Development of a Rhodium Tetrathioether Bombesin Analogue and Investigation of Cyclic and Acyclic Ligand Systems for $^{105}$Rh(III)

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Doctor of Philosophy

by

VALERIE CARROLL

Dr. Silvia Jurisson and Dr. Timothy Hoffman, Dissertation Supervisors

MAY 2013
The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

DEVELOPMENT OF A RHODIUM TETRATHIOETHER BOMBESIN ANALOGUE AND INVESTIGATION OF CYCLIC AND ACYCLID LIGAND SYSTEMS FOR $^{105}$RHODIUM (III)

presented by Valerie Carroll,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

__________________________________________
Dr. Silvia Jurisson

__________________________________________
Dr. Timothy Hoffman

__________________________________________
Dr. Cathy Cutler

__________________________________________
Dr. Susan Lever

__________________________________________
Dr. Timothy Glass
This work is dedicated to my loving Husband,
with my deepest appreciation.
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ABSTRACT

Rhodium-105 is an attractive nuclide for radiotherapeutic applications due to its nuclear properties (566 keV $\beta^-$, 319 keV [19%], 306 keV [5%]) and the kinetic stability of Rhodium (III) complexes with soft sulfur donor atoms. Extension of previous research involving tetrathioether chelate systems to include a targeting molecule may have implications for prostate cancer therapy.

This work reports on the synthesis and evaluation of a new bombesin peptide targeted Rh (III) tetrathioether analogue, [Rh-S4-8Aoc-BBN(7-14)NH$_2$]$^+$, which shows high affinity for the BB2r receptor on PC-3 cancer cells (IC$_{50}$ = 2.2 ± 0.3 nM). However, multiple $^{105}$Rh labeled species were obtained under the radiolabeling conditions investigated.

To better understand the results observed for [${^{105}}$Rh-8Aoc-BBN(7-14)NH$_2$]$^+$, the chemistries of previously investigated [Rh-S4-Diol]$^+$ and [Rh-S4-(COOH)$_2$]$^+$ were re-evaluated using more recently available techniques. A quantitative evaluation of the [Rh-S4-Diol]$^+$ and [Rh-S4-(COOH)$_2$]$^+$ systems using NMR, ESI-MS and HPLC reveals formation of multiple species resulting from both exchange of the coordinated chlorides at the metal center and esterification of pendant carboxylate groups. While a predominate trans-chloro Rh(III)-S4 species may be favored by addition of excess NaCl, both ethanol and acid are required for radiolabeling. Thus, ligand systems utilizing pendant carboxylate groups are not compatible with traditional $^{105}$Rh radiolabeling techniques. Future studies involving a $^{105}$Rh tetrathioether bombesin analogue without pendant carboxylate groups are recommended.
Chapter 1: Introduction

1.1 Introduction to Radiopharmaceuticals

The use of radiopharmaceuticals has changed the way we diagnose and treat disease by allowing doctors to image a specific biological process or to kill cancer cells while only injecting nanomolar concentrations of the radioactive drug. In general any drug that contains a radioactive nuclide can be considered a radiopharmaceutical. Based on the radioactive properties of the radionuclide these drugs are used for different applications.

For diagnostic purposes it is necessary to use nuclides that have penetrating emissions such as gamma ($\gamma$) rays or photons from positron ($\beta^+$) annihilation. In this case, once the pharmaceutical is administered, the radiation can be detected externally and recorded to generate an image. For gamma emissions images are obtained using a technique called Single Photon Emission Computed Tomography (SPECT). Positron Emission Tomography or PET is used to create images from positron annihilation photons. This technique allows researchers and clinicians to monitor the behavior of the radiotracer in vivo. $^{18}$F-Fluorodeoxyglucose (Figure 1a) is a well-known example of an FDA approved diagnostic radiopharmaceutical that is used routinely. The $\beta^+$ emitting $^{18}$F labeled Fluorodeoxyglucose mimics endogenous glucose allowing clinicians to assess changes in glucose metabolism.\(^1\)

For therapy, particulate emissions such as alpha ($\alpha$) or beta ($\beta^-$) particles are used to deliver a therapeutic dose to the diseased tissue. $^{153}$Sm-EDTMP (commercial name Quadramet\textsuperscript{\textregistered}) is an example of a radiotherapeutic drug commonly used for pain palliation
associated with metastatic bone cancer (Figure 1b).\textsuperscript{[2-4]} In this case the natural affinity of phosphonate groups on the chelate for hydroxyapatite is employed to allow uptake at the site of bone metastases.

![Diagram of 18FDG and SmEDTMP]

**Figure 1:** Common radiopharmaceuticals including imaging agent $^{18}$FDG (a) and therapeutic agent $^{153}$SmEDTMP (b).

Currently the majority of radiopharmaceuticals are used for imaging purposes with about 95% used for diagnostic procedures and the remaining 5% for therapeutic purposes. However the availability of more selective molecular carriers has spurred a revival in interest towards the development of targeted radiotherapeutic agents.\textsuperscript{[5-7]} By coupling a radionuclide to a tumor specific peptide or antibody, it is possible to create a drug which is able to circulate through the patient’s body after injection and become localized at the tumor site in order to deliver a lethal radiation dose to the diseased cancer cells while limiting exposure to non-target tissue. One method for coupling the radiometal to a targeting agent is the bifunctional chelate approach. In this system, a multidentate bifunctional chelate is employed to bind to the metal while linking it to targeting moiety (Figure 2). This approach takes advantage of proteins on the surface of the cell membrane that specific signaling peptides may attach to.
Figure 2: A bifunctional chelate binds to the radiometal while linking it to a biomolecule which targets over expressed receptors on the tumor cells’ surface.

While there are many examples of imaging agents that utilize the bifunctional chelate technique there are currently only two traditional targeted radiotherapeutic agents that are FDA approved. They are Zevalin® ($^{90}$Y ibritumomab tiuxetan)$^{[8]}$ and Bexxar® ($^{131}$I tositumomab)$^{[9, 10]}$. Both utilize a monoclonal antibody which targets the CD20 antigen present in B-cell non-Hodgkin’s lymphomas. The successful translation of the bifunctional chelate technique from imaging to therapeutic agents in the clinic leaves an opening for the development of more agents which use this technology to treat a variety of diseases.

1.2 Radiopharmaceutical Design

Many factors must be considered during the design of a targeted radiopharmaceutical. First one must consider the nuclear properties of the nuclide of interest. The radioactive emissions must be appropriate for the desired application. As discussed above, particulate emissions are required for therapy. The energy of the resulting emissions and their range in tissue should also be paired to the type of targeted tissue and tumor size. Next, the half-life of the radioisotope under consideration must be well matched with the biological half-life of the drug in order to allow sufficient activity to be present once the drug becomes localized at the tumor site yet not so long as to cause...
an unnecessary prolonged radiation dose. Finally the availability of specific activity of the desired isotopes should be considered. Specific activity refers to the activity of the desired isotope divided by the mass of the sample. When a target is irradiated only some of the desired radioisotope will be produced in the reaction. A large amount of the non-radioactive target material or “carrier” isotope will remain. In order to achieve high specific activity, the isotope of interest must be chemically separable from the target. This is necessary for pharmaceutical purposes since the presence of target isotope would dilute the activity of the final drug.\[7\]

In addition to radioactive properties it is also vital to carefully consider the kinetic stability of the resulting metal-ligand complex. While it is common during radiolabeling, to run the reaction with an excess of ligand, once the radiolabeled complex is purified and injected it becomes very dilute. In this case it is essential not only for the rate of complexation, $k_{on}$ (Figure 3) to be very large but also for the reverse rate of decomplexation to be virtually non-existent. Otherwise, there is a potential in vivo for loss of the radiolabel from the bifunctional chelate molecule.

\[
\begin{align*}
M + L & \xrightleftharpoons[k_{off}]{k_{on}} ML \\
\text{Figure 3: Preferred chemical kinetics of a metal-ligand (ML) complex for radiopharmaceutical use.}
\end{align*}
\]

1.3 Rhodium-105 as a Potential Radiotherapeutic Nuclide

Rhodium-105 was first proposed for radiotherapeutic use by David Troutner in 1988,\[11\] Since then many researchers have continued to investigate rhodium complexes for this purpose.\[12-23\] Its moderate 566 keV $\beta^-$ emission is well suited for therapy of small solid tumors (up to $\sim$5 $\mu$m in diameter) and the accompanying $\gamma$
emissions (319 KeV [19%], 306 KeV [5%]) can be used for tracking purposes (Figure 4). In addition to its favorable nuclear properties, rhodium-105 is also of particular interest due to the kinetic inertness of rhodium (III) complexes, which is owed to its low spin d⁶ electron configuration.\cite{11, 18, 24-27} It is available in high specific activity (842 Bq/kg based on 100% separation) from neutron irradiation of a $^{104}$Ru target at the University of Missouri Research Reactor (MURR) (Figure 5).

![Rhodium-105 decay scheme](image.png)

**Figure 4:** Rhodium-105 decay scheme.\cite{28}

![Carrier free production of $^{105}$Rh](image.png)

**Figure 5:** Carrier free production of $^{105}$Rh.
1.4 Production and Separation of Rhodium-105

Rhodium-105 is produced by neutron activation of a 99% enriched ruthenium-104 target with a thermal neutron flux of $\sim 3 \times 10^{14}$ n/cm$^2$/s at the University of Missouri Research Reactor (MURR). Ruthenium-105 subsequently undergoes beta decay with a half-life of 4.4 hours generating rhodium-105. After allowing a decay time of 24 hours from end of bombardment, the target is dissolved in 2 M NaOH. Chlorine gas is bubbled through the sodium hydroxide solution generating sodium hypochlorite in situ which oxidizes the ruthenium metal to ruthenium tetroxide (RuO$_4$). The RuO$_4$ is then removed by distillation and collected in an HCl trap (Figure 6). The remaining rhodium-105 is then converted to a mixture of chloride complexes with the addition of HCl.$^{[6, 11]}$

\[\text{Cl}_2 \text{ tank} \quad \text{Air} \quad \text{Impinger} \quad \text{Heating block} \quad \text{Dry bath} \quad \text{Ru metal in NaOH} \quad \text{HCl Trap} \quad \text{NaOH Trap} \quad \text{NaOH Trap} \]

\textbf{Figure 6:} Distillation setup for separation of Rh-105.$^{[29]}$
1.5 Introduction to Rhodium Chemistry

Discovered in 1803 by W. H. Wollaston\cite{30}, rhodium is a group 9 transition metal. It is present in the earth’s crust in only 0.0001 ppm. The most common sources of this rare metal are found in Sudbury, Canada. Rhodium is a borderline soft Lewis acid preferring complexes with moderately soft Lewis base donor atoms, where S $>$ C $>$ N $>$ O.

Many oxidation states from -1 to +6 are available for rhodium. However the most important of these is the +3 oxidation state, providing a variety of kinetically inert low spin octahedral complexes with high crystal field stabilization energies (CFSE).\cite{31} This kinetic inertness can be observed in the ligand exchange rate of $[\text{Rh(H}_2\text{O)}_6]^3+$. In fact, next to $[\text{Ir(H}_2\text{O)}_6]^3+$, $[\text{Rh(H}_2\text{O)}_6]^2+$ is the second most inert aqua ion known with a mean life time of a single water molecule corresponding to approximately 30 years.\cite{26,32}

The formation of aqueous Rh$^{3+}$ complexes is typically assisted by catalytic reduction to Rh$^{1+}$ to facilitate ligand exchange. The use of alcohols to catalyze ligand exchange was first observed as early as 1929 by M. Delphine.\cite{33} This mechanism involves a 2 electron reduction of Rh$^{3+}$ to an intermediate Rh$^{1+}$ generating ethoxide, from ethanol which is later deprotonated to give acetaldehyde (Figure 7). The mechanism was confirmed by detection of trace amounts of acetaldehyde.\cite{34}

\[
\begin{align*}
\text{Rh(H}_2\text{O)}_3\text{Cl}_3 & \xrightarrow{\text{reduction}} \text{Rh (I)} \\
\text{Rh (I)} + 4 \text{ L} & \xrightarrow{\text{fast}} [\text{Rh(L)}_4]^+ \\
[\text{Rh(L)}_4]^+ + \text{Rh(H}_2\text{O)}_3\text{Cl}_3 & \xrightarrow{\text{slow}} [\text{Rh(L)}_4\text{-Cl-Rh(H}_2\text{O)}_3\text{Cl}_2]^+ \\
[\text{Rh(L)}_4\text{-Cl-Rh(H}_2\text{O)}_3\text{Cl}_2]^+ & \xrightarrow{\text{fast}} [\text{Rh(L)}_4\text{(H}_2\text{O)}\text{Cl}]^{2+} + \text{Rh(I)} \\
[\text{Rh(L)}_4\text{(H}_2\text{O)}\text{Cl}]^{2+} + \text{Cl}^- & \rightarrow [\text{Rh(L)}_4\text{Cl}_2]^+ + \text{H}_2\text{O}
\end{align*}
\]

**Figure 7:** Ligand exchange process catalyzed by reduction of Rh (III).
The formation of Rh\(^{3+}\) complexes in aqueous solutions is highly sensitive to changes in pH. In basic solutions rhodium tends to form an insoluble Rh(OH)\(_3\), \(K_{sp} = 4.8 \times 10^{-23}\) (Figure 8).\(^{[35]}\) Thus ligand exchange reactions are usually carried out under acidic conditions. It has been shown that in dilute conditions the Rh(OH)\(_3\) precipitate can be converted back to Rh(ClO\(_4\))\(_3\) 3H\(_2\)O with the addition of perchloric acid.\(^{[35, 36]}\) However, in more concentrated solutions rhodium will form hydroxide bridged polymers under basic conditions (Figure 9). Due to the kinetic inertness of Rh\(^{3+}\), once such polymers are formed they cannot be easily converted back to a soluble species.\(^{[37]}\)

\[
\begin{align*}
\text{Rh}^{3+} + \text{H}_2\text{O} & \xrightarrow{K_1} \text{Rh(OH)}^{2+} + \text{H}^+ \quad K_1 = 3.7 \times 10^{-4} \\
\text{Rh(OH)}^{2+} + 2 \text{OH}^- & \xrightarrow{K_2} \text{Rh(OH)}_3 \quad K_2 = 2.1 \times 10^{-22}
\end{align*}
\]

**Figure 8:** Formation of solid Rh(OH)\(_3\) in dilute solutions under basic conditions.\(^{[35]}\)

**Figure 9:** Formation of rhodium oxide and hydroxide bridged species.\(^{[35]}\)
1.6 Previously Investigated Chelate Systems for Rhodium-105

Several ligand systems have been investigated with $^{105}$Rh for use as a bifunctional chelate system (BFC).\cite{12-23,25,38} Table 1 provides a summary of some of the notable ligands that have previously been used for complexation of $\text{Rh}^{3+}$. Much of the early work towards development of $\text{Rh}^{3+}$ ligands focused on nitrogen donor atoms and particularly using cyclam, cyclen and their derivatives. Kruiper et al. reported the synthesis of three cyclam derivatives BA-cyclam, BA-2,3,2-tet, and BA-N-cyclen (Table 1 a, b, c). Radiolabeling was accomplished by heating a solution of $^{105}\text{Rh}$ with excess ligand for 1h at 90 °C, pH 6. Radiochemical yields of >85% were achieved.\cite{19}

Next N and O donor, amine oxime, amine phenol, and amine porphyrin ligands were investigated by the Troutner group (Table 1 d, e, f). The Rhodium (III) dichloride amine oxime and amine phenol complexes were both synthesized on the macroscopic scale with non-radioactive Rh and characterized using X-ray crystallography. In each case, the ligands formed stable octahedral complexes with donor atoms coordinated along the equatorial sites, while chlorine atoms occupied the axial positions. The amine oxime, amine phenol and the porphyrin ligands were then radiolabeled by heating at 90 °C for 2 h. Radiochemical yields were estimated at 95% for the amine oxime complex, 80% for the amine phenol and 60% for the porphyrin complex.\cite{14,22} For the amine oxime and amine phenol the authors went on to couple the pre-labeled complex with the human immunoglobulin G (IgG) for antibody receptor targeting.\cite{14}
Table 1: A brief summary of current Rh(III) chelate systems\textsuperscript{[13]}

<table>
<thead>
<tr>
<th>Structure</th>
<th>Radiolabeling YIELD</th>
<th>Structure</th>
<th>Radiolabeling YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>\textbf{~85%}\textsuperscript{[20]}</td>
<td>(i)</td>
<td>\textbf{81%}\textsuperscript{[21,39]}</td>
</tr>
<tr>
<td>(b)</td>
<td>\textbf{~85%}\textsuperscript{[20]}</td>
<td>(j)</td>
<td>\textbf{59%}\textsuperscript{[21,39]}</td>
</tr>
<tr>
<td>(c)</td>
<td>\textbf{~85%}\textsuperscript{[20]}</td>
<td>(k)</td>
<td>\textbf{94%}\textsuperscript{[21,22]}</td>
</tr>
<tr>
<td>(d)</td>
<td>\textbf{95%}\textsuperscript{[15,23]}</td>
<td>(l)</td>
<td>\textbf{&lt;90%}\textsuperscript{[16,17,18]}</td>
</tr>
<tr>
<td>(e)</td>
<td>\textbf{80%}\textsuperscript{[15,23]}</td>
<td>(m)</td>
<td>\textbf{&lt;90%}\textsuperscript{[16,17,18]}</td>
</tr>
<tr>
<td>(f)</td>
<td>\textbf{60%}\textsuperscript{[15,23]}</td>
<td>(n)</td>
<td>\textbf{60%}\textsuperscript{[13]}</td>
</tr>
<tr>
<td>(g)</td>
<td>\textbf{&gt;90%}\textsuperscript{[24]}</td>
<td>(o)</td>
<td>\textbf{&lt;90%}\textsuperscript{[14]}</td>
</tr>
<tr>
<td>(h)</td>
<td>\textbf{94%}\textsuperscript{[38,21]}</td>
<td>(p)</td>
<td>\textbf{&lt;90%}\textsuperscript{[14]}</td>
</tr>
</tbody>
</table>
Then, Blake et al. reported the synthesis of a series of kinetically inert Rh(III) complexes with crown thioethers. In 1996, Venkatesh et al. reported the carrier free synthesis of a $^{105}$Rh-[16]ane-S$_4$-diol with >90% radiochemical yields (Table 1 g). These studies shifted the focus from harder N and O donors to more soft S donor atoms. Ning Li continued this area of investigation by studying a series of 14-membered(108,682),(921,916)

Li continued with this work to synthesize a carboxylic acid functionalized S$_4$ macrocycle (Table 1 k), which was coupled to a bombesin derivative. Rh-S$_4$-X-Ava-BBN(7-14)NH$_2$ complexes were studied where the Rh-S$_4$ chelate was coupled either directly to the binding sequence (X = 0) or through a 5 carbon spacer (X = 5) (Figure 10). In a competitive binding displacement assay compared to $^{125}$I-Tyr$^4$-BBN, IC$_{50}$ values of 37.5 ± 10.5 nM for the Rh-S$_4$-BBN and 4.76 ± 0.79 nM for the Rh-S$_4$-5-Ava-BBN were reported. A radiochemical yield of 95 ± 5% of the $^{104}$Rh-S$_4$-5-Ava-BBN complex was obtained by heating 2.5 x 10$^{-4}$ M ligand with ~1 mCi $^{105}$Rh chloride in 20% ethanol for 3h at 80°C. Biodistribution studies were carried out in Sprague-Dawley rats. A GRP receptor specific uptake of 1.01 ± 0.14 %ID/organ and 2.25 ± 1.02 %ID/organ were found in the normal pancreas at 2 h for the Rh-S$_4$-BBN and Rh-S$_4$-5-Ava-BBN
complexes respectively, however large uptake was also noted in the liver, kidney, and intestines (Table 2). The primary route of clearance was through the bladder.

![Chemical structure](image)

**Figure 10:** Rh-S$_4$-BBN(7-14)NH$_2$ (a) and Rh-S$_4$-5-Ava-BBN(7-14)NH$_2$ (b).[20]

<table>
<thead>
<tr>
<th>Complex</th>
<th>Rh-S$_4$-BBN</th>
<th>Rh-S$_4$-5-Ava-BBN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>% Injected Dose per Organ</td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>2 h</td>
<td>0.5 h</td>
</tr>
<tr>
<td>Organ</td>
<td>Brain</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>4.48 ± 1.24</td>
<td>0.99 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>0.13 ± 0.03</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.08</td>
<td>0.15 ± 0.09</td>
</tr>
</tbody>
</table>

**Table 2:** Biodistribution Data for Rh-S$_4$-BBN and Rh-S$_4$-5-Ava-BBN. [20, 21]

Later that year a series of acyclic tetrathioether (S$_4$) Rh(III) complexes were also reported.[15, 16] 2,2,2-S$_4$, 2,3,2-S$_4$, 3,3,3-S$_4$, and 3,2,3-S$_4$ ligands were synthesized with either dicarboxylic acid pendant groups or dibenzyl pendant groups. Goswami studied the effect of backbone length of the ligand on the conformation of the final Rh(III) molecule. The Rh-S$_4$-(Bz)$_2$ complexes were analyzed using x-ray crystallography and
Rh-S$_4$-(COOH)$_2$. Out of the four complexes studied only the 2,2,2-S$_4$ and the 3,3,3-S$_4$ ligands resulted in a single isomer upon complexation with Rh(III) on the macroscopic scale. The 2,2,2-S$_4$ ligand resulted in a cis-dichloro Rh(III) complex and the 3,3,3-S$_4$ ligand resulted in the formation of only the trans dichloride species, while both the 2,3,2-S$_4$ and 3,2,3-S$_4$ ligands resulted in a mixture of cis and trans-dichloro Rh(III) complexes.$^{[15, 16]}$ The 2,2,2-S$_4$ and 3,3,3-S$_4$ ligands (Table 1 l, m) were labeled by refluxing a solution of the ligand in a 40% ethanol with a weakly acidic pH 4 -5 solution of $^{105}$RhCl$_3$ producing radiochemical yields of $>95\%$. Biodistribution studies in normal mice showed rapid clearance from all major organs for both $^{105}$Rh-S$_4$ compounds (Table 3)$^{[15, 17]}$.

<table>
<thead>
<tr>
<th>Organs</th>
<th>% Injected Dose per Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 h</td>
</tr>
<tr>
<td>Brain</td>
<td>0.09 ± 0.07</td>
</tr>
<tr>
<td>Blood</td>
<td>4.01 ± 0.71</td>
</tr>
<tr>
<td>Heart</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Lung</td>
<td>0.25 ± 0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>9.91 ± 0.84</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>Intestines</td>
<td>9.50 ± 2.48</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.96 ± 0.29</td>
</tr>
<tr>
<td>Urine</td>
<td>53.65 ± 3.42</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.07 ± 0.06</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.20 ± 0.01</td>
</tr>
</tbody>
</table>

Table 3: Biodistribution of $^{105}$RhCl$_2$-3,3,3-S$_4$ in normal mice.$^{[15, 17]}$

Acyclic diaminodithioether (DADTE) ligand systems have also been investigated for use as bifunctional chelates for $^{105}$Rh.$^{[12]}$ A series of DADTE ligands were synthesized to study the effect of backbone length and substitution on the conformation of the Rh(III) complex. The Rh-DADTE complexes were analyzed using NMR
spectroscopy and X-ray crystallography. Of the ligand systems tested only the 2,3,2 backbone length complexes with geminal dimethyl substitution (Table 1 n) resulted in a single isomer upon complexation with the Rh$^{3+}$. This ligand was labeled with $^{105}$Rh by heating at 80ºC with a 15% ethanolic solution at pH 4 for 1 h. Radiochemical yields of 60% were achieved. For use as a BFC system, one of the secondary amine groups on this ligand was activated and coupled to a bombesin derivative Ahx-NLeu$^{14}$-BBN(7-14), (Ahx-QWAVGHLNLeu-NH$_2$) as a targeting moiety (Figure 11).$^{[12]}$

![Figure 11: Rh-N$_2$S$_2$- Ahx-NLeu$^{14}$-BBN(7-14)NH$_2$.][12]

More recently, phosphine containing chelates have been investigated using phosphines as internal reducing agents in an attempt to decrease the concentration of ethanol required for $^{105}$Rh labeling.$^{[13]}$ 2,3,2-P$_2$N$_2$ and 2,3,2-P$_2$S$_2$ ligands were synthesized with phenyl substituted phosphines. Labeling conditions were optimized for pH, temperature, time, ligand concentration, and ethanol concentration. Radiochemical yields of $>$90% were achieved with a notable decrease in ethanol concentration (10% EtOH for P$_2$N$_2$ and 5% for P$_2$S$_2$) compared to 20% - 40% for similar S$_4$ systems. However a much larger excess of ligand concentration (mM concentrations for P$_2$N$_2$ and P$_2$S$_2$ compared to µM concentrations for S$_4$ systems) was needed to achieve high yields.
1.7 Introduction to Bombesin

Bombesin (BBN), originally isolated from the skin of the Bombina bombina frog, is a 14 amino acid peptide analogue of the human gastrin releasing peptide (GRP).[^39] The bombesin receptor family consists of four receptor subtypes: neuromedin B (BB1), the gastrin releasing peptide receptor (BB2r), the orphan receptor (BB3r) and bombesin receptor (BB4r).[^40] Of these four subtypes we are primarily interested in the BB2r subtype. BB2 receptors have been shown to be over-expressed on the surface of prostate[^41, 42], breast[^43, 44], pancreatic[^45], small cell lung cancers[^46, 47], and gastrointestinal stromal tumor cells (GIST).[^48] As a result, bombesin analogues have been heavily investigated for tumor specific in vivo delivery of a radionuclide both for imaging and for therapeutic applications.[^49, 50]

1.8 Bombesin Targeted Radiopharmaceuticals

Several groups have focused efforts on preclinical development of bombesin targeted analogues utilizing the bifunctional chelate approach with radiometals such as $^{99m}$Tc[^51-65], $^{111}$In[^66-69], $^{68/67}$Ga[^70, 71], $^{64}$Cu[^72-80], $^{177}$Lu[^70, 81, 82] and $^{18}$F[^83, 84]. Many analogues exhibit high binding affinity (nM IC$_{50}$ values) with GRP receptors and reasonable uptake in tumor tissues. A handful of these analogues have resulted in phase 1 clinical trials. $^{99m}$Tc-N$_3$S-Gly-5Ava-BBN(7-14), $^{99m}$Tc-RP257 was the first radiolabeled bombesin analogue to be studied in humans. Van de Wiele et al. monitored uptake of $^{99m}$Tc-RP527[^85] in the tumors of 4 patients exhibiting metastatic prostate cancer and 6 women with breast carcinomas.[^86, 87] Patients were monitored at 1 h and 5 – 6 h p.i. using a planar gamma scan and single photon emission computed tomography.
(SPECT). $^{99m}$Tc-RP527 uptake was observed in one out of 4 prostate carcinomas and 4 out of 6 breast carcinomas with high tumor to normal ratios ($7.23 \pm 8.46$ at 5 – 6 h p.i.) despite a diffuse uptake in normal breast tissue. The compound was primarily cleared through the kidneys however the authors suggest that a smaller amount, which is cleared by hepatobiliary excretion, may be problematic for imaging of tumors in the abdominal region.

Another $^{99m}$Tc labeled bombesin analogue $^{99m}$Tc-cys-(6-amino-$n$-hexanoic acid)BN(2-14), $^{99m}$Tc-Leu$^{12}$-BN1 was synthesized by the Scopinaro group$^{[88]}$ and has been clinically studied for diagnosing breast, prostate and colon cancers. In 5 patients with breast cancer, all 5 primary tumors were detected with $^{99m}$Tc-Leu$^{13}$-BN1 and exhibited higher tumor to normal ratios when compared head to head with $^{99m}$Tc-sestamibi, a compound that is already in clinical use for staging auxiliary lymph node involvement in breast cancer.$^{[89]}$ In a group of 10 prostate cancer patients 2 with benign tumors and 8 with confirmed cancer SPECT images showed uptake of $^{99m}$Tc-Leu$^{13}$-BN1 in all 8 patients with cancer and true negative for the two patients with benign tumors. In three cases the images were able to diagnose invasion of the pelvic lymph nodes.$^{[90]}$

Next in 13 patients with known or suspected colorectal cancer $^{99m}$Tc-Leu$^{13}$-BN1 scan was able to detect 16 out of 17 confirmed tumor locations and lymph node invasion in 5 cases. However out of 6 benign tumor locations 2 false positives were observed.$^{[91]}$ Also in a study involving 26 patients with various cancers (15 breast, 3 prostate, 5 colo-rectal, 1 pancreas, 2 small cell lung and 1 gastrinoma) the authors demonstrated rapid uptake of $^{99m}$Tc-Leu$^{13}$-BN1 in the tumor tissue. On average across all tumor types 80% maximum uptake of the activity in tumor tissue was achieved with-in $2.68 \pm 1.03$ min for
tumors, which have invaded lymph nodes (N+), and 5.5 ± 0.82 min for cancers, which have not involved lymph nodes (N-). In all cases uptake of $^{99m}$Tc-Leu$^{13}$-BN1 was faster than 10 min.[92]

Based on the RP527 patent, another bombesin analogue $^{177}$Lu-DOTA- Gly-4-aminobenzyl-BBN(7-14), $^{177}$Lu-AMBA was developed by Bracco Diagnostics.[82, 93] In SPECT images 5 out of 7 patients with prostate cancer exhibited specific uptake of $^{177}$Lu-AMBA. High uptake was also observed in the pancreas however a detailed description of the formulation and purification of the final drug has not been published.

Antonia Dimitrakopoulou-Strauss et al. have also done a number of clinical studies using a $^{68}$Ga labeled bombesin analogue $^{68}$Ga-DOTA-PEG$_2$-[D-Tyr$^6$,$\beta$-Ala$^{11}$,Thr$^{13}$,Nle$^{14}$]BBN(6-14), $^{68}$Ga-BZH3.[71, 94] Less than 50% of patients treated exhibited tumor uptake of the compound, however presence of the BB2 receptor was not confirmed. In a comparison with $^{18}$F-FDG, a small minority of patients with histologically confirmed aggressive (WHOIV) tumors did exhibit enhanced uptake of $^{68}$Ga-BZH3 while no uptake of $^{18}$F-FDG was observed.[94]

Until recent years most bombesin targeted radiopharmaceutical research was focused toward the development of bombesin agonist peptides. An agonist peptide is internalized after binding to a receptor and activates a biological response. In the case of BB2r receptors this triggers cell proliferation and release of gastrointestinal hormones. On the other hand, an antagonist does not trigger any response when binding to receptors. Previously agonists had been pursued due to their ability to internalize and accumulate in the cell, theoretically leading to increased tumor uptake. However recent studies suggest that antagonist bombesin analogues may exhibit similar or even better in vivo behavior.
In 2003 Nock et al. investigated the antagonist analogue $^{99m}$Tc-N$_4$-[D(Phe)$_6$-Leu-NH$_2$]-des-Met$_{14}$]BN(6-14), Demobesin 1. This analogue showed a high specific uptake (16.2 ± 3.1 %ID/g at 1h p.i.) in the tumors of PC-3 xenografted Swiss nu/nu mice, which was retained over time (15.61 ± 1.19 %ID/g at 4h p.i.).$^{[58]}$ In a subsequent study,$^{[51]}$ Demobesin 1 was directly compared to a similar agonist analogue $^{99m}$Tc-N$_4$-[Pro$_1$-Leu-Tyr$_4$-Nle$_{14}$]bombesin, Demobesin 4. Although the two analogues had comparable IC$_{50}$ values (2.1 ± 0.5 nM Demobesin 1 and 0.8 ± 0.1 nM Demobesin 4), Demobesin 1 exhibited a significantly higher tumor uptake (22.66 ± 2.20 %ID/g) than Demobesin 4 (5.19 ± 0.59 %ID/g). This resulted in improved tumor to non-target tissue ratios (tumor:kidney ratios 4 h p.i: 5.23 Demobesin 1 and 0.70 Demobesin 4), which suggests that BB2r receptor antagonists may actually be superior to antagonists.

Despite excellent tumor uptake and retention Demobesin 1 also showed significant accumulation in the stomach (2.12 ± 0.48 %ID/g at 4h p.i.),$^{[51]}$ which is often indicative of free TcO$_4^-$ having dissociated from the chelate. This observation led Abd-Galiel et al.$^{[66]}$ to investigate $^{111}$In-Bomproamide, $^{111}$In-DOTA-aminohexanoyl-[D(Phe)$_6$-Leu-NH$_2$CH$_2$CH$_2$CH$_3$-des Met$_{14}$]-BBN(6-14). This analogue exhibited an initial high uptake in the PC-3 tumors of SCID mice (6.9 ± 1.06 %ID/g at 15 min p.i.) and was not accumulated in the stomach (1.91 ± 0.29%ID/g at 15min p.i. and 0.05 ± 0.03 %ID/g at 4h p.i.). While this compound had faster wash out from the tumors than Demobesin 1, it also cleared more rapidly from nontarget tissues leading to diagnostically useful ratios.

More recently Mansi et al. synthesized RM2 a DOTA-4-amino-1-carboxymethyl-piperidine-(D)Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH$_2$ bombesin antagonist analogue utilizing radiotracers $^{111}$In and $^{68}$Ga. Both $^{111}$In-RM2 and $^{68}$Ga-RM2 exhibited high
uptake and retention in tumor tissues of PC-3 nude mice (15.23 ± 4.78 %ID/g at 1 h p.i., 11.75 ± 2.43 %ID/g at 4 h p.i. for $^{111}$In-RM2, and 5.50 ± 0.39 %ID/g at 1 h p.i, 8.18 ± 1.89 %ID/g at 2 h p.i. for $^{68}$Ga-RM2) with rapid clearance from non-target tissues.$^{[95]}$

Many groups have since focused their efforts on development of novel bombesin antagonist radiotracers.$^{[96-98]}$

For this project we have chosen to focus on a bombesin(7-14) agonist peptide as a viable starting point for the proof-of-concept synthesis of a GRP targeted $^{105}$Rh conjugate.

1.9 Objectives

The goal of this project is to develop a new bombesin(7-14) targeted radiotherapeutic agent using $^{105}$Rh. In pursuit of this objective, we have extended the work of Goswami et al. to include a bombesin targeting vector, which may have implications for prostate cancer therapy. We have also investigated the use of cyclic and acyclic tetrathioether chelates with $^{105}$Rh and the implications this chelate choice may have on radiopharmaceutical development.
Chapter 2: Experimental

2.1 General Materials and Instrumentation

Fmoc-8Aoc-BBN(7-14) was prepared using automated solid phase Fmoc chemistry with an Omega AAPPTEC 396 peptide synthesizer on a Rink Amide resin support. Rink Amide resin, Fmoc amino acids and coupling reagents were purchased from EMD Biosciences (San Diego, CA, USA). $^{125}$I-Tyr$^4$-BBN was purchased from Perkin Elmer (Waltham, MA, USA). All other reagents were purchased from either Fisher Scientific (Pittsburg, PA, USA) or Sigma Aldrich (St. Louis, MO, USA). Triethyl amine was dried over calcium sulfate overnight. All other reagents were used without further purification.

Fluka Silica gel purchased from Sigma Aldrich (St. Louis, MO, USA, 60 Å pore size 220 – 440 mesh) was used for column chromatographic purification of the synthesized chelate and intermediates. Selecto Scientific plastic-backed silica gel TLC plates purchased from Fisher Scientific (Pittsburg, PA, USA) were used to radio TLC.

The synthesized chelate and intermediates were analyzed by NMR spectroscopy using either a Bruker DRX 500 MHz or a DRX 300 MHz widebore spectrometer (Billerica, MA, USA). Analytical HPLC was performed using a Shimadzu SCL-10A (Koyoto, Japan) system with a binary gradient system [solvent A = DI water with 0.1% TFA, B = Acetonitrile with 0.1% TFA] and an in line SPD-10A UV absorbance detector ($\gamma = 280$ nm, 220 nm).

2.2 Ligand Synthesis

Two bifunctional chelate systems were initially considered for this project. The first was a backbone functionalized acyclic tetrathioether, 8-(4-carboxybutyl)-3,7,11,15-
tetrathiaheptadecanedioic acid (Figure 12). The other bifunctional chelate pursued,
3,7,11,15-tetrathiaheptadecanedioic acid, 3,3,3-S4(COOH)2 was functionalized from one
of the two carboxylate pendant groups (Figure 13). Both chelate systems were pursued
simultaneously, however it proved difficult to isolate the pure back bone functionalized
chelate. Meanwhile, a pendant coupled bombesin bifunctional chelate had been
successfully prepared using the synthesis outlined in Figure 13. The backbone
functionalized approach was abandoned.
Figure 12: Synthesis of backbone functionalized tetrathioether chelate.
Intermediate 1: methyl 2-((3-chloropropyl)thio)acetate

Intermediate 2: dimethyl 3,7,11,15-tetrathiaheptadecane-1,17-dioate

Figure 13: Synthesis of 3,3,3-S4-(COOH)₂ chelate.
2.2.1 Synthesis of Intermediate 1: methyl 2-((3-chloropropyl)thio)acetate

The dicarboxylic acid functionalized ligand 3,3,3-S₄-(COOH)₂ was synthesized according to previously reported methods,[16] as shown in Figure 13. Methylthioglycolate (4.0 mL, 42 mmol) and an excess of 1,3-dichloropropane (40 mL, 410 mmol) were brought to reflux at 84°C under nitrogen followed by the dropwise addition of dry triethylamine (TEA, 6.6 mL, 47 mmol). The triethylammonium chloride precipitate was removed by filtration and excess dichloropropane was removed under vacuum at 60°C yielding a yellow oil. The separation of intermediate 1 using the vacuum distillation technique described in Goswami et al[16] did not result in sufficient purification. The resulting oil was further purified by silica gel column chromatography with a dry mass of silica gel to sample ratio of 80:1. The sample was loaded in a 50% mixture of hexanes and diethyl ether and then eluted with the same solution. The loaded solution was yellow in color. As the yellow band began to elute, fractions were collected and monitored by silica TLC developed in 50% hexanes/diethyl ether. The fractions containing intermediate 1 (Rf = 0.56) were collected and the mobile phase was removed under nitrogen gas. The purified intermediate 1, recovered in 71% yield, was characterized by ¹H NMR spectroscopy. ¹H NMR (300 MHz, CDCl₃): δ ppm: 3.75 (s, -O-CH₂, 3H), 3.65 (t, -CH₂-Cl, 2H), 3.24 (s, -CH₂-COO, 2H), 2.80 (t, -CH₂-CH₂-S, 2H), 2.07 (p, CH₂-CH₂-CH₂, 2H).

2.2.2 Synthesis of Intermediate 2: dimethyl 3,7,11,15-tetrathiaheptadecane-1,17-dioate

Two molar equivalents of intermediate 1 (3.58 g, 19.6 mmol) were combined with 2.8 mL (20 mmol) of dry triethylamine (TEA). 1,3-propane dithiol (0.96 mL, 9.56 mmol) was added to the mixture dropwise, and the solution was refluxed under nitrogen.
at 94°C for 17 hours. The mixture was then cooled and slurried with ~5 mL of diethyl ether. Triethyl ammonium chloride was removed by filtration and diethyl ether removed under nitrogen. The intermediate 2 oil was purified by silica gel chromatography as described above. A yield of 60% was isolated, 1H NMR (500 MHz, CDCl3): δ ppm: 3.75 (s, -O-CH₃, 6H), 3.24 (s, -CH₂-COO, 4H), 2.75 (t, -CH₂-S-CH₂-COO, 4H), 2.62 (t, CH₂-S-CH₂, 8H), 1.87 (m, CH₂-CH₂-CH₂, 6H).

2.2.3 Synthesis of 3,3,3-S₄-(COOH)₂: 3,7,11,15-tetrathiaheptadecane-1,17-dioic acid

Intermediate 2 (2.046 g, 5.1 mmol) was dissolved in an alkaline solution of KOH (1.194 g) in 6 mL of methanol and heated to 90°C for 55 min. The solid product was filtered, washed with methanol and dissolved in 10 mL of water. Hydrochloric acid (6 M) was added to the solution dropwise resulting in the precipitation of the desired product 3,3,3-S₄-(COOH)₂ at pH 2. The precipitate was filtered and dried under vacuum. A 71% yield of the final product was isolated, 1H NMR (500 MHz, CD₃CN): δ ppm: 3.22 (s, -CH₂-COO, 4H), 2.69 (t, -CH₂-S-CH₂-COO, 4H), 2.58 (t, CH₂-S-CH₂, 8H), 1.81 (m, CH₂-CH₂-CH₂, 6H)

2.2.4 Synthesis of coupled 3,3,3-S₄-BBN(7-14)

8-Aoc-BBN(7-14)NH₂ was synthesized on a solid rink amide resin support using Fmoc protected amino acids. The resin bound amino acids were deprotected with a 0.1 M solution of hydroxybenzotriazole (HOBT) and 20% piperidine. Then the carboxyl groups were activated with 0.5 M of o-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluorophosphate (HBTU) in DMF, and a mixture of 34.8 mL (0.2 mol) diisopropylethylamine (DIEA) in 65.2 mL (0.7 mol) N-methyl-2-pyrrolidone (NMP).
The activated end of the growing resin-bound peptide was flushed with an excess of Fmoc-amino acid resulting in the formation of an amide bond.

Manual coupling was used to conjugate the tetrathioether chelate by adding 60 μmol of Fmoc-8Aoc-BBN(7-14)NH₂ on the resin support to a stirring solution containing DIEA (50 μL, 300 μmol), NMP (100 μL, 130 μmol), 3,3,3-S₄-COOH₂ (65 mg, 17 μmol), HBTU (45.5 mg, 120 μmol), and HOBT (27 mg, 200 μmol). The solution was stirred at 60°C for 30 min, cooled and then filtered.

S₄-8Aoc-BBN(7-14)NH₂ was cleaved from the resin support in a solution of 5% water, 5% triisopropyl silane (TIS), and 5% phenol in trifluoroacetic acid (TFA), filtered and precipitated in 12 mL of cold t-butyl ether. The desired product was HPLC purified using a Prep Nova-Pak HR C₁₈ Waters column (6 μm, 7.8 x 300 mm, 60 Å) and a binary solvent gradient (80% A, 20% B shifted to 50% A, 50% B over 60 min). The purified product was then analyzed via LC-MS using a Kromasil C18 HPLC column (5 μm, 150 x 4.6 mm, 100 Å) and a Finnigan TSQ7000 mass spectrometer with the same solvent gradient. (m/z = 1436.15 Da, calc = 1435.68 Da)

2.2.5 Synthesis of [RhCl-S₄-8Aoc-BBN(7-14)NH₂]⁺ TFA⁻ complex

S₄-8Aoc-BBN (0.5 mg, 3.1 x 10⁻⁴ mmol) was dissolved in 5 mL of 4% ethanol/acetonitrile solution. The solution was brought to reflux at 90°C and 40 μL of RhCl₃·3H₂O (0.9 mg, 3.4 x 10⁻⁴ mol) in acetonitrile was added dropwise. The mixture was refluxed at 90°C for 1 h, cooled and then lyophilized. The resulting pale yellow solid was analyzed by LC-MS using a Kromasil C18 HPLC column (5 μm, 150 x 4.6 mm, 100 Å) with a Finnigan TSQ7000 mass spectrometer using a solvent gradient of 90% A , 10% B shifted to 50% A, 50% B over a period of 30 min. The product was also analyzed by
MALDI TOF MS with an AB Sciex4700 mass spectrometer. \((m/z = 1571.8 \text{ Da, calc } = 1571.02 \text{ Da for [Rh-S4-8Aoc-BBN(7-14)NH}_2]\)^+\) \((m/z = 786.3 \text{ Da, calc } = 786.51 \text{ Da for [Rh-S4-8Aoc-BBN(7-14)NH}_2]^{2+}\).

2.2.6 In vitro analysis of \([\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^{+}\) TFA

The affinity of \([\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^{+}\) for the GRP receptor was evaluated using a competitive binding assay compared to \(^{125}\text{I-Tyr}^4\text{-BBN with GRP receptor positive PC-3 human prostate cancer cells. In a micro-well plate approximately 3 x 10}^5\text{ PC-3 cells were suspended in Roswell Park Memorial Institute (RPMI) medium at pH 7.4 with 4.8 mg/mL HEPES, and 2 mg/mL BSA. The mixture was incubated at 37°C for 1 h with 30,000 cpm of }^{125}\text{I-Tyr-BBN and increasing concentrations of [RhCl-S4-BBN(7-14)NH}_2]^{+}\text{ from 3.3x10}^{-13}\text{ to 3.3x10}^{-6}\text{ M. The cells were washed four times with media to release any non-specitically bound BBN and then counted on a MultiWiper (Laboratory Tecnologies, Maple Park, IL, USA) multiwell NaI gamma scintillation detector. The inhibitory concentration 50% (IC}\_50\) was derived from the average of three experiments. The IC\_50 curve (Figure 15) was obtained by plotting the % of \(^{125}\text{I-Tyr-BBN bound to the cell as a function of the concentration of [RhCl}_2\text{-S4-BBN(7-14)NH}_2]^{+}\) added using GraphFit software version 4 (Erithacus Software Limited, Middlesex, UK).

2.3 Synthesis and Evaluation of Rh-S4-Diol and \(^{105}\text{Rh-S4-Diol}

As previously discussed the macrocyclic 3,3,3,3-S4-Diol investigated by Venketesh et al.\(^{123}\) is a well-known chelate for \(^{105}\text{Rh. This complex has been shown to provide >90\% yields with }^{105}\text{Rh via a quick labeling procedure and has previously been
well characterized by silica gel TLC. Until recently 3,3,3,3-S4-Diol has been available commercially. For this reason many researchers studying new chelate systems for $^{105}$Rh have used the $^{105}$Rh-S4-Diol labeling procedure and analysis as a quick quality control procedure to determine the labeling efficiency of $^{105}$Rhodium Chloride. We have elaborated on the previous TLC evaluation to include an HPLC method of evaluation.

2.3.1 *Development of an HPLC Rh-S4-Diol QC Procedure*

Non-radioactive Rh-S4-Diol$^+$ was prepared following the procedure reported by Venketesh$^{23}$. Briefly, 0.80 mL (3.0 x $10^{-6}$ mol) of a 1.0 mg/mL solution of RhCl$_3$·3H$_2$O in acetonitrile was added to 1.0 mL (3.0 x $10^{-6}$ mol) of a 1.0 mg/mL solution of 3,3,3,3-S4-Diol in either 10% Ethanol/H$_2$O or 10% Ethanol/Saline at pH 4. The solution was heated at 80 °C for 1 h. Formation of chelated Rh-S4-Diol was confirmed by mass spectrometry. The macroscopic Rh-S4-Diol complex was evaluated by HPLC using a Waters Symmetry Shield RP-18 column (5 µm, 4.6 x 250 mm) with binary gradient where A is increased from 1% to 90% over 8 min, remains linear at 90% until 9 min and is decreased from 90% back to 1% by 10 min.

2.3.2 *Synthesis of radiolabeled $^{105}$Rh-S4-Diol*

The radiolabeled $^{105}$Rh-S4-Diol was synthesized according to the previously published procedure.$^{23}$. Briefly, 100 µL (3.0 x $10^{-4}$ mol) of a 0.1 mg/mL solution of 3,3,3-S4-Diol in 15% Ethanol/H$_2$O was added to 500 µL (1 - 2 mCi) of $^{105}$Rh Chloride at pH 4 and heated for 1 h at 80 °C. The resulting solution was spotted on a silica gel TLC plate and developed in 0.9% saline. The labeling solution was also evaluated using the HPLC method described above and compared to macroscopic results.

A 40 µL aliquot of the reaction mixture described above was also spiked with 20 µL (3.8 x $10^{-5}$ mol) of cold 1 mg/mL RhCl$_3$·3H$_2$O and heated for an additional hour at 80
Again this mixture was analyzed using HPLC allowing for in situ confirmation of radio chromatogram peaks with species observed via UV detection.

2.4 Synthesis and Evaluation of Rh-S4-(COOH)$_2$ and $^{105}$Rh-S4-(COOH)$_2$

Goswami et al. reports formation of a single radiolabeled $^{105}$Rh-S4-(COOH)$_2$ species as evaluated by silica gel thin layer chromatography.$^{[15]}$ These results were further substantiated based on X-ray crystallography of the analogous macroscopic complex with benzyl pendant groups Rh-S4-diBz and NMR studies involving Rh-S4-(COOH)$_2$ species. However no HPLC data was reported on either a macroscopic or radiotracer scale.$^{[15-17]}$ This is the first reported HPLC evaluation for the 3,3,3-S4-(COOH)$_2$ ligand system.

2.4.1 Characterization of Rh-S4-(COOH)$_2$

Non-radioactive Rh-S4-(COOH)$_2$ was prepared according to the procedure of Goswami$^{[15]}$ by adding 825 µL (2.82 x $10^{-6}$ mol) of a 1.0 mg/mL solution of RhCl$_3$·3H$_2$O to 500 µL (2.82 x $10^{-6}$ mol) of a 2.1 mg/mL solution of 3,3,3-S4-(COOH) in ethanol. The solution was heated at 80 °C for 1 h. Formation of chelated trans-[RhX$_2$-S4-(COOH)$_2$]$^+$ was confirmed by mass spectrometry. The macroscopic Rh-S4-(COOH)$_2$ complex was evaluated using the same HPLC procedure described in section 2.3.1. Peaks were observed at retention times 6.14 min and 6.87 min.

2.4.2 Synthesis of Radiolabeled $^{105}$Rh-S4-(COOH)$_2$

The radiolabeled $^{105}$Rh-S4-(COOH)$_2$ was synthesized according to the previously reported method$^{[15]}$ by adding 100 µL (3.0 x $10^{-4}$ mol) of a 0.1 mg/mL solution of 3,3,3-S4-(COOH)$_2$ in ethanol and 50 µL acetonitrile to 100 µL (~ 3 mCi) of $^{105}$Rh Chloride at pH 4 and heated for 1 h at 80 °C. The resulting solution was spotted on a silica gel TLC
plate and developed in 0.9% saline. TLC results were consistent with those previously reported.\textsuperscript{[15]} The labeling solution was also evaluated using the HPLC method described in section 2.3.1 and compared to macroscopic results. Multiple peaks were observed with retention times 4.87 min, 5.71 min, 6.24 min, and 6.82 min.

A 40 μL aliquot of the reaction mixture described above was also spiked with 20 μL (3.8 x 10\textsuperscript{-5} mol) of non-radioactive 1 mg/mL RhCl\textsubscript{3}•3H\textsubscript{2}O and heated for an additional hour at 80°C. Again this mixture was analyzed using HPLC allowing for \textit{in situ} confirmation of radio chromatographic peaks with species observed via UV detection.

2.4.3 \textit{Macroscopic Rh-S4-(COOH)}\textsubscript{2} \textit{Studies}

In order to characterize multiple potential Rh-S4-(COOH)\textsubscript{2} species, the macroscopic complex was synthesized under various reaction conditions at pH 3 as follows;

1.) 3.07 x 10\textsuperscript{-6} M S4-(COOH)\textsubscript{2} and 3.80 x 10\textsuperscript{-6} M RhCl\textsubscript{3}•3H\textsubscript{2}O in 50% ethanol/acetonitrile. (t\textsubscript{r} = 6.20 min)

2.) 3.07 x 10\textsuperscript{-6} M S4-(COOH)\textsubscript{2} and 3.80 x 10\textsuperscript{-6} M RhCl\textsubscript{3}•3H\textsubscript{2}O in a 50% ethanol/acetonitrile solution saturated with NaCl. (t\textsubscript{r} = 6.19 min)

3.) 3.07 x 10\textsuperscript{-6} M S4-(COOH)\textsubscript{2} and 3.80 x 10\textsuperscript{-6} M RhCl\textsubscript{3}•3H\textsubscript{2}O in 25% water, 25% ethanol, 50% acetonitrile. (t\textsubscript{r} = 5.68 min, 6.21 min, ~ 5.06 – 6.54 min multi)

4.) 3.07 x 10\textsuperscript{-6} M S4-(COOH)\textsubscript{2} and 3.80 x 10\textsuperscript{-6} M RhCl\textsubscript{3}•3H\textsubscript{2}O in a 25% water, 25% ethanol, 50% acetonitrile solution with 0.1 g, 1.7 x 10\textsuperscript{-3} mol NaCl. (t\textsubscript{r} = 6.18 min)

Each solution was heated at 85°C for 1 h and then analyzed by HPLC. After HPLC analysis the solutions were lyophilized and then taken up in 50% acetonitrile/water for mass spectrometry or 50% D\textsubscript{3}-acetonitrile/D\textsubscript{2}O for NMR.

2.5 \textit{Synthesis of radiolabeled} \textsuperscript{105}Rh-S4-BBN
Ethanolic solutions of 3,3,3-S4-BBN were added to 0.5 - 1 mCi of rhodium-105 chloride in pH 3 - 4 dilute HCl. Labeling conditions were varied from 2.5% - 57% ethanol and $5.8 \times 10^{-5}$ M - $1.16 \times 10^{-3}$ M 3,3,3-S4-BBN. In general, the following labeling conditions were attempted:

- 500 µL (~ 1 mCi) of Rh-105, 100 µL of 0.5 mg/mL 3,3,3-S4-BBN in 15% ethanol. Total ligand concentration: $5.8 \times 10^{-2}$ M, Total ethanol: 2.5%
- 500 µL (~ 1 mCi) of Rh-105, 100 µL of 0.67 mg/mL 3,3,3-S4-BBN in 15% ethanol. Total ligand concentration: $7.8 \times 10^{-5}$ M, Total ethanol: 2.5%
- 20 µL (~ 50 µCi) of Rh-105, 200 µL of 0.67 mg/mL 3,3,3-S4-BBN in 15% ethanol. Total ligand concentration: $4.2 \times 10^{-4}$ M, Total ethanol: 13.6%
- 0.05 mg lyophilized 3,3,3-S4-BBN in 50 µL acetonitrile, 100 µL ethanol 200 µL (~ 1 mCi) of Rh-105 stock Total ligand concentration: $9.46 \times 10^{-4}$ M, Total ethanol: 28.5%
- 0.067 mg lyophilized 3,3,3-S4-BBN in 50 µL acetonitrile, 100 µL ethanol 100 µL (~ 0.5 mCi) of Rh-105 stock Total ligand concentration: $1.84 \times 10^{-4}$ M, Total ethanol: 40%
- 0.067 mg lyophilized 3,3,3-S4-BBN in 50 µL acetonitrile, 200 µL ethanol 100 µL (~ 1 mCi) of Rh-105 stock. (200 µL was concentrated to 100 µL at 90°C, N₂) Total ligand concentration: $1.33 \times 10^{-4}$ M, Total ethanol: 57.1%
- 0.5 mg lyophilized 3,3,3-S4-BBN in 100 µL acetonitrile, 100 µL ethanol 100 µL (~ 0.5 mCi) of Rh-105 stock Total ligand concentration: $1.16 \times 10^{-3}$ M, Total ethanol: 33%

The reaction solutions were heated at 80°C for 1h and analyzed by HPLC. For all samples analyzed less than 10% yield (for peaks with $t_r = -18.6$ min) was observed. After heating 2 h many peaks were observed with $t_r = 15 – 25$ min.
Chapter 3: Results and Discussion

3.1 Synthesis and Evaluation of [Rh-S4-8Aoc-BBN(7-14)NH2]+

3.1.1 Synthesis of [Rh-S4-8Aoc-BBN(7-14)NH2]+TFA-

The synthesis of 3,3,3-S4-(COOH)2 was carried out analogously to reported methods with modifications to the isolation procedure as noted. In particular, an improved separation procedure using silica gel chromatography to isolate pure Intermediate 1, methyl 2-((3-chloropropyl)thio)acetate, as determined by NMR. NMR characterization of the final product was consistent with previously reported data. The chelate was then manually coupled to 8Aoc-BBN(7-14)NH2 with HOBT/HBTU and then cleaved from the resin support. It should be noted that thiol containing scavengers must be omitted from the cleavage solution as they may react with the chelate thioethers. LC-MS analysis (Figure 14) confirmed formation of the 94.66% pure 3,3,3-S4-8Aoc-BBN(7-14)NH2 with a retention time of 42.26 min and m/z of 1436.2 Da (calculated = 1435.68 Da).

The m/z =2154.57 Da peak observed can be explained as a cluster of 3 target molecules with a +2 charge formed in the ion source where the calculated molecular weight = (3TM)/2 = ((3)(1436.2))/2 = 2154.3 Da. This phenomena is not uncommon for this type of analysis.[99]
Figure 14: LC-MS of 3,3,3-S4-8Aoc-BBN(7-14)NH₂.
The metal complex was formed by adding RhCl$_3$·3H$_2$O in acetonitrile to a refluxing solution of S4-8Aoc-BB(7-14)NH$_2$ in an ethanolic solution. LC-MS analysis supports the formation of a [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ complex based on the proposed structure (Figure 15b) in which the Rh(III) is coordinated to each of the four sulfur atoms, one chloride and a carboxylate pendant group as evidenced by a m/z of 1571.8 Da (calculated = 1571.02 Da) for the [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ and a m/z of 786.3 Da (calculated = 786.51 Da) for the protonated [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^{2+}$ species (Figure 16). This configuration was unexpected based on previous studies with S4-(COOH)$_2$ in which the Rh(III) was coordinated to two chlorides in addition to the four sulfur atoms but not with either of the two pendant carboxylic acid groups (Figure 15a).\textsuperscript{[16]} We expect that the dichloro configuration would dominate in the presence of excess chloride ions in the reaction mixture. This synthesis was done without NaCl added, however during the $^{105}$Rh separation significant amounts of NaCl are generated by the addition of HCl to a mixture of NaOCl and NaOH.

The two overlapping LC peaks (Figure 16) at retention times 20.12 min and 20.30 min suggest two chemically similar species. MS analysis of the entire peak area (both 20.12 min and 20.30 min) results in only one m/z value as discussed above, and suggests the presence of a single molecule. As each S is chiral on coordination to the metal center, it is possible that 2 isomers of the target molecule exist. This observation may suggest the presence of two isomers. Since the previous author\textsuperscript{[16]} observed only one trans-dichloro isomer for the 3,3,3-S4-(COOH)$_2$ ligand system, the existence of two isomers would further indicate that the expected dichloro species (Figure 15 a) is not present.
Figure 15: Expected configuration of $[\text{RhCl}_2\text{-S}_4\text{-8Aoc-BBN(7-14)NH}_2]^+$ (a)\textsuperscript{[16]} and proposed configuration of $[\text{RhCl-S}_4\text{-8Aoc-BBN(7-14)NH}_2]^+$ (b).
**Figure 16:** LC-MS of [RhCl-S4-8Aoc-BBN(7-14)]^+
3.1.2 *In Vitro Binding Assays with [Rh-S4-8Aoc-BBN(7-14)NH2]*

Competitive binding displacement assays using $^{125}$I-Tyr$^4$-BBN and [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ were used to determine the affinity of [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ for the BB2r expressed on PC-3 human prostate cancer cells (Figure 17). The average concentration of [RhCl-S4-8Aoc-BB(7-14)NH$_2$]$^+$ needed to inhibit $^{125}$I-Tyr$^4$-BBN by 50% (IC$_{50}$) was determined to be 2.2 ± 0.3 nM. This value represents a significant improvement over the previously reported Rh-S$_4$-BBN(7-14)NH$_2$ (IC$_{50}$ = 37.5 ± 10.5 nM) and Rh-S$_4$-5-Ava-BBN(7-14)NH$_2$ (IC$_{50}$ = 4.76 ± 0.79 nM)$^{[20]}$ and indeed is well within the range (0.06 – 32.0 nM)$^{[51-84]}$ of recently investigated pre-clinical bombesin analogues for targeting prostate cancer.

![Figure 17: [RhCl-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ vs $^{125}$I-Tyr$^4$-BBN competitive binding Assay.](image)

3.1.2 $^{105}$Rh Radiolabelling with S4-8Aoc-BBN(7-14)NH$_2$

Based on this the IC$_{50}$ value determined, it appears that [Rh-S4-8Aoc-BBN(7-14)NH$_2$]$^+$ may have a BB2r affinity that is suitable for further investigation for clinical prostate cancer therapy. However, repeated attempts to synthesize $^{105}$Rh-S4-8Aoc-
BBN(7-14)NH₂ under various radiolabeling conditions have failed to result in a single species with significant yield (>10%) of the radiolabeled complex. Ethanol concentrations were varied from 10% - 57% and ligand concentrations were varied from and 5.8 x 10⁻⁵ M - 1.16 x 10⁻³ M with heating at 80 °C for 1 h. All of these conditions resulted in low labeling yields (< 5 - 10%) as measured by analytical HPLC (Figure 18, a). Additional heating (85 °C, 2 h) resulted in formation of many radiolabeled species also observed by HPLC (Figure 18 b and c). The multiple products likely resulted from both hydrolysis of amide bonds on the peptide due to excess heat and existence of multiple ¹⁰⁵Rh-S₄ species. For comparison, analytical HPLC chromatograms of non-radioactive 3,3,3-S₄-8Aoc-BBN(7-14)NH₂ (Figure 19, a) and [RhCl-S₄-8Aoc-BBN(7-14)NH₂]⁺ (Figure 19, b) using the same solvent gradient, column and system are included below. To better understand the results we encountered with radiolabeling ¹⁰⁵Rh-S₄-8Aoc-BBN(7-14)NH₂, the ¹⁰⁵Rh-S₄ chemistry with cyclic 3,3,3-S₄-Diol[²³] and acyclic 3,3,3-S₄-(COOH)₂[¹⁵-¹⁷] chelate systems was revisited.
Figure 18: Representative HPLC radio chromatogram of $^{105}$Rh-S4-8Aoc-BBN(7-14)NH$_2$ for (a) 1 h heat at 80ºC, (b) 2 h heat at 80ºC, and (c) 2h heat with a spike of non-radioactive RhCl$_3$·3H$_2$O.
Figure 19: HPLC chromatogram of (a) 3,3,3-S4-8Aoc-BBN(7-14)NH₂ and (b) [RhCl-S4-8Aoc-BBN(7-14)NH₂]⁺ prepared on a macroscopic scale.
3.2 Synthesis and evaluation of 3,3,3,3-S4-Diol 3,3,3-S4-(COOH)₂ chelate systems

3.2.1 Rh-S₄-Diol: Development of an HPLC QC Procedure

As discussed earlier, the easy labeling of ¹⁰⁵Rh-S₄-Diol and silica gel TLC analysis of the radiochemical yield has provided previous researchers with a useful tool for determining the labeling efficiency of ¹⁰⁵Rh chloride. While the TLC technique is useful as a rapid QC method, a detailed analysis of the trans-[RhCl₂-S₄-Diol]⁺ complex has not been published with modern techniques. We have expanded the previous studies to include an HPLC and mass spectrometry analysis of trans-[RhCl₂-S₄-Diol]⁺.

HPLC analysis of the non-radioactive Rh-S₄-Diol complex prepared as previously reported²³ in a pH 3 aqueous ethanolic solution resulted in a single peak with a retention time of 5.65 min using a gradient of 1% B – 90% B over 8min (Figure 20, c). However analysis of the radiolabeled ¹⁰⁵Rh-S₄-Diol exhibited two peaks under the same HPLC conditions, one at 5.81 min and a second peak at 5.53 min (Figure 20, d). A second non-radioactive Rh-S₄-Diol complex was prepared in pH 3 ethanolic solution with excess NaCl. HPLC analysis of this solution revealed two peaks at 5.54 min and 5.80 min (Figure 20, e). ESI-MS evaluation (Figure 21) of the macroscopic solutions indicates the presence of both trans-[Rh(OH)₂-S₄-Diol]⁺ (m/z = 464.92 Da, calc = 464.98 Da) (Figure 22a) and trans-[RhCl₂-S₄-Diol]⁺ (m/z = 500.97 Da, calc = 500.91 Da) (Figure 22 b) based on the proposed structures. On an expanded scale, there are three peaks (m/z = 500.97 Da, 502.94 Da, 504.94 Da), which can be assigned to the RhCl₂-S₄-Diol structure. Each peak is separated by 2 Da and the relative abundance of
the three is typical for a compound containing two chloride atoms and 4 sulfur atoms due to the isotopic abundance of the two principal isotopes of chlorine (\(^{35}\text{Cl} = 75.78\%, \ ^{37}\text{Cl} = 24.22\%)\) and sulfur (\(^{32}\text{S} = 95.02\%, \ ^{34}\text{S} = 4.21\%)\).

This new data provides valuable information about the impact of reaction conditions on the species of \textit{trans}-\(\text{RhX}_{2}\)-S4-Diol formed and indicates that the radiotracer chemistry of \(^{105}\text{Rh}\) complexes must be carefully evaluated for multiple isomers when halides are coordinated to the metal center. In the presence of a reducing agent such as ethanol, the coordinated halides are fairly labile and may be exchanged. Additionally, the HPLC method developed represents a new quantitative QC method for future researchers to analyze \(^{105}\text{Rh}\) chloride labeling efficiency.
Figure 20: Summary of Rh-S4-Diol HPLC analysis.
Figure 21: ESI-MS evaluation of Rh-S4-Diol prepared in saline.

Figure 22: Macroscopic species, Rh(OH)$_2$-S4-Diol (a) and RhCl$_2$-S4-Diol (b).
Figure 23: ESI-MS evaluation of RhCl$_2$-S$_4$-Diol showing isotopic analysis.
3.2.2 Synthesis and Evaluation of Rh-S4-(COOH)₂

Goswami et al. reports formation of a single radiolabeled trans-[^105RhCl₂-S₄-(COOH)₂]⁺ species as evaluated by silica gel thin layer chromatography (TLC) in which the product does not move from the origin; only ^105Rh chloride moves with the solvent front in saline.[15] Based on the results observed for trans-RhX₂-S₄-Diol, it was suspected that a number of Rh-S₄-(COOH)₂ species were possible for this preparation as well and therefore a more quantitative HPLC analysis was performed.

Non-radioactive Rh-S₄-(COOH)₂ was prepared in ethanolic solutions with and without excess NaCl analogous to the Rh-S₄-Diol preparation described above and analyzed by the same HPLC procedure. In the absence of excess NaCl the primary Rh(III) species formed is the mono chloride species, [RhCl-S₄(COOH)(COO⁻)]⁺ with one coordinated pendant carboxylate group (m/z = 508.83 Da, calc = 508.92 Da) (Figure 24a). This species is observed at an HPLC retention time of 6.14 min (Figure 25, b). When prepared with excess NaCl present the two species observed are trans-[RhCl₂-S₄-(COOH)₂]⁺ (m/z = 544.93 Da, calc = 544.90 Da) (Figure 24b) with a retention time of 6.87 min (Figure 25, c) and [RhCl-S₄(COOH)(COO⁻)]⁺ with a retention time of 6.26 min (Figure 25, c). As previously discussed, in the presence of ethanol the coordinated chlorides are readily exchanged. If chloride ions (i.e. salt, NaCl) are not present in sufficient concentration the coordinated chloride may exchange for another nearby donor atom, in this case a pendant carboxylate.
**Figure 24:** Initial species observed in initial macroscopic preparations.
Figure 25: Summary of trans-RhX₂-S₄-(COOH)₂ HPLC analysis
3.2.3 $^{105}$Rh$X_2$-S$_4$-(COOH)$_2$ Evaluation

Since the existence of at least two $trans$-[Rh$X_2$-(COOH)$_2$]$^+$ species has been confirmed on a macroscopic scale, it is necessary to evaluate the radiotracer behavior. The $^{105}$Rh chloride stock solution is in dilute HCl (pH ~ 1) following separation from the $^{104}$Ru target material at MURR. Before labeling, this sample is adjusted to pH 3-4 with 0.1 M NaOH, which generates NaCl. We hypothesized that radiolabeling of the S$_4$-(COOH)$_2$ ligand under these conditions would result in predominately the $trans$-[RhCl$_2$-S$_4$-(COOH)$_2$]$^+$ species. However HPLC analysis of the radiolabeling reaction mixture revealed at least 4 different peaks with retention times of 4.87 min, 5.71 min, 6.24 min and 6.82 min (Figure 25, d). The radiolabeled mixture was spiked with non-radioactive RhCl$_3$·3H$_2$O and heated for an additional hour. This test generates “carrier” Rh-S$_4$-(COOH)$_2$ compounds in macroscopic amounts, which can be observed by UV ($\lambda = 220$ nm) to confirm a radiochromatogram peak and will highlight any differences between chemistry that occurs on the tracer level and chemistry that occurs on the macroscopic level under the same conditions. The UV trace confirmed all 4 of the tracer peaks observed (Figure 25, e). It is clear that under these conditions it is possible to make multiple Rh-S$_4$-(COOH)$_2$ species.

At this point, we hypothesized that the additional peaks by HPLC may be due to any combination of dichloro, dihydroxo, and pendant carboxylate coordinated $trans$-Rh$X_2$-S$_4$-(COOH)$_2$ species.

3.2.4 Macroscopic Rh$X_2$-S$_4$-(COOH)$_2$ Synthesis and HPLC Analysis

During the initial macroscopic evaluation of $trans$-[Rh$X_2$-S$_4$-(COOH)$_2$]$^+$ two species were observed where either $X_2 = Cl_2$ ($t_r = 6.87$ min) or $X_2 = Cl$, pendant COO$^-$ ($t_r$
= 6.14 min) depending on the concentration of NaCl present in solution. Using the published radiolabeling conditions more than two trans-[^105RhX_2-S_4-(COOH)_2]^+ species were observed by HPLC (t_r = 4.87 min, 5.71 min, 6.24 min and 6.82 min). It was suspected that the trans-[Rh(OH)_2-S_4-(COOH)_2]^+ species might also be possible in a manner analogous to the observed trans-[Rh(OH)_2-S_4-Diol]^+ when water is present. Therefore reaction conditions varying the amount of water present and the amount of salt present were investigated.

In order to identify as many Rh-S_4-(COOH)_2 species as possible, the complex was formulated under a series of reaction conditions all at pH 3: (1) all organic solvent reaction conditions (50% ethanol/acetonitrile), (2) an organic solvent system with excess NaCl (saturated), (3) an aqueous-organic solvent mixture (25% water, 25% ethanol, 50% acetonitrile) and (4) an aqueous solution with excess NaCl (0.1 g). All reaction mixtures were heated for 1 h at 80°C, cooled, and then analyzed using the same HPLC method described above. The reaction mixtures were then lyophilized and taken up in either 50% acetonitrile/water for ESI-MS evaluation or 50% d_3-acetonitrile/D_2O for NMR studies.

HPLC evaluation (Waters RP-18, 1% B – 90% B over 8 min) of sample (1) prepared in 50% ethanol/acetonitrile resulted in a primary peak with a retention time of 6.20 min. Sample (2) prepared in 50% ethanol/acetonitrile saturated with NaCl resulted in a primary peak with a retention time of 6.19 min. The solubility of NaCl in ethanol and acetonitrile is low, 0.65 g/kg and 0.003 g/kg respectively. Thus the conditions in these two preparations are quite similar and similar HPLC results are not surprising.

The macroscopic synthesis of trans-[RhCl_2-S_4-(COOH)_2]^+ previously reported was carried out in acetonitrile/ethanol solution. The radiotracer synthesis is carried out
quite differently because of the aqueous starting solution available for $^{105}\text{Rh}$-chloride following separation from its target. Our macroscopic preparation of samples (3) prepared in 25% water, 25% ethanol, 50% acetonitrile and (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl are more similar to radiolabeling conditions.

HPLC evaluation of sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile resulted in a broad peak (or group of overlapping peaks) with retention time of $\sim 5.06 - 6.54$ min. Within this region two significant peaks are observed with retention times of 5.68 min and 6.21 min. Under these conditions it appears that many species are formed.

Evaluation of sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl resulted in a primary peak with a retention time of 6.18 min. This result suggests that addition of excess NaCl may be used to encourage formation of a single predominate species.

The amount of NaCl present during radiotracer formulation of trans-$[^{105}\text{Rh}X_2\text{-S}_4\text{-}}^{\text{COOH}}_2$ is currently unknown. During separation of $^{105}$Rh from $^{104/105}$Ru, significant amounts of NaCl are generated by the addition of HCl to a mixture of NaOCl and NaOH. The NaOCl was generated insitu by bubbling of Cl$_2$ gas into NaOH. Since it is unknown how much NaOCl is generated at this step, the amount of NaCl generated by addition of acid to the separation solution is also unknown. It is possible that the concentration of NaCl present in our radiolabelling formulation is less than the amount present (0.025 mg/mL) in sample (4). Addition of more salt to the radiolabeling solution may improve the yield of a single (or more predominate) species.
Sample (1) 50% Ethanol/Acetonitrile

Sample (2) 50% Ethanol/Acetonitrile + NaCl

Sample (3) 25% Water/25% Ethanol/50% Acetonitrile

Sample (4) 25% Water/25% Ethanol/50% Acetonitrile + NaCl

Figure 26: HPLC analysis of samples prepared under reaction conditions 1-4.
3.2.5 NMR Characterization of Rh-S4-(COOH)$_2$ Samples

The lyophilized trans-[RhX$_2$-S4-(COOH)$_2$]$^+$ was taken up in d$_3$-acetonitrile and evaluated by NMR. The presence of multiple isomers of the product will result in a complex spectrum with overlapping peaks, especially in the regions for the three propylene backbone protons. However, the methylene group on the terminal thioethers should be singlets if the carboxylate group is not coordinated and a doublet of doublets (each proton unique) if it is coordinated to the Rh center.

Based on $^1$HNMR and COSY analysis it is evident that multiple Rh-S4-(COOH)$_2$ species are present in each of the samples. 3,3,3-S4-(COOH)$_2$ is a symmetrical molecule. Each H is chemically equivalent to the corresponding H in the other half of the molecule (across the plane of symmetry). Thus the $^1$HNMR spectrum of the uncomplexed 3,3,3-S4-(COOH)$_2$ chelate shows relatively few peaks (Figure 26) (see also Chapter 2.2.3). Upon complexation with Rh chloride, if there were only one trans-RhCl$_2$-S4-(COOH)$_2$ species produced with a single isomer, as previously believed, one would not expect any increase in the number of peaks observed, only a change in chemical shifts. $^1$HNMR evaluation of the RhX$_2$-S4-(COOH)$_2$ complexes formed when prepared in aqueous solutions results in the appearance of many additional peaks. This is indicative of the presence of multiple RhX$_2$-S4-(COOH)$_2$ species (Figure 27), some of which may involve coordination to a pendant carboxylate group. Species with a coordinated carboxylate do not have a plane of symmetry. Each H on the molecule is chemically unique, and therefore many more peaks can be expected.
It is not possible to identify any species based on this $^1$HNMR evaluation, but it is clear that multiple species are present. The multiple overlapping peaks can be explained by either formation of additional trans-RhX$_2$-S$_4$-(COOH)$_2$ species (all differing in X$_2$ coordinated atoms) and/or the existence of more than one isomer of trans-RhX$_2$-S$_4$-(COOH)$_2$.

**Figure 27:** $^1$HNMR of 3,3,3-S$_4$-(COOH)$_2$ ligand.
Figure 28: $^1$HNMR analysis of trans-[RhX$_2$-S$_4$-(COOH)$_2$]$^+$ prepared in an aqueous solution.

A useful signal to evaluate is that from the methylene protons on the carbon adjacent to the carboxylic acid (-CH$_2$-COO). There are no H’s on either of its nearest neighbors so it should be a singlet if the two protons are equivalent, and the most downfield shifted peak in the spectrum. These methylene protons are observed as a singlet at 3.22 ppm in the S$_4$-(COOH)$_2$ ligand. Many of $^1$HNMR methylene proton peaks in the 3.2 – 4.5 ppm region for the trans-[RhX$_2$-S$_4$-(COOH)$_2$]$^+$ complex are somewhat obscured by the large water peak (Figure 28). Thus a $^1$H-$^1$H COSY spectrum was obtained. This allows us to look at correlation peaks for each proton that is coupled to a neighboring proton on the same molecule. Now the methylene proton peaks that had
been buried under the water peak are visible. Using this technique it is still not possible to determine which species are present or exactly how many species are present, but we can see that in sample 1 (prepared in 50% ethanol/acetonitrile) there are at least 4 dominate peaks (Figure 28), which correspond to chemically unique (-CH$_2$-COO) protons. In the case of only one conformer and one species of RhCl$_2$-$\text{S}_4$-(COOH)$_2$ we would expect to see only one peak in this region. Again, for sample 2 (prepared in 50% ethanol/acetonitrile with excess NaCl) the spectrum is similar. There are 4 dominate peaks for chemically unique protons. Two additional peaks seem to be present, perhaps in slightly lower concentration. It is worthy of noting that sample 3 prepared in aqueous solution (25% water, 25% ethanol, 50% acetonitrile) appears to have many peaks in this region.

An NMR analysis of sample (4) was not obtained. The large amount of NaCl (0.1 g) and the low solubility of salt resulted in the formation of immiscible d$_3$-acetonitrile and D$_2$O brine layers.
Figure 29: $^1$H – $^1$H COSY Sample (1) prepared in 50% ethanol/acetonitrile.

Figure 30: $^1$H – $^1$H COSY Sample (2) prepared in 50% ethanol/acetonitrile with excess NaCl.
3.2.6 Mass Spectrometry of Rh-S4-(COOH)$_2$ Samples

Using ESI-MS several compounds were identified based on proposed structures as summarized in Figure 32 and Table 4. Samples employing reaction conditions (1) and (2) were very similar. Both were prepared in 50% ethanol/acetonitrile, sample (2) with the addition of excess NaCl. Under these conditions, RhCl$_2$-S4-(COOH)$_2$ (m/z = 544.91 Da, calc = 544.90 Da) (Figure 32 a) and RhCl$_2$-S4-(COOH)(COOEt) (m/z = 572.89 Da, calc = 572.93 Da) (Figure 32 b) were predominate and to a somewhat lesser extent RhCl-S4-(COOH)(COO-) where a pendant carboxylate group is coordinated to the metal center (m/z = 508.87 Da, calc =508.92 Da) (Figure 32 d) is also observed (Figures 33 and 34). The ethyl ester (Figure 32, b) is formed by acid catalyzed esterification in the presence of ethanol.

Figure 31: $^1$H – $^1$H COSY Sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile.
In sample (4) under aqueous conditions with excess NaCl the formation of RhCl$_2$-S$_4$-(COOH)$_2$ (m/z = 544.91 Da, calc = 544.90 Da) (Figure 32 a) is dominate. However small amounts of RhCl-S$_4$-(COOH)(COO-) (m/z = 508.87 Da, calc = 508.92 Da) (Figure 32 d), RhCl$_2$-S$_4$-(COOH)(COOEt) (m/z = 572.89 Da, calc = 572.93 Da) (Figure 32 b) and RhCl$_2$-S$_4$-(COOEt)$_2$ (m/z = 600.91 Da, calc = 600.96 Da) (Figure 32 c) are also present (Figure 36).

The presence of all five species was observed in sample (3) prepared in aqueous conditions (25% water, 25% ethanol, 50% acetonitrile no salt). A significant yield of all three species RhCl$_2$-S$_4$-(COOEt)$_2$ (m/z = 600.91 Da, calc = 600.96 Da) (Figure 32 c), RhCl-S$_4$-(COOH)(COO-) (m/z = 508.87 Da, calc = 508.92 Da) (Figure 32 d), and RhCl-S$_4$-(COOEt)(COO-) (m/z = 536.90 Da, calc = 536.95 Da) (Figure 32 e) was observed (Figure 35). This is the only sample in which the monochloro pendant carboxylate coordinated RhCl-S$_4$-(COOEt)(COO-) species was observed with a pendant ethyl ester. We had hypothesized that a Rh(OH)$_2$-S$_4$-(COOH)$_2$ species analogous to the observed Rh(OH)$_2$-S$_4$-Diol may be present in aqueous solutions without NaCl. However this species was not observed.

There is a significant difference between sample (3) which was prepared in a solution of 25% water, 25% ethanol, 50% acetonitrile and sample (4) which was prepared in the same solution with the addition of 0.1 g NaCl. Without the presence of excess chloride ions in solution the pendant coordinated monochloride species RhCl-S$_4$-(COOH)(COO-) and RhCl-S$_4$-(COOEt)(COO-) are favored in addition to other species. With the presence of excess chloride ions in solution the dichloro species especially RhCl$_2$-S$_4$-(COOH)$_2$ are more favored. The solvents used in radiolabeling are aqueous
and it is likely that the concentration of NaCl present during radiolabeling is in between the conditions in samples (3) and (4). Therefore the addition of more NaCl might favor the production of a more dominate single $^{105}$RhX$_2$-S$_4$-(COOH)$_2$ species.

The pendant ethyl esters on species (b), (c) and (e) (Figure 32) are formed by acid catalyzed Fischer esterification of the pendant carboxylic acid (Figure 37). As previously discussed (Chapter 1.5), an acidic solution is required to prevent the formation of $^{105}$Rh(OH)$_3$ and ethanol is required as a reducing agent. If neither acid nor ethanol can be eliminated, the formation of ethyl esters will continue to be a competing reaction for this formulation.

**Figure 32:** Rh-S$_4$-(COOH)$_2$ species identified in samples 1-4.
### Table 4: Products observed for various reaction conditions

| Sample | Reaction Conditions                                      | Products observed
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50% ethanol/acetonitrile</td>
<td>a, b, d</td>
</tr>
<tr>
<td>2</td>
<td>50% ethanol/acetonitrile + NaCl</td>
<td>a, b, d</td>
</tr>
<tr>
<td>3</td>
<td>25% water, 25% ethanol, 50% acetonitrile</td>
<td>a, b, c, d, e</td>
</tr>
<tr>
<td>4</td>
<td>25% water, 25% ethanol, 50% acetonitrile + NaCl</td>
<td>a, b, c, d</td>
</tr>
</tbody>
</table>
Figure 33: Rh-S4-(COOH)$_2$ Sample (1) prepared in 50% ethanol/acetonitrile.

Figure 34: Rh-S4-(COOH)$_2$ Sample (2) prepared in 50% ethanol/acetonitrile + NaCl.
Figure 35: Rh-S4-(COOH)$_2$ Sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile.

Figure 36: Rh-S4-(COOH)$_2$ Sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile + NaCl.
Figure 37: Fischer Esterfication of a pendant carboxylic acid.\[1\]
Chapter 4: Conclusions

We have successfully synthesized a novel rhodium bombesin conjugate, [RhCl-S4-8Aoc-BBN(7-14)NH2]⁺. In vitro evaluation indicates high affinity for PC-3 human prostate cancer cells however low radiochemical yields of a single [¹⁰⁵Rh-S4-8Aoc-BBN(7-14)NH₂]⁺ species on the radiotracer scale may preclude its usefulness as a radiotherapeutic agent. The pendant carboxylic acids were thought to be useful for maintaining reasonable hydrophilicity and thus clearance through the renal system. However, pendant carboxylic acids resulted in both complexes with a coordinated carboxylate and in esterification, the latter particularly a problem at the radiotracer level where acidic ethanolic reaction conditions are needed. In place of an acyclic bifunctional chelate (BFC) system with a pendant carboxylic acid, it is recommended to change the direction of research to investigate cyclic BFC’s or acyclic BFC’s with a methyl ester or methyl ether pendant group. This will prevent the formation of ethyl esters during radiolabeling.

The macrocyclic tetrathiother BFC system, S₄-5-Ava-BBN(7-14)NH₂ reported by Ning Li et al.¹²⁰, ³⁸ exhibited properties that show potential for therapeutic applications (IC₅₀ = 4.76 ± 0.79 nM, BB2r specific pancreas uptake 2.25 ± 1.02 % ID/organ). It would be interesting to see this work extended to a PC-3 tumor bearing animal model. However yields > 90% required 3 h heating at 80°C. It has been hypothesized that switching to an acyclic ligand system such as the one investigated here may result in higher radiolabelling yields with shorter reaction times. This data is inconclusive in that regard. After one hour heating at 80°C less that 10% yield was achieved. After heating
for 2 h, multiple products were observed. This could be due to either formation of multiple Rh-S4 species and/or hydrolysis of the peptide.

Further work in the direction of acyclic S4-BBN analogues will need to be investigated using a chelate that does not involve pendant carboxylate groups. The pendant group on current analogue S4-8Aoc-BBN could be protected by the formation of a methyl ester prior to radiolabelling. Another option would be to pursue the backbone derivatized analogue initially proposed in this work (section 2.2, figure 12).

Another viable research direction would be to eliminate the use of ethanol by using an alternate reducing agent such as stannous chloride which may also facilitate catalytic reduction of Rh(III) to Rh (I) while allowing. Previously the use of ethanol as a reducing for Rh(III) has been sought because small concentrations of ethanol can be tolerated in animal models. For a system where there is >95% of the desired $^{105}$Rh complex, the product does not necessarily require further purification. However for a bombesin targeted radiopharmaceutical the radiolabeled complex would need to be separated from excess ligand by HPLC purification anyway because any excess bombesin-chelate would block BB2 receptors on the cell surface.

We have also developed a new, more quantitative $^{105}$Rh-S4-Diol QC procedure based on HPLC analysis that will allow future researchers to quickly evaluate the labeling efficiency of $^{105}$Rh chloride. HPLC analysis of the acyclic 3,3,3-S4-(COOH)$_2$ chelate on the tracer and macroscopic scale has provided useful information regarding chemistry that occurs at the pendant carboxylate group and halide-water exchange at the Rh center. This new information adds to the current knowledge of $^{105}$Rh ligand systems and will aid future researchers in the selection criteria for viable chelate systems.
References


20. Li, N., Synthesis and characterization of \(^{105}\)Rh-labeled thiamacrocycles for use to formulate peptide receptor agents, in Department of Chemistry. 1996, University of Missouri. p. 141.


Gallazzi, F., as per communication, V. Carroll and S. Jurisson, Editors. Thursday, April 25, 2013
APPENDIX A: Supplementary Data
Figure A-1: NMR characterization of Intermediate 1: methyl 2-(2-chloropropyl)thioacetate.
Figure A-2: NMR characterization of Intermediate 2: dimethyl 3,7,11,15-tetrathiaheptadecane-1,17-dioate
Figure A-3: NMR Characterization of 3,3,3-S4-(COOH)$_2$; 3,7,11,15-tetrathiaheptadecane-1,17-dioic acid
**Figure A-4:** ESI-MS evaluation of [Rh(OH)$_2$-S$_4$-Diol]$^+$ showing isotopic abundance due to thioether sulfurs.
Figure A-5: ESI-MS evaluation of [RhCl₂S₄(COOH)₂]⁺ showing isotopic abundance.
Figure A-6: ESI-MS evaluation of [RhCl$_2$-S4-(COOH)(COOEt)]$^+$ showing isotopic abundance.
Figure A-7: ESI-MS evaluation of $[\text{RhCl}_2\text{S}_4\text{-}(\text{COOEt})_2]^+$ showing isotopic abundance.
Figure A-8: ESI-MS evaluation of \([\text{RhCl-S4-(COOH)(COO}^-])^+\) showing isotopic abundance.
Figure A-9: ESI-MS evaluation of $[\text{RhCl}_2\text{S}_4\text{-(COOH)}_2]^+$ showing isotopic abundance.
Multiple chlorides based on multiple isotope peaks
Perhaps a bridged $[\text{RhCl}_x]_n$ species
Difference in m/z between each is 40.93 Da suggest acetone (41.03 Da)

Figure A-10: ESI-MS analysis of m/z = 718.48, 759.21, 800.14 Da peaks.
Figure A-11: Bioscan count of $^{105}$Rh chloride on silica TLC developed in saline.

Figure A-12: Bioscan count of $[^{105}\text{Rh-S4-Diol}]^+$ on silica TLC developed in saline.

Figure A-13: Bioscan count of $[^{105}\text{Rh-S4-(COOH)$_2$}]^+$ on silica TLC developed in saline.
### Table A-1: Comparison of TLC and HPLC techniques.

<table>
<thead>
<tr>
<th>Complex</th>
<th>TLC: Activity at Origin</th>
<th>HPLC: Area Total (peaks t_r &gt; 4 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{105}\text{Rh}X_2\text{-S4-Diol}]^+$</td>
<td>61.0 %</td>
<td>45.4%</td>
</tr>
<tr>
<td>$[^{105}\text{Rh}X_2\text{-S4-(COOH)}_2]^+$</td>
<td>64.9 %</td>
<td>52.0%</td>
</tr>
</tbody>
</table>

*(unoptimized labeling conditions)*
APPENDIX B: Synthesis of $^{52}$Fe Core Labeled Superparamagnetic Iron Oxide Nanoparticles for Dual PET/MR Imaging.

Valerie Carroll, Dmitri Medvedev, Joanna Fowler, David Schlyer, Leonard Mausner

Dual modality imaging is a valuable tool in nuclear medicine research. Imaging techniques such as CT (x-ray computed tomography) and MRI (magnetic resonance imaging) provide researchers and clinicians with anatomical data while techniques such as SPECT and PET are able to provide functional data about a specific biological process. Physiological images, when fused with the anatomical data can provide researchers with valuable information correlating form with function. This technique allows for patient specific mapping of biologic function. Dual SPECT/CT imaging has already been common place in the clinics for a number of years however MR imaging may provide enhanced spatial and contrast resolution. At the time of this work (December 2008) dual PET/MR technology was still under investigation. Since then combined PET/MR cameras have been marketed by Philips, Siemens and GE. With the rise of this technology there will be a need for development of new probes that take advantage of characteristics of both imaging modalities.

This project was done during a three month internship at Brookhaven National Laboratory completed as part of my NIBIB training experience. The goal of this project was to produce core labeled $^{52}$Fe super paramagnetic iron oxide ($\text{Fe}_2\text{O}_3$) nanoparticles and to evaluate their properties for potential dual PET/MR imaging. As a comparison for this project we used Feridex® (AMAG Pharmaceuticals) an already FDA approved dextran stabilized iron oxide nanoparticle MRI contrast agent used for detection of liver lesions. Two radioisotopes of iron (Table 4) were used for different aspects of this
For PET studies we used $^{52}$Fe a positron emitting isotope with a half-life of 8.28 h. For many of the laboratory studies we used $^{59}$Fe, a beta particle emitter with a half-life of 44.5 d as a surrogate for $^{52}$Fe. This longer half-life allowed us to study the particles for a longer period of time.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>$t_{1/2}$</th>
<th>Emissions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-52</td>
<td>8.28 h</td>
<td>$0.80$ MeV $\beta^+$, $168.7$ keV $\gamma$</td>
</tr>
<tr>
<td>Fe-59</td>
<td>44.5 d</td>
<td>$0.466$ MeV $\beta^-$, $1099$ keV $\gamma$</td>
</tr>
</tbody>
</table>

Table B-1: Radioactive properties of $^{52}$Fe and $^{59}$Fe.

A variety of literature preparations were investigated to synthesize the nanoparticles by co-precipitation of iron salts in a solution of excess dextran stabilizer. The best MRI T2 relaxation signal was obtained with a preparation according to Molday et al.\cite{100} Briefly a 50% Dextran solution was mixed with an equal volume of a 5% $\text{Fe}^{2+}/\text{Fe}^{3+}$ solution with a 2:1 molar ratio of $\text{Fe}^{2+}:/\text{Fe}^{3+}$. The pH was then shifted to $>10$ with the addition of 1.0 mL of 15% NH$_3$ resulting in the precipitation of iron oxide colloid. The solution was then sonicated at 60°C for 30 min and centrifuged to remove aggregates. The nanoparticles were separated from excess dextran using a 100,000 Da molecular weight centrifuge filter and washed with deionized water. The prepared nanoparticles were diluted to 50 ppm solutions with deionized water. Sealed vials containing the 50 ppm nanoparticle solutions were placed in a plastic holder, submerged in water and T2 weighted images were obtained using a 4 Tesla MRI instrument (TE = 0.01 ms, TR = 0.1 ms).
Figure B-1: MRI comparison of literature iron oxide nanoparticle preparations.

The Molday synthesis resulted in a comparable signal (Figure B1) to commercial Feridex®; however, this preparation is typically used to prepare a large amount of nanoparticles in a highly concentrated solution. To accommodate the $^{52}$Fe radio label it was necessary to scale down this preparation. It was found that scaling down this preparation by preparing nanoparticles in a more dilute solution lead to formation of nanoparticles that were smaller in size and did not exhibit desired magnetic properties. Thus, nanoparticles were synthesized in small amounts of concentrated solution according to the following preparation. An iron/dextran stock solution was prepared containing 0.064 g FeCl$_2$ 4H$_2$O and 0.093 g FeCl$_3$ in 1.0 mL 50% Dextran. The stock solution (200 µL) was added to 200 µL of water for non-radioactive preparations (or 200 µL of either the $^{59}$Fe or $^{52}$Fe radiolabel). The pH was adjusted by adding 200 µL of 15% NH$_3$ and the solution was sonicated for 30 min at 60°C. Again they were centrifuged to remove aggregates and purified using multiple washings with a 100,000 Da molecular weight filter. MRI images were obtained for 50 ppm solutions of the non-radioactive nanoparticles (Figure B-2). 50 ppm solutions of the same preparation of nanoparticles on the same day seem to vary, but all samples tested provide good contrast that is
comparable or better than the commercial feridex®. This variance in contrast is likely due to error in pipetting small amounts of the very viscous dextran stock solution during synthesis. The nanoparticles were studied using a dynamic light scattering instrument (Malvern) to measure hydrodynamic radius over time (Figure B-3) and TEM images (Figure B-4) were collected using a high-resolution TEM microscope (JEOL JEM2100F). Nanoparticles have a core radius of 2-5 nM and do not exhibit aggregation after 4 h incubation in phosphate buffered saline.

**Figure B-2:** MRI of scaled down synthesis based on Molday et. al.[100]
Figure B-3: Change in hydrodynamic radius over time of 50 ppm iron oxide nanoparticle solutions in pH 7 phosphate buffered saline.

Figure B-4: TEM image of iron oxide nanoparticles compared to Feridex®.

A biodistribution study was carried out using $^{59}$Fe-iron oxide nanoparticles prepared as described above. Swiss/Webster mice ($n = 4$) were injected with a dose containing 0.1 mg total Fe/mouse and 1.3 µCi/mouse. The mice were sacrificed at $t = 5$, 10, 30, 60 and 180 min time points. Organs were collected and counted using a sodium
iodide detector. High uptake was noted in both the liver and spleen (Figure B-5). The nanoparticles cleared from the blood over 180 min. High liver uptake was expected as this was an untargeted nanoparticle probe based on a commercial MRI probe used for imaging the liver. PET images (Figure B-6) were obtained of core labeled $^{52}$Fe-iron oxide nanoparticle solutions prepared according to the same procedure. This initial data suggests that iron oxide nanoparticles and core labeled $^{52}$Fe-iron oxide nanoparticles synthesized using this preparation method may have both magnetic and nuclear properties needed however additional MRI data is needed to verify that the $^{52}$Fe-iron oxide nanoparticles retain super paramagnetic properties with the incorporated radiolabel.

**Figure B-5:** Biodistribution data for core labeled $^{59}$Fe-iron oxide nanoparticles in Swiss/Webster mice.
VITA

Valerie Carroll was born in St. Louis Missouri to Berry and Jo Ellen Rahing. She received her bachelor’s degree in Chemical Engineering from the University of Missouri in May 2008. In 2008 she married her sweetheart Joshua Carroll. In August 2008 she was accepted as a Ph.D. student in the Chemistry program at University of Missouri where she joined the Jurisson research group and earned her Ph.D. degree in 2013. She will pursue her postdoctoral research at University of California, San Francisco.