The Development of Metal Complexes as Components of Fluorescent and Nuclear Imaging Probes

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Graduate Program in Chemistry

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Abstract

The advent of molecular imaging as a discipline has drastically improved our ability to understand the biochemical and cellular events that drive life, death and disease. This thesis will document the discovery of new metal chelators for Re/\(^{99m}\)Tc, new methods of incorporating these metals into peptide structures, as well as new fluorescent compounds. These novel methods and compounds may be used in the development of molecular imaging probes for single photon emission computed tomography (SPECT) and optical imaging techniques, with applications for differentiating cancerous tissue from benign and healthy.

Chapter 2 discusses the development of a dual modality Re/\(^{99m}\)Tc imaging probe for fluorescence/SPECT imaging for evaluating CXCR4 expression in cancer cells. The T140 derivative contains a 4-amino-1,8-naphthalimide chelator to bind rhenium or technetium-99m for fluorescence or SPECT imaging. The rhenium coordinated peptide retained high affinity for CXCR4 and was able to detect cells expressing the receptor by fluorescence microscopy. However, little uptake of the technetium-99m labelled peptide was observed in CXCR4 expressing tumors in a murine model.

Chapter 3 aims to address the shortcomings of the naphthalimide chelation system discussed in chapter 2. Three 4-amino-1,8-naphthalimides containing chelators with a positive, neutral or negative charge were synthesized and coordinated with rhenium. All three complexes had photophysical properties that were compatible with confocal fluorescence microscopy and showed uptake in cells that was dependent on charge. All three ligands were radiolabelled with technetium-99m in high radiochemical yield and purity.

Chapter 4 discusses the development of a \(\beta\)-hairpin forming peptide containing a 2,2'\(^{-}\)bipyridine moiety to chelate Re/\(^{99m}\)Tc. A novel amino acid based on 3,3'-diamino-2,2'\(^{-}\)bipyridine was synthesized, and incorporated into a peptide. Circular dichroism spectroscopy indicated a lack of secondary structure, but formation of secondary structures resembling a \(\beta\)-sheet was possible upon the addition of 2,2,2-trifluoroethanol (TFE). However, metal coordination resulted in unstable complexes that were unable to be isolated.
Chapter 5 discusses the development of 2,2′-bipyridine-based fluorophores for the detection of intracellular Zn(II). Two benzimidazole functionalized bipyridines were found to have a fluorescence response to Zn(II) that was compatible for detection by confocal fluorescence microscopy. Both ligands displayed fluorescence in benign prostate cells due to Zn(II) coordination but not in prostate cancer cells. These ligands are promising candidates for the detection of Zn(II) in ex vivo prostate tissues for differentiating malignant tissue from benign and healthy.

Keywords

Molecular Imaging Probes, CXCR4, Rhenium, Technetium-99m, Zinc, Fluorescence, SPECT, Bipyridine, Naphthalimide, Peptide
Co-Authorship Statement

Chapter 2 is a manuscript in preparation. All work was performed by William Turnbull except for the *ex vivo* biodistribution studies which were performed by Dr. Lihai Yu, and the fluorescence quantum yield measurements and spectra collection which were performed by Dr. Mark Milne.

Chapter 3 is a manuscript in preparation. Synthesis of **L1**, **L2**, **L3**, **Re-L1**, **Re-L2** and **Re-L3**, acquisition of UV/Vis and fluorescence spectra, and all cell culture and *in vitro* cell experiments were performed by William Turnbull. Synthesis of compounds **1-3** and radiolabelling with technetium-99m was performed by Emily Murrell. Determination of fluorescence quantum yields, compound characterization by NMR & MS, and development of synthetic methodology was performed by Mariel Bulcan-Gnirss.

Chapter 5 was adapted from a manuscript published in *Chem Eur. J*. 2018 (DOI 10.1002/chem.201803051). All work was performed by William Turnbull.
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# Table of Contents

Abstract ........................................................................................................................................... i
Co-Authorship Statement .................................................................................................................. iii
Acknowledgments ............................................................................................................................ iv
Table of Contents ............................................................................................................................. v
List of Tables ..................................................................................................................................... ix
List of Figures .................................................................................................................................... x
List of Schemes ............................................................................................................................... xvii
List of Abbreviations ...................................................................................................................... xviii
Chapter 1 ......................................................................................................................................... 1
  1 Introduction ................................................................................................................................. 1
    1.1 Molecular Imaging .................................................................................................................. 1
    1.2 Molecular Imaging Probes .................................................................................................... 2
      1.2.1 Targeting Entity ............................................................................................................. 2
      1.2.2 Signaling Entity .............................................................................................................. 4
    1.3 Single Photon Emission Computed Tomography ................................................................. 7
    1.4 Technetium-99m .................................................................................................................... 8
    1.5 Rhenium ............................................................................................................................... 12
    1.6 C-X-C Chemokine Receptor 4 ............................................................................................ 13
    1.7 Biological Roles of Zinc ......................................................................................................... 18
      1.7.1 Zinc in Prostate Cancer ................................................................................................. 19
      1.7.2 Fluorescent Probes Responsive to Zinc ...................................................................... 20
    1.8 Summary ............................................................................................................................... 25
    1.9 References ............................................................................................................................. 26
Chapter 2 ........................................................................................................................................ 36
A Dual Modality $^{99m}$Tc/Re(I)-Labelled T140 Analogue for Imaging of CXCR4 Expression .................................................................36

2.1 Introduction ..............................................................................36

2.2 Results and Discussion..........................................................38

  2.2.1 Design and Synthesis..........................................................38

  2.2.2 In Vitro Evaluation of Re-7 .................................................40

  2.2.3 $^{99m}$Tc-labelling and In Vitro Cellular Uptake ....................42

  2.2.4 Ex Vivo Biodistribution Studies ..........................................43

2.3 Conclusions ..............................................................................44

2.4 Experimental ...........................................................................45

  2.4.1 General Experimental .......................................................45

2.5 References ..............................................................................53

Chapter 3 ..........................................................................................56

3 A Study of $^{99m}$Tc/Re-Tricarbonyl Complexes of 4-Amino-1,8-Naphthalimides ....56

3.1 Introduction ..............................................................................56

3.2 Results and Discussion..........................................................58

  3.2.1 Synthesis and Structural Evaluation ....................................58

  3.2.2 Photophysical Properties ..................................................60

  3.2.3 Confocal Microscopy .........................................................62

  3.2.4 Radiolabelling with Technetium-99m .................................63

3.3 Conclusions ..............................................................................65

3.4 Experimental ...........................................................................65

  3.4.1 General Experimental .......................................................65

3.5 References ..............................................................................72

Chapter 4 ..........................................................................................74

4 The Development of $^{99m}$Tc/Re(I) Tricarbonyl [2+1] Complexes with Peptides Containing 2,2’-Bipyridine Residues as $\beta$-Turn Mimics ........................................74
4.1 Introduction .............................................................................................................. 74
  4.1.1 Protein Secondary Structure ........................................................................... 74
  4.1.2 Imaging Probe Design: Integrated Versus Pendant ........................................ 75
  4.1.3 2,2'-Bipyridine as a Bidentate Chelator ......................................................... 76
4.2 Results and Discussion .......................................................................................... 78
  4.2.1 Synthesis of 3,3’-Diamino-2,2’-Bipyridine Amino Acid ................................. 78
  4.2.2 Design of a β-Hairpin Peptide ......................................................................... 79
  4.2.3 Synthesis of a β-Hairpin Peptide ...................................................................... 81
  4.2.4 Structural Evaluation by Circular Dichroism Spectroscopy ............................. 82
  4.2.5 Formation of the [2+1] Rhenium Complex and Radiolabelling with Technetium-99m .......................................................... 83
4.3 Conclusions ............................................................................................................. 85
4.4 Experimental .......................................................................................................... 85
4.5 References ................................................................................................................ 90

Chapter 5 ......................................................................................................................... 92

5 Amino-Substituted 2,2’-Bipyridine Ligands as Fluorescent Indicators for Zn(II) and Applications for Fluorescence Imaging of Prostate Cells ........................................... 92

5.1 Introduction ............................................................................................................. 92
5.2 Results and Discussion .......................................................................................... 93
  5.2.1 Synthesis .......................................................................................................... 93
  5.2.2 UV/Vis Absorption Studies ............................................................................... 94
  5.2.3 Fluorescence Studies ........................................................................................ 100
  5.2.4 Effects of Other Metal Ions on the Absorption and Emission Spectra ......... 105
  5.2.5 Confocal Microscopy of Prostate Cell Lines .................................................. 106
5.3 Conclusions ............................................................................................................. 108
5.4 Experimental .......................................................................................................... 109
5.5 References ................................................................................................................ 112
Chapter 6.................................................................................................................. 115

6 Conclusions ............................................................................................................. 115

6.1 References ......................................................................................................... 115

Appendix A: Additional Data for Chapter 2.............................................................. 122

Appendix B: Additional Data for Chapter 3.............................................................. 126

Appendix C: Spectra of Compounds from Chapter 4................................................. 141

Appendix D: Additional Data for Chapter 5.............................................................. 147

Curriculum Vitae......................................................................................................... 161
List of Tables

Table 2.1: Photophysical Properties of DPA-Naph Peptide Conjugates..................................................39

Table 3.1: Photophysical properties of the naphthalimide ligands and their Re(I)-tricarbonyl complexes. ..........................................................................................................................................................62

Table 5.1: UV/Vs absorption characteristics of bipyridine ligands and their Zn(II) complexes in methanol .........................................................................................................................................................95

Table 5.2: Photophysical properties of compound 2 in various solvents.................................................96

Table 5.3: Photophysical properties of compound 4 in various solvents.................................................97

Table 5.4: Photophysical properties of compound 7 in various solvents.................................................98

Table 5.5: Fluorescence properties of the bipyridine ligands and their Zn(II) complexes in methanol. .........................................................................................................................................................104

Table A1: Ex vivo biodistribution data for $^{99m}$Tc-7 in NOD/SCID mice two hours post-injection .................................................................................................................................125

Table C1: Photophysical Properties of Compound 6 in Various Solvents............................................159
List of Figures

Figure 1.1: Comparison of spatial resolution and molecular sensitivity between imaging modalities......................................................................................................................................................................................2

Figure 1.2: Examples of molecular imaging probes for PET ([18F]FDG), SPECT ([99mTc]Tc-HYNIC-TOC) and OI (RGD-Cy5.5)..................................................................................................................................................................................................................3

Figure 1.3: Structures of some common fluorophores (A-C), bifunctional chelators (D-F) and prosthetic groups (G and H) used in signaling components of molecular imaging probes......5

Figure 1.4: Methods for incorporating a radioisotope into PET and SPECT molecular imaging probes..................................................................................................................................................................................................................7

Figure 1.5: Radioactive decay scheme of 99Mo to the stable isotope 99Ru.................................................9

Figure 1.6: Examples of "99mTc-essential" and "99mTc-tagged" radiopharmaceuticals. ............10

Figure 1.7: 99mTc(I)-tricarbonyl complexes formed with a wide variety of ligands. ...............11

Figure 1.8: Two-component binding of a carbonyl ligand to a metal center.................................12

Figure 1.9: Examples of luminescent Re(I)-tricarbonyl complexes with polypyridyl ligands. ..................................................................................................................................................................................................................13

Figure 1.10: The development of T140 from polyphemusin II .......................................................15

Figure 1.11: Peptide and small molecule inhibitors of CXCR4. ......................................................16

Figure 1.12: PET and SPECT molecular imaging agents targeting CXCR4.................................17

Figure 1.13: Biological distribution and functions of zinc.................................................................................19

Figure 1.14: Evolution of zinc responsive fluorescent probes: TSQ, to the more cell membrane permeable ZnAF-R2, and the visible light excitable FluoZin-1, and the cell membrane permeable variant FluoZin-1 AM. ........................................................................................................................................................................................................21

Figure 1.15: The process of PeT as applied to Zn(II) indicators..........................................................23
Figure 1.16: ICT mechanism in non-polar and polar solvents (top), and upon coordination to Zn(II) (bottom). ...................................................................................................................................................................................24

Figure 1.17: Process of ESIPT occurring in HBO, and Zn(II) coordination suppressing ESIPT...................................................................................................................................................................................25

Figure 2.1: Structure of T140 (6) and the newly synthesized derivative DPA-Naph-T140 (7). ........................................................................................................................................................................................................38

Figure 2.2: Representative competitive binding curves of T140 (black curve) and Re-7 (grey curve) using U87.CD4.CXCR4 cells with [125I]-SDF-1 as the competing radioligand. ...........40

Figure 2.3: Confocal microscope images of U87.CD4.CXCR4 cells (A, C) and U87.CD4 cells (B, D) incubated with Re-7...................................................................................................................................................................................41

Figure 2.4: Confocal microscope images of BPH-1 (A, D), PC-3 (B, E) and DU 145 cells (C, F) incubated with Re-7 ...................................................................................................................................................................................42

Figure 2.5: (Left) UV-HPLC chromatogram of Re-7 (blue) overlaid with the radio-HPLC chromatogram of [99mTc]Tc-7 (black). (Right) In vitro cellular uptake of [99mTc]Tc-7 in U87.CD4.CXCR4 cells in comparison to U87.CD4 cells..........................................................................................................................................................43

Figure 2.6: Ex vivo biodistribution of [99mTc]Tc-7 in U87.CD4.CXCR4 tumor bearing mice at 2 hours post-injection (n = 3). ...................................................................................................................................................................................44

Figure 3.1: Structures of the three naphthalimide ligands coordinated to Re or 99mTc...........57

Figure 3.2: 1H NMR spectroscopy chemical shifts of the chelator arm methylene (red) and linker ethylene (black) protons of L1 (top left) and L3 (top right) and changes upon rhenium coordination (bottom). ...................................................................................................................................................................................60

Figure 3.3: UV/Vis absorption (A-C) and fluorescence excitation/emission spectra (D-F) of the ligands (λEx = 440 nm) and their rhenium complexes (λEx = 425 nm) in CH3CN (20 µM). ...................................................................................................................................................................................61
Figure 3.4: Confocal fluorescence microscope images of OVCAR-8 cells (A-C 40X, D-F 100X) incubated with: (A, D) Re-L1; (B, E) Re-L2; and (C, F) Re-L3 at a concentration of 20 µM (λ_ex = 458 nm).

Figure 3.5: HPLC chromatograms (System II, 20-80% Solvent A) of the 99mTc-labelled ligands (blue) and their Re-coordinated standards (pink).

Figure 4.1: Parallel (left) versus anti-parallel (right) β-sheets.

Figure 4.2: Pendant (left) versus integrated (right) design.

Figure 4.3: Square planar Cu(II) complexes nucleating β-sheet formation.

Figure 4.4: Synthesis of isostructural [2+1] complexes of Re and 99mTc.

Figure 4.5: Proposed Fmoc-protected 2,2′-bipyridine amino acid.

Figure 4.6: β-Hairpin peptide BH8 (top). 2,2′-Bipyridine β-hairpin peptide 7 (bottom).

Figure 4.7: Circular dichroism spectra of peptide 7 in water (blue) and 30% TFE (orange).

Figure 4.8: Coordination of peptide 7 and 4-DMAP with rhenium to form a [2+1] chelation system.

Figure 4.9: UHPLC chromatograms of the Re coordinated peptide 7 (top) and 99mTc-labelled peptide 7 (bottom).

Figure 5.1: Amine substituted 2,2′-bipyridine ligands.

Figure 5.2: Proposed ICT excited state of compound 2.

Figure 5.3: UV/Vis absorption spectra of compound 4 in (A) non-polar and (B) polar solvents.

Figure 5.4: UV/Vis absorption spectra of compound 1 (20 µM) (A) compound 2 (B) compound 4 (C) compound 6 (D) and compound 7 (E) at a concentration of 20 µM in methanol upon titration with ZnSO₄.
Figure 5.5: Fluorescence emission spectra of compound 4 in a range of solvents. ..........101

Figure 5.6: Lippert plots of compound 2 (orange), compound 4 (blue) and compound 7 (green) ..........................................................................................................................................................................................102

Figure 5.7: Fluorescence emission spectra of compound 1 (A) compound 2 (B) compound 4 (C) compound 6 (D) and compound 7 (E) at a concentration of 20 µM in methanol upon titration with ZnSO$_4$ ................................................................................................................................................................................................103

Figure 5.8: UV/Vis absorbance and fluorescence emission spectra of compound 6 (A and D, black) and compound 7 (B and D, black) in the presence of Fe(III) (brown), Cu(II) (blue), Mg(II) (magenta), Ca(II) (green) and Zn(II) (red). ..........................................................................................................................................................................................106

Figure 5.9: (A) BPH-1, (B) DU 145 and (C) PC-3 cells incubated with compound 6 (20 µM) for 60 minutes. (D) BPH-1, (E) DU 145 and (F) PC-3 cells incubated with compound 6 (20 µM) for 60 minutes, followed by the addition of ZnSO$_4$ (200 µM) for 60 minutes........107

Figure 5.10: (A) BPH-1, (B) DU 145 and (C) PC-3 cells incubated with compound 7 (20 µM) for 60 minutes. (D) BPH-1, (E) DU 145 and (F) PC-3 cells incubated with compound 7 (20 µM) for 60 minutes, followed by the addition of ZnSO$_4$ (200 µM) for 60 minutes......108

Figure A1: $^1$H NMR spectrum (400 MHz; CDCl$_3$) of compound 5 (DPA-Naph-OH). ..........122

Figure A2: $^{13}$C NMR spectrum (100 MHz; CDCl$_3$) of compound 5 (DPA-Naph-OH). ......123

Figure A3: HPLC chromatogram (10-70% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide 6 (T140). ..................................................................................................................................................................................................................................................................................123

Figure A4: HPLC chromatogram (20-80% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide 7 (DPA-Naph-T140). ..................................................................................................................................................................................................................................................................................124

Figure A5: HPLC chromatogram (20-80% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide Re-7 (Re(CO)$_3$-DPA-Naph-T140). ..................................................................................................................................................................................................................................................................................124

Figure B1: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of compound 3.................................126

Figure B2: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of compound 3.................................127
Figure B3: $^1$H NMR spectrum (400 MHz; CDCl$_3$) of L1. ..................................................128

Figure B4: $^{13}$C NMR spectrum (100 MHz; CDCl$_3$) of L1. ..................................................129

Figure B5: $^1$H NMR spectrum (400 MHz; acetone-$d_6$) of Re-L1 .............................................130

Figure B6: $^{13}$C NMR spectrum (100 MHz; acetone-$d_6$) of Re-L1 .............................................131

Figure B7: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of L2 .....................................................132

Figure B8: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of L2 .....................................................133

Figure B9: $^1$H NMR spectrum (400 MHz; CD$_3$CN) of Re-L2 ...................................................134

Figure B10: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of L3 .....................................................135

Figure B11: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of L3 .....................................................136

Figure B12: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of Re-L3 ...................................................137

Figure B13: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of Re-L3 ...................................................138

Figure B14: Confocal microscope images of OVCAR-8 cells incubated with Re-L1 (A, D) Re-L2 (B, E) and Re-L3 (C, F) at a concentration of 20 $\mu$M ($\lambda_{ex} = 458$ nm, 40x objective). .................................................................................................................................139

Figure B15: Confocal microscope images ($\lambda_{ex} = 458$ nm, 100x objective) of OVCAR-8 cells incubated with Re-L1 (left), Re-L2 (middle) and Re-L3 (right) .........................................................................................140

Figure C1: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 1 ..................................................141

Figure C2: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 1 ..................................................141

Figure C3: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 2 ..................................................142

Figure C4: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 2 ..................................................142

Figure C5: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 3 ..................................................143
Figure C6: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 3 .................................. 143

Figure C7: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 4 ....................................... 144

Figure C8: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 4 ....................................... 144

Figure C9: $^1$H NMR spectrum (DMSO-$d_6$; 400 MHz) of compound 5 .................. 145

Figure C10: $^{13}$C NMR spectrum (DMSO-$d_6$; 100 MHz) of compound 5 .................. 145

Figure C11: $^1$H NMR spectrum (DMSO-$d_6$; 400 MHz) of compound 6 .................. 146

Figure C12: $^{13}$C NMR spectrum (DMSO-$d_6$; 100 MHz) of compound 6 .................. 146

Figure D1: $^1$H NMR Spectrum (CDCl$_3$; 400 MHz) of Compound 1 .................. 147

Figure D2: $^{13}$C NMR Spectrum (CDCl$_3$; 100 MHz) of Compound 1 .................. 148

Figure D3: $^1$H NMR Spectrum (DMSO-$d_6$; 400 MHz) of Compound 2 .................. 149

Figure D4: $^{13}$C NMR Spectrum (DMSO-$d_6$; 100 MHz) of Compound 2 .................. 150

Figure D5: $^1$H NMR Spectrum (DMSO-$d_6$; 400 MHz) of Compound 4 .............. 151

Figure D6: $^{13}$C NMR Spectrum (DMSO-$d_6$; 100 MHz) of Compound 4 .............. 152

Figure D7: $^1$H NMR Spectrum (1:1 CDCl$_3$:TFA-$d$; 400 MHz) of Compound 6 .......... 153

Figure D8: $^{13}$C NMR Spectrum (1:1 CDCl$_3$:TFA-$d$; 100 MHz) of Compound 6 .......... 154

Figure D9: $^1$H NMR Spectrum of (CDCl$_3$; 400 MHz) Compound 7 .................. 155

Figure D10: $^{13}$C NMR Spectrum (CDCl$_3$; 100 MHz) of Compound 7 .................. 156

Figure D11: UV/Vis absorption spectra of: (A) compound 2; (B) compound 4; (C) compound 6; and (D) compound 7 in various solvents. ................................................................. 157

Figure D12: Fluorescence emission spectra of compound 2 (20 µM) in non-polar (left) and polar solvents (right) with 40 µM DIPEA ................................................................. 157
Figure D13: Fluorescence emission spectra of compound 6 (20 µM) (top) and compound 7 (20 µM) in non-polar (bottom left) and polar (bottom right) solvents.................................158

Figure D14: Fluorescence excitation and emission spectra of compound 2 in methanol (black), upon the addition of base (blue) and upon the addition of acid (red)......................159

Figure D15: Fluorescence excitation and emission spectra of the Zn(II) complexes of: (A) compound 1; (B) compound 2; (C) compound 4; (D) compound 6; and (E) compound 7 in methanol. ........................................................................................................................................160
List of Schemes

Scheme 2.1: Synthesis of compound 5 (DPA-Naph-OH). .................................................................39

Scheme 3.1: Synthetic route to the three naphthalimide ligands and their Re(I)-tricarbonyl complexes. ..........................................................................................................................59

Scheme 4.1: Initial synthesis of 3,3'-diamino-2,2'-bipyridine. .........................................................78

Scheme 4.2: Synthesis of 3,3'-diamino-2,2'-bipyridine amino acid (6). ............................................79

Scheme 5.1: Syntheses of bipyridine derivatives 4, 6 and 7...............................................................94
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>4-DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>4-FB</td>
<td>4-fluorobenzoyl</td>
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<td>Alloc</td>
<td>allyloxy carbonyl</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxy methyl</td>
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<td>1,4-dioxane</td>
</tr>
<tr>
<td>EDG</td>
<td>electron donating group</td>
</tr>
<tr>
<td>EI</td>
<td>electron ionization</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ESIDPT</td>
<td>excited-state intramolecular double proton transfer</td>
</tr>
<tr>
<td>ESIPT</td>
<td>excited-state intramolecular proton transfer</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>EWG</td>
<td>electron withdrawing group</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDG</td>
<td>2-fluoro-2-deoxyglucose</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethylcarbonyl</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate</td>
</tr>
<tr>
<td>HBO</td>
<td>2-(2’-hydroxyphenyl)benzoxazole</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HCTU</td>
<td>O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>HYNIC</td>
<td>hydrazinonicotinic acid</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICT</td>
<td>intramolecular charge transfer</td>
</tr>
<tr>
<td>LUMO</td>
<td>lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MAG₃</td>
<td>mercaptoacetyl triglycine</td>
</tr>
<tr>
<td>MBHA</td>
<td>methylbenzhydryl amine</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MLCT</td>
<td>metal-to-ligand charge transfer</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSAP</td>
<td>multifunctional single attachment point</td>
</tr>
<tr>
<td>Naph</td>
<td>1,8-naphthalimide</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidinone</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>non-obese diabetic/severe combined immunodeficiency</td>
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</tbody>
</table>
OI  optical imaging
OTf  trifluoromethanesulfonate
Pbf  2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PBS  phosphate buffered saline
PCa  prostate cancer
PeT  photoinduced electron transfer
PET  positron emission tomography
RPMI  Roswell Park Memorial Institute medium
ROS  reactive oxygen species
RT  room temperature
SAAC  single amino acid chelate
SDF-1  stromal cell-derived factor 1
SFB  $N$-succinimidyl 4-fluorobenzoate
SPECT  single photon emission computed tomography
SPPS  solid-phase peptide synthesis
SS  Stoke’s shift
Suc  succinamide
TAMRA  5-carboxytetramethylrhodamine
TBME  tert-butylmethyl ether
TFA  trifluoroacetic acid
TFE  2,2,2-trifluoroethanol
TIPS  triisopropylsilane
TLC  thin-layer chromatography
TOC  Tyr$^3$-Octreotide
Trt  triphenylmethyl
TSQ  $N$-(6-methoxyquinolin-8-yl)-p-toluenesulfonamide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHPLC</td>
<td>ultra high-performance liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible</td>
</tr>
<tr>
<td>ZIP</td>
<td>zrt-like, irt-like protein</td>
</tr>
<tr>
<td>ZnT</td>
<td>zinc transporter</td>
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</tbody>
</table>
Chapter 1

1 Introduction

1.1 Molecular Imaging

Living organisms are a complex medley of synergistic biochemical and cellular events, and unveiling these processes that drive life, death and disease is a formidable task. In the past, our progress towards this goal had been impaired by our inability to observe these processes in living organisms as they occur, without perturbing the natural environment in which they take place. The advent of molecular imaging as a discipline has drastically improved our ability to understand these processes. While the precise definition of the term molecular imaging may be different amongst various researchers and scientists, the definition put forth by Gambhir & James will be used here: the non-invasive, real-time visualization of biochemical processes at the cellular and molecular level within living cells, tissues or organisms.¹ Not only do molecular imaging techniques allow for fundamental research into biochemical and cellular processes, they also have use in a clinical setting, allowing for the diagnosis and monitoring of disease which can be used to devise treatments that may preclude the need for invasive procedures such as biopsies.

Molecular imaging is an incredibly broad field that encompasses a number of imaging modalities including, but not limited to: positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and optical fluorescence imaging (OI). Each of these imaging modalities has its own inherent strengths and weaknesses in terms of molecular sensitivity and spatial resolution (Figure 1.1) as well as depth of tissue penetration and cost.² Nuclear imaging modalities such as PET and SPECT offer remarkable sensitivity and unlimited tissue penetration at the cost of requiring ionizing radiation. MRI techniques do not require ionizing radiation but suffer from low sensitivity. OI has limited clinical use due to the limited depth of tissue penetration.
1.2 Molecular Imaging Probes

Molecular imaging modalities often require a method to create a signal or image contrast that is detectable by external detectors. In order for this signaling entity to reach the organs, tissues or cellular compartments of interest it must be chemically attached to a targeting entity that is able to interact with molecular targets in the region of interest. These molecular targets may be receptors, transporters or enzymes. The combination of a targeting entity and signaling entity for the purpose of molecular imaging is called a molecular imaging probe, though other terms such as molecular imaging agent are often used. Figure 1.2 depicts three examples of molecular imaging probes. Further details about the targeting and signaling entities will be presented in this section.

1.2.1 Targeting Entity

The choice of targeting entity is critical, as this component can determine many of the pharmacological properties of the molecular imaging agent. While it is difficult to predict the pharmacokinetics of an imaging agent, it is widely acknowledged that molecular size,
charge, lipophilicity and binding affinity to the target of interest are all contributing factors. The most common targeting entities are derived from small molecules, peptides or monoclonal antibodies (mAbs).

Figure 1.2: Examples of molecular imaging probes for PET ($^{[18F]}$FDG), SPECT ($^{[99mTc]}$Tc-HYNIC-TOC) and OI (RGD-Cy5.5).

Small molecules (usually <500 Da) typically offer good pharmacokinetic properties such as fast accumulation in the target tissues, and quick clearance from the vasculature, giving good signal-to-background contrast. However, due to their small size, they are often limited in the type of signaling components that may be incorporated, as large fluorophores and metal chelators can negatively impact the small molecule’s ability to bind to and reach its target. On the opposite end of the spectrum, mAbs are typically on the size range of 150 kDa, and due to their large size, may be appended with large fluorophores and metal chelators without affecting the incredibly high affinity and specificity that they have for their molecular targets. However, mAbs have long biological half-lives (typically days to
weeks) and are retained in the blood for long periods of time which can result in low signal-to-background ratios, and therefore poor quality images. Peptides have found a place as the “best of both worlds” between small molecules and mAbs. They offer the benefits of small molecules such as membrane permeability and fast clearance kinetics, but retain the high stability, target specificity and tolerance towards modification that mAbs possess. Peptides are also easily synthesized by solid-phase peptide synthesis, and are easily modified to improve in vivo stability, pharmacokinetics and affinity for the molecular target of interest.

1.2.2 Signaling Entity

A wide variety of signaling entities are available depending on the chosen imaging modality. Optical imaging requires the addition of a fluorescent molecule to the targeting entity. For in vitro cellular microscopy, fluorophores with emission wavelengths ranging from 400-600 nm are most widely used such as fluorescein isothiocyanate (FITC), 7-amino-4-methylcoumarin (AMC) and 5-carboxytetramethylrhodamine (TAMRA) (Figure 1.3, A-C). For in vivo imaging applications however, wavelengths in the near infrared (NIR) window (650-900 nm) are required due to signal attenuation by tissues in the visible range. Far-red cyanine dyes are the most commonly used fluorophores for this purpose. Various chemical methods such as amidation, thiourea formation, and click chemistry may be used to conjugate fluorophores to targeting entities such as peptides and mAbs.
**Figure 1.3:** Structures of some common fluorophores (A-C), bifunctional chelators (D-F) and prosthetic groups (G and H) used in signaling components of molecular imaging probes. (A) Fluorescein isothiocyanate (FITC), (B) 7-amino-4-methylcoumarin (AMC), (C) 5-carboxytetramethylrhodamine (TAMRA), (D) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), (E) diethylenetriaminepentaacetic acid (DTPA), (F) [bis(pyridine-2-ylmethyl)amino]acetic acid (BPA), (G) N-succinimidyl $[^{18}\text{F}]$4-fluorobenzoate ($[^{18}\text{F}]$SFB), (H) $[^{11}\text{C}]$methyl iodide.

Four common strategies are employed for incorporating radioisotopes into targeting molecules: pendant labelling, prosthetic group labelling, direct labelling and integrated labelling (Figure 1.4).\(^9\) Pendant labelling is commonly employed with radiometals for labelling of peptides and mAbs. This method involves the attachment of a bifunctional chelator to the targeting entity in a location away from the biologically active site. Examples of bifunctional chelators include 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), diethylenetriaminepentaacetic acid (DTPA), and [bis(pyridine-2-ylmethyl)amino]acetic acid (BPA) (Figure 1.3, D-F). For a peptide, these chelators are
typically connected to one of the termini, or an amino acid side-chain such as a lysine side-chain. Some examples of molecules labelled by this method include somatostatin derivatives such as $[^{99}\text{Tc}]\text{Tc-HYNIC-TOC}^{14}$ and the CXCR4 targeting peptide $[^{68}\text{Ga}]\text{Ga-Pentixafor}$.\textsuperscript{15} This method can be problematic as it results in an increase in the molecular weight and size of the molecule, and can affect binding to the target of interest. An integrated labelling approach may be used to mitigate the effects of pendant labelling. This method attempts to “hide” a radioisotope within the framework of the targeting entity, often using a radiometal to induce the formation of secondary structure in a protein or peptide, or stabilize already present secondary structures.\textsuperscript{16–18} An integrated approach has also been employed in steroid mimics targeting the estrogen receptor.\textsuperscript{19–21} This approach of concealing the radioisotope would ideally have little effect on the affinity of the targeting entity to the molecular target. Radionuclides such as $^{18}\text{F}$ and $^{11}\text{C}$ are most commonly incorporated into imaging agents through the prosthetic group labelling approach. This involves the radiolabelling of a small molecule “prosthetic group” containing a reactive group which may then be conjugated to the targeting entities. $N$-succinimidyl $[^{18}\text{F}]4$-fluorobenzoate ($[^{18}\text{F}]\text{SFB}$)\textsuperscript{22} and $[^{11}\text{C}]\text{methyl iodide}$\textsuperscript{23} (Figure 1.3, G and H) are common prosthetic groups employed for radiolabelling of peptides, proteins and small molecules. Direct labelling approaches are typically involved in the radiosynthesis of small molecules such as $[^{18}\text{F}]2$-fluoro-2-deoxyglucose ($[^{18}\text{F}]\text{FDG}$),\textsuperscript{24} where a leaving group is directly displaced with the radionuclide. There are however, several published methods for the direct labelling of peptides and other biomolecules.\textsuperscript{25–27}

A wide range of methodologies are available for combining a signaling entity with a targeting entity for the creation of molecular imaging probes. The choice of method strongly depends on the molecular target of interest, the desired pharmacokinetic properties, and the available ligands that can bind to the molecular target.
Figure 1.4: Methods for incorporating a radioisotope into PET and SPECT molecular imaging probes. Pendant and integrated methods are often employed when using radiometals such as $^{68}$Ga and $^{99m}$Tc, while prosthetic group and direct labelling are employed with non-metal radioisotopes such as $^{11}$C and $^{18}$F.

1.3 Single Photon Emission Computed Tomography

Single photon emission computed tomography (SPECT) imaging is the most widely used imaging modality in nuclear medicine due to the availability of on-site generators to produce the required radionuclides. Gamma-emitting radionuclides such as $^{99m}$Tc ($t_{1/2} = 6$ hrs), $^{111}$In ($t_{1/2} = 2.8$ days) and $^{123}$I ($t_{1/2} = 13.2$ hrs) are employed for this modality. Their decay energies, which range from 100 to 300 keV, are optimal for tissue penetration, while being relatively innocuous to the subject. These isotopes are typically produced in an on-site generator or by a cyclotron. SPECT radioisotopes tend to be longer lived than PET radioisotopes, with half-lives ranging from 6 hours to 3 days. This is sufficient time for radiosynthesis, purification and administration to patients, followed by biodistribution and
imaging. The longer half-lives of SPECT isotopes also provide the opportunity for imaging over longer periods of time.

Gamma-emitting isotopes produce gamma-rays unidirectionally, requiring the detector (a gamma camera) to be rotated around the subject, collecting images from multiple angles for 3D reconstruction. Signal attenuation is a common problem in SPECT, as collimators are required to exclude gamma rays that have been scattered. This results in lower sensitivity than PET, and makes quantitative information nearly impossible to gather.\textsuperscript{29,30} Despite these shortcomings, SPECT imaging is an integral technique in nuclear medicine due to its wide availability, and relatively low cost.

1.4 Technetium-99m

Technetium-99m is considered a workhorse radionuclide in nuclear medicine. In the late 1990s to early 2000s, it accounted for approximately 80\% of radiopharmaceuticals used clinically and 85\% of diagnostic nuclear imaging procedures in hospitals.\textsuperscript{31,32} Technetium-99m emits gamma rays with a decay energy of 140 keV, which is considered optimal for imaging with gamma cameras, and has a half-life of 6 hours. This half-life is long enough for preparation and administration of the imaging agent, while minimizing the absorbed radiation dose. Technetium-99m is typically produced by radioactive decay of molybdenum-99 in a generator system.\textsuperscript{33,34} In recent years, cyclotron production of technetium-99m has been explored as an alternative.\textsuperscript{35} From the more common generator system, molybdenum-99 decays by $\beta$- decay ($t_{1/2} = 66$ h) to technetium-99m. This metastable nuclear isomer decays to the ground state technetium-99 predominantly by emission of a 140 keV gamma ray. Technetium-99 subsequently decays with an exceptionally long half-life of $2.12 \times 10^5$ years to the stable ruthenium-99 by $\beta$- decay (Figure 1.5).

The technetium-99m is eluted from a $^{99}$Mo/$^{99m}$Tc generator in the form of sodium pertechnetate ($[^{99m}\text{Tc}\text{NaTcO}_4]$), where the technetium is in the synthetically inaccessible $+7$ oxidation state and must be reduced to a lower oxidation state for incorporation into radiopharmaceuticals. Oxidation states ranging from -1 to +7 are accessible for technetium,
though for medical applications the +1, +3 and +5 oxidation states are used most commonly.\textsuperscript{36}

Figure 1.5: Radioactive decay scheme of $^{99}$Mo to the stable isotope $^{99}$Ru.

Technetium-99m radiopharmaceuticals can be classified into two categories: $^{99m}$Tc-essential and $^{99m}$Tc-tagged radiopharmaceuticals. In the former, the technetium-99m is an integral component of the radiopharmaceutical. Neither the technetium-99m nor the ligand on its own can reach the target of interest, only after metal coordination to the ligand does the agent have the desired pharmacological properties. Perfusion agents such as $[^{99m}\text{Tc}]\text{Tc-MAG}_3$\textsuperscript{37} and $[^{99m}\text{Tc}]-\text{Sestamibi}$\textsuperscript{38} are classical examples of $^{99m}$Tc-essential radiopharmaceuticals (Figure 1.6, Top). $^{99m}$Tc-labelled molecular imaging probes labelled through the integrated method may be considered as $^{99m}$Tc-essential as well.

$^{99m}$Tc-tagged radiopharmaceuticals are in the same vein as pendant labelled molecular imaging probes, where a bifunctional chelator is conjugated to a targeting entity that guides the radioisotope to the molecular target of interest. $[^{99m}\text{Tc}]\text{Tc-HYNIC-TOC}$\textsuperscript{14} $[^{99m}\text{Tc}]\text{TROD-AT-1}$\textsuperscript{39} and $[^{99m}\text{Tc}]\text{Tc(CO)}_3(\text{MIP-1404})$\textsuperscript{40} (Figure 1.6, Bottom) are some notable examples of $^{99m}$Tc-tagged radiopharmaceuticals. $^{99m}$Tc-tagged radiopharmaceuticals commonly contain metal complexes with $^{99m}$Tc in the +3 or +5 oxidation states.
oxidation states due to their ease of labelling and purification, as well as the availability of kit formulations.

**Figure 1.6:** Examples of "$^{99m}$Tc-essential" and "$^{99m}$Tc-tagged" radiopharmaceuticals.

Many of these complexes however, suffer from low stability in solution and in vivo or contain multiple isomeric forms that may differ in their biological activity. In the late 1990’s Schubiger et al developed the fac-technetium(I) tricarbonyl core as an alternative. $^{44}$ fac-[Tc(H$_2$O)$_3$(CO)$_3$]$^+$ was produced in a single step from NaTcO$_4$ by reduction with NaBH$_4$ in aqueous solution under CO atmosphere. Other reagent systems have been developed that do not require reaction under CO atmosphere and are therefore more suitable for the synthesis of radiopharmaceuticals in a clinical setting. $^{45}$ fac-[Tc(H$_2$O)$_3$(CO)$_3$]$^+$ was demonstrated to be stable in aqueous solution at a pH range from 1 to 13 for several hours, and the water ligands were shown to be quite labile, allowing for
substitution with tridentate ligands to form complexes with exceptional stability (>48 hours in serum). This opened the doors for the development of a wide range of methodologies for the incorporation of $^{99m}$Tc into imaging probes (Figure 1.7).\textsuperscript{46–50}

\textbf{Figure 1.7:} $^{99m}$Tc(I)-tricarbonyl complexes formed with a wide variety of ligands. Examples include tridentate ligands such as the amino acid histidine, cyclopentadienyl ligands coordinated in an $\eta^3$ fashion, and [2+1] systems where a bidentate and monodentate ligand occupy the 3 coordination sites.

The high stability and facial orientation of the carbonyl ligands can be attributed to the two-component bonding of the ligand to the metal center (Figure 1.8). The first component is a simple sigma bond where the electrons in the sp-orbital of the carbon atom of the triple bonded carbonyl ligand is donated to a vacant d-orbital on the metal. The second component is a $\pi$ back-bond. The partially filled d-orbital of the metal center can donate electron density to the empty $\pi^*$ orbital of the carbonyl ligand. While this decreases the CO bond order, the metal-CO bond order is increased, resulting in a strong metal-ligand bond. The facial orientation results from the orientation of the d-orbitals that are back-bonding to the carbonyl ligands.
As described in the previous section, the chemistry of technetium is vast, with a variety of oxidation states and ligands available. The development of $^{99m}$Tc-labelled radiopharmaceuticals can be challenging, as technetium has no stable isotopes. While technetium-99 complexes may be produced on milligram to gram scales to study the macroscopic properties of the technetium-99m complexes, the radioactive nature of the isotope limits its availability and use. A more common approach is to use the non-radioactive rhenium as a surrogate for technetium. As both are group VII elements, they share similar chemical reactivity, and tend to form isostructural complexes with similar physicochemical properties such as size and lipophilicity. This allows not only for structural characterization of the metal complexes by standard spectroscopic methods such as X-ray crystallography and infrared spectroscopy, but also allows for *in vitro* evaluation of $^{99m}$Tc radiopharmaceuticals for their ability to bind a molecular target without the use of radioactive material. Rhenium exists in nature as a mixture of two isotopes, $^{185}$Re (37%) and $^{187}$Re (63%). Radioactive isotopes of rhenium $^{186}$Re and $^{188}$Re undergo $\beta$-decay, and have seen clinical use as radiotherapeutics.\textsuperscript{52,53}

Though rhenium has seen wide use as a surrogate for technetium-99m in the development of radiopharmaceuticals, rhenium complexes are also useful in other aspects. Many Re(I)-tricarbonyl complexes display photoluminescence properties and have been developed as cellular imaging agents for microscopy applications (Figure 1.9).\textsuperscript{48,49,54–56} While these photoluminescence properties generally result from metal-to-ligand charge transfer
(MLCT) electronic transitions, complexation to rhenium has also been shown to modulate the fluorescence properties of fluorophores adjacent to the metal complex.\textsuperscript{57,58}

Luminescent Re(I)-tricarbonyl complexes typically have large Stokes shifts, long luminescent lifetimes, high photostability and high cellular permeability, making them ideal imaging agents for cellular microscopy. They also have the potential to aid in the preclinical development of \(^{99m}\text{Tc}\) radiopharmaceuticals by allowing the visualization of tissue uptake and cellular localization of targeted imaging agents without the need for using radioactive isotopes. They may also be helpful in the analysis of \textit{ex vivo} tissue samples for studying and diagnosing disease.

![Figure 1.9: Examples of luminescent Re(I)-tricarbonyl complexes with \(\pi\)-accepting polypyridyl ligands. Suitable ligands include 2,2'-bipyridine, phenanthroline, quinoline and phenanthrodine.](image)

### 1.6 C-X-C Chemokine Receptor 4

The C-X-C chemokine receptor 4 (CXCR4, also called LESTR or Fusin), a G protein-coupled receptor (GPCR), was originally discovered as a co-receptor to CD4 for HIV-1
infection into T-lymphocytes.\textsuperscript{59–63} In addition to T-lymphocytes, it was also shown to be expressed on B-cells and monocytes.\textsuperscript{60} The only known natural ligand to CXCR4, stromal cell-derived factor 1 (SDF-1\(\alpha\), also called CXCL12) has been shown to support the proliferation of B-cells\textsuperscript{64} and is a strong chemoattractant for lymphocytes and monocytes, due to the interaction of SDF-1\(\alpha\) with CXCR4.\textsuperscript{65} Together, CXCR4 and SDF-1\(\alpha\) also play a role in the recruitment of T-lymphocytes to sites of infection and inflammation within the body,\textsuperscript{66,67} hematopoiesis,\textsuperscript{68} and embryonic development.\textsuperscript{69} In recent years, there has been a surge in research on CXCR4/SDF-1\(\alpha\) focusing on their role in cancer. CXCR4 is expressed in at least 23 different types of cancer including breast, prostate, lung, colon, glioma and multiple myeloma.\textsuperscript{70–77} Expression of CXCR4 in cancers has been shown to promote metastasis,\textsuperscript{78–81} particularly to sites containing high expression of SDF-1\(\alpha\) such as lymph nodes, bone marrow, lungs and liver.\textsuperscript{71,82–85} Most healthy tissues contain very low expression of CXCR4, but in cancerous tissue the expression is greatly enhanced. It may also be noted, that while prostate cancer tissue overexpresses CXCR4, expression remains low in benign prostatic hyperplasia (BPH) and healthy prostate tissue.\textsuperscript{86} This provides a diagnostic biomarker that may be used to differentiate healthy and benign tissue from malignant. The roles of CXCR4/SDF-1\(\alpha\) in diseases such as cancer and HIV prompted the development of inhibitors as therapeutics and molecular imaging agents for evaluation of CXCR4 expression \textit{in vivo}.

A turning point in the development of CXCR4 inhibitors occurred upon the discovery of the antimicrobial self-defense peptides tachyplesin I \& II and polyphemusin I \& II from the hemocytes of the horseshoe crabs \textit{Tachypleus tridentatus} and \textit{Limulus polyphemus}.\textsuperscript{87,88} These peptides are structurally intriguing as they contain 17 or 18 amino acids for tachyplesin and polyphemusin respectively, an amidated C-terminus, and two disulfide bridges stabilizing an anti-parallel \(\beta\)-sheet structure. Not only did these peptides demonstrate antimicrobial activity, but were also shown to inhibit the infectivity of HIV-1 into MT-4 cells.\textsuperscript{89} Though they were effective at inhibiting HIV-1 infection, they were also found to be relatively cytotoxic. As polyphemusin II was shown to have the greatest inhibitory effect on HIV, it was used as a scaffold to reduce cytotoxic effects and improve potency, which led to the development of the peptide T22 ([Tyr\textsuperscript{5,12}, Lys\textsuperscript{7}]-polyphemusin
II), which had much greater potency, and reduced cytotoxicity.\textsuperscript{90,91} It wasn’t until 5 years after the development of T22 that it was discovered the inhibitory effect on HIV was due to the peptide’s interaction with CXCR4.\textsuperscript{92} Further efforts to reduce the molecular size of the peptide while retaining high potency and low cytotoxicity resulted in the peptide TW70, which reduced the size of the peptide to 14 amino acids, and removed one of the disulfide bridges while retaining the β-sheet structure and potency, and reducing cytotoxicity.\textsuperscript{93} It was observed that cell membrane permeability and the number of positive charges had a great effect on the selectivity index (SI, ratio of cytotoxicity measure (CC\textsubscript{50}) to potency (EC\textsubscript{50})), resulting in the peptides T134, and finally T140, which replaced the amidated C-terminus with a carboxylic acid, and reduced the charge of the molecule to +7, giving the highest SI values thus far.\textsuperscript{94,95} Figure 1.10 highlights the structural changes made from polyphemusin II to T140.

<table>
<thead>
<tr>
<th></th>
<th>polyphemusin II</th>
<th>T22</th>
<th>TW70</th>
<th>T134</th>
<th>T140</th>
</tr>
</thead>
</table>

**Figure 1.10:** The development of T140 from polyphemusin II. Black bars indicate disulfide bridges (top bars pertain to polyphemusin II and T22, bottom bar pertains to TW70, T134 and T140), colours indicate structural changes made in the subsequent peptide.

Alanine scans of T140 determined that there were 4 residues that were indispensable to the binding affinity of the peptide to CXCR4: Arg\textsuperscript{2}, Nal\textsuperscript{3}, Tyr\textsuperscript{5} and Arg\textsuperscript{14}.\textsuperscript{95} This eventually led to the development of the cyclic pentapeptide FC131 (cyclo[Nal\textsuperscript{1}-Gly\textsuperscript{2}-D-Tyr\textsuperscript{3}-Arg\textsuperscript{4}-Arg\textsuperscript{5}]), which retained similar affinity for CXCR4 as T140.\textsuperscript{96}

Small molecule CXCR4 inhibitors have also been developed separately from the peptide inhibitors. It was noticed that molecules containing two cyclam rings (bicyclams) were able to inhibit HIV infection in human T cells with varying potencies, without any noticeable cytotoxic effects.\textsuperscript{97} It was eventually determined that a p-xylylene spacer linking the two cyclam rings gave the compound with the highest potency out of all of the synthesized analogues, and was denoted JM3100.\textsuperscript{98} This compound now goes by the name
AMD3100 or Plerixafor, and it has been shown to inhibit HIV infection through interaction with CXCR4. AMD3100 has also seen clinical use as a mobilizer of hematopoietic stem cells. Figure 1.11 depicts the structures of the described CXCR4 inhibitors T140, FC131 and AMD3100.

**Figure 1.11:** Peptide (T140, FC131) and small molecule (AMD3100) inhibitors of CXCR4.

While CXCR4 inhibitors show great promise as therapeutics for HIV, they have also shown potential as anti-metastatic agents in several cancers. The ability to target CXCR4 in cancer has garnered much interest in developing molecular imaging agents containing radioactive and fluorescent tags for evaluation of CXCR4 expression both in tissue samples, and in vivo. As healthy tissue surrounding the diseased site does not express high amounts of CXCR4, the diseased tissue may be specifically targeted by molecular imaging agents. Figure 1.12 depicts the structures of several radiolabelled molecular imaging agents targeting CXCR4. A wide range of T140 derivatives have been developed containing imaging labels. These labels are typically placed on the peptide’s N-terminus, or from the D-Lys side chain. It has been demonstrated that the turn region lies outside of the CXCR4 binding pocket, making the D-Lys side chain ideal for conjugation of an imaging moiety. Amidation of the N-terminus has also been shown to have little effect on binding affinity, often improving it, though bulky substituents may have negative effects on receptor
binding. The first reported PET imaging agent based on the T140 scaffold was $^{[18}F]4$-FB-TN14003 (Figure 1.12, Top Right). This peptide contains an $^{[18}F]4$-fluorobenzoyl ($^{[18}F]4$-FB) group on the N-terminus, which improved CXCR4 affinity for the receptor. However, addition of the 4-FB group had the unwanted side effect of causing non-specific binding to red blood cells, which made targeting CXCR4 expressing tumors rather challenging in vivo. Inclusion of a non-radioactive, fluorine-19 containing N-terminal 4-FB group in other radiolabelled T140 derivatives showed similarly high blood uptake in in vivo animal models. Several other radiolabelled T140 derivatives have mitigated the problem of binding to blood cells, but often give poor tumor uptake or high accumulation in the liver and kidneys. Despite the shortcomings of these agents, a $^{68}$Ga-labelled T140 derivative has been tested for imaging of CXCR4 expression in glioma patients. This imaging agent showed specific uptake of the tracer in the cancerous tissue, with a much lower background signal in comparison to $^{[18}F]$FDG. Fluorescently labelled T140 derivatives have been used successfully as an alternative to antibody staining to evaluate CXCR4 expression in Ewing sarcoma and high-grade bladder cancer.

**Figure 1.12**: PET and SPECT molecular imaging agents targeting CXCR4.

Structure-activity relationship studies on FC131 led to the development of the Pentixafor (previously named CPCR4-2) scaffold (Figure 1.12). Coordination of Ga(III) to the appended DOTA chelator resulted in an imaging probe with similar affinity to CXCR4 as FC131. The $^{68}$Ga-labelled peptide has been used in humans for imaging of CXCR4
expression in a wide variety of cancers, and has great promise as a clinically useful PET imaging agent.\textsuperscript{15,114,115} Several other derivatives of Pentixafor have been explored in \textit{in vitro} models, and also show promise for \textit{in vivo} imaging as well as radiotherapy.\textsuperscript{116–119} It was previously demonstrated that coordination of AMD3100 with metal ions such as Cu(II), Zn(II) and Ni(II) improved the binding affinity of the molecule to CXCR4.\textsuperscript{120} This concept has been used in the development of radiolabelled AMD3100 analogues. \textsuperscript{[64Cu]}Cu-AMD3100 was used to successfully differentiate CXCR4-positive tumors in an \textit{in vivo} animal model.\textsuperscript{121,122} \textsuperscript{99mTc}Tc-labelled AMD3100, and the derivative AMD3465 have also shown promise as SPECT imaging agents for imaging of CXCR4 expressing tumors \textit{in vivo}.\textsuperscript{123,124}

Targeting of the CXCR4/SDF-1α axis for therapeutic and diagnostic purposes in HIV and cancer is a large and rapidly growing field. Despite all of the work in the field, the need for imaging agents with improved pharmacokinetic and biodistribution profiles is still an area of research that is actively being developed. While radiolabelled agents targeting CXCR4 are being evaluated in humans for PET imaging, there is still a need for imaging agents compatible with SPECT imaging, as well as PET agents with improved pharmacokinetics.

### 1.7 Biological Roles of Zinc

Zinc is a ubiquitous element in the human body. It is the second most abundant transition metal, second only to iron. Under physiological conditions, zinc is found in the form of Zn(II). While total zinc concentration in cells is in the mM range, free zinc concentrations are tightly regulated. This is achieved through active transport by the zinc transporter (ZnT) and zrt-like, irt-like protein (ZIP) classes of transporters,\textsuperscript{125} buffering of zinc by metallothioneins,\textsuperscript{126} and sequestering of zinc into organelles such as zincosomes.\textsuperscript{127,128} This results in free zinc concentrations in the nM range.\textsuperscript{129}

Zinc has a broad range of cellular functions and roles (Figure 1.13). Zn(II) ions function as co-factors for a number of zinc-dependent enzymes.\textsuperscript{130–132} Enzymes such as carbonic anhydrase,\textsuperscript{133} carboxypeptidase A,\textsuperscript{134} and alkaline phosphatase\textsuperscript{135} require the Zn(II) ion to activate electrophilic sites so that enzymatic hydrolysis can occur. Zn(II) ions act as structural elements of many proteins such as zinc finger transcription factors.\textsuperscript{136} Insulin is
also stored as a hexamer through coordination with Zn(II), and metal ions such as Zn(II) and Cu(II) have been implicated in the formation of amyloid plaques in Alzheimer’s disease. Zn(II) signaling plays a key role in neurotransmission, as well as the activation and inhibition of transcription factors. Disruption of the tightly regulated zinc homeostasis has been implicated in a number of diseases such as diabetes, Alzheimer’s disease and prostate cancer. The following section will discuss the role of zinc in the latter.

**Figure 1.13:** Biological distribution and functions of zinc. Abbreviations: IAP, inhibitor of apoptosis protein; ROS, reactive oxygen species; ER, endoplasmic reticulum; ZIP, Zrt-like, Irt-like protein; ZnT, zinc transporter.

### 1.7.1 Zinc in Prostate Cancer

It was documented as early as the 1920s that the human prostate has the highest zinc concentration of any soft tissue (~1000 µg/g of dry tissue), though the exact reason for
this is not entirely clear. It has been noted that prostate epithelial cells have characteristically high aerobic glycolysis, low respiration rates, and high citrate secretion, and it has been proposed that high zinc concentrations are required to maintain these characteristics.\textsuperscript{145,146} Zinc was shown to inhibit \textit{m}-aconitase, an enzyme responsible for citrate oxidation, which may be responsible for the high citrate secretion and low respiration in prostate tissue.\textsuperscript{147,148} It has also been demonstrated that in malignant prostate tissue there is a nearly ten-fold reduction in zinc concentration.\textsuperscript{149} Decreases in cellular zinc may lead to an increase in cellular respiration favouring growth and differentiation, which may lead to malignancy.\textsuperscript{150} Zinc has also been shown to regulate apoptosis in prostate cells.\textsuperscript{151} Lower levels of zinc can be attributed to an increase in the expression of ZnT proteins, responsible for zinc export, and a decrease in the expression of ZIP proteins, which are responsible for zinc import.\textsuperscript{152} Also to be noted, benign hyperplastic prostate tissue retains the high zinc accumulation observed in healthy prostate tissue.\textsuperscript{149}

Due to the characteristic decrease in zinc concentration in malignant prostate tissue, there has been much clinical interest in zinc as a biomarker for prostate cancer, allowing for the differentiation of malignant prostate tissue from benign and healthy. Evaluation of prostatic zinc levels has mostly been limited to \textit{ex vivo} analysis of biopsies through a variety of methods.\textsuperscript{153–155} More recently, the development of molecular imaging agents for \textit{in vivo} analysis of prostatic zinc levels has been investigated.\textsuperscript{156} However, for the pre-clinical study of zinc as it relates to disease, the development of fluorescent, cell permeable indicators for zinc has been a large area of investigation.\textsuperscript{157,158} The following section will discuss the mechanisms behind the function of zinc-sensitive fluorescent probes.

\subsection*{1.7.2 Fluorescent Probes Responsive to Zinc}

The detection of zinc by spectroscopic methods can be challenging, as the 3d\textsuperscript{10} electron configuration makes the metal ion alone insensitive to spectroscopic methods such as NMR and UV/Vis. However, ligand-centric photophysical processes may be exploited as a method of fluorescence detection of Zn(II). Quantum yields, fluorescence lifetimes, and excitation/emission energies of a ligand/fluorophore can be manipulated by coordination to Zn(II). The appropriate choice of chelator and fluorophore that imparts selectivity to
Zn(II) over other metal ions would give a fluorescent probe with a signal unique to the presence of Zn(II).

Fluorescent stains for biological zinc began gaining wide acceptance in the 1980s with histochemical stains based on 8-aminoquinoline such as N-(6-methoxyquinolin-8-yl)-p-toluenesulfonamide (TSQ). Further research has focused on improving cellular permeability and shifting excitation and emission wavelengths further into the visible range (Figure 1.14).

![Chemical structures](image)

**Figure 1.14:** Evolution of zinc responsive fluorescent probes: TSQ, to the more cell membrane permeable ZnAF-R2, and the visible light excitable FluoZin-1, and the cell membrane permeable variant FluoZin-1 AM.

A variety of ligand-centric photophysical processes have been exploited in the development of zinc-responsive fluorescent probes. These include: photoinduced electron transfer (PeT), intramolecular charge transfer (ICT), excited-state intramolecular proton transfer (ESIPT), Förster resonance energy transfer (FRET), and excimer/exciplex formation. In this section, PeT, ICT and ESIPT will be discussed in further detail.

Photoinduced electron transfer (PeT, not to be confused with positron emission tomography (PET)) is a fluorescence quenching mechanism that occurs in molecules containing a fluorophore that is appended with an electron donor. Note that there is no conjugated π-system involved (i.e. there is no ground state mixing of the respective
molecular orbitals). Upon promotion of the fluorophore to the excited state, the electron donor may donate an electron into the half-filled HOMO of the fluorophore (a redox process), affording a radical cation/anion pair. This prevents the electron located in the LUMO of the fluorophore from returning to the ground state, effectively quenching fluorescence emission. As PeT applies to Zn(II) indicators, the electron donor is typically a chelating moiety, capable of selectively binding to Zn(II). Chelators containing tertiary amino groups are often employed due to their oxidizable nature. The HOMO of the chelator is of a higher energy than that of the fluorophore, allowing for donation of an electron from the chelator to the fluorophore. In the free ligand state, fluorescence is efficiently quenched (Figure 1.15, Top).

However, coordination of the chelating moiety to Zn(II) lowers the HOMO energy of the electron donor, preventing electron transfer to the excited state fluorophore HOMO. This restores the fluorescence emission pathway (Figure 1.15, Bottom). PeT sensors are therefore “turn-on” sensors. Coordination to Zn(II) typically causes little change in the excitation and emission energies, but an increase in fluorescence quantum yield ($\phi_f$) is observed. The fluorescence intensity may then be correlated to the concentration of Zn(II). FluoZin-1 (Figure 1.14, right) is an example of a Zn(II) indicator operating by a PeT mechanism.
Figure 1.15: The process of PeT as applied to Zn(II) indicators. Coordination of Zn(II) prevents the metal chelator from acting as an electron donor, increasing fluorescence intensity from the fluorophore.

In contrast to PeT where an electron donor and acceptor are separated, ICT fluorophores contain a $\pi$-conjugated electron donor and acceptor. Upon photoexcitation, charge separation occurs in the fluorophore, resulting in a polar excited state. Fluorophores displaying ICT characteristics are positively solvatochromic in nature (i.e. excitation and emission energies decrease with increasing solvent polarity). In non-polar solvents, a “locally excited” state occurs in which the excited fluorophore has little effect on solvent ordering. In polar solvents, the solvent molecules may align their dipoles with the dipole of the excited fluorophore, effectively decreasing the energy of the excited state, and shifting the fluorescence emission wavelength further into the visible range (Figure 1.16, Top).
Figure 1.16: ICT mechanism in non-polar and polar solvents (top), and upon coordination to Zn(II) (bottom).

In the case of the zinc indicator ZnAF-R2 (Figure 1.14, middle) where Zn(II) binds to the electron donor site, the ability of the donor to transfer electron density to the acceptor is reduced and the excited state is destabilized, which hypsochromically shifts the excitation and emission wavelengths. However, coordination of Zn(II) to the acceptor site, as is the case in many 2,2'-bipyridine based zinc indicators, results in a stabilization and a decrease in polarity of the excited state due to the cationic Zn(II) (Figure 1.16, Bottom). As a result, a bathochromic shift in the excitation and emission wavelengths occurs. As the free ligand and the Zn(II) complex will typically display fluorescence, in theory the ratio of the fluorescence emission from the ligand and the Zn(II) complex may be used to determine Zn(II) concentrations. This is usually not done in practice however, as the absorption wavelengths of the free ligands often occur in the UV range, and are not ideal for use in live cells and tissues.

ESIPT occurs in molecules where intramolecular hydrogen bonds are present, and tautomerization is possible. Phototautomerization may occur in the excited state of a molecule, with a concomitant transfer of a proton from a photoacid to a photobase. 2-(2’-Hydroxyphenyl)benzoxazole (HBO) is an example of an ESIPT fluorophore (Figure 1.17). In non-polar solvents, ESIPT occurs at a faster rate than fluorescence emission. Therefore, the lower energy emission from the phototautomeric form dominates. As solvent polarity increases, the rate of proton transfer decreases due to hydrogen bonding with the solvent, and the emission from the phototautomer decreases. If the photobasic and photoacidic sites
become coordinated to a metal ion such as Zn(II), ESIPT is no longer possible (Figure 1.17, Left). The emission energy becomes intermediate between the energies of the phototautomeric forms. As with ICT fluorophores, the ratio between emission from the free ligand and the metal complex may be used to determine Zn(II) concentrations.

Figure 1.17: Process of ESIPT occurring in HBO, and Zn(II) coordination suppressing ESIPT.

While there is certainly a plethora of Zn(II) sensitive fluorescent probes to choose from, there is still plenty of room for advancement in the field. There is still a need to develop sensors with useful ratiometric fluorescence emission, so that the probe may be visualized in the free ligand, and Zn(II) coordinated states, improvement in cellular permeability and cellular localization as well as a lack of toxicity to the target cells or tissues that are being interrogated.\textsuperscript{164}

1.8 Summary

This thesis will document the discovery of new metal chelators for Re\textsuperscript{99mTc}, new methods of incorporating these metals into peptide structures, as well as new fluorescent compounds. These novel methods and compounds may be used in the development of molecular imaging probes for SPECT and OI techniques, with applications for differentiating cancerous tissue from benign and healthy. Chapter 2 will discuss the
development of a dual modality Re/$^{99m}$Tc imaging probe for fluorescence/SPECT imaging derived from T140 for the evaluation of CXCR4 expression in cancer cells, with applications to differentiating malignant prostate cells from benign. Chapter 3 contains a study evaluating the fluorescence properties and radiolabelling of Re/$^{99m}$Tc-containing 4-amino-1,8-naphthalimides towards tuning the pharmacokinetic and biodistribution properties of their bioconjugates. Chapter 4 highlights the challenges in developing integrated molecular imaging probes. The development of 3,3’-diamino-2,2’-bipyridine containing peptides as SPECT imaging agents will be discussed. Chapter 5 will discuss the development of amino-substituted 2,2’-bipyridine ligands as intracellular Zn(II) sensors, and their application in differentiating malignant and benign prostate cells by their differences in Zn(II) uptake. Chapter 6 will summarize the findings of the thesis and how they advance the field of molecular imaging as a whole.

1.9 References


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Chem. 2013, 52, 13521–13528.


Chapter 2

2 A Dual Modality $^{99m}$Tc/Re(I)-Labelled T140 Analogue for Imaging of CXCR4 Expression

2.1 Introduction

The C-X-C chemokine receptor 4 (CXCR4) and its natural ligand CXCL12 are involved in developmental, immune and inflammatory processes in healthy tissues.\textsuperscript{1,2} It was first discovered as a co-receptor for HIV infection into T lymphocytes.\textsuperscript{3–5} CXCR4 has been shown to be overexpressed in at least 23 types of cancer,\textsuperscript{6} including prostate cancer (PCa),\textsuperscript{7} where it and CXCL12 are involved in metastasis,\textsuperscript{7–9} angiogenesis,\textsuperscript{10} and adhesion of PCa cells to bone marrow endothelial cells.\textsuperscript{11,12} While CXCR4 expression is higher in PCa and associated metastases, expression is low in benign prostatic hyperplasia and normal prostate tissue.\textsuperscript{13} This gives the potential for CXCR4 to be a biomarker to aid in the diagnosis of PCa and differentiating malignant and benign tissue. CXCR4 expression was shown to be a superior predictor of bone metastasis to the Gleason score, and that 94\% of patients with metastatic prostate cancer showed expression of the protein. Patients with high CXCR4 expression had an overall lower cancer-specific survival than those with low expression of the receptor.\textsuperscript{8} Therefore, CXCR4 is a potential diagnostic and prognostic factor for prostate cancer. CXCR4 targeted imaging agents may aid in the visualization of metastatic lesions, and CXCR4 antagonists have therapeutic potential for metastatic prostate cancer.

Several CXCR4 antagonists have been reported, including the biscyclam AMD3100,\textsuperscript{14} 14 amino-acid β-hairpin peptide T140,\textsuperscript{15} and cyclic pentapeptide FC131.\textsuperscript{16} These three compounds have been widely used as scaffolds for the development of positron emission tomography (PET), single photon emission computed tomography (SPECT), and fluorescence imaging probes targeting CXCR4, and several published reviews discuss this topic.\textsuperscript{17–19} While the development of CXCR4 targeted nuclear imaging probes has previously been focused on PET agents containing $^{68}$Ga, $^{64}$Cu and $^{18}$F, only a few SPECT imaging agents had been developed. Recently there has been a surge in the number of
publications on CXCR4 targeted SPECT imaging agents containing $^{\text{99m}}$Tc as the radionuclide.$^{20-23}$

Multi-modality imaging agents are of great interest, as a single compound may be used for various clinical applications and can combine the advantages of different modalities, while mitigating some of the disadvantages.$^{24-26}$ These agents typically contain a separate label for each modality (i.e. a radionuclide for PET/SPECT, and a fluorophore for fluorescence). This presents many design challenges, as fluorophores and metal chelators can be quite large in size and molecular weight. Conjugation of multiple large moieties to biomolecules such as peptides can have a negative impact on their ability to bind a biological target. For example, a dual-modality CXCR4 targeted imaging agent has previously been reported for dual SPECT/fluorescence imaging.$^{27}$ The T140 analogue Ac-TZ14011 was derivatized with an $^{111}$In labelled diethylenetriaminepentaacetate (DTPA) chelator for SPECT imaging and a Cy5.5 dye for fluorescence imaging. Introduction of these large moieties resulted in a 20-fold reduction in binding affinity to CXCR4 due to increased steric bulk. Due to this decrease in binding affinity, it would therefore be advantageous to reduce the size of the appended labels by incorporating a fluorophore and radioisotope into a single, more compact entity.$^{28}$ Rhenium(I) tricarbonyl complexes can exhibit fluorescence and replacing rhenium with technetium-$^{99m}$Tc would allow for single photon emission computed tomography (SPECT) imaging. 4-Amino-1,8-naphthalimides appended with a di-(2-picolyl)amine binding unit coordinated to Re(I) have been reported as cellular imaging agents with ideal fluorescence properties.$^{29}$ However, their incorporation into biological molecules and labelling with $^{99m}$Tc has not been explored.

Herein, we report the synthesis and evaluation of a dual modality imaging agent derived from T140 containing a 4-amino-1,8-naphthalimide appended with a di-(2-picolyl)amine (DPA) binding unit (Figure 2.1). The peptide was coordinated to Re(I) for evaluation of CXCR4 binding and photophysical properties. CXCR4 expression in various cell lines, including prostate cell lines, was observed by confocal fluorescence microscopy. Labelling with $^{99m}$Tc(I) was performed for evaluation in a murine xenograft model.
Figure 2.18: Structure of T140 (6) and the newly synthesized derivative DPA-Naph-T140 (7).

2.2 Results and Discussion

2.2.1 Design and Synthesis

A carboxylic acid functionalized dipicolylamine-naphthalimide (DPA-Naph) fluorophore (5) was synthesized in five steps from commercially available 4-chloro-1,8-naphthalic anhydride (scheme 2.1) and conjugated to T140 through solid-phase peptide synthesis. Naphthalimide 1 was synthesized by reacting 4-chloro-1,8-naphthalic anhydride and ethyl 3-aminopropionate in refluxing ethanol. This was then heated in DMSO with tert-butyl (2-aminoethyl)carbamate giving the Boc-protected amine 2. Deprotection of the Boc group with TFA yielded the free amine 3, which underwent reductive alkylation with 2-pyridinecarboxaldehyde to form the tridentate chelator 4. Saponification of the ethyl ester gave the carboxylic acid 5 (DPA-Naph-OH), which could then be conjugated to a peptide by standard peptide coupling methods.
Scheme 2.1: Synthesis of compound 5 (DPA-Naph-OH).

The D-Lys\textsuperscript{8} side chain of T140 was chosen as the site of conjugation, as previous reports show that this residue is located outside of the CXCR4 binding pocket.\textsuperscript{30} Peptides were synthesized by standard Fmoc solid-phase peptide synthesis techniques. The D-Lys\textsuperscript{8} side chain was protected with an allyloxycarbonyl (Alloc) protecting group for selective deprotection and coupling of 5. Compound 5 was conjugated to the peptide through standard HCTU coupling procedures. After disulfide bridge formation by oxidation with I\textsubscript{2} and final Fmoc deprotection, the peptide was cleaved from the resin and purified by preparative HPLC-MS to give the peptide with the free chelator (7). We then synthesized the rhenium tricarbonyl complex, by reacting the peptide with [Re(H\textsubscript{2}O\textsubscript{3})(CO)\textsubscript{3}]OTf under microwave conditions. The peptide was then purified by preparative HPLC-MS to give the peptide Re-7. UV/Vis absorption and fluorescence data are summarized in Table 2.1. Reported values are very similar to those given by Pope et al. for the four rhenium naphthalimides they had synthesized, confirming that the fluorophore retains its fluorescent properties upon conjugation to a peptide, and subjected to aqueous conditions.
**Table 2.1:** Photophysical Properties of DPA-Naph Peptide Conjugates.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$a \lambda_{\text{max}}$</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$</th>
<th>$b \phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>450 nm</td>
<td>14,100</td>
<td>545 nm</td>
<td>0.28</td>
</tr>
<tr>
<td>Re-7</td>
<td>439 nm</td>
<td>12,370</td>
<td>527 nm</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*a*18 µM in H$_2$O. *b*Relative to [Ru(bpy)$_3$](PF$_6$)$_2$ in aerated H$_2$O ($\phi$ = 0.04)

### 2.2.2 *In Vitro* Evaluation of Re-7

To investigate the influence of the DPA-Naph moiety and metal complex on the peptide’s ability to bind to CXCR4, competitive binding assays were performed using stably transfected U87.CD4.CXCR4 cells with $[^{125}\text{I}]$-SDF-1 as the competing ligand. Under our assay conditions, the parent peptide T140 (6) gave an IC$_{50}$ value of 1.0 nM, while Re-7 only showed a modest increase in IC$_{50}$ to 1.9 nM (Figure 2.2). This indicates that conjugation of the DPA-Naph moiety, and formation of the rhenium tricarbonyl complex has little influence on the ability of the peptide to bind the receptor, as the D-Lys$^8$ side chain is located outside of the CXCR4 binding pocket.

![Figure 2.19](image)

**Figure 2.19:** Representative competitive binding curves of T140 (black curve) and Re-7 (grey curve) using U87.CD4.CXCR4 cells with $[^{125}\text{I}]$-SDF-1 as the competing radioligand.

Confocal fluorescence microscopy was then performed to validate Re-7 as a fluorescent imaging agent specific for CXCR4. U87.CD4.CXCR4 and U87.CD4 cells were incubated with Re-7 and were then fixed and mounted on slides for imaging. U87.CD4.CXCR4 cells...
showed strong fluorescence, while U87.CD4 cells did not (Figure 2.3), indicating that Re-7 can selectively detect CXCR4 in cells expressing the receptor.

**Figure 2.20:** Confocal microscope images of U87.CD4.CXCR4 cells (A, C) and U87.CD4 cells (B, D) incubated with Re-7. $\lambda_{\text{Ex}} = 458$ nm, $\lambda_{\text{Em}} = 500-550$ nm, 40x objective.

It has been previously reported in the literature that PCa cell lines such as PC-3 and DU 145 express CXCR4 to a much higher extent than benign prostate cells such as BPH-1. These three cell lines were also incubated with Re-7 and observed by confocal microscopy to evaluate CXCR4 expression (Figure 2.4). BPH-1 cells showed no discernable fluorescence. Both PC-3 and DU 145 cells however, displayed observable fluorescence. The fluorescence intensity was not as strong as that seen in U87.CD4.CXCR4 cells due to the endogenous expression of CXCR4 being lower in the PCa cells than in the transfected cells. As CXCR4 expression was observable in the PCa cells and not in the BPH cells, Re-7 may be useful in *ex vivo* analysis of prostate tissue to differentiate benign and malignant prostate tissue by their CXCR4 expression.
2.2.3 $^{99m}$Tc-labelling and In Vitro Cellular Uptake

Peptide 7 was also radiolabelled with $^{99m}$Tc to give $[^{99m}$Tc]$Tc-7$. The peptide was labelled by reaction with $[^{99m}$Tc]$Tc$(CO)$_3$(H$_2$O)$_3^+$ under microwave conditions. The radiolabelled peptide was purified by semi-preparative HPLC, giving decay corrected radiochemical yields ranging from 60-85%, radiochemical purities of >95% (Figure 2.5, left) and molar activities of 36-44 GBq/µmol.

Cellular uptake of $[^{99m}$Tc]$Tc-7$ was evaluated in U87.CD4.CXCR4 cells in comparison to U87.CD4 cells (Figure 2.5, right). Approximately two-fold higher uptake was observed in the CXCR4 expressing cells (10.4 ± 0.8%) compared to the U87.CD4 cells (5.6 ± 0.1%). While there was a relatively high amount of non-specific binding observed as seen by the high uptake in U87.CD4 cells, the radiolabelled peptide shows specific uptake in CXCR4 expressing cells.
2.2.4  *Ex Vivo* Biodistribution Studies

*Ex vivo* biodistribution studies were performed with $[^{99m}\text{Tc}]{\text{Tc}}$-7 in NOD/SCID mice bearing U87.CD4.CXCR4 tumors. The animals were sacrificed at 2 hours post-injection, and the activity was determined in the organs of interest (Figure 2.6). Uptake in the liver and kidneys was quite high (22.67 ± 5.02 and 25.69 ± 15.19 % ID/g respectively), which is consistent with several other radiolabelled T140 derivatives due to hepatic metabolism and urinary clearance.\textsuperscript{27,31,32} Moderate uptake was also observed in the lung, spleen, intestines and stomach. The stomach is known to express CXCR4 to a small degree,\textsuperscript{33} so this may account for some of the uptake. However, previous reports also suggest that hydrophobic imaging labels may increase non-specific uptake in undesired tissues,\textsuperscript{34} including the stomach.\textsuperscript{27} Therefore, the uptake may be non-specific, driven by the relatively non-polar naphthalimide metal complex. The lung, spleen and intestinal uptake is likely caused by specific binding to CXCR4, as these organs are known to have moderate expression of the receptor,\textsuperscript{22} and previously reported CXCR4-targeted radiotracers are known to specifically accumulate in these organs. Disappointingly, tumor uptake was quite low (0.51 ± 0.09 % ID/g). This may be due to a combination of higher blood uptake (1.91
± 0.16 % ID/g) compared to many other T140-based radiotracers along with specific accumulation in other organs. Optimization of the biodistribution of the peptide is required to improve the tumor uptake. Several T140 derived peptides have been reported that retain affinity for CXCR4 such as TN14003 and Ac-TZ14011, so exploration of other derivatives may help to improve the biodistribution. Alteration of the structure of the metal chelator to reduce lipophilicity may also lead to a peptide with an improved biodistribution and pharmacokinetic profile.

Figure 2.23: Ex vivo biodistribution of $[^{99m}Tc]Tc$-7 in U87.CD4.CXCR4 tumor bearing mice at 2 hours post-injection (n = 3).

### 2.3 Conclusions

In summary, we have developed a novel T140 derivative (7) containing a 4-amino-1,8-naphthalimide appended with a di-(2-picolyl)amine (DPA) binding unit to chelate Re(I)-tricarbonyl for use in confocal fluorescence microscopy, and $^{99m}$Tc(I)-tricarbonyl for use as a SPECT imaging agent. Re-7 showed specific uptake in U87.CD4.CXCR4 cells, as well as PC-3 and DU 145 PCa cells, while minimal uptake was observed in U87.CD4 and BPH-1 cells. This fluorescent peptide may be useful in differentiating malignant tissue from healthy and benign, as malignant tissue tends to express CXCR4 to a much higher extent. The technetium-99m labelled variant $[^{99m}Tc]Tc$-7 was prepared in high yield, purity
and molar activity, and was shown to have two-fold higher uptake in CXCR4 expressing cells. However, in a murine xenograft model, \[^{99mTc}\text{Tc-7}\] showed very low uptake in CXCR4 expressing tumors. Alteration of the metal chelation system appended to the naphthalimide to one with lower lipophilicity may provide a SPECT imaging agent with improved biodistribution.

2.4 Experimental

2.4.1 General Experimental

All reagents were purchased from commercial sources and used without further purification. \([\text{Re}(\text{H}_2\text{O})_3(\text{CO})_3]\text{OTf}\) was synthesized according to a published procedure.\(^{35}\) NMR spectra were recorded on a Bruker AvIII HD 400 spectrometer. All chemical shifts are reported in ppm and referenced to the residual solvent peaks. High resolution mass spectra for small molecules were recorded on a Waters Xevo QToF mass spectrometer with an electrospray ion source in positive mode. For peptides, high resolution mass spectra were recorded on a Bruker micrOTOF II mass spectrometer with an electrospray ion source in positive mode. Analytical reversed-phase HPLC-MS was performed on a system consisting of an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 150 mm), Waters 600 controller and Binary Solvent Pump, Waters Inline degasser, Waters Quattro Micro API mass spectrometer and Waters Mass Lynx software. Mass spectra were collected using an ESI source in positive ion mode. The gradient solvent system comprised of solvent A (CH\(_3\)CN + 0.1 % TFA) and solvent B (H\(_2\)O + 0.1 % TFA) at a flow rate of 1.5 mL/min over 10 minutes with a 5-minute wash cycle at 95% solvent A. The UV absorbance was detected using a Waters 2998 Photodiode array detector. Peptides were purified by reversed-phase preparative HPLC-MS on the same system with an Agilent Zorbax PrepHT SB-C18 column (5 µm, 21.2 x 150 mm) at a flow rate of 20 mL/min. After purification, the collected fractions were frozen at -78 °C, and lyophilized. Analytical radio-HPLC was performed on a system comprising of an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 150 mm), Waters 600 controller and Binary Solvent Pump, Waters Inline degasser with a Carroll & Ramsey radiometric detector connected to a waters e-Sat/IN module and Waters 2498 dual absorbance UV-Vis detector. The gradient solvent system comprised of solvent A (CH\(_3\)CN + 0.1 % TFA) and solvent B (H\(_2\)O + 0.1 % TFA) at a flow rate of 1.5 mL/min
over 10 minutes with a 5-minute wash cycle at 95% solvent A. Semi-preparative radio-
HPLC was performed on the same system with an Agilent Zorbax SB-C18 column (3.5
µm, 4.6 mm x 150 mm) at a flow rate of 4 mL/min. UV-Vis spectra were recorded on an
Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a
Photon Technologies International, Inc. Quanta Master – 7/2005. Quantum yields were
determined in aerated H₂O solutions of the peptides using [Ru(bpy)₃](PF₆)₂ in H₂O (ϕ =
0.04) as a standard.³⁶

**Ethyl 3-(6-chloro-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propanoate (1)**

Triethylamine (1.8 mL, 12.9 mmol) and 4-chloro-1,8-naphthalic anhydride (2.00 g, 8.6
mmol) were added to a solution of β-alanine ethyl ester hydrochloride (2.00 g, 12.9 mmol)
in ethanol (80 mL). The resulting orange suspension was heated to reflux for 16 hours,
which became a clear orange solution upon heating. The solution was cooled to room
temperature, resulting in precipitation of the product as bright yellow needles, which were
collected by filtration, washed with cold ethanol and diethyl ether and dried *in vacuo.*
Yield: 2.17 g, 6.53 mmol, 76%. ¹H NMR (CDCl₃, 400 MHz) δ 8.65 (d, J = 7.1 Hz, 1H),
8.59 (d, J = 8.5 Hz, 1H), 8.49 (d, J = 7.9 Hz, 1H), 7.83 (m, 2H), 4.48 (t, J = 7.4 Hz, 2H),
4.14 (q, J = 7.1 Hz, 2H), 2.75 (t, J = 7.4 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H) ppm. ¹³C NMR
(CDCl₃, 100 MHz) δ 171.2, 163.6, 163.3, 139.2, 132.1, 131.2, 130.8, 129.3, 129.1, 127.9,
127.4, 122.9, 121.4, 60.7, 36.3, 32.7, 14.1 ppm. HR-MS (ESI+): m/z calculated for
C₁₇H₁₅NO₄Cl⁺ [M+H⁺]⁺ 332.0690; found 332.0710.

**Ethyl 3-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-1,3-dioxo-1H-
benzo[de]isoquinolin-2(3H)-yl)propanoate (2)**

To a suspension of 1 (1.5 g, 4.52 mmol) in DMSO (10 mL) was added tert-butyl (2-
aminoethyl)carbamate (2.17 g, 13.56 mmol), and the resulting suspension heated to 70 °C
under a nitrogen atmosphere for 16 hours. The resulting orange suspension was cooled to
room temperature and the pH adjusted to 6-7 with 0.1 M HCl. The resulting yellow
precipitate was taken up into DCM, washed with water and brine, dried over MgSO₄ and
filtered. The orange solution was reduced to a minimum volume, and precipitation was
induced by the addition of hexanes. The product was collected by filtration as an orange-
yellow solid. Yield: 1.42 g, 3.12 mmol, 69%. $^1$H NMR (CDCl$_3$, 400 MHz) δ 8.56 (d, $J=7.5$ Hz, 1H), 8.42 (d, $J=8.4$ Hz, 1H), 8.26 (d, $J=8.4$ Hz, 1H), 7.6 (t, $J=7.5$ Hz, 1H), 7.08 (br. s, 1H), 6.55, (d, $J=8.4$ Hz, 1H), 5.13, (t, $J=6.1$ Hz, 1H) 4.47 (t, $J=7.5$ Hz, 2H), 4.14, (q, $J=7.1$ Hz, 2H), 3.64 (m, 2H), 3.44 (m, 2H), 2.74 (t, $J=7.5$ Hz, 2H), 1.47 (s, 9H), 1.22 (t, $J=7.1$ Hz, 3H) ppm.

$^{13}$C NMR (CDCl$_3$, 100 MHz) δ 171.6, 164.6, 164.0, 158.6, 150.3, 134.7, 131.2, 129.9, 127.2, 124.7, 122.7, 120.4, 109.7, 103.3, 80.8, 60.6, 46.8, 39.5, 35.9, 32.9, 29.7, 28.4, 14.2 ppm. HR-MS (ESI+): $m/z$ calculated for C$_{24}$H$_{30}$N$_3$O$_6$ [M+H]$^+$ 456.2135; found 456.2123.

**Ethyl 3-(6-((2-aminoethyl)amino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propanoate (3)**

Compound 2 (1.38 g) was dissolved in DCM (5 mL), followed by the addition of trifluoroacetic acid (5 mL), and stirred for 24 hours. The solvent was removed under reduced pressure. The resulting orange semi-solid was dissolved in water, the pH adjusted to 9 with 1 M NaOH, and extracted with DCM. The organic layer was washed with water and brine, dried over MgSO$_4$, filtered and evaporated under reduced pressure to give the product as a yellow-orange solid. Yield: 0.60 g, 1.69 mmol, 56% $^1$H NMR (CDCl$_3$, 400 MHz) δ 8.51 (d, $J=7.3$ Hz, 1H), 8.39 (d, $J=8.4$ Hz, 1H), 8.14 (d, $J=8.3$ Hz, 1H), 7.56 (t, $J=7.9$ Hz, 1H), 6.64 (d, $J=8.5$ Hz, 1H), 6.22 (m, 1H), 4.46 (t, $J=7.5$ Hz, 2H), 4.14 (q, $J=7.1$ Hz, 2H), 3.39 (dt, $J=5.1$ Hz, 6.2 Hz, 2H), 3.17 (t, $J=6.2$ Hz, 2H), 2.74 (t, $J=7.5$ Hz, 2H), 1.46 (br. s, 2H), 1.22 (t, $J=7.1$ Hz, 3H) ppm. $^{13}$C NMR (CDCl$_3$, 100 MHz) δ 171.6, 164.5, 163.9, 149.8, 134.6, 131.2, 129.8, 126.4, 124.6, 122.8, 120.4, 109.9, 104.4, 60.6, 44.9, 40.1, 35.9, 32.9, 14.2 ppm. HR-MS (ESI+): $m/z$ calculated for C$_{19}$H$_{22}$N$_3$O$_4$ [M+H]$^+$ 356.1610 found; 356.1669.

**Ethyl 3-((2-(bis(pyridin-2-ylmethyl)amino)ethyl)amino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propanoate (DPA-Naph-OEt) (4)**

To a solution of 3 (0.55 g, 1.55 mmol) in 1,2-dichloroethane (20 mL) was added 2-pyridinecarboxaldehyde (0.295 mL, 3.10 mmol), and the solution stirred for 2 hours under a nitrogen atmosphere. The solution was cooled to 0 °C on an ice-water bath, and sodium triacetoxyborohydride (0.75 g, 3.56 mmol) was added. The solution was allowed to warm
to RT, and then stirred for 48 hours. Water was added, and the product was extracted with chloroform. The organic layer was washed with water and brine, dried over MgSO₄, filtered and concentrated to a minimum volume. The product was precipitated by the addition of hexanes and collected by filtration as a bright yellow solid. Yield: 0.65 g, 1.21 mmol, 78%. ¹H NMR (CDCl₃, 400 MHz) δ 8.84 (d, J = 8.4 Hz, 1H), 8.63 (d, J = 7.3 Hz, 1H), 8.59 (d, J = 4.6 Hz, 2H), 8.42 (d, J = 8.4 Hz, 1H), 7.93 (br. s, 1H), 7.70 (t, J = 7.8 Hz, 2H), 7.39 (d, J = 7.8 Hz, 2H), 7.17 (dd, J = 7.8 Hz, 5.9 Hz, 2H), 6.55 (d, J = 8.5 Hz, 1H), 4.5 (t, J = 7.6 Hz, 2H), 4.16, (q, J = 7.1 Hz, 2H), 4.02 (s, 4H), 3.41 (m, 2H), 3.07 (t, J = 5.5 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H), 1.24 (t, J = 7.1 Hz, 3H) ppm. ¹³C NMR (CDCl₃, 100 MHz) δ 171.6, 164.7, 163.9, 158.8, 150.6, 149.3, 136.7, 134.9, 131.1, 130.1, 127.8, 124.3, 123.3, 122.7, 122.3, 120.8, 108.9, 104, 60.5, 59.7, 51, 41, 35.8, 32.9, 29.7, 14.2 ppm. HR-MS (ESI+): m/z calculated for C₃₁H₃₂N₅O₄ [M+H]+ 538.2454; found 538.2449.

3-(6-((2-(Bis(pyridin-2-ylmethyl)amino)ethyl)amino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propanoic acid (DPA-Naph-OH) (5)

To a solution of 4 (0.60 g, 1.12 mmol) in methanol (10 mL) was added NaOH (0.14 g, 3.40 mmol) in H₂O (10 mL). The solution was heated to reflux overnight. Upon cooling to RT, the solvent was removed under reduced pressure, and water (30 mL) was added. The pH of the solution was adjusted to 7 with 1 M HCl, and the product extracted with DCM. The organic layer was washed with water and brine, dried over MgSO₄, filtered and evaporated to give the product as a yellow-orange solid. Yield: 0.48 g, 0.94 mmol, 84%. ¹H NMR (CDCl₃, 400 MHz) δ 8.65 (d, J = 8.3 Hz, 1H), 8.59 – 8.56 (m, 2H), 8.53 (d, J = 7.3 Hz, 1H), 8.34 (d, J = 8.4 Hz, 1H), 7.65 – 7.53 (m, 4H), 7.37 (d, J = 7.8 Hz, 2H), 7.19 – 7.13 (m, 2H), 6.47 (d, J = 8.6 Hz, 1H), 4.47 (t, J = 7.2 Hz, 2H), 4.01 (s, 4H), 3.39 (s, 2H), 3.03 (dd, J = 5.6, 5.2 Hz, 2H), 2.78 (dd, J = 7.1 Hz, 2H). ¹³C NMR (CDCl₃, 100 MHz) δ 164.8, 164.1, 158.41, 150.6, 149.0, 136.9, 135.0, 131.2, 130.0, 127.8, 124.3, 123.6, 122.5, 122.4, 120.6, 108.6, 104.0, 77.3, 77.0, 76.7, 59.6, 51.2, 40.9, 35.8. HR-MS (ESI+): m/z calculated for C₂₉H₂₈N₅O₄ [M+H]+ 510.2144; found 510.2141.
General Peptide Synthesis

Peptides were synthesized by standard Fmoc solid-phase peptide chemistry on Rink Amide MBHA resin (0.1 mmol scale, 0.52 mmol/g resin loading) in an automated peptide synthesizer (Syrowave, Biotage Inc.) with deprotection and coupling steps carried out at room temperature in DMF using HCTU as a coupling agent and N,N-diisopropylethylamine (DIPEA) as a base. The N-terminus was left Fmoc-protected for subsequent modification of the peptides. Alloc deprotection was achieved by treating the resin with Pd(PPh$_3)_4$ (0.1 equiv.) in the presence of PhSiH$_3$ (20 equiv.) in dry DCM for 45 minutes. Disulfide bridges were formed by reaction with I$_2$ (10 equiv.) in 4:1 DMF/H$_2$O for 1 hour, followed by washing of the resin with DMF (3 x 4 mL), 2% ascorbic acid in DMF (3 x 4 mL), then again with DMF (3 x 4 mL). The final Fmoc deprotection was achieved by treatment of the resin with 20% piperidine in DMF for 5 and 15 minutes. Upon completion of synthesis, the resin was rinsed with DCM and allowed to dry. Peptides were cleaved from the resin using 4 mL of 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% distilled water for 5 h. The cleavage cocktail was collected into a 50-mL falcon tube where 20 mL of ice cold tert-butylmethylether (TBME) was slowly added to precipitate the crude peptide. Falcon tubes were centrifuged at 3000 rpm for 10 minutes resulting in a peptide pellet. The remaining TBME was decanted and peptide pellet was re-suspended in 20 mL of fresh ice-cold TBME before being centrifuged again. The resulting peptide pellet was dissolved in water, frozen in dry ice and freeze-dried overnight using a lyophilizer. The crude peptides were analyzed by analytical HPLC-MS.

Synthesis of T140 (6)

The D-Lys side chain was protected with a Boc group. The peptide was cleaved from the resin following disulfide bridge formation and Fmoc deprotection. Purification was performed by preparative HPLC-MS (linear gradient 10-70% Solvent A in B) and was obtained as a fluffy white powder with a purity of 95% after freeze drying of the collected fractions. HPLC (10-70% Solvent A in B) $t_R = 9.32$ min. HRMS (ESI+): $m/z$ calculated for C$_90$H$_{143}$N$_{34}$O$_{17}$S$_2$ [M+H]$^+$ 2036.0807; found 2036.0819.
Synthesis of DPA-Naph-T140 (7)
The D-Lys side chain was protected with an Alloc group. Following Alloc deprotection, DPA-Naph-OH (3 equiv.) was coupled to the peptide by activation with HCTU (3 equiv.) in the presence of DIPEA (6 equiv.) in DMF for 2 hours. This was followed by disulfide bridge formation, Fmoc deprotection and cleavage of the peptide from the resin. Purification was performed by preparative HPLC-MS (linear gradient 20-80% Solvent A in B) and was obtained as a fluffy bright-yellow powder with a purity of 96% (by HPLC) after freeze drying of the collected fractions. HPLC (20-80% Solvent A in B) \( t_R = 8.03 \) min. HRMS (ESI+): \( m/z \) calculated for \( C_{119}H_{168}N_{39}O_{20}S_2 \) \([M+H]^+\) 2527.2764; found 2527.2671.

Synthesis of Re(CO)\(_3\)-DPA-Naph-T140 (Re-7)
To a solution of 7 in dH\(_2\)O (3 mL) in a 5 mL microwave vial was added a 0.1 M solution of [Re(CO)\(_3\)(H\(_2\)O)\(_3\)]OTf (1.2 equiv.) in H\(_2\)O. The reaction was heated to 110 °C for 15 minutes under microwave irradiation. The peptide was then purified by preparative HPLC-MS (linear gradient 20-80% Solvent A in B) and was obtained as a fluffy, bright-yellow powder with a purity of >98% (by HPLC) after freeze drying of the collected fractions. HPLC (20-80% Solvent A in B) \( t_R = 9.63 \) min. HR-MS(ESI+): \( m/z \) calculated for \( C_{122}H_{167}N_{39}O_{23}^{185}\)ReS\(_2\) \([M]^+\) 2795.2063; found 2795.2077.

\( ^{99m}\)Tc-Labelling of 7
Sodium boranocarbonate (10.0 mg, 0.10 mmol), sodium carbonate (15.0 mg, 0.14 mmol), sodium borate (20 mg, 0.05 mmol), and sodium potassium tartrate (22 mg, 0.08 mmol) were dissolved in dH\(_2\)O (1 mL). This solution was transferred to a sterile vial containing 500-1000 MBq \(^{99m}\)TcNaTcO\(_4\) from a \(^{99}\)Mo/\(^{99m}\)Tc generator, and then transferred to a 2 mL microwave vial, which was heated in a microwave reactor for 3.5 minutes at 110 °C to form \(^{99m}\)Tc(Tc(CO)\(_3\)(H\(_2\)O)\(_3\))^+. The mixture was neutralized by the addition of 1 M HCl (0.2 mL). Approximately 200-500 MBq of \(^{99m}\)Tc(Tc(CO)\(_3\)(H\(_2\)O)\(_3\))^+ was added to 7 (0.1 mg) in dH\(_2\)O (0.1 mL) in a 2 mL microwave vial and heated to 100 °C for 12 minutes in the microwave reactor. Upon cooling, the reaction mixture was purified by semipreparative HPLC (linear gradient 20-70% Solvent A in B) to give the labelled peptide in decay
corrected radiochemical yields ranging from 60-85%, radiochemical purities >95% and molar activities of 36-44 GBq/µmol.

**Cell Culture**

U87.CD4 and U87.CD4.CXCR4 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Hong Kui Deng and Dr. Dan R. Littman.37 PC-3 and DU 145 cells were purchased from ATCC. BPH-1 cells were a generous gift from Dr. John Lewis (University of Alberta). U87.CD4.CXCR4 cells were maintained in DMEM – high glucose (Sigma) containing 15% fetal bovine serum (FBS), 1 µg/mL puromycin, 300 µg/mL G418, and 1X penicillin-streptomycin. U87.CD4 cells were maintained in DMEM – high glucose (Sigma) containing 10% FBS, 300 µg/mL G418, and 1X penicillin-streptomycin. PC-3 cells were maintained in F-12K medium (Wisent) supplemented with 10% FBS and 1X penicillin-streptomycin. DU 145 cells were maintained in EMEM (Sigma) supplemented with 10% FBS, 1X MEM non-essential amino acids (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate and 1X penicillin-streptomycin. BPH-1 cells were maintained in RPMI 1640 medium (Wisent) containing 10% FBS and 1X penicillin-streptomycin. All cell lines were cultured at 37°C in humidified atmosphere with 5% CO2 and passaged 2 to 3 times per week.

**Confocal Microscopy**

48 hours prior to the experiment, cells were seeded onto coverslips in 12-well plates (100,000 cells/well). The culture media was removed, and each well was then washed with phosphate buffered saline (PBS, 1 mL). Re-7 was dissolved in culture media at a concentration of 1 µM, and 1 mL of this solution was added to each well. The cells were incubated at 37 °C for 1 hour. After the incubation, each well was washed with PBS (2 x 1 mL) and fixed by the addition of 4% paraformaldehyde in PBS for 15 minutes. Each well was then washed with PBS (2 x 1 mL), and the cover slips mounted on slides with ProLong® Diamond antifade mountant (Molecular Probes). After allowing the slides to cure for 24 hours, cells were imaged by confocal microscopy on an Olympus Fluoview FV1000 confocal microscope using a 40x objective. The excitation wavelength was set at 458 nm and emission collected from 500-550 nm.
**Competitive Binding Assays**

CXCR4 affinities were determined through competitive binding assays using U87.CD4.CXCR4 cells with $[^{125}\text{I}]-\text{SDF}-1$ as the radioligand. T140 was used as a reference to ensure the validity of the results. The peptide of interest (at concentrations ranging from $10^{-12}$ to $10^{-6}$ M) and $[^{125}\text{I}]-\text{SDF}-1$ (20 pM) were mixed with the binding buffer (20 mM HEPES, 0.5% BSA in PBS, pH 7) in 1.5 mL Eppendorf Protein LoBind vials. A suspension of U87.CD4.CXCR4 cells (50,000 cells) was added to each vial to give a final volume of 300 µL. The vials were shaken at 550 rpm for 20 minutes at 37 °C. Immediately after the incubation, the vials were centrifuged at 13000 rpm for 5 minutes and the supernatant removed. The cell pellet was washed with 500 µL of 50 mM Tris buffer (pH 7) and centrifuged again. The amount of $[^{125}\text{I}]-\text{SDF}-1$ bound to the cells was measured using a gamma counter (Perkin Elmer). IC$_{50}$ values were determined by non-linear regression analysis to fit a 4-parameter dose response curve using GraphPad Prism (Version 6.0c).

**Cellular Uptake Assay**

48 hours prior to the experiment, U87.CD4.CXCR4 and U87.CD4 cells were seeded in 6-well plates at a density of 3 x 10$^5$ cells per well. On the day of the experiment, the cell media was aspirated, each well rinsed with Hank’s Balanced Salt Solution (HBSS, 2 x 1 mL)), and an additional 2 mL of HBSS added to each well. 0.5 MBq of $[^{99}\text{mTc}]\text{Tc-7}$ in 100 µL of HBSS was added to each well and the cells were incubated at 37 °C for 1 hour. The supernatant was removed, and each well rinsed with HBSS (1 mL). The combined supernatant and washes represented the free radioligand. The cells were then scraped in PBS (1 mL) and transferred to tubes for counting. The wells were then rinsed with PBS (2 x 1 mL) and combined with the cells. The bound and unbound radioactivity was counted on a Perkin-Elmer Wizard 1470 gamma counter. Cell uptake was expressed as percentage of cell bound activity over total activity.

**Biodistribution Studies**

The Western University Animal Use Subcommittee of the Canadian Council on Animal Care approved the protocols for all mouse handling and treatment procedures described in this study (protocol 2012-033). Male NOD/SCID mice aged 4-6 weeks were
subcutaneously injected with U87.CD4.CXCR4 cells (2.5 x 10^6 cells). After 4-6 weeks, when the tumors had grown to a size of ~0.8 cm, 1.5-2.5 MBq of [^{99m}Tc]Tc-7 was intravenously administered into the tail vein of isoflurane-anesthetized animals (n = 3). The animals were sacrificed at two hours post-injection. The tissues and organs of interest were removed and weighed before being counted for radioactivity in a gamma counter.

2.5 References


1246.


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Chapter 3

3 A Study of $^{99m}$Tc/Re-Tricarbonyl Complexes of 4-Amino-1,8-Naphthalimides

3.1 Introduction

Functionalized 1,8-naphthalimides are an incredibly useful class of molecules that have applications as anticancer drugs\cite{1,2} and as fluorescent cellular imaging agents.\cite{3,4} Substitution of the naphthyl ring with electron donating substituents creates a naphthalimide with an excited state resulting from intramolecular charge transfer (ICT). The resulting fluorophore possesses visible light excitation, solvatochromic properties, and large Stokes shifts.\cite{4} Photophysical properties such as absorption and emission wavelengths, quantum yields, and fluorescent lifetimes can be modulated by appending metal complexes to the naphthalimide. Metals such as Gd(III),\cite{5} Zn(II),\cite{6,7} Au(I),\cite{8} and Re(I)\cite{9,10} have been used to this end, and have shown exceptional utility in various cellular imaging applications. Rhenium complexes are of particular interest, as a fluorescent ligand that can bind rhenium may also bind technetium-99m for the purpose of single photon emission computed tomography (SPECT) imaging. Fluorescent rhenium complexes may aid in the preclinical development of SPECT imaging agents by providing insight into cellular localization and tissue uptake, as well as having uses in staining of \textit{ex vivo} tissue samples.\cite{11-14}

The fac-Re/$^{99m}$Tc(I)-tricarbonyl core is a convenient and robust system that allows for the use of a wide variety of tridentate ligands to coordinate the metal, resulting in kinetically stable complexes.\cite{15} The choice of ligand is critical for determining the overall charge of the metal complex, which can have an impact on hydrophilicity, cellular uptake and localization, as well as biodistribution in an \textit{in vivo} setting. Pope \textit{et al.} have recently developed a set of 4-amino-1,8-naphthalimide ligands containing a di-(2-picolyl)amine binding unit to coordinate Re(I)-tricarbonyl and varying napththalimide substituents.\cite{9} This chelation system imparts an overall positive charge to the Re(I) complex. Coordination of the ligand to Re(I) caused a hypsochromic shift of the absorption and fluorescence wavelengths, as well as an increase in the fluorescence quantum yields due to suppression
of quenching by photo-induced electron transfer (PeT). The complexes also showed increased cellular uptake over the free ligand in most cases. The effect of changing the naphthalimide substituent showed tunability of cellular uptake of these naphthalimides. For the purpose of targeted imaging agents, we wished to observe instead the impact that the charge of the metal complex has on the photophysical and cellular uptake characteristics. This information could be used for the development and optimization of bioconjugates containing Re/\textsuperscript{99m}Tc naphthalimide complexes for fluorescence and SPECT imaging.

In this work, we report the synthesis of three naphthalimide ligands that have been appended with different chelation systems. First, \textbf{L1} contains a di-(2-picolyl)amine ligand and is comparable to those developed by Pope \textit{et al.} to serve as a reference. Two novel ligands were synthesized: \textbf{L2}, containing a binding unit with one picolyl and one carboxyl arm which results in a neutral metal complex, and \textbf{L3}, with an iminodiacetate ligand resulting in a negatively charged metal complex (Figure 3.1). We have compared the photophysical properties of the ligands and their Re(I) complexes and evaluated the cellular uptake of the Re(I) complexes by confocal microscopy. Finally, we demonstrate for the first time, the radiolabelling of the three ligands with technetium-99m to highlight their utility as components of SPECT imaging agents.

\textbf{Figure 3.24:} Structures of the three naphthalimide ligands coordinated to Re or \textsuperscript{99m}Tc.
3.2 Results and Discussion

3.2.1 Synthesis and Structural Evaluation

The three naphthalimide ligands (L1-L3) were chosen to afford Re(I) complexes with three different charge states; positive (Re-L1), neutral (Re-L2) and negative (Re-L3). All three ligands were derived from the precursor amine 3, which was synthesized in three steps from commercially available 4-chloro-1,8-naphthalic anhydride (Scheme 3.1). The chloro-substituted naphthalimide 1 was formed in 84% yield by reaction of the anhydride with glycine methyl ester hydrochloride in ethanol. The Boc protected amine 2 was made in quantitative yield by reaction of 1 with tert-butyl (2-aminoethyl)carbamate in DMSO. This was followed by deprotection of the Boc group with TFA to afford the amine 3 in 41% yield. L1 was then formed by reaction of 3 with 2-picoly chloride in DMF in 68% yield. L2 was synthesized in 37% yield via a one-pot asymmetric reductive alkylation of 3 with 2-pyridinecarboxaldehyde and glyoxylic acid monohydrate using sodium cyanoborohydride. Similarly, L3 was synthesized by reductive alkylation of 3 with glyoxylic acid monohydrate using sodium cyanoborohydride in 50% yield.

The ligands were fully characterized by NMR and mass spectrometry. The Re(I)-tricarbonyl complexes were then formed by reaction of the ligands with fac-[Re(CO)₃(CH₃CN)₃]OTf in chloroform at 60 °C. As with the free ligands, the complexes were characterized by NMR and mass spectrometry. In the ¹H NMR spectra of Re-L1, the ethylene protons from the linker and the methylene protons of the picolyl arms were deshielded in comparison to that of L1 (Figure 3.2, left). A characteristic pair of signals were observed due to the diastereotopic splitting of the methylene protons of the picolyl arms. The coupling constant was 16.4 Hz, corresponding to a geminal coupling. This data confirms that the di-(2-picoly)amine is indeed bound to the rhenium atom as previously reported by Pope et al.
Scheme 3.2: Synthetic route to the three naphthalimide ligands and their Re(I)-tricarbonyl complexes.

The $^{13}$C NMR of Re-L1 showed two new resonances in comparison to L1 at 197.0 and 196.1 ppm which are characteristic of the equatorial and axial carbonyl ligands respectively. In the $^1$H NMR spectrum of Re-L3, the ethylene protons from the linker were deshielded as with Re-L1, but to a much lesser extent. The diastereotopic methylene protons on the carboxyl arms also show a geminal coupling of 15.8 Hz. However, one pair of the protons is slightly deshielded, while the other is slightly shielded in comparison to L3 (Figure 3.2, right). For Re-L2 the picolyl methylene protons shifted similarly to that of
**Re-L1** and the carboxyl methylene protons shifted similarly to those of **Re-L3**. Therefore, the methylene proton shift is characteristic of the chelating arm and not of the overall complex charge. Both pairs of methylene protons showed geminal couplings, again confirming the binding mode.

![Figure 3.25: $^1$H NMR spectroscopy chemical shifts of the chelator arm methylene (red) and linker ethylene (black) protons of L1 (top left) and L3 (top right) and changes upon rhenium coordination (bottom).](image)

### 3.2.2 Photophysical Properties

The UV/Vis absorption and fluorescence properties of the ligands were compared to those of the rhenium complexes (Table 3.1). **L1** had a broad absorption band with a maximum at 439 nm. This band is assigned to an intramolecular charge transfer (ICT) of an N→π* character. **L2** and **L3** had a similar broad band, however the maximum for both ligands was at 436 nm, slightly higher in energy in comparison to **L1**. Interestingly, the molar extinction coefficient of **L1** was significantly higher than those of **L2** and **L3**. It is known that the nature of the amino substituent on the naphthalimide ring can impact the absorbance and energy of the ICT band due to orientation effects.\(^{10,16}\) The absorption band for **Re-L1** experienced a hypsochromic shift and a reduction in the molar extinction
coefficient in comparison to \textbf{L1}, similar to the analogues previously reported by Pope \textit{et al.} The ICT absorption band for \textbf{Re-L2} and \textbf{Re-L3} underwent similar hypsochromic shifts, but an increase in the molar absorption coefficients was observed.

Fluorescence emission maxima varied between the three ligands (Table 3.1). \textbf{L1} had the lowest energy emission at 528 nm, while \textbf{L3} had the highest energy emission at 516 nm. Rhenium coordination caused a hypsochromic shift of the emission maximum for all three ligands, however the extent of the shift depended on the charge of the complex. The positively charged \textbf{Re-L1} induced the largest shift from 528 nm to 506 nm, while the negatively charged \textbf{Re-L3} induced the smallest shift from 516 to 512 nm. The excitation and emission wavelengths for all three complexes are highly compatible with confocal fluorescence microscopy. The UV/Vis and fluorescence spectra are shown in Figure 3.3.

The quantum yield of \textbf{L1} increased upon rhenium coordination due to suppression of PeT quenching pathways as previously reported for this type of complex. However, metal coordination had little effect on the quantum yields of \textbf{L2} and \textbf{L3}. This suggests that either quenching pathways are not suppressed in \textbf{Re-L2} and \textbf{Re-L3}, or the quenching pathways were not present in \textbf{L2} and \textbf{L3}. The quantum yields for both \textbf{Re-L2} and \textbf{Re-L3} were lower than that of \textbf{Re-L1}, however they are still sufficiently high for use in confocal fluorescence microscopy.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spectra.png}
\caption{UV/Vis absorption (A-C) and fluorescence excitation/emission spectra (D-F) of the ligands ($\lambda_{\text{Ex}} = 440$ nm) and their rhenium complexes ($\lambda_{\text{Ex}} = 425$ nm) in CH$_3$CN (20 $\mu$M).}
\end{figure}
Table 3.2: Photophysical properties of the naphthalimide ligands and their Re(I)-tricarbonyl complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{abs}$ (nm)$^a$</th>
<th>$\varepsilon$ (L·mol$^{-1}$·cm$^{-1}$)$^a$</th>
<th>$\lambda_{Ex}$ (nm)$^a$</th>
<th>$\lambda_{Em}$ (nm)$^a$</th>
<th>$\phi$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>439</td>
<td>14500</td>
<td>444</td>
<td>528</td>
<td>0.27</td>
</tr>
<tr>
<td>L2</td>
<td>436</td>
<td>9950</td>
<td>438</td>
<td>520</td>
<td>0.24</td>
</tr>
<tr>
<td>L3</td>
<td>436</td>
<td>8300</td>
<td>438</td>
<td>516</td>
<td>0.13</td>
</tr>
<tr>
<td>Re-L1</td>
<td>424</td>
<td>14100</td>
<td>422</td>
<td>506</td>
<td>0.45</td>
</tr>
<tr>
<td>Re-L2</td>
<td>425</td>
<td>14750</td>
<td>424</td>
<td>510</td>
<td>0.23</td>
</tr>
<tr>
<td>Re-L3</td>
<td>428</td>
<td>15000</td>
<td>424</td>
<td>512</td>
<td>0.13</td>
</tr>
</tbody>
</table>

$^a$In CH$_3$CN (20 µM). $^b$In aerated CH$_3$CN relative to [Ru(bpy)$_3$](PF$_6$)$_2$ in aerated CH$_3$CN ($\phi_e = 0.018$).

3.2.3 Confocal Microscopy

The cellular permeability of the three Re(I) complexes was evaluated in OVCAR-8 ovarian cancer cells by confocal fluorescence microscopy (Figure 3.4, see Appendix B for DIC images). The cells were incubated with the complexes at a concentration of 20 µM and were then fixed and mounted on slides for imaging. The ethyl ester variant of Re-L1 previously reported by Pope et al was shown to have good cellular permeability in human osteoarthritic cells, with cytoplasmic and mitochondrial staining. Re-L1 showed similar uptake and distribution in the OVCAR-8 cells, with strong signal emanating from the cytoplasm, and organelles (Figure 3.4, D). Re-L2 showed decreased permeability in comparison to Re-L1 (Figure 3.4, B). Less cytoplasmic uptake was observed, though there was still a granular staining pattern, likely due to uptake in mitochondria (Figure 3.4, E). The positive charge of Re-L1 along with the increased lipophilicity of the picolyl ligands likely are responsible for the high permeability across the negatively charged phospholipid membrane in comparison to the neutral complex Re-L2. Due to the negative charge on Re-L3, it showed poor cellular permeability (Figure 3.4, C). A weak fluorescent signal was, however, observed emanating from some structures within the cells (Figure 3.4, F). It has previously been reported that hydrophobic imaging labels may increase non-specific uptake in an in vivo setting. Therefore, the higher polarity of Re-L2 and Re-L3 suggests that the technetium-99m labelled variants may be ideal for conjugation to biomolecules for the purpose of SPECT imaging in order to reduce off-target accumulation of an imaging agent.
Figure 3.27: Confocal fluorescence microscope images of OVCAR-8 cells (A-C 40x, D-F 100x) incubated with: (A, D) Re-L1; (B, E) Re-L2; and (C, F) Re-L3 at a concentration of 20 µM (λEx = 458 nm).

While the cellular uptake of Re-L1 was the highest, the high cytoplasmic localization could obscure fluorescent signals from other cellular structures. For applications where specific targeting of cellular structures is required, Re-L2 might be a more suitable imaging agent. In any case, we have demonstrated that changing the polarity and charge of the rhenium complex drastically alters the cellular permeability and localization. This provides the opportunity to create bioconjugates of these complexes with the potential to tune the pharmacokinetic properties, cellular permeability and biodistribution depending on the charge of the chosen complex.

3.2.4 Radiolabelling with Technetium-99m

The previously reported di-(2-picolyl)amine substituted 4-amino-1,8-naphthalimides had not been labelled with technetium-99m, only with the non-radioactive analogue rhenium to exploit the fluorescence properties for fluorescence microscopy applications. To demonstrate the applicability of these ligands as components of SPECT imaging agents,
we radiolabelled them with technetium-99m. Under aqueous conditions, the ligands were reacted with $[^{99m}\text{Tc}][\text{Tc(CO)}_3(\text{H}_2\text{O})_3]^+$ to form the $\text{fac}^{-99m}\text{Tc(I)-tricarbonyl complexes.}$ Initially, we chose a temperature of 100 °C for 15 minutes under microwave irradiation to radiolabel L1. A single radiolabelled product was observed by analytical HPLC of the reaction mixture; however, the retention time was significantly shorter than for that of the rhenium complex. We surmised that the methyl ester was hydrolyzed in the aqueous environment under the high temperatures. Reduction of the temperature to 70 °C for 15 minutes prevented hydrolysis from occurring, and a single radiolabelled product was observed that matched the retention time of the corresponding rhenium complex (Figure 3.5). These same conditions were applied to L2 and L3. The labelled products were isolated by semi-preparative HPLC to remove any unlabelled ligand, resulting in radiochemical yields of the three complexes ranging from 60-95% and radiochemical purities of greater than 95%. The ease of labelling and purification of the ligands under mild aqueous conditions makes them suitable as components of SPECT imaging agents.

![HPLC chromatograms](image)

**Figure 3.28:** HPLC chromatograms (System II, 20-80% Solvent A) of the $^{99m}\text{Tc}$-labelled ligands (blue) and their Re-coordinated standards (pink).
3.3 Conclusions

In summary, we have reported the synthesis and photophysical properties of two new naphthalimide ligands and their Re(I)-tricarbonyl complexes, and compared them to an analogue of a previously reported complex. The photophysical properties of the three complexes are ideal for confocal fluorescence microscopy. Altering the charge and polarity of the metal complex drastically altered the cellular permeability of the ligands. Re-L1 showed the highest cellular permeability. The neutral complex, Re-L2, although having decreased cellular permeability compared to Re-L1, had similar cellular distribution, except with lower cytoplasmic uptake. The cellular uptake of the negatively charged complex, Re-L3, was limited due to its high polarity, but this property may be ideal for technetium-99m labelled SPECT imaging agents to reduce non-specific binding in vivo. The differing cellular permeability of the three complexes demonstrates their vastly different polarity, which may be exploited for use in in vivo imaging studies. We have also demonstrated the 99mTc-labelling of the three ligands in high yields and radiochemical purities, making them suitable as components of SPECT imaging agents. Bioconjugates of these naphthalimides may have applications as dual modality imaging agents for in vitro and ex vivo tissue staining by fluorescence, and as in vivo SPECT imaging agents.

3.4 Experimental

3.4.1 General Experimental

All reagents were purchased from commercial sources and used without further purification. NMR spectra were recorded on a Bruker AvIII HD 400 spectrometer. All chemical shifts are reported in ppm and referenced to the residual solvent peaks. High resolution mass spectra were recorded on a Thermo Scientific Double Focusing Sector mass spectrometer for EI or a Bruker micrOTOF II for ESI in either positive or negative mode. Analytical reversed-phase HPLC-MS was performed on a system consisting of an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 150 mm), Waters 600 controller and binary solvent pump and a Waters Quattro Micro API mass spectrometer (System I). Mass spectra were collected using an ESI source in positive ion mode. The linear gradient solvent system comprised of solvent A (CH$_3$CN + 0.1 % TFA) and solvent B (H$_2$O + 0.1 % TFA) at a flow
rate of 1.5 mL/min over 10 minutes with a 5-minute wash cycle at 95% solvent A. The UV/Vis absorbance was detected using a Waters 2998 photodiode array detector. Preparative reversed-phase HPLC-MS was performed on the same system using an Agilent Zorbax PrepHT SB-C18 column (5 µm, 21.2 x 150 mm) at a flow rate of 20 mL/min. After purification, the collected fractions were frozen at -78 °C, and lyophilized. Analytical radio-HPLC was performed on a system comprising of an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 150 mm), Waters 600 controller and Binary Solvent Pump, a Carroll & Ramsey radiometric detector connected to a Waters e-Sat/IN module and Waters 2498 dual absorbance UV/Vis detector (System II). The linear gradient solvent system comprised of solvent A (CH₃CN + 0.1 % TFA) and solvent B (H₂O + 0.1 % TFA) at a flow rate of 1.5 mL/min over 10 minutes with a 5-minute wash cycle at 95% solvent A. Semi-preparative radio-HPLC was performed on the same system with an Agilent Zorbax SB-C18 column (3.5 µm, 4.6 mm x 150 mm) at a flow rate of 4 mL/min. UV-Vis spectra were recorded on an Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technologies International, Inc. Quanta Master – 7/2005. Quantum yields were determined on aerated acetonitrile solutions using [Ru(bpy)₃](PF₆)₂ in acetonitrile (ϕᵣ = 0.018) as a reference.¹⁸ fac-[Re(CO)₃(CH₃CN)₃]OTf was prepared according to the literature.¹⁹

**Methyl 2-(6-chloro-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)acetate (1)**

4-Chloro-1,8-napthalic anhydride (2.00 g, 8.6 mmol), triethylamine (0.85 g, 8.5 mmol) and glycine methyl ester hydrochloride (1.53 g, 17.2 mmol) were added to ethanol (50 mL) and heated to reflux for 16 hours. The orange solution was cooled to room temperature, resulting in the formation of the product as an off-white precipitate that was collected by filtration, washed with ice cold ethanol and then dried under vacuum (2.2 g, 84%).¹¹ H NMR (400 MHz; CDCl₃) δ 8.64 (dd, J = 7.3, 1.0 Hz, 1H), 8.58 (dd, J = 8.5, 1 Hz, 1H), 8.48 (d, J = 7.3 Hz, 1H), 7.89 – 7.76 (m, 2H), 4.94 (s, 2H), 3.78 (s, 3H).¹³ C NMR (CDCl₃; 100 MHz) δ 168.5, 163.5, 163.2, 139.7, 132.5, 131.6, 131.2, 129.5, 129.3, 128.0, 127.5, 122.7, 121.2, 52.7, 41.4. HRMS (EI): m/z calculated for C₁₅H₁₀ClNO₄ [M]+ 303.0298; found 303.0284.
Methyl 2-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)acetate (2)

*tert*-Butyl (2-aminoethyl)carbamate (3.5 g, 21.7 mmol) and compound 1 (2.2 g, 7.2 mmol) were dissolved in DMSO (10 mL) and heated to 70 °C for 16 hours. The resulting deep red solution was cooled to room temperature and the pH neutralized with 0.1 M HCl. The solution was diluted with dichloromethane and washed with water. After drying with MgSO₄ the dichloromethane layer was reduced to a minimal volume under vacuum. Hexanes was added, resulting in the precipitation of the product as an orange solid which was collected by filtration, washed with cold hexanes, and dried under vacuum (3.07 g, quant.). ¹H NMR (400 MHz; CDCl₃) δ 8.45 (d, J = 7.3 Hz, 1H), 8.36 (d, J = 8.4 Hz, 1H), 8.24 (d, J = 8.4 Hz, 1H), 7.49 (t, J = 7.3 Hz, 1H), 7.04 (br. s, 1H), 6.51 (d, J = 8.5 Hz, 1H), 4.95 (s, 2H), 3.79 (s, 3H), 3.61 (q, J = 5.6 Hz, 2H), 3.43 (q, J = 4.4 Hz, 2H), 1.48 (s, 9H). ¹³C NMR (100 MHz; CDCl₃) δ 169.7, 164.6, 163.8, 158.4, 150.8, 135.1, 131.5, 127.7, 124.7, 120.5, 109.1, 103.6, 80.7, 52.6, 46.4, 41.2, 39.6, 29.8, 28.5. HRMS (ESI⁺): m/z calculated for NaC₂₂H₂₅N₃O₆ [M+Na]⁺ 450.1641; found 450.1644.

Methyl 2-(6-((2-aminoethyl)amino)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)acetate (3)

Compound 2 (3.0 g, 7.02 mmol) was dissolved in dichloromethane (10 mL) and trifluoroacetic acid (10 mL) and the solution was stirred for 24 hours at room temperature. The solvent was removed under vacuum and the resulting TFA salt was dissolved in water and the pH was neutralized with saturated sodium bicarbonate. The product was extracted into DCM, the organic layer was collected and dried with Na₂SO₄. The solvent was then removed under reduced pressure producing a yellow solid (0.95 g, 41%). ¹H NMR (400 MHz; CD₃OD) δ 8.50 – 8.38 (m, 2H), 8.29 (d, J = 8.5 Hz, 1H), 7.61 (dd, J = 8.3, 7.5 Hz, 1H), 6.80 (d, J = 8.5 Hz, 1H), 4.86 (s, 2H), 3.78 (s, 3H), 3.75 (t, J = 6.2 Hz, 2H), 3.34 (t, J = 6.2 Hz, 2H). ¹³C NMR (100 MHz; CD₃OD) δ 169.7, 165.6, 165.1, 151.9, 135.8, 132.5, 131.1, 129.6, 126.0, 123.0, 122.2, 110.4, 105.3, 52.9, 42.0, 41.7, 39.2. HRMS (ESI⁺): m/z calculated for C₁₇H₁₈N₃O₄ [M+H]⁺ 328.1297; found 328.1284.
L1
To a solution of compound 3 (50 mg, 0.15 mmol) in DMF (4 mL) was added K$_2$CO$_3$ (84 mg, 0.61 mmol), KI (51 mg, 0.31 mmol) and 2-picolychloride hydrochloride (50 mg, 0.31 mmol). The orange solution was heated to 50 °C for 48 hours. The solution was partitioned into water (30 mL) and chloroform (50 mL). The aqueous layer was extracted 3 times with chloroform, and the combined organics were dried over Na$_2$SO$_4$ and concentrated under reduced pressure to give the product as a yellow-orange solid (L1) (53 mg, 68%). UV/Vis (CH$_3$CN): $\lambda_{max}$/nm (ε/L·mol$^{-1}$·cm$^{-1}$) = 439 (14 500) 340 (2750) 324 (3450) 282 (19 000) 269 (29 500) 263 (33 000) 256 (32 000). $^1$H NMR (400 MHz; CDCl$_3$) δ 8.85 (dd, $J$ = 8.4, 1.0 Hz, 1H), 8.63 (dd, $J$ = 7.4, 1.0 Hz, 1H), 8.59 – 8.56 (m, 2H), 8.42 (d, $J$ = 8.5 Hz, 1H), 7.98 (s, 1H), 7.69 (dd, $J$ = 8.4, 7.4 Hz, 1H), 7.56 (td, $J$ = 7.8, 1.8 Hz, 2H), 7.37 (d, $J$ = 7.8 Hz, 2H), 7.15 (ddd, $J$ = 7.5, 4.9, 1.0 Hz, 2H), 6.55 (d, $J$ = 8.5 Hz, 1H), 4.96 (s, 2H), 4.01 (s, 4H), 3.77 (s, 3H), 3.43 – 3.36 (m, 2H), 3.09 – 3.02 (m, 2H).

fac-[Re(CO)$_3$(L1)]OTf (Re-L1)
L1 (53 mg, 104 µmol) and fac-[Re(CO)$_3$(CH$_3$CN)$_3$]OTf (56 mg, 104 µmol) were added to chloroform (5 mL) and heated to 60 °C for 18 hours. The solution was cooled to room temperature and diethyl ether was added to induce precipitation of the product as a yellow solid, which was collected by filtration, washed with diethyl ether and dried under vacuum giving Re-L1 (72 mg, 74%). UV/Vis (CH$_3$CN): $\lambda_{max}$/nm (ε/L·mol$^{-1}$·cm$^{-1}$) = 424 (14 100) 338 (4150) 321 (5700) 276 (25 500) 268 (34 800) 259 (35 500) 253 (34 000). $^1$H NMR (400 MHz; acetone-$d_6$) δ 8.99 – 8.96 (m, 2H), 8.67 (d, $J$ = 8.5 Hz, 1H), 8.50 (dd, $J$ = 7.3, 1.0 Hz, 1H), 8.38 (d, $J$ = 8.5 Hz, 1H), 8.05 (td, $J$ = 7.3, 1.6 Hz, 2H), 7.74 – 7.67 (m, 3H), 7.48 (t, $J$ = 6.0 Hz, 2H), 7.43 (t, $J$ = 5.3 Hz, 1H), 7.14 (d, $J$ = 8.5 Hz, 1H), 5.54 (d, $J$ = 16.4 Hz, 2H), 5.28 (d, $J$ = 16.4 Hz, 2H), 4.85 (s, 2H), 4.51 (t, $J$ = 6.6 Hz, 2H), 4.33 – 4.27 (m, 2H), 3.73 (s, 3H). $^{13}$C NMR (100 MHz; acetone-$d_6$) δ 197.0, 196.1, 169.7, 164.6, 163.7, 161.9, 152.9, 151.0, 141.5, 135.1, 131.9, 130.8, 129.1, 126.7, 125.8, 124.7, 123.2, 121.9,
Compound 3 (50 mg, 0.15 mmol) sodium acetate (50 mg, 0.61 mmol), acetic acid (70 µL, 1.22 mmol) and 2-pyridinecarboxaldehyde (16 mg, 0.15 mmol) were dissolved in methanol (5 mL) and heated to 65 °C for 16 hours. Upon cooling to room temperature, glyoxylic acid monohydrate (21 mg, 0.23 mmol) and NaBH₃CN (21 mg, 0.34 mmol) were added, and the solution stirred at room temperature for 24 hours. The solvent was removed under reduced pressure and the crude product was purified by preparative HPLC (System I, 20-80%) to give the product as a hygroscopic orange solid (27 mg, 37%). UV/Vis (CH₃CN): \( \lambda_{\text{max}}/\text{nm} (\varepsilon/L \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}) = 436 \) (9950) 339 (1930) 322 (2350) 279 (13\,000) 268 (22\,700) 261 (25\,500) 255 (24\,500). \(^1\)H NMR (400 MHz; CD₃OD) \( \delta \) 8.53 – 8.47 (m, 2H), 8.33 – 8.29 (m, 1H), 8.22 (d, \( J = 8.5 \) Hz, 1H), 8.05 (td, \( J = 7.9, 1.6 \) Hz, 1H), 7.76 (d, \( J = 7.9 \) Hz, 1H), 7.67 (dd, \( J = 8.5, 7.4 \) Hz, 1H), 7.51 – 7.46 (m, 1H), 6.64 (d, \( J = 8.6 \) Hz, 1H), 4.88 (s, 2H), 4.42 (s, 2H), 3.92 (s, 2H), 3.78 (s, 3H), 3.63 (t, \( J = 6.0 \) Hz, 2H), 3.34 (t, \( J = 6.0 \) Hz, 2H). \(^{13}\)C NMR (100 MHz; CD₃OD) \( \delta \) 173.7, 170.9, 165.7, 165.1, 155.4, 152.0, 145.0, 144.4, 135.9, 132.6, 131.3, 129.6, 126.6, 126.0, 125.9, 123.1, 122.0, 109.6, 105.3, 58.2, 56.5, 54.1, 52.9, 42.0, 41.4. HRMS (ESI+): \( m/z \) calculated for C₂₅H₂₅N₄O₆ \([\text{M}+\text{H}]^+ \) 477.1774; found 477.1776.

**fac-Re(CO)₃(L2)** (Re-L2)

Prepared as for **Re-L1** but using L2 (22 mg, 46 µmol) and fac-[Re(CO)₃(CH₃CN)₃]OTf (25 mg, 46 µmol). Precipitation with diethyl ether gave crude product which was purified by preparative HPLC (System I, 35-85%) to give the pure product as a yellow solid (10 mg, 29%). UV/Vis (CH₃CN): \( \lambda_{\text{max}}/\text{nm} (\varepsilon/L \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}) = 425 \) (14\,750) 340 (3800) 323 (4950) 277 (23\,500) 268 (29\,000) 262 (34\,500) 255 (34\,000). \(^1\)H NMR (400 MHz; CD₃CN) \( \delta \) 8.80 (d, \( J = 5.6 \) Hz, 1H), 8.56 – 8.53 (m, 1H), 8.47 – 8.40 (m, 2H), 8.09 – 8.02 (m, 1H), 7.77 – 7.70 (m, 1H), 7.63 (d, \( J = 8.0 \) Hz, 1H), 7.53 – 7.46 (m, 1H), 6.94 (d, \( J = 8.5 \) Hz, 1H), 6.36 (br. s, 1H), 4.83 (d, \( J = 16 \) Hz 1H), 4.83 (s, 2H) 4.61 (d, \( J = 16 \) Hz, 1H), 4.09 –
3.98 (m, 2H), 3.95–3.85 (m, 3H), 3.72 (s, 3H), 3.58 (d, J = 16.6 Hz, 1H). HRMS (ESI+): m/z calculated for C_{28}H_{24}N_{4}O_{9}{^{185}}Re [M+H]^+ 745.1073; found 745.1082.

**L3**

Compound 3 (50 mg, 0.15 mmol), sodium acetate (50 mg, 0.61 mmol), acetic acid (70 µL, 1.22 mmol) glyoxylic acid monohydrate (42 mg, 0.46 mmol) and NaBH₃CN (21 mg, 0.34 mmol) were dissolved in methanol (5 mL) and stirred at room temperature for 48 hours. The solvent was removed under reduced pressure and the crude product purified by preparative HPLC (System I, 15-70%) to give the product as a yellow solid (34 mg, 50%). UV/Vis (CH₃CN): λ_{max}/nm (ε/L·mol⁻¹·cm⁻¹) = 436 (8700) 339 (1900) 279 (11 300) 269 (20 900) 255 (21 500). ¹H NMR (400 MHz; CD₃OD) δ 8.64 (dd, J = 8.4, 1 Hz, 1H), 8.48 (dd, J = 7.4, 1.0 Hz, 1H), 8.33 (d, J = 8.6 Hz, 1H), 7.62 (dd, J = 8.4, 7.4 Hz, 1H), 6.77 (d, J = 8.6 Hz, 1H), 4.87 (s, 2H), 3.87 (s, 4H), 3.77 (s, 3H), 3.58 (t, J = 5.8 Hz, 2H), 3.37 (t, J = 5.8 Hz, 2H). ¹³C NMR (100 MHz; CD₃OD) δ 173.3, 170.8, 165.9, 165.2, 152.5, 136.2, 132.5, 131.4, 130.0, 125.7, 122.9, 122.0, 109.3, 105.3, 55.8, 53.5, 52.9, 42.0, 41.3. HRMS (ESI-): m/z calculated for C_{21}H_{20}N_{3}O₈ [M-H]⁻ 442.1250; found 442.1262.

**fac-Na[Re(CO)₃(L3)] (Re-L3)**

L3 (25 mg, 56 µmol) and fac-[Re(CO)₃(CH₃CN)₃]OTf (31 mg, 56 µmol) and sodium bicarbonate (10 mg, 113 µmol) were added to chloroform (5 mL) and heated to 60 °C for 18 hours then cooled to room temperature. The solvent was removed, and the residue taken up in methanol (2 mL) and the residual salts were removed by filtration. The product was precipitated by the addition of diethyl ether (10 mL) to give the product as a yellow-orange solid that was collected by filtration (16 mg, 39%). UV/Vis (CH₃CN): λ_{max}/nm (ε/L·mol⁻¹·cm⁻¹) = 428 (15 000) 340 (2500) 324 (3200) 278 (22 500) 269 (31 500) 262 (33 000) 256 (32 000). ¹H NMR (400 MHz; CD₃OD) δ 8.53 – 8.44 (m, 2H), 8.37 (d, J = 8.6 Hz, 1H), 7.64 (dd, J = 8.4, 7.4 Hz, 1H), 6.89 (d, J = 8.6 Hz, 1H), 4.89 (s, 2H), 3.94 (d, J = 15.8 Hz, 2H), 3.88 – 3.72 (m, 9H). ¹³C NMR (100 MHz; CD₃OD) δ 198.8, 197.6, 182.7, 171.0, 165.8, 165.1, 152.1, 136.1, 132.5, 131.3, 129.6, 125.9, 123.1, 122.1, 110.0, 105.5, 67.9,
64.2, 52.9, 42.0, 40.7. HRMS (ESI-): m/z calculated for C_{24}H_{19}N_{3}O_{11}^{185}\text{Re [M]}\ 710.0549; found 710.0540.

**General procedure for radiolabelling with technetium-99m**

A lyophilized powder containing sodium boranocarbonate (10.0 mg, 0.10 mmol), sodium carbonate (15.0 mg, 0.14 mmol), sodium borate (20 mg, 0.05 mmol), and sodium potassium tartrate (22 mg, 0.08 mmol) was dissolved in 1 mL of deionized water. This solution was transferred to a sterile vial containing 1 GBq of $[^{99}\text{mTc}]\text{NaTcO}_4$ from a $^{99}\text{Mo}/^{99}\text{mTc}$ generator, and then transferred to a 2 mL microwave vial. The reaction mixture was heated in a microwave reactor (Biotage Inc.) for 3.5 minutes at 110 °C to form $[^{99}\text{mTc}][\text{Tc(CO)}_3(\text{H}_2\text{O})_3]^+$. The pH of the mixture was neutralized by the addition of 1 M HCl (0.2 mL). 150-250 MBq of the $[^{99}\text{mTc}][\text{Tc(CO)}_3(\text{H}_2\text{O})_3]^+$ was transferred to a 2 mL microwave vial containing the ligand in methanol (0.1 mL), and the volume made up to 1 mL with deionized water. The resulting solution was heated in the microwave reactor at 70 °C for 15 minutes. The radiolabelled products were then directly purified by semi-preparative HPLC (System II, 20-80%). The identity of the radiolabelled product was confirmed by comparison of the retention time with the corresponding rhenium coordinated ligand.

$[^{99}\text{mTc}]\text{fac-[Tc(CO)}_3(\text{L1})]\ ^+\ ([^{99}\text{mTc}]\text{Tc-L1})$

**L1** (0.5 mg) was used for labelling. Radiochemical yields: 74 ± 13%. Radiochemical purities: 99 ± 1%. HPLC (System II, 20-80 %) $t_R = 10.0$ min.

$[^{99}\text{mTc}]\text{fac-Tc(CO)}_3(\text{L2})\ ([^{99}\text{mTc}]\text{Tc-L2})$

**L2** (0.1-0.2 mg) was used for labelling. Radiochemical yields: 66 ± 10%. Radiochemical purities: 98 ± 2%. HPLC (System II, 20-80%) $t_R = 9.3$ min.

$[^{99}\text{mTc}]\text{fac-[Tc(CO)}_3(\text{L3})]\ ^-\ ([^{99}\text{mTc}]\text{Tc-L3})$

**L3** (0.5 mg) was used for labelling. Radiochemical yields: 90 ± 5%. Radiochemical purities: 96 ± 4%. HPLC (System II, 20-80%) $t_R = 7.8$ min.
Cell Culture
OVCAR-8 cells were cultured in RPMI 1640 culture medium (Wisent) supplemented with 10% fetal bovine serum (Wisent) and 1X penicillin-streptomycin (Wisent) in a 37 °C incubator with a 5% CO$_2$ atmosphere. Cells were passaged 2-3 times per week.

Fluorescence Confocal Microscopy
OVCAR-8 cells were seeded at a density of 100,000 cells per well in 12-well plates containing glass coverslips and allowed to adhere for 24 hours. The culture medium was removed, and the wells washed with phosphate buffered saline (PBS). A stock solution of each rhenium complex in DMSO (80 µL, 1 mM) was diluted to 4 mL in culture medium giving a 20 µM solution. 1 mL of solution was added to each well (n = 2 per rhenium complex), and the cells incubated for 30 minutes at 37 °C. The culture medium was removed, and the cells were washed twice with PBS. The cells were then fixed by the addition of 1 mL of a 4% paraformaldehyde solution in PBS for 15 minutes. After washing with PBS, the cells were mounted on glass slides using ProLong® Diamond Antifade Mountant (Invitrogen) for imaging. Confocal fluorescence microscopy was performed on an Olympus FluoView FV1000 confocal microscope with a 40x or 100x objective, a 458 nm laser for excitation and the emission filter set to 500-550 nm.

3.5 References

(2) Kilpin, K. J.; Clavel, C. M.; Edafe, F.; Dyson, P. J. Organometallics 2012, 31, 7031–7039.


Chapter 4

4 The Development of $^{99m}$Tc/Re(I) Tricarbonyl [2+1] Complexes with Peptides Containing 2,2’-Bipyridine Residues as β-Turn Mimics

4.1 Introduction

4.1.1 Protein Secondary Structure

β-Sheets are a common secondary structural motif in proteins, accounting for >30% of all protein structures.$^1$ β-sheets are composed of multiple β-strands linked together by hydrogen bonds in either a parallel or anti-parallel fashion (Figure 4.1), with the latter being more common. Often, β-strands are linked by a U-shaped turn region called a β-hairpin, which reverses the overall direction of the peptide strand, arranging them in an anti-parallel fashion.

![Parallel (left) versus anti-parallel (right) β-sheets.](image)

β-hairpins are important in biological recognition events such as protein-protein and protein-peptide interactions.$^{2,3}$ Their study for the purpose of molecular imaging is important, as they could be utilized to target medically relevant biological entities with a propensity to interact with β-hairpins. One such example is the peptide T140, which binds to the C-X-C chemokine receptor 4 (CXCR4). T140 was derived from the antimicrobial self-defense peptides tachyplesin and polyphemusin, which were extracted from the hemocytes of horseshoe crabs. Structurally, T140 contains a disulfide bridge that stabilizes the anti-parallel β-sheet structure.$^4$ This structure is essential for the peptide’s interaction with CXCR4, but the turn region is located outside of the CXCR4 binding pocket.$^5$ Modification of the turn region has been shown to be possible without affecting the binding
of the peptide to the receptor. Therefore, the development of peptides containing \( \beta \)-hairpin mimicking structures may help create diagnostic and therapeutic agents for diseases involving the CXCR4 receptor such as HIV and cancer.

### 4.1.2 Imaging Probe Design: Integrated Versus Pendant

Metal-based molecular imaging probes require a method of connecting a targeting component, such as a peptide, and signaling component, such as a radiometal. This is typically done through a metal chelator. Two methodologies are employed when designing metal-containing peptide-based imaging probes (Figure 4.2). The most common approach is the pendant design in which a radiometal is connected to a peptide through a bifunctional chelator, which is attached to the peptide either through a modified side-chain, or through one of the termini of the peptide, typically through an amide linkage. By this method, the metal complex is placed away from the biologically active site. The pendant approach can be problematic, as modification of the original peptide may result in decreased affinity for the biological target of interest, and removal of the metal complex from the targeting entity \textit{in vivo} can occur by trans-chelation, or enzymatic cleavage. The latter can be mitigated through the use of single amino acid chelates (SAAC), or through conjugation of the complex to the peptide through linkages other than amide bonds; however these do not address the issue of decreased affinity to the target of interest.

![Diagram of Pendant and Integrated Designs](image)

**Figure 4.30:** Pendant (left) versus integrated (right) design.

An integrated approach may be able to remedy these issues. With this approach, the metal complex is integrated into the peptide backbone, effectively “hiding” the radiometal within the framework of the peptide, resulting in increased stability and making for a more compact metal complex. It has been shown that smaller technetium complexes have a
higher likelihood of maintaining the biological activity of the original ligand.\textsuperscript{10} An example of an integrated design is the use of a tridentate 2,6-bis(aminomethyl)pyridine ligand coordinated to rhenium and integrated into a macrocyclic peptide structure as a replacement for the turn region.\textsuperscript{11} Integration of the radiometal into the peptide structure did not interfere with the already present β-sheet secondary structural elements, effectively integrating the metal complex into the β-hairpin mimic, with the potential for applications in radiopharmaceuticals.

4.1.3 2,2’-Bipyridine as a Bidentate Chelator

Examples in the literature have shown that peptides containing 2,2’-bipyridine (bpy) can form β-hairpin structures upon coordination with a metal. A notable example was by Schneider and Kelly, in which square planar Cu(II) complexes were used to nucleate β-hairpin formation.\textsuperscript{12} Their peptides contain a 6,6’-bis(acylamino)-2,2’-bipyridine residue, which exists in a transoid conformation prior to metal coordination (Figure 4.3 – left). Upon coordination with Cu(II), the bipyridine residue forms a cisoid structure (Figure 4.3 – right). This allows for interstrand hydrogen bonds to form, resulting in a β-hairpin structure.

The ability of bipyridine based peptides to complex with a metal and form a β-turn structure introduces the possibility of incorporating a radiometal into the peptide structure. Since β-sheets are important in biological recognition,\textsuperscript{2} a bipyridine based β-turn peptide labelled with a radiometal could allow for their use in nuclear imaging techniques such as SPECT or PET for the purpose of imaging cancer and other disease states.

![Figure 4.31: Square planar Cu(II) complexes of 2,2’-bipyridine nucleating β-sheet formation.](image)
There are many examples in the literature of rhenium complexes with bipyridine. A bidentate bipyridine ligand and a monodentate ligand such as pyridine or imidazole can form a Re(I) tricarbonyl complex. Isostructural complexes can also be formed with technetium-99m to form a radioactive imaging probe. These can be synthesized efficiently using a microwave reactor in a one-pot, two-step synthesis (Figure 4.4).

**Figure 4.32:** Synthesis of isostructural [2+1] complexes of Re and $^{99m}$Tc. A bidentate bipyridine ligand and monodentate pyridine ligand fill the 3 coordination sites on the metal center.

This chapter will focus on the design and synthesis of β-hairpin mimicking peptides containing a bidentate 2,2′-bipyridine chelator for the purpose of coordinating Re/$^{99m}$Tc towards the development of SPECT imaging agents. The incorporation of a bipyridine moiety into a peptide would most easily be accomplished by Fmoc solid-phase peptide synthesis (SPPS), therefore requiring the design of an unnatural Fmoc-amino acid containing the desired bipyridine moiety (Figure 4.5). After the amino acid has been incorporated into the peptide, the structure of the peptide will be evaluated by circular dichroism (CD) spectroscopy. The peptide will then be coordinated to Re/$^{99m}$Tc for the purpose of nucleating β-sheet formation, and the structure evaluated by CD spectroscopy.

**Figure 4.33:** Proposed Fmoc-protected 2,2′-bipyridine amino acid.
4.2 Results and Discussion

4.2.1 Synthesis of 3,3'-Diamino-2,2'-Bipyridine Amino Acid

To synthesize the bipyridine amino acid, 3,3'-diamino-2,2'-bipyridine was used as a building block, as the two amino groups could be used for further elaboration. The first synthetic approach taken for the synthesis of 3,3'-diamino-2,2'-bipyridine was the Ullmann coupling of compound 1 to make the bipyridine 2, followed by hydrolysis of the acetyl groups to yield the diamine 3 (Scheme 4.1). In the Ullmann coupling, free amines greatly decrease the yield of the desired product, due to the formation of byproducts. 3-Amino-2-chloropyridine was protected with an acetyl group by dissolving it in neat acetic anhydride and stirring overnight. The acetylated product 1 was obtained in an 85% yield. Synthetic procedures published in the literature gave a yield of 77% for the Ullmann coupling of 1, followed by a 95% yield for the hydrolysis of the acetyl groups. However, yields obtained from this reaction were quite low. A 27% yield of 2 was obtained on one attempt, while all attempts afterwards only yielded recovered starting material.

![Scheme 4.3: Initial synthesis of 3,3'-diamino-2,2'-bipyridine.](image)

The low yields obtained in the coupling reaction can be attributed to the lack of reactivity of the starting material. Electron donating groups are typically inhibiting in Ullmann couplings. Electron withdrawing groups (EWG) ortho to the halogen are typically activating towards the formation of the arylcopper(I) intermediate, accelerating the reaction rate and allowing for coupling at lower temperatures. A nitro group in the 3-position would be ideal as an EWG in this position, as it may be reduced to the amine after homocoupling. 3-Nitro-2-chloropyridine was used in the coupling reaction with activated bronze powder as the copper source. TLC indicated that the starting material was consumed after only 2 hours at 150 °C. After optimization of the reaction and workup conditions, yields of 40-48% were obtained. Reduction of the nitro groups was then performed with
\[
\text{SnCl}_2 \cdot 2\text{H}_2\text{O} \text{ at reflux in concentrated HCl. The diamine 3 was obtained in 90-95% yield.}
\]

Formation of the monosuccinamide 5 was achieved by stirring 3 in DCM with succinic anhydride in yields ranging from 75-90%. Protection of the amine with an Fmoc group gave 6 in 68-72% yield. This novel amino acid was then suitable for use in SPPS. Scheme 4.2 highlights the synthesis of compound 6.

4.2.2 Design of a β-Hairpin Peptide

Designing a peptide that is optimal for β-hairpin formation is important, as secondary structures may not form if the chosen sequence does not stabilize them. Ideally, with a peptide containing 2,2'-bipyridine, coordination with a metal such as rhenium or technetium-99m should nucleate β-hairpin formation or stabilize already present secondary structure. In 1996, Ramírez-Alvarado et al. reported on the design and structural characterization of a peptide system optimized to readily undergo β-hairpin formation.\footnote{Ramírez-Alvarado et al. (1996)}

Using a program called WHATIF\footnote{WHATIF (1990)} and the protein structure database included with the program consisting of 279 proteins with less than 50% homology, they determined that peptides containing two-residue turns with three-residue strands were most common. They developed a peptide designated as BH8, which was shown to form a β-sheet structure by NMR and CD spectroscopy in water, and was stabilized in 30% 2,2,2-trifluoroethanol (TFE). Residues on the N-terminal end were defined as -B3, -B2 and -B1, while those on the C-terminal end were defined as +B1, +B2 and +B3.
We elected to use this scaffold in the design of our bipyridine β-hairpin peptide. The 2,2’-bipyridine based amino acid will emulate the two-residue turn region, with the succinamide group on the C-terminal end, and a β-alanine on the N-terminal end, which will allow the two strands to align in an anti-parallel fashion. Due to the trans conformation of 2,2’-bipyridine ligands, the non-coordinated peptide should not contain any ordered secondary structure. Metal coordination should induce formation of a β-sheet. The remaining residues were mainly chosen due to statistical considerations; however, there were other factors that were considered as well. The -B2 and +B2 positions were chosen to be threonine residues, as previous work showed that when present together in a non-hydrogen bonded site, they had the highest intrinsic propensity of all amino acids for formation of β-hairpins.\textsuperscript{21} It was found that positively charged residues are commonly found in the +B1 position, so lysine was chosen for this position. +B3 was chosen to be tyrosine, as aromatic residues were found to be quite abundant in that position. Isoleucine and valine were found together at the -B3 and -B1 positions respectively, twice as often as random chance might predict. A previous study also determined that interactions between tyrosine and isoleucine at the +B3 and -B3 positions respectively, aided in the stabilization of the β-hairpin when in a hydrogen bonded site.\textsuperscript{22} In order to prevent lateral oligomerization of the peptide strands, arginine residues were added to the termini to cause electrostatic repulsion between the individual peptide strands. However, this could have the potential side effect of causing intrasstrand electrostatic repulsion. In order to counteract this, glycine was added as a spacer in between the arginine residues and the -B3 and +B3 residues. Since glycine has few conformational restrictions, it tends to break secondary structure. This should prevent the arginine and glycine residues from forming a part of the β-hairpin structure, reducing the repulsion between the two β-strands. The C-terminus of the peptide will also be modified with an amide in place of the carboxylic acid in order to improve stability. Figure 4.6 shows the peptide BH8 compared to our newly designed bipyridine β-hairpin peptide (7).
4.2.3 Synthesis of a β-Hairpin Peptide

Synthesis of the diamino-bipyridine peptide was performed by both manual and automated methods. The peptide was built on Rink amide MBHA resin in order to afford an amide terminus upon cleavage from the resin. The first five amino acids, KTYGR, were coupled by automated synthesis using the microwave reactor in a Biotage Syrowave peptide synthesizer. Couplings were all performed at 75 °C through microwave irradiation. Compound 6 was coupled manually at room temperature. The bipyridine amino acid was poorly soluble in DMF, so N-methylpyrrolidinone (NMP) was used instead. Once this was coupled, the remaining amino acids RGITV-(β-Ala) were coupled using the microwave reactor. Once cleaved from the resin, the crude peptide was analyzed by HPLC-MS. The desired peptide was observed as determined by mass spectrometry; however, there were many other species present in the crude product. One of the most prominent side products was the peptide containing the first five amino acids plus the bipyridine amino acid. It was determined that the amino group on the bipyridine amino acid was less reactive than that of a standard amino acid, due to conjugation with the aromatic system. In order to improve
the coupling efficiency, Fmoc-β-Ala-OH was coupled twice for 45 minutes at 75 °C in the microwave reactor, using HATU as the coupling reagent. After these coupling cycles, approximately equal amounts of peptide with and without the β-Ala residue were present. After a final coupling at 75 °C for 2 hours, only small amounts of peptide without the β-Ala residue were observed. Complete coupling was observed with a coupling of 2 hours, followed by a second 1-hour coupling, both at 75 °C with HATU as the coupling agent. The remainder of the peptide was synthesized using standard protocols. After the peptide was cleaved from the resin and purified by reversed-phase HPLC, the pure peptide 7 was isolated in 14% yield.

4.2.4 Structural Evaluation by Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was used to evaluate the secondary structure of peptide 7 (Figure 4.7). The UV-CD spectrum was first acquired on peptide 7 in water. A negative band was observed with a minimum at 194 nm, which is consistent with a random coil structure as was expected for the peptide. A positive band was also observed at <180 nm. The spectrum was also acquired in 30% TFE, a secondary structure stabilizing solvent, to determine if it was possible for secondary structure to form. For a peptide/protein containing a β-sheet, a negative band in the 210-220 nm range and a positive band in the 190-200 nm range are expected, though some variation in these numbers can be expected. A shift of the negative band from 194 nm to 203 nm occurred, and a positive band with a maximum of 186 nm was observed. The bands observed in the CD spectrum of 7 in 30% TFE suggest the presence of a certain population of peptide folded into a β-sheet structure, with the rest existing in a random coil conformation. This indicates that the peptide is capable of forming secondary structure. Unfortunately, the effect of rhenium coordination on the secondary structure could not be characterized, as the pure complex could not be obtained in sufficient quantities, as will be discussed in the next section.
4.2.5 Formation of the [2+1] Rhenium Complex and Radiolabelling with Technetium-99m

Coordination of peptide 7 to rhenium was performed in a one-pot two-step synthesis to form the [2+1] complex. First, 7 was reacted with one equivalent of [Re(CO)\(_3\)(H\(_2\)O)\(_3\)]OTf under aqueous conditions at 100 °C for 12 minutes under microwave irradiation to form Re(CO)\(_3\)(H\(_2\)O)(7\(^+\)). Some uncoordinated peptide was still observed by HPLC-MS, but longer reaction times nor additional equivalents of the rhenium precursor resulted in consumption of the peptide. Two peaks of approximately equal intensity were observed in the HPLC chromatogram that corresponded to Re(CO)\(_3\)(H\(_2\)O)(7\(^+\)), resulting from the formation of a pair of coordination isomers due to the asymmetric nature of the peptide ligand (Figure 4.8). An excess of 4-dimethylaminopyridine (4-DMAP) was added to displace the H\(_2\)O ligand, as 4-DMAP was previously shown to form stable [2+1] rhenium-bipyridine complexes.\(^{24}\) This step had to be carefully optimized, as long reaction times at higher temperatures (100 °C) resulted in 4-DMAP displacing 7 to form Re(CO)\(_3\)\((4\text{-DMAP})\(_3\)\(^+\)), while low temperatures (40 °C) and shorter reaction times resulted in incomplete formation of the desired Re(CO)\(_3\)\((4\text{-DMAP})(7\(^+\)). Nearly complete conversion was observed when the reaction was performed at 75 °C for 45 minutes.
**Figure 4.36:** Coordination of peptide 7 and 4-DMAP with rhenium to form a [2+1] chelation system.

Purification of the complex was attempted by solid-phase extraction on a Waters C-18 Sep-pack. While this was successful in removing remaining 4-DMAP, it was unable to remove Re(CO)$_3$(4-DMAP)$_3^{3+}$ resulting from the reaction of 4-DMAP with unreacted [Re(CO)$_3$(H$_2$O)$_3$]OTf. Purification was then attempted by preparative HPLC. However, this was also unsuccessful in removing Re(CO)$_3$(4-DMAP)$_3^{3+}$ as well as uncoordinated 4-DMAP. It was also noted that upon standing in solution at room temperature, an increase in the amount of uncoordinated peptide was observed over time due to displacement of the peptide by the solvent. Previous reports suggest that 3,3’-disubstituted 2,2’-bipyridine ligands form a twisted geometry upon metal coordination due to steric clash of the 3,3’ substituents, preventing the pyridyl rings from forming a planar geometry.$^{17}$ This is likely exacerbated by the long peptide strands in 7, causing instability of the metal complex. Isolation of the pure peptide metal complex was not possible due to its instability. Attempts to radiolabel the peptide with technetium-99m gave similar results as for coordination to rhenium (Figure 4.9). Analysis by radio-UHPLC showed a peak corresponding to the [2+1] technetium-99m complex with 7 and 4-DMAP as observed by comparison to the retention time of the rhenium complex. Numerous other peaks were observed as well, including one corresponding to the [99mTc]Tc(CO)$_3$(4-DMAP)$_3^{3+}$ complex.
Figure 4.37: UHPLC chromatograms of the Re coordinated peptide 7 (top) and $^{99m}$Tc-labelled peptide 7 (bottom).

4.3 Conclusions

A novel Fmoc-protected amino acid containing a 2,2’-bipyridine residue (compound 6) was synthesized in four steps from 3-nitro-2-chloropyridine. The amino acid was incorporated into a peptide (peptide 7) by solid-phase peptide synthesis. Analysis by CD spectroscopy confirmed that the peptide exists in a disordered structure but can fold into a structure resembling a β-sheet in 30% TFE. Coordination of the peptide to Re/$^{99m}$Tc in a [2+1] fashion yields unstable complexes that were unable to be purified. While peptides containing the bipyridine amino acid (6) are not suitable for coordination to Re/$^{99m}$Tc for the purpose of developing molecular imaging agents, they may be more suitable for applications such as metal ion sensing where isolation of the formed peptide metal complex is not necessary. Alternatively, amino acids containing 4,4’-disubstituted bipyridines may allow for the formation of more stable complexes with Re/$^{99m}$Tc.

4.4 Experimental

**General Procedures and Materials:** All reagents and solvents were purchased from commercial sources and used without further purification unless otherwise stated. NMR spectra were recorded on either an Agilent Mercury VX 400 or Inova 400. All chemical
shifts are reported in ppm and referenced to the residual solvent peaks. Circular dichroism spectra were obtained on a Jasco J-810 circular dichroism spectropolarimeter.

**Manual Peptide Synthesis:** Fully protected resin bound peptides were synthesized by standard Fmoc solid phase peptide synthesis methods. Fmoc protected Rink amide MBHA resin (loading 0.34 mmol/g or 0.54 mmol/g) was employed as the solid support. Resin was swollen by suspension in DCM for 15 minutes. Fmoc deprotection was achieved using 20% piperidine in DMF over two cycles (2 minutes, then 15 minutes). Amino acids were pre-activated for 5 minutes using 3 equivalents of the Fmoc protected amino acid with 3 equivalents of either HCTU or HATU and 6 equivalents of DIPEA (N,N-diisopropylethylamine) in DMF. The activated ester was added to the resin and coupling was performed over 60 minutes. The resin was washed consecutively with DMF then DCM. Cleavage of the side chain protecting groups and the peptides from the resin was performed using 95% (v/v) TFA, 2.5% (v/v) TIPS and 2.5% (v/v) H2O over a period of 3-4 hours. The peptides were precipitated from solution with tert-butylmethyl ether (TBME) and centrifuged for 5 minutes at 3000 rpm. After decanting of the mother liquor, addition of TBME was repeated and the peptide centrifuged again. H2O was then added, the solution frozen at -78 °C and lyophilized.

**Automated Peptide Synthesis:** Automated peptide synthesis was performed using a Biotage Syrowave automated microwave peptide synthesizer. Fmoc protected Rink amide MBHA resin (loading 0.34 mmol/g or 0.54 mmol/g) was employed as the solid support. Resin was swollen by suspending in DCM for 15 minutes prior to inserting the resin into the automated synthesizer. Fmoc deprotection was achieved by addition of a 40% solution of piperidine in DMF over two cycles (30 seconds and 12 minutes). Fmoc protected amino acids were coupled by addition of 4 equivalents of Fmoc protected amino acid in DMF, 4 equivalents of HCTU in DMF and 8 equivalents of DIPEA in NMP (N-methyl pyrrololidone). Couplings were performed over 5 minutes at 75 °C by microwave irradiation. More difficult couplings were performed using HATU with longer periods of microwave irradiation. Resin cleavage and purification were performed as per manual peptide synthesis.
Purification by Reverse-Phase HPLC-MS: Reactions were analyzed using reversed-phase analytical HPLC-MS (Agilent Zorbax SB-C18 Column, 5 µm, 4.6 x 150 mm). The setup consisted of a Waters 600 controller, Waters prep degasser and Waters Mass Lynx software. The gradient solvent system comprised of solvent A (H₂O+0.1%TFA) and solvent B (ACN + 0.1%TFA) at a flow rate of 1.5 mL/min over 10 minutes with a 5-minute wash cycle at 95% solvent B. The UV absorbance was detected using a Waters 2998 photodiode array detector. Peptides were purified using reverse phase preparative HPLC column (Agilent Zorbax PrepHT SB-C18 Column, 5 µm, 21.2 x 150 mm). The flow rate for preparative HPLC was 20 mL/min. After purification, the collected fractions were frozen at -78 °C, lyophiilized and analyzed by reversed-phase analytical HPLC-MS.

3-acetylamino-2-chloropyridine (1): Compound 1 was synthesized by a published method from 3-amino-2-chloropyridine (5 g, 38.9 mmol,) and Ac₂O (50 mL). The pure acetylated product was collected as colourless needles (5.6 g, 85%). ¹H NMR (400 MHz, CDCl₃): δ = 8.71 (dd, J = 8.2 Hz, J = 1.7 Hz, 1 H), 8.10 (dd, J = 4.7 Hz, 1 H), 7.65 (br. s, 1 H), 7.26 (dd, J = 8.2 Hz, J = 4.7 Hz, 1 H), 2.27 (s, 3 H). ¹³C NMR (100 MHz, CDCl₃) δ = 186.8, 143.8, 139.8, 131.9, 129.3, 123.3, 24.8. MS (ESI+): m/z calculated C₇H₈ClN₂O⁺ [M+H]⁺ 171.0; found 171.0.

3,3'-diacetylamino-2,2'-bipyridine (2): Compound 2 was synthesized by a modification of a published procedure. Bronze powder (10 g) was activated by stirring in 100 mL of a 2% solution of I₂ in acetone for 15 minutes. After filtration, the solid was then treated with 10 mL conc. HCl in 90 mL of acetone for 15 minutes. After filtration and rinsing with acetone, the bronze powder was dried under high vacuum and then used immediately. Compound 1 (4.1 g, 24 mmol) was dissolved in dry DMF (25 mL) under N₂ atmosphere. Activated bronze powder (4.0 g, 63 mmol) was suspended in the solution, and heated to 110 °C for 24 hours. The reaction was quenched by the addition of 20 mL of H₂O. The resulting grey solid was filtered, washed with 20 mL H₂O, 40 mL conc. NH₄OH and then an additional 20 mL of H₂O. The grey cake was suspended in 100 mL of DCM and stirred vigorously. After filtration, the solvent was removed, and the pure product was isolated as an off-white solid (0.91g, 27 %). ¹H NMR (400 MHz, CDCl₃) δ = 9.08 (d, J = 8.3 Hz, 2 H), 8.32 (d, J = 4.2 Hz, 2 H), 7.36 (dd, J = 8.5 Hz, J = 4.6 Hz, 2 H), 2.23 (s, 6 H). ¹³C NMR
(100 MHz, CDCl$_3$) $\delta = 169.3, 141.9, 140.4, 136.6, 129.9, 124.0, 25.5$. MS (ESI+): $m/z$ calculated C$_{14}$H$_{15}$N$_4$O$_2^+$ [M+H]$^+$ 271.1; found 271.1.

3,3'-diamino-2,2'-bipyridine (3)

**Method A:** Compound 3 was synthesized by a published procedure from compound 2 (0.9 g, 3.29 mmol). The product was isolated as a bright yellow solid (0.47 g, 77%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.98$ (dd, $J = 4$ Hz, $J = 2.1$ Hz, 2 H), 7.04 (m, 4 H), 6.28 (br. s, 4 H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 143.8, 140.6, 135.8, 124.0, 123.0. MS (ESI+): $m/z$ calculated C$_{10}$H$_{11}$N$_4^+$ [M+H]$^+$ 187.1; found 187.1.

**Method B:** Compound 3 was synthesized from compound 4 (3 g, 12.2 mmol) by a published procedure. The pure product was isolated as a bright yellow solid (2.1 g, 93 %).

3,3'-dinitro-2,2'-bipyridine (4): Compound 4 was synthesized by modification of a published procedure. Activated bronze powder (10 g, 157.4 mmol) was suspended in a solution of 3-nitro-2-chloropyridine (10 g, 63.3 mmol) in 120 mL of dry DMF under N$_2$. The mixture was heated to 150 °C for 3.5 hours, at which point no more starting material was observed as indicated by TLC. The hot reaction mixture was then filtered over Celite. 40 mL of conc. NH$_4$OH and 40 mL H$_2$O was added to the resulting solution which was then extracted multiple times with EtOAc. The combined organic layers were dried over Na$_2$SO$_4$ and concentrated under reduced pressure until all of the EtOAc was removed and only DMF was left. Approximately 100 mL of H$_2$O was added to precipitate the crude product, which was collected by vacuum filtration. The pure product was obtained as a golden yellow solid by recrystallization from EtOH (3.23 g, 41%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.88$ (dd, $J = 4.8$ Hz, $J = 1.5$ Hz, 2 H), 8.59 (dd, $J = 8.4$ Hz, $J = 1.5$ Hz, 2 H), 7.65 (dd, $J = 8.4$ Hz, $J = 4.8$ Hz, 2 H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 153.1, 151.4, 143.9, 133.0, 124.2. MS (ESI+): $m/z$ calculated C$_{10}$H$_7$N$_4$O$_4^+$ [M+H]$^+$ 247.0; found 247.0.

4-((3'-amino-[2,2'-bipyridin]-3-yl)amino)-4-oxobutanoic acid (H-Bpy-Suc-OH) (5): Compound 3 (2 g, 10.75 mmol) was dissolved in DCM (40 mL) and succinic anhydride was added (1.08 g, 10.75 mmol). After a few moments of stirring at room temperature the
product began to precipitate. After 45 minutes, the reaction mixture was filtered by vacuum filtration to yield the pure product as a yellow solid (2.67g, 87%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ = 12.16 (br. s, 1 H), 8.85 (dd, J = 8.4 Hz, J = 1.6 Hz, 1 H), 8.28 (dd, J = 4.6 Hz, J = 1.6 Hz, 1 H), 7.91 (dd, J = 4.3 Hz, J = 1.6 Hz, 1 H), 7.29 (dd, J = 8.4 Hz, J = 4.6 Hz, 1 H), 7.26 (dd, J = 8.4 Hz, J = 1.6 Hz, 1 H) 7.15 (dd, J = 8.4 Hz, J = 4.3 Hz, 1 H), 2.58 (m, 4 H).

$^{13}$C NMR (100 MHz, DMSO-d$_6$) δ = 174.1, 170.9, 146.1, 143.4, 141.2, 136.9, 135.4, 128.1, 125.2, 124.8, 122.7, 32.8, 29.2.

MS (ESI+): m/z calculated C$_{14}$H$_{15}$N$_4$O$_3^+$ [M+H]$^+$ 287.1; found 287.1.

4-((3'-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-[2,2'-bipyridin]-3-yl)amino)-4-oxobutanoic acid (Fmoc-Bpy-Suc-OH) (6): Compound 5 (2.5 g, 8.74 mmol) was dissolved in a solution of NaHCO$_3$ (2.2 g, 26.2 mmol) in H$_2$O (150 mL) and cooled to 0 °C in an ice bath. Fmoc-Cl (3.38 g, 13.1 mmol) was added dropwise as a solution in 1,4-dioxane over a period of 1 hour. The solution was warmed to room temperature and stirred overnight. The pH of the solution was adjusted to 1 with concentrated HCl. The resulting solid was filtered off and recrystallized from toluene to give the pure product as an off-white solid (2.39 g, 68%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ = 12.64 (s, 1 H), 12.23 (s, 1 H), 12.16 (s, 1 H), 8.87 (dd, J = 8.5, 1.6 Hz, 1 H), 8.48 (d, J = 8.6 Hz, 1 H), 8.37 (dd, J = 4.6 Hz, J = 1.6 Hz, 1 H), 8.27 (dd, J = 4.6, J = 1.6 Hz, 1 H), 7.83 (d, J = 7.5 Hz, 2 H), 7.61 (d, J = 7.4 Hz, 2 H), 7.49 (dd, J = 8.5, J = 4.6 Hz, 1 H), 7.44 (dd, J = 8.5, J = 4.6 Hz, 1 H), 7.35 (t, J = 7.4 Hz, 2 H), 7.27 (t, J = 7.4 Hz, 1 H), 4.40 (d, J = 6.9 Hz, 2 H), 4.27 (t, J = 6.8 Hz, 1 H), 2.61 (t, J = 6.6 Hz, 2 H), 2.51 (t, J = 6.9 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ = 173.99, 171.08, 153.36, 144.02, 142.03, 141.84, 141.38, 141.21, 136.18, 136.00, 129.97, 128.82, 128.11, 127.52, 125.47, 124.70, 120.60, 120.54, 110.81, 66.77, 46.85, 32.60, 29.16. MS (ESI+): m/z calculated C$_{29}$H$_{25}$N$_4$O$_5^+$ [M+H]$^+$ 509.2; found 509.1.

H-Arg-Gly-Ile-Thr-Val-(β-Ala)-(Bpy-Suc)-Lys-Thr-Tyr-Gly-Arg-NH$_2$ (7): The peptide was synthesized and isolated by standard automated methods. Fmoc-β-Ala-OH was coupled twice at 75 °C under microwave irradiation for 2 hours and 1 hour, respectively. Purification was performed by preparative reversed-phase HPLC (17% to 50% ACN/H$_2$O + 0.1% TFA). The peptide was obtained as a white powder (20.4 mg, 14%). MS (ESI+): m/z calculated C$_{67}$H$_{106}$N$_{23}$O$_{16}^+$ [M+H]$^+$ 1488.8; found 1488.7.
4.5 References


Chem. 2013, 52, 13521–13528.


(20) Vriend, G. J. Mol. Graph. 1990, 8, 52–56.


Chapter 5

5 Amino-Substituted 2,2’-Bipyridine Ligands as Fluorescent Indicators for Zn(II) and Applications for Fluorescence Imaging of Prostate Cells

5.1 Introduction

As a divalent cation, zinc has several biological functions including storage and secretion of insulin from pancreatic beta cells, and the maintenance of prostate health through various mechanisms such as suppression of overall energy production, controlling apoptosis, and suppressing tumor progression. The prostate has been shown to have the highest Zn(II) concentration of any soft tissue. Downregulation of zinc transporters in cancerous prostate tissue results in greatly reduced levels of Zn(II) in comparison to healthy prostate tissue. In contrast, in benign prostatic hyperplasia (BPH) Zn(II) levels are maintained. To this end, Zn(II) has garnered much interest as a potential biomarker of prostate cancer (PCa) and may be useful in differentiating malignant tissue from healthy and benign tissue. Fluorescence has become the foremost method for the detection of Zn(II) in biological systems, as Zn(II) ions are unresponsive to other analytical techniques due to its electronic nature. Fluorescence offers the flexibility for use in in vitro biological assays and cellular imaging by fluorescence microscopy. A 3-hydroxychromone based fluorescent Zn(II) sensor has recently shown promise for differentiating PCa cells from normal prostate epithelial cells in vitro.

Fluorescence of ligands coordinated to Zn(II) may be modulated by a variety of mechanisms, including intramolecular charge transfer (ICT), photo-induced electron transfer (PeT) and excited-state intramolecular proton transfer (ESIPT). Fluorescent ICT agents have been used extensively as sensors for a wide variety of metal ions. An ICT fluorophore is an organic ligand containing a conjugated electron donor and acceptor which undergo charge separation upon photoexcitation. Upon binding of Zn(II) to either the electron donor or acceptor sites, the fluorescence excitation and emission wavelengths shift, giving a fluorescent signal unique to Zn(II). Substituted 2,2’-bipyridine ligands have shown great promise as Zn(II) sensors. When substituted with electron donating groups, the imine nitrogen atoms act as electron acceptors. Coordination
of Zn(II) at the acceptor site causes a bathochromic shift of excitation and emission due to stabilization of the excited state.

In this work, we have prepared several derivatives of 2,2’-bipyridine with electron-donating amine substituents (Figure 5.1). In order to determine the characteristics that make for an optimal fluorescent Zn(II) indicator, we have characterized the UV/Vis absorption and fluorescence properties of the ligands, and changes in these properties upon coordination to Zn(II). We have also applied this knowledge to Zn(II) sensing in a biological system by comparing the ability of two of the ligands to differentiate PCa cells from BPH cells by Zn(II) mediated fluorescence.

![Figure 5.38: Amine substituted 2,2’-bipyridine ligands.](image)

5.2 Results and Discussion

5.2.1 Synthesis

Five amine-substituted bipyridine ligands were synthesized for evaluation as fluorescent Zn(II) sensors (Figure 5.1). The series of five ligands were chosen to compare compounds with differing excited states, and different strengths of electron donating substituents to determine the characteristics best suited for Zn(II) sensing in a biological system. Compounds 1 and 2 were synthesized by previously reported methods. Scheme 5.1 highlights the syntheses of compounds 4, 6, and 7. Compound 4 was synthesized in 25% yield by a ligand-free Ni(II) catalyzed coupling reaction from compound 3, which was synthesized by Suzuki-Miyaura coupling of 5-bromo-2-chloropyridine with 4-
aminophenylboronic acid pinacol ester. Compound 6 was synthesized in two steps from 5,5’-dimethyl-2,2’-bipyridine, which was oxidized with potassium permanganate by a previously reported protocol to form the diacid 5,27 which then underwent a condensation reaction with o-phenylenediamine in polyphosphoric acid to form the benzimidazole 6 in 75% yield. Methylation of 6 with methyl iodide gave compound 7 in a moderate 43% yield.

Scheme 5.5: Syntheses of bipyridine derivatives 4, 6 and 7.

5.2.2 UV/Vis Absorption Studies

5.2.2.1 Solvent Polarity Effects

The effects of solvent polarity on the UV/Vis absorption spectra were determined for the bipyridine ligands to aid in characterizing the excited state. The UV/Vis spectra of 1 in n-hexane, n-dodecane and acetonitrile had previously been reported by Glasbeek and Toele.28 They described two strong absorption bands around 370 nm and 265 nm corresponding to $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions respectively, with maxima that were insensitive to solvent polarity. The $S_0 \rightarrow S_1$ transition was shown to primarily involve $\pi \rightarrow \pi^*$ HOMO-LUMO transitions of the bipyridine group. We observed similar results in methanol for compound 1 (Table 5.1) with two absorption bands appearing at 364 nm and 265 nm.
Table 5.3: UV/Vis absorption characteristics of bipyridine ligands and their Zn(II) complexes in methanol.

<table>
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<tr>
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<td>4</td>
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<td>Zn-1(^{[a]})</td>
<td>366, 265</td>
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<td>Zn-2</td>
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<td>14000, 22500</td>
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<td>Zn-7</td>
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</table>

\(^{[a]}\)In the presence of 30 equivalents of ZnSO\(_4\).

Compound 2 also showed two absorption bands, one centered around 325 nm and the other around 290 nm. The UV/Vis absorption spectra were taken in a range of solvents with different polarities to help determine the excited state characteristics. The absorption maxima for both bands showed a slight dependence on solvent polarity (Table 5.2), with polar solvents displaying a lower energy absorption than non-polar solvents (330 and 295 nm in DMSO versus 316 and 284 nm in cyclohexane). This positive solvatochromism is characteristic of an N\(\rightarrow\)\(\pi^*\) ICT excited state. Figure 5.2 depicts the ICT excited state of compound 2. The \(S_0\rightarrow S_1\) transition is of a higher energy than for compound 1 likely due to the lack of intramolecular hydrogen bonding in compound 2.
Figure 5.39: Proposed ICT excited state of compound 2.

Table 5.4: Photophysical properties of compound 2 (20 µM) in various solvents.\[^{[a]}\] Only the lowest energy absorption band is shown for clarity.

<table>
<thead>
<tr>
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\[^{[a]}\] Abbreviations: CyH, cyclohexane; DX, 1,4-dioxane; CHCl₃, chloroform; DCM, dichloromethane; EtOH, ethanol; MeOH, methanol; CH₃CN, acetonitrile; DMF, \(N,N\)-dimethylformamide; DMSO, dimethylsulfoxide; \(\varepsilon\), solvent dielectric constant; SS, Stoke’s Shift \((\nu_{Ex} - \nu_{Em})\).

Compound 4 showed a lower energy absorption band in comparison to compound 2 due to extended conjugation of the aromatic system. A single broad absorption band was observed around 360 nm. Compound 4 displayed strongly positive solvatochromism of the absorption band (Figure 5.3) in comparison to 2, likely due to increased charge separation in the excited state of the molecule. In cyclohexane, the absorption maximum appears at 342 nm but is significantly red shifted to 375 nm in DMSO (Table 5.3).
**Figure 5.40:** UV/Vis absorption spectra of compound 4 in (A) non-polar and (B) polar solvents.

**Table 5.5:** Photophysical properties of compound 4 (20 µM) in various solvents.

<table>
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<tr>
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UV/Vis spectra were not obtainable in the full range of solvents for compound 6 due to poor solubility, however, synthesis of the N,N’-dimethylated derivative 7 allowed for studies in the full range (Table 5.4, See Appendix C, Table C1 for compound 6 data). Little solvatochromism was observed in the absorption spectra for both compounds. This is likely due to the weaker electron donating ability of the benzimidazole groups in comparison to the amino and aminophenyl substituents of 2 and 4, resulting in a smaller degree of charge separation in the excited state. This effect was also observed by Zhu *et al.* when altering the electron donating ability of aryl groups on their 5-arylvinyl-5’-methyl-2,2’-bipyridyl (AVMB) fluorophores.¹⁴ Lack of solvatochromism in the absorption spectrum does not necessarily indicate a lack of ICT.
Table 5.6: Photophysical properties of compound 7 (20 μM) in various solvents.

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<td>344</td>
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5.2.2.2 Zinc Titration

The ligands were then titrated with ZnSO₄ in methanol to determine the changes in the UV/Vis absorption spectrum over a range of Zn(II) concentrations. This also aids in characterizing the excited state of the molecules. If an ICT character is observed, a bathochromatic shift of the absorption spectrum is expected upon Zn(II) coordination. Ideally, the absorption spectrum of the Zn(II) complex should be in the visible portion of the electromagnetic spectrum (greater than 400 nm) for use as a Zn(II) indicator in biological systems and for imaging by confocal microscopy. This is because fluorescence excitation in the UV region is prone to causing autofluorescence, and can be damaging to live cells in imaging experiments. As well, many confocal microscopes are only compatible with excitation wavelengths at 405 nm or greater.

Titration of compound 1 with ZnSO₄ in methanol (0.1-30 equivalents) showed very little change in the absorption spectrum (Figure 5.4A). The molar absorption coefficient of the lowest energy band showed a slight increase, while that of the highest energy band decreased slightly. The lowest energy band showed a slight red shift (Table 5.1). Zn(II) coordination would likely not stabilize the excited state of compound 1 due to the lack of ICT character, therefore showing little change in the absorption characteristics. The absorption spectrum of the Zn(II) complex is mostly at wavelengths less than 405 nm, so this ligand is not ideal for imaging by confocal microscopy.

In contrast to compound 1, compound 2 showed a large change in the spectrum upon the addition of even 0.1 equivalents of ZnSO₄ in methanol (Figure 5.4B). Absorption bands with maxima at 326 nm and 285 nm both showed large bathochromic shifts to 375 nm and
313 nm respectively. Bathochromic shifts of the absorption spectra upon binding to a cationic species are indicative of an ICT excited state. However, further addition of Zn(II), from 0.2 up to 30 equivalents, did not result in further changes to the absorption maxima, and only a slight increase to the molar extinction coefficients was observed up to the addition of 5 equivalents of Zn(II). With an absorption spectrum entirely below 405 nm, compound 2 is also not ideal for Zn(II) sensing in biological systems.

Titration of compound 4 with ZnSO₄ in methanol resulted in a bathochromic shift of the absorption maximum from 355 nm to 405 nm (Figure 5.4C), also indicating an ICT excited state. More subtle transitions in the absorption spectrum were observed for 4 in comparison to 2, with the most significant changes occurring upon the addition of 0.1-1 equivalents of Zn(II), and no further changes occurring after the addition of 5 equivalents. With the Zn(II) complex of compound 4 having an absorption maximum at 405 nm, it may be suitable for imaging cellular Zn(II).

Both compounds 6 and 7 showed very subtle changes to the absorption wavelengths upon titration with ZnSO₄ in methanol (Figure 5.4D and 5.4E), in contrast to the sharp changes observed in compounds 2 and 4. For both compounds, 30 equivalents of Zn(II) were required before no further changes were observed. The Zn(II) complexes show significant absorption at 405 nm. While this is not near the absorption maximum for either compound, it is likely to be sufficient for detecting fluorescence from intracellular Zn(II). As compound 6 has a stronger absorption at 405 nm upon coordination to Zn(II) in comparison to compound 7, it may be more suitable for the detection of intracellular Zn(II).
Figure 5.41: UV/Vis absorption spectra of compound 1 (20 µM) (A) compound 2 (B) compound 4 (C) compound 6 (D) and compound 7 (E) at a concentration of 20 µM in methanol upon titration with ZnSO₄ (0.1-1 equivalents in intervals of 0.1 equivalents, then 2, 3, 5, 10, 20 and 30 equivalents).

5.2.3 Fluorescence Studies

5.2.3.1 Solvent Polarity Effects

The fluorescence emission spectra of compound 1 in n-hexane, n-dodecane and acetonitrile were previously reported by Glasbeek and Toele, showing two emission bands in the steady-state emission spectra. A higher energy emission around 425 nm corresponds to the excited Franck-Condon state, and a lower energy emission around 585 nm results from an excited state intramolecular double proton transfer (ESIDPT), with subsequent emission from the tautomeric state. Solutions of compound 1 in methanol were also observed to be weakly fluorescent. However, only a single emission band was observed at 425 nm. It is known that ESIPT is less favourable in polar solvents, which could result in weaker or absent emission from the tautomeric form of the molecule in methanol.

For compound 2, 40 µM of N,N'-diisopropylethylamine (DIPEA) was added to each solution for the fluorimetry experiments, as in certain solvents (DX, CHCl₃, DCM and MeOH) a second excitation and emission band was observed corresponding to the protonated form of the molecule due to traces of acid present in these solvents. The addition
of DIPEA caused these bands to disappear. The addition of acid in methanol caused the 
first emission band to disappear, and the second emission band to increase in intensity (See 
Appendix D, Figure D14). This evidence supports that the second band is from the 
protonated species. Compound 2 displayed solvatofluorochromism, with emission maxima 
ranging from 375 nm in cyclohexane to 405 nm in DMSO (Table 5.2) indicative of an ICT 
excited state.

Compound 4 displayed a high degree of positive emission solvatofluorochromism (Table 
5.3), suggesting a polar emissive excited state. In non-polar solvents, emission maxima 
were at 402 nm in cyclohexane and 423 nm in 1,4-dioxane and Stokes shifts were relatively 
small. In cyclohexane, the emission band has some vibronic band structure which is 
indicative of a locally excited state.19 With a slight increase in solvent polarity, the band 
becomes more rounded and unstructured, indicating a shift to a solvent stabilized ICT state. 
This is consistent with the observations made by Zhu et al. for an ICT excited state with a 
strongly donating amino group. In polar aprotic solvents, Stokes shifts were greater than in 
non-polar solvents and emission maxima were shifted to 482-493 nm (Figure 5.5). In polar 
protic solvents, methanol and ethanol, Stokes shifts were much greater, and emission 
maxima were observed at 514 and 498 nm respectively. Stokes shifts for compound 4 were 
also greater than those observed for compound 2, likely due to greater solvent interactions 
in polar solvents.

![Fluorescence emission spectra of compound 4 in a range of solvents.](image)

**Figure 5.42:** Fluorescence emission spectra of compound 4 in a range of solvents.

Compounds 6 and 7 (Table 5.4) also showed positive solvatofluorochromism. Stokes shifts
were generally smaller in comparison to compound 4, due to the weaker electron donating nature of the benzimidazole. Compound 6 showed structured vibronic bands in 1,4-dioxane and chloroform, while compound 7 additionally showed structured bands in cyclohexane (See Appendix D, Figure D13). Compound 7 displayed greater Stokes shifts than compound 6 in all cases.

For qualitative comparison of the excited-state dipole moments, the Stokes shifts of compounds 2, 4 and 7 were plotted against the orientation polarizabilities of the solvents to afford the Lippert plots (Figure 5.6). While the linearity of the plots is generally poor due to specific solvent interactions (such as hydrogen bonding, and locally excited versus charge transfer excited states) causing deviation from the Lippert-Mataga model, a correlation between solvent polarity and Stokes shift is observed. The slope of the Lippert plot of compound 4 is certainly greater than that of compounds 2 and 7, indicating a higher degree of polarization in the excited state due to the presence of stronger electron donating groups.

![Figure 5.43: Lippert plots of compound 2 (orange), compound 4 (blue) and compound 7 (green).](image)

5.2.3.2 Zinc Titration and Quantum Yield

A change in either the intensity of the fluorescence emission, or the emission wavelength upon Zn(II) coordination is essential for being able to differentiate fluorescence from the
free ligand to that of the Zn(II) complex. A high quantum yield for the Zn(II) complex is also essential in order to have a detectable fluorescence signal.

In stark contrast to the UV-Vis absorption spectrum, the fluorescence wavelengths of 1 undergo significant change upon the addition of Zn(II). The excitation and emission maxima both undergo a bathochromic shift, however, the Stokes shift decreases slightly. The emission intensity was also very sensitive to the number of equivalents of Zn(II), with very subtle changes occurring upon the addition of up to 30 equivalents of the metal ion (Figure 5.7A). Glasbeek and Toele\textsuperscript{28} estimated that quantum yields for compound 1 were on the order of $10^{-5}$ in $n$-hexane, $n$-dodecane and acetonitrile.

While we have found a rather low quantum yield for 1 in this study as well, a drastic 70-fold increase in the quantum yield was observed upon coordination to Zn(II) in aerated methanol (Table 5.5). This fluorescence enhancement is likely due to the suppression of quenching pathways observed in the free ligand by restricting rotation and by removing the intramolecular hydrogen bonding. While this fluorescence enhancement is certainly desirable for a Zn(II) indicator, the absorption spectrum is primarily in the UV region. Further elaboration of this ligand to decrease the excitation energy may result in a Zn(II) sensor suitable for cell imaging by confocal microscopy.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fluorescence_spectra}
\caption{Fluorescence emission spectra of compound 1 (A) compound 2 (B) compound 4 (C) compound 6 (D) and compound 7 (E) at a concentration of 20 µM in \ldots}
\end{figure}
methanol upon titration with ZnSO₄ (0.2-1 equivalents in intervals of 0.2 equivalents, then 2, 3, 5, 10, 20 and 30 equivalents).

**Table 5.7:** Fluorescence properties of the bipyridine ligands and their Zn(II) complexes in methanol.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{Ex}$ [nm]</th>
<th>$\lambda_{Em}$ [nm]</th>
<th>SS [cm⁻¹]</th>
<th>$^a$Φᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>342</td>
<td>425</td>
<td>5710</td>
<td>0.0027</td>
</tr>
<tr>
<td>2</td>
<td>337</td>
<td>406</td>
<td>5043</td>
<td>0.058</td>
</tr>
<tr>
<td>4</td>
<td>360</td>
<td>514</td>
<td>8323</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>358</td>
<td>433</td>
<td>4838</td>
<td>0.93</td>
</tr>
<tr>
<td>7</td>
<td>346</td>
<td>438</td>
<td>6071</td>
<td>0.92</td>
</tr>
<tr>
<td>Zn-1</td>
<td>366</td>
<td>442</td>
<td>4698</td>
<td>0.19</td>
</tr>
<tr>
<td>Zn-2</td>
<td>377</td>
<td>422</td>
<td>2829</td>
<td>0.18</td>
</tr>
<tr>
<td>Zn-4</td>
<td>406</td>
<td>583</td>
<td>7478</td>
<td>0.0097</td>
</tr>
<tr>
<td>Zn-6</td>
<td>371</td>
<td>471</td>
<td>5723</td>
<td>0.71</td>
</tr>
<tr>
<td>Zn-7</td>
<td>351</td>
<td>481</td>
<td>7700</td>
<td>0.36</td>
</tr>
</tbody>
</table>

$^a$Aerated methanol, compared to quinine sulfate in 0.05 M H₂SO₄ ($\phi = 0.52$)²⁹

[b]In the presence of 30 equivalents of ZnSO₄.

As with the absorption spectrum for compound 2, the fluorescence emission spectrum showed a sharp change upon the addition of 0.2 equivalents of Zn(II) in methanol. The emission band was bathochromically shifted from 406 nm to 422 nm. Further addition of Zn(II) resulted in an increase in the emission intensity, with no changes observed after the addition of 5 equivalents (Figure 5.7B). As with the absorption spectrum for the Zn(II) complex, two excitation maxima were observed at 380 and 320 nm. The quantum yield of compound 2 was higher than that of compound 1 (Table 5.5). However, the Zn(II) complex of compound 2 had a remarkably similar quantum yield to that of the Zn(II) complex of compound 1, giving only a 3-fold increase. A decrease in the Stokes shift was also observed for the Zn(II) complex of compound 2. While compound 2 was responsive to Zn(II) concentration, absorption in the UV range prevents its use in Zn(II) sensing in biological systems.

The quantum yield for compound 4 was similar to that of compound 2 in aerated methanol.
We had hoped that a similar increase in quantum yield would be observed for compound 4 as well upon Zn(II) coordination, however, the Zn(II) complex of compound 4 showed significantly quenched fluorescence. This finding is in agreement with other ligands containing strongly electron donating amino groups such as aminophenyl-substituted AVMBs\textsuperscript{14} and phenanthroline\textsuperscript{30} ligands upon coordination to Zn(II). A bathochromic shift of the emission band was observed for the Zn(II) complex as expected, however the Stokes shift was also seen to decrease. As with the absorption spectrum, titration with Zn(II) showed no further changes to the emission spectrum after the addition of 5 equivalents of Zn(II) (Figure 5.7C). While the absorption characteristics of compound 4 are ideal, the very low quantum yield of the Zn(II) complex would make visualizing the metal ion in biological systems challenging.

The quantum yield for compound 6 had previously been reported as $\phi = 0.93$ in aerated methanol,\textsuperscript{31} which we have confirmed here (Table 5.5). The quantum yield for compound 7 was almost identical to that of compound 6. However, the quantum yields for both compounds differ greatly upon coordination to Zn(II), which had not been previously reported on. Compound 6 only showed a marginal decrease in quantum yield while compound 7 showed a more drastic decrease. However, both Zn(II) complexes were still highly fluorescent. The Zn(II) complexes of compounds 6 and 7 showed increased Stokes shifts over the free ligands, and bathochromic shifts of the excitation and emission maxima. For both compounds 6 and 7, titration of 30 equivalents of Zn(II) was required before no further change to the emission spectrum was observed (Figure 5.7D and 5.7E). Since the Zn(II) complexes of compounds 6 and 7 both had significant absorption at 405 nm, while the free ligands did not, and the quantum yields of the complexes were high, we elected to further explore these two ligands for sensing Zn(II) in a biological system by confocal microscopy.

5.2.4 Effects of Other Metal Ions on the Absorption and Emission Spectra

In order to detect Zn(II) in a complex biological system, it is essential that the fluorescence response be specific for Zn(II) over other metal ions. The UV/Vis absorption spectra and fluorescence emission spectra for compounds 6 and 7 were recorded in the presence of
Zn(II), Fe(III), Cu(II), Mg(II) and Ca(II). These metal ions represent the most abundant metals in a cellular environment that are likely to cause interference. For both ligands, the addition of Ca(II) and Mg(II) had no effect on the absorption maximum, and minimal effect on the extinction coefficient. The addition of Cu(II) resulted in a larger red shift of the absorption spectrum than did Zn(II). The addition of Fe(III) had minimal effect on the position of the absorption maximum, but greatly increased the extinction coefficients. For both ligands Ca(II) and Mg(II) also had no effect on the fluorescence emission spectrum, Cu(II) caused complete quenching of fluorescence, and Fe(III) caused a slight bathochromic shift with significant quenching (Figure 5.8).

**Figure 5.45:** UV/Vis absorbance and fluorescence emission spectra of compound 6 (A and D, black) and compound 7 (B and D, black) in the presence of Fe(III) (brown), Cu(II) (blue), Mg(II) (magenta), Ca(II) (green) and Zn(II) (red).

### 5.2.5 Confocal Microscopy of Prostate Cell Lines

PC-3 and DU 145 prostate cancer cells have been previously shown to have an impaired ability to uptake Zn(II). In contrast BPH-1 cells retain the ability to accumulate high levels of intracellular Zn(II). All cell lines were incubated with either compound 6 or 7 at a concentration of 20 µM for 60 minutes. After the incubation period, cells were fixed with...
4% paraformaldehyde and mounted on slides for imaging by confocal microscopy. Weak fluorescence was observed in BPH-1 cells after incubation with either compound 6 (Figure 5.9) or 7 (Figure 5.10) for 60 minutes when excited with a 405 nm laser, and emission collected from 475-575 nm. While the free ligands do show some fluorescence emission in this range, neither one displays any absorbance at 405 nm. Fluorescence emission then should be exclusively from the Zn(II) complexes. Therefore the observed background fluorescence is likely due to cellular uptake of Zn(II) present in the culture medium. This has been observed with similar fluorescent Zn(II) indicators as well.²³ BPH-1 cells incubated with either compound 6 or 7 followed by a 60 minute incubation with Zn(II) at a concentration of 200 µM showed a large increase in fluorescence intensity. PC-3 and DU 145 cells displayed no fluorescence upon incubation with compound 6 or 7, and no increase in fluorescence intensity was observed upon the addition of Zn(II). These results are promising, as bipyridine ligands have the possibility to differentiate benign and malignant tissue by Zn(II) mediated fluorescence. Compounds 6 and 7 also have the potential to be used for Zn(II) sensing in *ex vivo* malignant, benign and healthy prostate tissues.

**Figure 5.46:** (A) BPH-1, (B) DU 145 and (C) PC-3 cells incubated with compound 6 (20 µM) for 60 minutes. (D) BPH-1, (E) DU 145 and (F) PC-3 cells incubated with compound 6 (20 µM) for 60 minutes, followed by the addition of ZnSO₄ (200 µM) for 60 minutes. \(\lambda_{\text{Ex}} = 405 \text{ nm}, \lambda_{\text{Em}} = 475-575 \text{ nm}\).
Figure 5.47: (A) BPH-1, (B) DU 145 and (C) PC-3 cells incubated with compound 7 (20 µM) for 60 minutes. (D) BPH-1, (E) DU 145 and (F) PC-3 cells incubated with compound 7 (20 µM) for 60 minutes, followed by the addition of ZnSO₄ (200 µM) for 60 minutes. λ<sub>Ex</sub> = 405 nm, λ<sub>Em</sub> = 475-575 nm.

5.3 Conclusions

In summary, we have shown that amino substituted bipyridine ligands are suitable for the detection of Zn(II) by fluorescence. We have investigated the UV/Vis and fluorescence properties of five bipyridine ligands in a range of solvents, showing that compounds 2, 4, 6, and 7 display characteristics of intramolecular charge transfer, while confirming previous reports that compound 1 does not. Quantum yields of compounds 1 and 2 were rather low but increased 70 and 3-fold respectively upon coordination to Zn(II). Compound 4 had a similar quantum yield to compound 2 but showed a drastic decrease upon Zn(II) coordination. Compounds 6 and 7 had very high quantum yields. While they decreased slightly upon Zn(II) coordination, the complexes were still highly fluorescent with bathochromically shifted UV/Vis and fluorescence spectra. The fluorescence response for compounds 6 and 7 was selective for Zn(II) over other biologically relevant metal ions. We have shown that these bipyridine ligands also respond to Zn(II) in a biological system, using prostate cells as a model system. BPH-1 cells, which are known to accumulate intracellular Zn(II) showed an increase in fluorescence in response to Zn(II) upon
incubation with either compound 6 or 7 and excitation at 405 nm. PC3 and DU 145 cells did not show an increase in fluorescence intensity, indicating low uptake of Zn(II). We have demonstrated the utility of compounds 6 and 7 in detecting Zn(II) in a cellular environment. Further investigation of these amino substituted bipyridine ligands may allow for quantitative determination of Zn(II) concentrations in ex vivo tissue samples, which may be used as a method for differentiating malignant from benign and healthy prostate tissue. Furthermore, structural modification of the ligands could allow for lower energy fluorescence excitation in order to reduce the possible effects of autofluorescence.

5.4 Experimental

General Experimental: All reagents and solvents were purchased from commercial sources and used without further purification unless otherwise noted. All reactions were performed in oven-dried glassware under nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was performed using plastic-backed TLC plates coated with silica gel 60 F254. Flash chromatography was performed using a Biotage Isolera Prime on KP-Sil cartridges. NMR spectra were recorded on a Bruker AvIII HD 400 spectrometer. All chemical shifts are reported in ppm and referenced to the solvent peaks. High resolution EI mass spectra were collected on a Thermo Scientific Double Focusing Sector mass spectrometer. UV/Vis spectra were recorded on an Agilent Cary 60 UV/Vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technologies International, Inc. Quanta Master – 7/2005. Quantum yields were determined on aerated methanol solutions using quinine sulfate in 0.05 M H$_2$SO$_4$ as a standard ($\phi = 0.52$). 3,3’-Diamino-2,2’-bipyridine (1), 5,5’-diamino-2,2’-bipyridine (2), and 2,2’-bipyridine-5,5’-dicarboxylic acid (5) were synthesized according to previously reported procedures.

4-(2-chloropyridin-5-yl)aniline (3): A mixture of 5-bromo-2-chloropyridine (500 mg, 2.6 mmol), 4-aminophenylboronic acid pinacol ester (570 mg, 2.6 mmol), Pd(PPh$_3$)$_4$ (150 mg, 0.13 mmol), and Na$_2$CO$_3$ (550 mg, 5.2 mmol) in 4:1 dioxane/water (25 mL) was heated at reflux for 24 hours. After cooling to room temperature, the solvent was evaporated, and the residue taken up in dichloromethane. The organic solution was washed with water and brine and filtered over an alumina plug. The solvent was evaporated, and the resulting brown semisolid was purified by automated flash chromatography (Snap 50 g KP-Sil, 10-
80% EtOAc/Hex) to give the pure product as a beige solid (380 mg, 72%). R_f = 0.38 (40% EtOAc/Hexanes); ^1H NMR (400 MHz, CDCl_3) δ = 8.54 (d, J = 2.3 Hz, 1H), 7.76 (dd, J = 8.3, 2.6 Hz, 1H), 7.41 – 7.30 (m, 3H), 6.82 – 6.71 (m, 2H), 3.83 (s, 2H); ^13C NMR (100 MHz, CDCl_3) δ = 149.1, 147.3, 146.9, 136.3, 135.6, 128.0, 126.4, 124.0, 115.5; HRMS (EI): m/z calculated for C_{11}H_{9}ClN_{2}^+ [M]^+ 204.0454; found 204.0451.

5,5'-bis-(4-aminophenyl)-2,2'-bipyridine (4): A mixture of 3 (100 mg, 0.49 mmol), NiCl_2·6H_2O (6 mg, 24 µmol), LiCl (21 mg, 0.49 mmol) and Zn dust (38 mg, 0.59 mmol) in anhydrous DMF (2 mL) was heated to 50 °C. The reaction was initiated by the addition of a small crystal of I_2 and one drop of AcOH, resulting in a colour change from blue to black. After 2 hours, all starting material had been consumed as indicated by TLC. After cooling to room temperature, ethyl acetate (10 mL) was added, resulting in the precipitation of the product as the Zn complex. This was collected by filtration and taken up in 1 M HCl (3 mL). The solution was then basified by the addition of conc. NH_4OH (1 mL), resulting in the precipitation of the crude product as a brown solid. The product was collected by filtration, triturated with 1,4-dioxane (5 mL), and once again collected by filtration to give the pure product as an orange-yellow solid (21 mg, 25%). ^1H NMR (400 MHz, DMSO-d_6) δ = 8.89 (d, J = 1.6 Hz, 2H), 8.37 (d, J = 7.6 Hz, 2H), 8.07 (dd, J = 8.3, 2.4 Hz, 2H), 7.55 – 7.47 (m, 4H), 6.73 – 6.63 (m, 4H), 5.40 (s, 4H); ^13C NMR (100 MHz, DMSO-d_6) δ = 152.9, 149.7, 146.5, 136.2, 133.6, 127.8, 123.9, 120.5, 114.8; HRMS (EI): m/z calculated for C_{22}H_{18}N_{4}^+ [M]^+ 338.1531; found 338.1530.

5,5'-bis(1H-benzimidazol-2-yl)-2,2'-bipyridine (6): To a 250 mL round bottomed flask containing 5 (500 mg, 2.05 mmol) and o-phenylenediamine (443mg, 4.09 mmol) was added polyphosphoric acid (~10 mL) and the syrupy suspension was heated to 180 °C in an oil bath for 24 hours. Upon cooling to 100 °C, deionized water (200 mL) was slowly added to the dark orange solution, resulting in precipitation of a bright yellow solid. The flask was removed from the oil bath and stirring continued until all of the viscous polyphosphoric acid was mixed with the water. Upon cooling to room temperature, the yellow phosphate salt was collected by filtration and dried under vacuum. The solid was suspended in deionized water (40 mL), and the pH adjusted to 11 with 6 M potassium hydroxide. The suspension was stirred at room temperature for 1 hour. The yellow solid
was collected by filtration, and dried in vacuo (594 mg, 75%). \(^1\)H NMR (400 MHz, 1:1 CDCl\(_3\):TFA-d) \(\delta = 9.92 - 9.79 (m, 2H), 9.32 - 9.19 (m, 2H), 9.00 - 8.85 (m, 2H), 7.98 - 7.86 (m, 5H), 7.86 - 7.74 (m, 5H); \(^{13}\)C NMR (100 MHz, 1:1 CDCl\(_3\):TFA-d) \(\delta = 148.4, 146.3, 143.3, 143.0, 131.7, 129.8, 125.5, 123.9, 114.8; HRMS (EI): \(m/z\) calculated for C\(_{24}\)H\(_{16}\)N\(_6\)\(^+\) [M]\(^+\) 388.1436; found 388.1435.

5,5'-bis(1-methyl-1\textit{H}-benzimidazol-2-yl)-2,2'-bipyridine (7): To a solution of 6 (100 mg, 0.26 mmol) in DMF (5 mL) was added Cs\(_2\)CO\(_3\) (78 mg, 0.57 mmol) and methyl iodide (81 mg, 0.57 mmol) and the resulting bright yellow solution was stirred at room temperature for 16 hours. The resulting pale-yellow suspension was then diluted with deionized water (30 mL), and the beige precipitate was collected by filtration and dried in vacuo (43 mg, 40%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta = 9.15 (dd, J = 2.2, 0.7 Hz, 2H), 8.69 (dd, \(J = 8.2, 0.7\) Hz, 2H), 8.33 (dd, \(J = 8.2, 2.2\) Hz, 2H), 7.91 – 7.85 (m, 2H), 7.48 – 7.44 (m, 2H), 7.42 – 7.33 (m, 4H), 3.98 (s, 6H); \(^{13}\)C NMR (10 MHz, CDCl\(_3\)) \(\delta = 156.2, 150.8, 149.6, 143.3, 138.0, 136.9, 126.9, 123.6, 123.1, 121.4, 120.3, 110.0, 32.0; HRMS (EI): \(m/z\) calculated for C\(_{26}\)H\(_{19}\)N\(_6\)\(^+\) [M-H]\(^+\) 415.1666; found 415.1665.

Cell Culture: PC-3 and DU 145 cells were purchased from ATCC. BPH-1 cells were a generous gift from Dr. John Lewis (University of Alberta, Edmonton, Alberta). PC-3 cells were maintained in F-12K medium (Wisent) supplemented with 10% FBS and 1X penicillin-streptomycin. DU 145 cells were maintained in EMEM (Sigma) supplemented with 10% FBS, 1X MEM non-essential amino acids (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate and 1X penicillin-streptomycin. BPH-1 cells were maintained in RPMI 1640 medium (Wisent) containing 10% FBS and 1X penicillin-streptomycin. All cell lines were cultured in a 37 °C incubator with a 5% CO\(_2\) atmosphere. Cells were passaged 2-3 times per week.

Fluorescence Imaging by Confocal Fluorescence Microscopy: In 12-well plates containing glass coverslips, PC-3, DU 145 and BPH-1 cells were seeded at a density of 100,000 cells per well and allowed to adhere and grow for 24-48 hours. The culture medium was removed, and the wells washed with PBS (2 x 1 mL). A stock solution of bipyridyl ligand (200 µL, 1 mM in DMSO) was diluted in culture media (10 mL) giving a
20 μM solution, which was filtered through a 0.2 μm syringe filter to remove any precipitates. This solution (1 mL) was added to each well, and the cells incubated for 60 minutes at 37 °C. The culture media was removed and each well washed with PBS (2 x 1 mL). Two wells were then incubated with a 200 μM solution of ZnSO₄·7H₂O in culture media (1 mL) for 60 minutes at 37 °C. The culture media was removed, and the cells washed twice with PBS. The cells were then fixed by the addition of 1 mL of a 4% paraformaldehyde solution in PBS (1 mL) for 15 minutes. After washing with PBS (2 x 1 mL), the cells were mounted on glass slides using ProLong® Diamond Antifade Mountant (Invitrogen) for imaging. Confocal fluorescence microscopy was performed on an Olympus FluoView FV1000 Confocal Microscope using a 40x objective with a 405 nm laser for excitation and the emission range set to 475-575 nm.

5.5 References


Chapter 6

6 Conclusions

The field of molecular imaging has provided clinicians and scientists a deeper understanding of disease, and the underlying cellular and molecular processes. A large tool palette of molecular imaging agents is available, ranging from PET, SPECT and MRI imaging probes for \textit{in vivo} diagnostic imaging, and fluorescent OI imaging probes for preclinical research applications in cells and tissues. However, there are still unmet needs in both clinical and research settings for molecular imaging probes that must be addressed.\textsuperscript{1-3} Therefore, novel methodologies and approaches to the design of molecular imaging probes must be taken in order to develop agents that improve upon those that exist or allow for imaging of new biological targets.

Molecular imaging agents that target GPCRs have become an approach to personalized medicine in a clinical setting. Determining expression of these receptors in disease allows for targeted therapeutic approaches, as well as more accurate and sensitive imaging of disease distribution in the body.\textsuperscript{4} The GPCR CXCR4 has garnered much attention as a target for molecular imaging probes in cancer. In chapter 2, the development of a novel dual modality molecular imaging probe targeting CXCR4 was explored. Dual modality imaging agents allow for a single entity to be used in different imaging applications, or for two modalities to be used simultaneously to overcome the disadvantages of each modality.\textsuperscript{5,6} It had previously been reported that 4-amino-1,8-naphthalimides containing a di-(2-picolyl)amine chelator coordinated to rhenium are suitable as fluorescent cellular imaging agents.\textsuperscript{7} Replacement of rhenium with technetium-99m opens the doors for the development of SPECT imaging agents. Though many fluorescent rhenium complexes have been reported that may also be coordinated to technetium-99m for SPECT imaging, these agents often suffer from low quantum yields, and the energy required for excitation is in the UV range.\textsuperscript{8} This is not ideal for imaging of live cells and tissues, so new agents with visible light excitation would help to improve the utility of fluorescent rhenium complexes for imaging applications.
A 4-amino-1,8-naphthalimide containing a carboxylic acid functionality was synthesized and conjugated to the D-Lys side chain of the CXCR4 antagonist peptide T140. The peptide was coordinated to rhenium for *in vitro* characterization. The rhenium coordinated peptide retained high fluorescence quantum yields. Through competition binding assays, it was determined that the rhenium coordinated peptide retained high affinity for CXCR4. By confocal fluorescence microscopy, it was shown that the peptide was taken up selectively by cells expressing CXCR4. This provides a possible route to differentiate malignant prostate cells from healthy and benign, as only malignant cells express CXCR4. Radiolabelling with technetium-99m was achieved in high radiochemical yield, purity and molar activity. To my knowledge, this is the first report of a $^{99m}$Tc(CO)$_3$-labelled CXCR4 imaging agent, and the first $^{99m}$Tc-labelled T140 derivative. Other technetium-99m labelled imaging agents targeting CXCR4 use the $^{99m}$Tc-HYNIC$^9$ or $^{99m}$TcO$_2^+$ scaffolds,$^{10,11}$ which tend to exhibit lower *in vivo* stability than the $^{99m}$Tc(CO)$_3$ system. These types of complexes also do not produce rhenium complexes that exhibit luminescence properties. Peptides such as T140 also provide the opportunity for optimizing structures to improve biodistribution and pharmacokinetics. Cellular uptake of the radiotracer in CXCR4 expressing cells was two-fold of that in cells that do not express the receptor. Disappointingly, in NOD/SCID mice bearing CXCR4 expressing tumors, uptake of the technetium-99m labelled tracer was rather low. A possible reason for the low uptake is the lipophilic nature of the naphthalimide entity, as this has been shown to cause non-specific binding in other organs. Even though the metal complex bears a positive charge, the non-polar nature of the aromatic pyridyl ligands appears to overpower the effects of the charge. There are several possibilities on how to improve the pharmacokinetic profile of the radiotracer. Previously reported CXCR4 targeting PET imaging probes that had low tumor uptake saw a reduction in non-specific binding upon lowering the molar activity of the radiotracer.$^{12,13}$ Another feasible approach is to reduce the lipophilicity of the naphthalimide by altering the metal chelation system. This may help to reduce non-specific binding *in vivo*. This newly developed dual modality imaging agent shows promise as a tool for evaluating CXCR4 expression in cells and tissues by fluorescence imaging, however improvements are required before it may be used as a SPECT imaging agent.
As seen in chapter 2, peptides containing technetium-99m labelled 4-amino-1,8-naphthalimides may suffer from poor pharmacokinetics in vivo due to their lipophilic nature. Therefore, the development of less lipophilic naphthalimide-metal complexes is required in order to address this issue. In order to be useful as dual modality imaging agents, the fluorescence properties must be suitable for fluorescence imaging. In chapter 3, the synthesis and evaluation of Re\(^{99m}\)Tc coordinated naphthalimides containing three different chelation systems was discussed. The three ligands were coordinated to rhenium to evaluate the fluorescence and cellular uptake properties. The fluorescence properties of the rhenium complexes were all suitable for use in confocal fluorescence microscopy. Upon incubation with OVCAR-8 cells, it was demonstrated that the cellular uptake of all three complexes was drastically different. The ligands containing carboxylate chelating arms showed much lower uptake into the cells, indicating a more polar metal complex. The three ligands were also radiolabelled with technetium-99m giving high radiochemical yields and purities. This demonstrates the three ligands’ utility as components of SPECT imaging agents. This may allow for the tuning of the pharmacokinetic profiles of bioconjugates for SPECT imaging, while still retaining utility as fluorescence imaging agents.

Cellular localization and uptake of luminescent metal complexes not only has implications for imaging, but for therapeutics as well. Fluorescence imaging of the cellular localization of metal complexes may allow for the determination of the mechanism of action of a potential therapeutic agent. Luminescent ruthenium-polypyridyl complexes bearing positive charges showed higher uptake in cancer cells than those bearing negative charges, and both complex types had very different cellular localization.\(^{14}\) While the positively charged complex displayed significant cytotoxicity in both dark and light conditions due to the mitochondrial localization, the negatively charged complex only displayed toxicity upon illumination. This provides the opportunity to develop the complex as an agent for photodynamic therapy. Visualization of the change in cellular localization upon modifying the complex charge allowed for the determination of the mechanism of action of this potential therapeutic agent. Therefore, luminescent metal complexes are a powerful tool for studying disease.
While pendant labelling approaches to molecular imaging probes such as that demonstrated in chapter 2 may not affect a peptide’s ability to bind to a receptor, in many cases pendant labels can be detrimental to binding affinity. Integrated labelling approaches may be beneficial for maintaining high affinity for receptors. However, as demonstrated in chapter 4, the design and implementation of integrated molecular imaging probes presents many challenges. An Fmoc-protected amino acid containing a 3,3’-diamino-2,2’-bipyridine residue was successfully synthesized and was incorporated into a peptide through SPPS. CD spectroscopy showed that the peptide exists in a random coil conformation in an aqueous environment. Addition of the secondary structure stabilizing solvent TFE resulted in a secondary structure that more closely resembles a β-sheet. However, coordination of the bipyridine peptide to rhenium and technetium-99m resulted in unstable metal complexes that could not be successfully purified or isolated. As previous reports have shown that bipyridine containing peptides can form β-sheet structures upon metal coordination, and bipyridine complexes of rhenium and technetium-99m are well known, it is feasible that they may be used in the development of SPECT imaging agents containing technetium-99m. The instability of the reported metal complexes results from steric clash of the 3,3’-substituents on the bipyridine rings, therefore, the use of either 6,6’- or 4,4’-disubstituted bipyridines may be more suitable for this use case. Other methods for incorporating Re/99mTc into turn mimics have been reported, so there is certainly promise for the field.

Chapter 5 discussed the development of amino-substituted 2,2’-bipyridine ligands as fluorescent indicators for Zn(II) in a cellular environment. 3,3’-Diamino- and 5,5’-diamino-2,2’-bipyridine ligands showed a strong fluorescent response to Zn(II), displaying a large increase in quantum yield in the presence of the metal ion. However, the excitation wavelengths required for fluorescence were in the UV region of the electromagnetic spectrum, which is not ideal for use in fluorescence imaging of live cells. The addition of aminophenyl substituents resulted in a ligand with ideal excitation wavelengths for live cell imaging upon coordination to Zn(II), however, the complex was only weakly fluorescent. Two benzimidazole substituted 2,2’-bipyridine ligands however, gave good quantum yields upon coordination with Zn(II) and were excitable with visible light for
cellular imaging. The fluorescence response of the ligands was selective for Zn(II) over other metal ions. The two bipyridine ligands were employed for cellular imaging of Zn(II) concentrations in prostate cell lines. A fluorescence response was observed in BPH-1 cells but not PC-3 and DU 145 PCa cell lines, due to the impaired Zn(II) uptake of the PCa cells. This allows for the differentiation of benign and malignant prostate tissues, which may have applications in imaging of ex vivo tissue samples.

Though the field of fluorescent Zn(II) indicators is certainly a crowded one, the need for new indicators still exists. A fluorescent 3-hydroxychromone Zn(II) sensor was previously shown to be able to differentiate between regular prostate epithelial cells and prostate cancer cells using two-photon excitation fluorescence microscopy. We have demonstrated that the same can be done with benign prostate cells using confocal fluorescence microscopy with visible light excitation. Previously reported 2,2’-bipyridine Zn(II) sensors have been used to detect intracellular Zn(II), but unfortunately could only be excited with UV light, or suffer from poor photostability. The 2,2’-bipyridine Zn(II) sensors reported in this thesis are excitable with visible light upon coordination to Zn(II), and are therefore more applicable for imaging of live cells. Fluorophores that are able to be used ratiometrically, and that are useful over a wide range of concentrations, as well as having defined cellular localization may improve the use of these sensors in the study and diagnosis of disease. Two-photon excitation microscopy has recently been employed for ratiometric sensing of intracellular Zn(II) with the sensor Chromis-1. Attachment of organelle targeting tags to Zn(II) sensitive probes has also been demonstrated to allow for the detection of Zn(II) in specific organelles such as mitochondria.

In summary, this thesis has highlighted the development of several novel approaches to molecular imaging agents. Novel methodologies and approaches to incorporating imaging moieties into molecular imaging agents can lead to the development of new classes of probes that target cellular and molecular entities that were not previously accessible. The development of new ways to diagnosis and treat disease may be uncovered through molecular imaging. Fluorescent naphthalimides containing a variety of metal chelators may allow for the development of new fluorescent imaging agents that could also be used for nuclear imaging applications. Alteration of the metal complex allows for the tuning of
the pharmacokinetic properties of the radiolabelled agent, while still retaining suitable fluorescence properties. The design of integrated radiopharmaceuticals presents many challenges, and while incorporation of metal chelators into the backbone of peptides is certainly possible, the coordination chemistry of the ligand must be carefully tuned in order to provide stable metal complexes that may be used for in vivo imaging applications. Though a metal chelator may not be suitable for one application, often other applications for the molecule may be explored. For example, the 3,3’-diamino-2,2’-bipyridine ligands developed in chapter 4 were not suitable for coordination to Re/99mTc. However, the discovery of their fluorescence properties upon coordination to Zn(II) paved the path for the development of the bipyridine ligands reported in chapter 5. These bipyridine ligands showed strong fluorescent responses to the presence of Zn(II), and are promising agents for diagnosing malignancies of the prostate.

6.1 References


Figure A48: $^1$H NMR spectrum (400 MHz; CDCl$_3$) of compound 5 (DPA-Naph-OH).
Figure A49: $^{13}$C NMR spectrum (100 MHz; CDCl$_3$) of compound 5 (DPA-Naph-OH).

Figure A50: HPLC chromatogram (10-70% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide 6 (T140).
Figure A51: HPLC chromatogram (20-80% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide 7 (DPA-Naph-T140).

Figure A52: HPLC chromatogram (20-80% CH$_3$CN/H$_2$O + 0.1% TFA) of peptide Re-7 (Re(CO)$_3$-DPA-Naph-T140).
Table A8: *Ex vivo* biodistribution data for $[^{99m}Tc]Tc-7$ in NOD/SCID mice two hours post-injection (n = 3).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Uptake (% ID/g ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>1.91 ± 0.16</td>
</tr>
<tr>
<td>heart</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>lung</td>
<td>2.80 ± 0.63</td>
</tr>
<tr>
<td>liver</td>
<td>22.67 ± 5.02</td>
</tr>
<tr>
<td>spleen</td>
<td>2.05 ± 1.37</td>
</tr>
<tr>
<td>pancreas</td>
<td>1.15 ± 0.54</td>
</tr>
<tr>
<td>stomach</td>
<td>4.67 ± 0.99</td>
</tr>
<tr>
<td>intestine</td>
<td>2.36 ± 1.40</td>
</tr>
<tr>
<td>kidney</td>
<td>25.69 ± 15.19</td>
</tr>
<tr>
<td>tumor</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>muscle</td>
<td>0.26 ± 0.07</td>
</tr>
<tr>
<td>brain</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>
Figure B53: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of compound 3.
Figure B54: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of compound 3.
Figure B55: $^1$H NMR spectrum (400 MHz; CDCl$_3$) of L1.
Figure B56: $^{13}$C NMR spectrum (100 MHz; CDCl$_3$) of L1.
Figure B57: $^1$H NMR spectrum (400 MHz; acetone-$d_6$) of Re-L1.
Figure B58: $^{13}$C NMR spectrum (100 MHz; acetone-$d_6$) of Re-L1.
Figure B59: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of L2.
Figure B60: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of L2.
Figure B61: ^1^H NMR spectrum (400 MHz; CD$_3$CN) of Re-L2.
Figure B62: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of L3.
Figure B63: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of L3.
Figure B64: $^1$H NMR spectrum (400 MHz; CD$_3$OD) of Re-L3.
Figure B65: $^{13}$C NMR spectrum (100 MHz; CD$_3$OD) of Re-L3.
Figure B66: Confocal microscope images of OVCAR-8 cells incubated with Re-L1 (A, D) Re-L2 (B, E) and Re-L3 (C, F) at a concentration of 20 µM ($\lambda_{Ex} = 458$ nm, 40x objective).
**Figure B67:** Confocal microscope images ($\lambda_{ex} = 458$ nm, 100x objective) of OVCAR-8 cells incubated with **Re-L1** (left), **Re-L2** (middle) and **Re-L3** (right). Top panel are fluorescence images, bottom panel are fluorescence images overlaid with DIC images.
Appendix C: Spectra of Compounds from Chapter 4

Figure C68: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 1.

Figure C69: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 1.
Figure C70: $^1\text{H}$ NMR spectrum (CDCl$_3$; 400 MHz) of compound 2.

Figure C71: $^{13}\text{C}$ NMR spectrum (CDCl$_3$; 100 MHz) of compound 2.
Figure C72: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 3.

Figure C73: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 3.
Figure C74: $^1$H NMR spectrum (CDCl$_3$; 400 MHz) of compound 4.

Figure C75: $^{13}$C NMR spectrum (CDCl$_3$; 100 MHz) of compound 4.
Figure C76: $^1$H NMR spectrum (DMSO-$d_6$; 400 MHz) of compound 5.

Figure C77: $^{13}$C NMR spectrum (DMSO-$d_6$; 100 MHz) of compound 5.
Figure C78: $^1$H NMR spectrum (DMSO-$d_6$; 400 MHz) of compound 6.

Figure C79: $^{13}$C NMR spectrum (DMSO-$d_6$; 100 MHz) of compound 6.
Appendix D: Additional Data for Chapter 5

Figure D80: $^1$H NMR Spectrum (CDCl$_3$; 400 MHz) of Compound 1.
Figure D81: $^{13}$C NMR Spectrum (CDCl$_3$; 100 MHz) of Compound 1.
Figure D82: $^1$H NMR Spectrum (DMSO-$d_6$; 400 MHz) of Compound 2.
Figure D83: $^{13}$C NMR Spectrum (DMSO-$d_6$; 100 MHz) of Compound 2.
**Figure D84:** $^1$H NMR Spectrum (DMSO-$d_6$; 400 MHz) of Compound 4.
**Figure D85**: $^{13}$C NMR Spectrum (DMSO-$d_6$; 100 MHz) of Compound 4.
Figure D86: $^1$H NMR Spectrum (1:1 CDCl$_3$:TFA-$d$; 400 MHz) of Compound 6.
Figure D87: $^{13}$C NMR Spectrum (1:1 CDCl$_3$:TFA-$d_4$; 100 MHz) of Compound 6.
Figure D88: $^1$H NMR Spectrum of (CDCl$_3$; 400 MHz) Compound 7.
Figure D89: $^{13}$C NMR Spectrum (CDCl$_3$; 100 MHz) of Compound 7.
**Figure D90**: UV/Vis absorption spectra of: (A) compound 2; (B) compound 4; (C) compound 6; and (D) compound 7 in various solvents.

**Figure D91**: Fluorescence emission spectra of compound 2 (20 µM) in non-polar (left) and polar solvents (right) with 40 µM DIPEA.
Figure D92: Fluorescence emission spectra of compound 6 (20 µM) (top) and compound 7 (20 µM) in non-polar (bottom left) and polar (bottom right) solvents.
Table C9: Photophysical Properties of Compound 6 in Various Solvents.

<table>
<thead>
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<th></th>
<th>CyH[a]</th>
<th>DX</th>
<th>CHCl₃</th>
<th>DCM[a]</th>
<th>EtOH</th>
<th>MeOH</th>
<th>CH₃CN[a]</th>
<th>DMF</th>
<th>DMSO</th>
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<tbody>
<tr>
<td>ε</td>
<td>2.02</td>
<td>2.22</td>
<td>4.81</td>
<td>8.93</td>
<td>25.3</td>
<td>33</td>
<td>36.6</td>
<td>38.3</td>
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</tr>
<tr>
<td>λₐbs (nm)</td>
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<td>359</td>
<td>356</td>
<td>-</td>
<td>364</td>
<td>366</td>
</tr>
<tr>
<td>λₑₓ (nm)</td>
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<td>362</td>
<td>355</td>
<td>-</td>
<td>359</td>
<td>358</td>
<td>-</td>
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<td>407</td>
<td>-</td>
<td>425</td>
<td>433</td>
<td>-</td>
<td>425</td>
<td>431</td>
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<tr>
<td>SS (cm⁻¹)</td>
<td>-</td>
<td>3528</td>
<td>3599</td>
<td>-</td>
<td>4326</td>
<td>4838</td>
<td>-</td>
<td>3943</td>
<td>4195</td>
</tr>
</tbody>
</table>

[a]Insoluble

Figure D93: Fluorescence excitation and emission spectra of compound 2 in methanol (black), upon the addition of base (blue) and upon the addition of acid (red).
Figure D94: Fluorescence excitation and emission spectra of the Zn(II) complexes of: (A) compound 1; (B) compound 2; (C) compound 4; (D) compound 6; and (E) compound 7 in methanol.
Curriculum Vitae

A. Professional History

Education

Doctoral Candidate
Ph.D. Organic Chemistry, University of Western Ontario, Canada 2013 – 2018
Research Supervisor – Prof. Dr. Leonard G. Luyt

Undergraduate
B.Sc. Chemistry Honours, Carleton University, Canada 2009 – 2013
Research Supervisor – Prof. Dr. Jeffrey M. Manthorpe

Research Experience

NSERC Undergraduate Student Research Award
Carleton University, Canada Summer 2012
Research Supervisor – Prof. Dr. Peter H. Buist

Awards and Scholarships
Western University Faculty of Science Graduate Student Teaching Award 2018
Ontario Graduate Scholarship 2014 – 2015
Ontario Graduate Scholarship 2013 – 2014
Senate Medal – Carleton University 2013
Canadian Society for Chemistry Medal 2012

B. Contributions

Publications


**Submitted Manuscripts**


**Manuscripts in Preparation**


**Published Abstracts**


**Conferences**


2. **Turnbull W.L.; Milne M.; Luyt L.G.,** Synthesis and Evaluation of a Bimodal $^{99m}$Tc/Re Labelled T140 Analogue for Determining CXCR4 Expression in Prostate


C. Training

Teaching Experience

Teaching Assistant 2013 – Present

University of Western Ontario, Canada


Teaching Assistant 2011 – 2013

Carleton University, Canada
Courses: CHEM 1101 - Chemistry for Engineers, CHEM 2203/2204 - Organic Chemistry I & II, CHEM 3205 - Advanced Organic Chemistry Laboratory

Mentorship

Chem 4491 Undergraduate Thesis Students
University of Western Ontario, London, Canada

Mariel Bulcan-Gnirss 2017 – 2018
Project - A Study of the Fluorescent Properties of Rhenium-Naphthalimide Conjugates

Natalie Liang 2016 – 2017
Project - Synthesis and Characterization of Rhenium/Technetium-99m(I) Tricarbonyl Complexes of Cyclen Derivatives

Dane D’Souza 2015 – 2016
Project - Synthesis of a Novel Cyclic Peptide Imaging Agent Targeting the CXCR4 Receptor