487, 1469, 1445, 1422, 1364, 1328, 1291, 1259, 1249, 1213, 1181, 1154, 1066, 1048, 1031, 1010, 1001, 930, 913, 888, 861, 839, 811, 756, 740, 684, 672.

6.3.35 'On-Column' ODN Coupling Reaction

The 5'-amino-CPG resin **42A/B/C** (0.5 μ mole) was placed in a screwcap eppendorf tube. Anhydrous dichloromethane (125 μ l), DIPEA (10 μ l) and succinimidyl 16-tritylmercaptohexadecanoate (**40**) were added. The mixture was agitated on an orbital shaker for 24 hours. The CPG resin was washed with dichloromethane and dried. The resin was treated with 28% ammonia for 1 hour and placed at 55 °C for 16 hours.

6.3.36 Preparation of *tert*-butyl-2-(3,4-dihydroxyphenethylamino)-2-oxoethoxycarbamate (**53**)

Dopamine hydrochloride (**54**) (0.113 g, 0.587 mmol) was reacted with NHS ester of N-(*tert*-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (**55**) (0.22 g, 0.763 mmol, 1.3 equivalents) and DIPEA (0.336 ml, 0.25 g, 1.93 mmol, 3.2 equivalents) in anhydrous DMF (5 ml), at room temperature under nitrogen. The solvent was removed on a high vacuum line to give a golden oil. The mixture was purified by flash chromatography on silica with eluent CHCl_3 :MeOH (9:1) and the solvent removed to yield 111 mg (58 %) of a white sticky solid. ^1H NMR (400 MHz, CDCl_3): δ = 8.51 (s, 1H, NH, O-NH-CO), 8.17 (s, 1H, NH, $\text{H}_2\text{C-NH-CO}$), 6.77 (d, 1H, C_5H), 6.75 (d, 1H, C_6H), 6.56 (dd, 1H, C_3H), 4.27 (s, 2H, $\text{CO-CH}_2\text{-O}$), 3.46 (m, 2H, $\text{CH}_2\text{-NH}$), 2.68 (t, 2H, CH_2), 1.44 (s, 9H, 3 $\times$ CH_3). ^{13}C NMR (100 MHz, CDCl_3): δ = 169.12 (C_9), 157.45 (C_{11}), 143.86 (C_2), 142.62 (C_1), 130.27 (C_4), 119.99 (C_5), 115.22 (C_3), 114.75 (C_6), 82.38 (C_{12}), 75.24 (C_{10}), 40.36 (C_7), 34.35 (C_8), 27.62 (C_{13-15}). MS-ESI: Calculated for $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_6$ m/z = 326.1478, Found 325.1396 $[\text{M-H}]^-$. IR (ATR, cm^{-1}): 3186, 2933, 1781, 1711, 1696, 1518, 1436, 1386, 1368, 1282, 1252, 1210, 1162, 1109, 1070, 997, 847, 814, 782, 712.

6.3.37 Deprotection of *tert*-butyl-2-(3,4-dihydroxyphenethylamino)-2-oxoethoxycarbamate

The sample **53** was placed stirring in DCM/TFA (50/50, v/v) with 5% triisopropylsilane for 2 hours at room temperature. Acetone was added to form the acetone oxime **56**. MS-ESI: Calculated for $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_4$ m/z = 266.1267, Found 289.1100 $[\text{M}+\text{Na}]^+$

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Chapter 7

Conclusions and Future Work

7.1 Conclusions

The overall aim of this thesis was to develop routes to a potential sensitive DNA detection probe by combining metal complexes (ruthenium complexes) with nanoparticles (triangular silver nanoplates) using DNA (oligonucleotides). The results described in this thesis outline the advances that have been made in the preparation of the individual components for this complex system.

Ruthenium complex oligonucleotide (ODN) conjugates were successfully prepared by oxime ligation. A phenanthroline ligand modified with an oxyamine, was synthesised and incorporated into a ruthenium complex. The Ru complex was reacted with a variety of aldehyde modified ODNs to give Ru-ODN conjugates in good yield. The method was found to be very efficient and improved on previous 'off-column' strategies. Initial experiments utilised a diol modified ODN, which necessitated oxidation with sodium periodate to yield the corresponding aldehyde. Further reactions incorporated a benzaldehyde directly into the ODN and the aromatic aldehyde was shown to be reactive enough for oxime ligation. This was a significant result extending the compatibility of the oxime approach with other chemistries. A bifunctionalised ODN with a 3'-disulfide and a 5'-Ru complex was successfully prepared. Previously this would have been impossible because the disulfide would not withstand the sodium periodate oxidation. There is still a great need for efficient methods for labelling nanoparticles. This approach offers a way to incorporate a wide range of modifications via oximation with control over the distance from the nanoparticle achievable by varying the length of the ODN chain.

The preparation of ODN conjugates with triangular silver nanoplates proved to be much more challenging than originally anticipated as: (1) Triangular silver nanoplates are very sensitive to degradation in the presence of anions. (2) The silver sulfur bond is weaker than that of gold (3) The surface packing on plate like structures is greatly reduced compared with spherical particles.

However triangular silver nanoplates were successfully stabilised using two approaches; thiol capping and titania coating. A long-chain alkyl thiol, 16-mercaptohexadecanoic

acid (MHA) was shown to form a surface assembled monolayer on the triangular silver nanoplates. Significantly the MHA capped nanoplates were shown to be stable even in 100 mM NaCl solutions. The MHA capped triangular silver nanoplates were successfully coated with a thin layer of titania and characterised. To the best of our knowledge this is the first report for the preparation of Ag@TiO₂ particles with anisotropic silver particles. The beauty of the adopted stabilisation approaches was that functionality was introduced to the nanoparticle surface, in each case, that could be used for ODN conjugation. A long-chain alkyl thiol modified ODN was prepared by an 'on-column' strategy. The successful preparation was confirmed by ESI-MS, but unfortunately the modified ODN was found to be extremely hydrophobic and excluded purification by HPLC. A catechol modified ODN was prepared by oximation. Alas due to time constraints the functionalisation of nanoparticles with these ODNs was not achieved.

The gold coating of the triangular silver nanoplates and subsequent formation of hollow triangular AuAg nanoboxes was developed by a colleague, Dr. Damian Aherne during the course of this PhD. Both spherical gold nanoparticles and AuAg nanoboxes were densely functionalised with ODNs. To the best of our knowledge, this is the first preparation of nanobox-ODN conjugates. Assemblies were formed from mixing complementary ODN conjugates and characteristic denaturing profiles were observed by UV-Vis and DLS for the 'disassembly' of the systems with increasing temperature. The results obtained for the spherical gold nanoparticles were in agreement with previous literature reports. Interestingly, for the nanobox system, a sharp melting temperature was obtained, but at a lower temperature than the spherical gold system and in comparison with literature reports involving gold nanoplates. Interesting assemblies were successfully prepared from mixing complementary spherical gold ODN conjugates with nanobox ODN conjugates.

7.2 Future Work

Much success has been achieved in preparing the components required for the assembly of a potential sensitive DNA detection probe. We are now at a stage where we can combine the nanoparticle-ODN conjugates and Ru-ODN conjugates to investigate their

potential in this field. We have a diverse toolkit that can be utilised to combine and investigate a number of systems. The combination of Ru-ODN conjugates with the AuAg nanoboxes could offer a route for the observation of MEF. An investigation into the DNA detection capabilities of the nanobox ODN conjugates should prove interesting as the nanobox systems should have an increased sensitivity compared with spherical gold nanoparticles. The efficient oximation strategy could be extended to incorporate other luminescent reporter groups. Chapter 5 detailed the preparation of ODNs for use with the stabilised triangular silver nanoplates described in Chapter 3, but the preparations require optimisation. Based on a recent publication the catechol modified ODN also has potential for the direct preparation of ODN conjugates with silver nanoparticles.¹

The field of plasmonics is a rapidly growing area of research, and as outlined in the introduction, it is exciting to think of the prospects of implementing the developed technologies from the research lab, into practical devices, for use in the clinic. A lot has been achieved in this field during the last decade and we can be sure that more is yet to come.

7.3 References

1. Lin, Y.; Chen, C.; Wang, C.; Pu, F.; Ren, J.; Qu, X. *Chem. Commun.* **2011**, 47, 1181.