Modular Synthesis of Dual- and Tri-modal Targeted Molecular Imaging Agents

Nicholas C. Schug
ncs2414@rit.edu

Follow this and additional works at: https://scholarworks.rit.edu/theses

Recommended Citation

This Thesis is brought to you for free and open access by RIT Scholar Works. It has been accepted for inclusion in Theses by an authorized administrator of RIT Scholar Works. For more information, please contact ritscholarworks@rit.edu.
Modular Synthesis of Dual- and Tri-modal
Targeted Molecular Imaging Agents

Nicholas C. Schug

A thesis submitted in partial fulfillment of the requirements for the degree
Master of Science in Chemistry

Supervised by
Dr. Hans Schmitthenner

School of Chemistry and Materials Science
College of Science
Rochester Institute of Technology

May 2020

Signature of the Author ______________________________

Accepted by __________________________________________
Director, M.S. Degree Program Date
The M.S. Degree Thesis of Nicholas C. Schug has been examined and approved by the thesis committee as satisfactory for the thesis required for the M.S. Degree in Chemistry.

Dr. Hans Schmitthenner, Thesis Advisor

Dr. Nathan Eddingsaas

Dr. Joseph Hornak

Dr. Michael Pierce

Date
Abstract

There are few reported methods for combining two or three different imaging dyes, metals, or dye-metal combinations, followed by the conjugation of a disease-targeting group to enable the use a given imaging system for multiple imaging applications. Previously, peptides have been used as scaffolds for dyes and metals used in diagnostic techniques such as optical molecular imaging (OMI), positron emission tomography (PET), and magnetic resonance imaging (MRI). The aim of this project was to reassemble peptide-based imaging agents through a modular method by coupling together modules comprised of amino acids with imaging agents attached to their side chains to form “imaging peptides,” followed by attaching a cancer-targeting group in the final step. This new modular approach for the synthesis of a diverse set of targeted molecular imaging agents (TMIAs) was optimized and exemplified by the synthesis of dual modal PET-MRI, dual OMI-MRI and dual metal (di-gadolinium for MRI), and the partial synthesis of a tri-modal OMI-PET-MRI agent. The dual modal imaging peptides were conjugated through a linker to targeting groups for lung cancer (A549 cells) and prostate cancer (PSMA positive C4-2) cells. In addition to imaging applications for early detection, active surveillance, image guided biopsies and surgery, this modular approach could potentially be used for creating therapeutic agents to treat cancer patients.
Acknowledgements

This research and academic journey would not have been possible without the support of many different people. Most importantly, I would like to thank my research advisor, Dr. Hans Schmitthenner, for his support through all stages of this project.

I am also grateful for all of my group members in the molecular imaging laboratory at RIT who have helped and supported me in my research endeavors. I would like to specifically thank a former group member, Kelsea Jones, for laying the framework for this specific research project. Osarhuwense Otasowie also deserves to be recognized for her help with the dual-gadolinium MRI contrast agent, for the many purifications, and for helping with other important synthetic steps along the way. Having a supportive group in the lab helped greatly in times where advice or assistance was needed.

Additionally, I would like to thank the RIT College of Science, specifically the School of Chemistry and Material Science for providing me with financial and academic support during my graduate studies at RIT. In particular, I would like to thank Dr. Michael Coleman, the Director of the Chemistry Graduate Program, and Dr. Paul Craig, the School of Chemistry and Materials Science Head.

Last but definitely not least, I would like to thank my family and friends who have supported me greatly during my time here at RIT. My parents, who have been there for me along every step of my educational journey, deserve the most recognition. I also could not have completed this research project and educational journey as successfully without support from my girlfriend, Lexi, who has always provided encouragement when things get hectic.
Abbreviations

Frequently Used Solvents
ACN: acetonitrile
AmAc: Ammonium Acetate, used as an aqueous buffer
DCM: dichloromethane
DI H₂O: deionized water, 18mΩ
DMF: n,n-dimethylformamide
DMSO: dimethyl sulfoxide
EtOAc: ethyl acetate
MeOH: methanol
NMP: n-methylpyrroolidone

Frequently Used Reagents and Compounds
BuAm: n-butylamine
DEA: diethylamine
DIPEA or DIPA: diisopropylethylamine
dLys, dK: the unnatural isomer of lysine
DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
Lys, K: lysine, a natural amino acid
NMM: n-methylmorpholine
NMP: N-methyl-2-pyrrolidone
TEA: triethylamine
TFA: tetrafluoroacetic acid

Other Instrumentation
HPLC-MS: high-pressure liquid chromatography and mass spectrometry
HPLC-prep: preparative high-pressure liquid chromatography
HRMS: high resolution mass spectrometry
NMR: nuclear magnetic resonance
SPE: solid-phase extraction
UPLC-MS: ultra-performance liquid chromatography and mass spectrometry

Molecular Imaging Terms
CA: contrast agent for MRI use
MRI: magnetic resonance imaging
PET: positron emission tomography
TCA: targeted contrast agent for MRI use
TMIA: targeted molecular imaging agent
# Table of Contents

Abstract .............................................................................................................................. iii  
Acknowledgements ........................................................................................................ iv  
List of Figures .................................................................................................................. vii  

1. Introduction .................................................................................................................. 1  
1.1. Cancer ..................................................................................................................... 1  
1.2. Molecular Imaging Methods Relevant to Cancer Detection .................................... 3  
1.3. Targeted Molecular Imaging Agents ...................................................................... 4  
1.4. Cancer Targeting Groups ....................................................................................... 6  
1.5. Relevant Imaging Agents ......................................................................................... 8  
1.5.1. MRI Imaging Agents .......................................................................................... 9  
1.5.2. PET Imaging Agents ......................................................................................... 11  
1.5.3. OMI and CFM Imaging Agents ......................................................................... 12  
1.5.4. Dual-modal Imaging Agents ............................................................................. 14  
1.5.5. Comparison of MRI, PET, and OMI .................................................................. 17  
1.6. Previous Literature Research in Imaging Agents ..................................................... 18  
1.6.1. Previous Literature Research in Dual-modal Imaging Agents ............................. 20  
1.7. Dual-Modal Imaging Instrumentation .................................................................... 26  

2. Modular Method for Multi-Modal Agents ................................................................... 29  
2.1. Modular Synthetic Approach .................................................................................. 30  
2.1.1. Linear Synthetic Approach ............................................................................... 32  
2.1.2. Convergent Synthetic Approach ...................................................................... 33  
2.2. Identification and Analysis of TMIA and Intermediates .......................................... 34  

3. Results and Discussion ............................................................................................... 39  
3.1. Synthetic Approach to Metal-Containing Imaging Modules .................................... 39  
3.2. Fmoc Deprotections .............................................................................................. 40  
3.3. NHS Ester Synthesis .............................................................................................. 42  
3.4. Synthesis of Dual-Modal TMIA for Prostate Cancer Using OMI-MRI .................... 42  
3.5. Synthesis of Dual-Modal TMIA for PET-MRI ....................................................... 46  
3.6. Synthesis of Dual-Gadolinium Targeted Contrast Agent for MRI ......................... 51  
3.7. Work Towards a Novel Tri-Modal Imaging Agent for OMI-PET-MRI .................. 54  

4. Conclusion ................................................................................................................... 56  

5. Experimental Procedures .......................................................................................... 57  

References ....................................................................................................................... 63  

Appendix I. HPLC-MS and HRMS Results .................................................................... 69
List of Figures

Figure 1. Schematic of a TMIA being used in PET showing a targeting biomolecule attached to a PET imaging agent binding to a tumor receptor on the membrane of a cancer. .......................................................... 5
Figure 2. c(RGDyK), a well-known cancer targeting peptide ........................................... 6
Figure 3. c(RGDyK)-Cy 5.5 TMIA (left) and CFM image of lung cancer cells targeted by the TMIA (right). There are about ten cells shown in this image, with the heart shaped cell in the middle as an example. The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis. .......... 7
Figure 4. DCL urea-based PSMA inhibitor ........................................................................ 7
Figure 5. DOTA, a well-known complexing agent used in cancer imaging.................... 10
Figure 6. Cy 5.5 NHS Ester Structure ........................................................................... 14
Figure 7. General schematic of a dual-modal TMIA ......................................................... 15
Figure 8. PET/MRI scan of patient with glioblastoma multiforme ................................. 16
Figure 9. DOTA conjugation with TRC105  ................................................................. 19
Figure 10. Structure of a DOTA-derived chelator coupled with a porphyrin moiety via a peptide bond to form a non-targeted molecular imaging agent .......................... 20
Figure 11. Metalation of the imaging agent in the final steps ......................................... 22
Figure 12. Final steps in the synthesis of EP-2104R, a Gd-based MRI contrast agent 44 23
Figure 13. Partial exchange of gadolinium for copper-64 in EP-2104R .......................... 24
Figure 14. Dual-modal molecular imaging agent design by Kumar et al. .......................... 25
Figure 15. Duet Optical Imaging module, U-OI from MILabs ............................... 26
Figure 16. Structure of L-lysine, the amino acid commonly used in our targeted molecular imaging agents ................................................................. 29
Figure 17. General schematic of the modular peptide synthesis of TMIA’s ...................... 30
Figure 18. Color-coded structure of final TMIA, c(RGDyK)-SMCC-dK(Cy5.5)-K(DOTA-Gd)-NH2 ......................................................... 31
Figure 19. Color-coded structure of final TMIA, DCL-DSS- dK(Cy 5.5)-K(DOTA-Gd)-NH2 ........................................................................................................ 32
Figure 20. General schematic showing linear synthetic method of TMIA’s, linker added in a separate linker step ........................................................................ 33
Figure 21. General schematic showing convergent synthetic method of TMIA’s, linker part of targeting module ................................................................. 34
Figure 22. Confocal fluorescence microscopy (CFM) of three A549 lung cancer cells targeted by the TMIA, c(RGDyK)-SMCC-dK(Cy5.5)-K(DOTA-Gd)-NH2 shown in Figure 18. There are about three cells shown in this image. The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis. The blue color is a result of staining of the cells by NucBlu, a widely used dye that stains only cell nuclei ............................................................................................ 36
Figure 23. Structure of B2 showing the single modal DCL-DSS-Lys(Cy5.5)-NH2 .......... 37
Figure 24. Left: Image of about ten C42 (PSMA+) prostate cancer cells illuminated by the TMIA B-2, DCL-DSS-Lys(Cy5.5). The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis. Middle: Same image of cells in which the nuclei are lit up with NucBlue. Right: About 20 PC3
(PSMA-) cells in a control experiment which were treated with the same concentration of TMIA, showing weak binding. ........................................................................................................ 37
Figure 25. Mechanism for Fmoc Deprotection ................................................................. 41
Figure 26. NHS Structure .................................................................................................. 42
1. Introduction

1.1. Cancer

Cancer refers to a group of diseases that are characterized by the uncontrolled growth and spread of abnormal cells. If left untreated, cancer is likely to result in death. More than 1.7 million new cancer cases were expected to be diagnosed in 2019 in the U.S. and approximately 606,880 Americans were expected to die of cancer in 2019.\textsuperscript{1} This translates to roughly 1660 deaths per day, making cancer the second most common cause of death in the United States, exceeded only by heart disease. Although our understanding and knowledge of cancer and its treatment methods continues to grow\textsuperscript{2}, the number of new cancer cases per year is also growing.\textsuperscript{3} Due to the ever-growing societal impact of cancer, improving cancer detection and treatment methods remains to be an important scientific research endeavor.

There are many different types of cancer. The commonality between all the different types is that cancer cells divide without stopping and begin to spread into surrounding tissues in the body.\textsuperscript{4} Cancer can start in almost any of the trillions of cells in the body, but usually begins in the more vulnerable tissue of the organs. When cancer develops, the typical orderly process of healthy cells growth breaks down. This causes old or damaged cells to survive when they should die and uncontrollably replicate into new cells. These extra cells will divide without stopping and form tumors. This makes cancer a genetic disease, since it causes changes in the DNA that, in turn, controls the way our cells function. Due to the nature of cancer and the way that it grows as its cells divide, it can sometimes be an extremely difficult or impossible disease to treat.
There are many different types of treatment methods used to combat cancer. Each method has varying levels of side-effects and the treatment method utilized typically depends on the type of cancer and how severe it is. Three of the most common treatment methods are surgery, radiation therapy, and chemotherapy. In surgery, a surgeon physically removes cancer from the body. Radiation uses high doses of radiation to irradiate cancer cells and to shrink tumors. Chemotherapy uses potent drugs to kill cancer cells. In most cases, each of these treatment methods have a significant physical toll on the human body and are not always successful in eliminating the disease. In addition to these three most common and well-known methods, there are other methods of treatment including immunotherapy and photodynamic therapy (PDT). Combinations of these different treatment methods are often used to increase the success and decrease the likelihood that the cancer returns.

Screening for cancer is an extremely important medical diagnostic tool. This refers to testing individuals who have no symptoms or early symptoms of cancer. Early detection has proved to reduce mortality from cancers of the colon and rectum, breast, uterine cervix, prostate, and lung. Early treatment of the disease is the most effective way to increase the chance of survival as it makes treatment methods more effective. Unfortunately, without early detection, most cancer treatments occur at a relatively advanced stage. Because cancer cells can quickly form tumors and spread if untreated, early diagnosis is important, to allow medical professionals to promptly begin treatment of the cancer.

Molecular imaging is a widely-used detection method that can be used in the diagnosis of cancer and provides the greatest hope for curing cancer. By targeting
specific biomarkers in cancer cells, molecular imaging that utilizes targeted molecular imaging agents (TMIAs) may vastly increase the effectiveness and success of cancer detection. The goal of this research project was to develop a synthetic route that other researchers and manufacturers can utilize to provide a wide variety of TMIAs. This new, easier and robust method could provide tailor-made TMIAs that could potentially be used in the diagnosis of many types of cancer with the overall goal of improving cancer detection which would ultimately decrease the mortality rate due to cancer.

1.2. Molecular Imaging Methods Relevant to Cancer Detection

Currently, there are limited methods used to screen for cancer. Methods can be invasive, such as a biopsy, where cell tissue is physically removed from a person’s body to test for the presence of the disease. In contrast, non-invasive methods such as molecular imaging prove to be more desirable in screening for cancer as they do not involve the surgical removal of tissue in the body which can be painful and carries risk of infection and other factors.  

There is conventional imaging with no contrast agents or non-targeted agents used in imaging methods such as PET and MRI. While these may detect large tumors, these methods are not ideal for the detection of cancer in its early stages.

The most common molecular imaging methods which employ contrast agents used in the detection and diagnosis of cancer are MRI, PET, computed tomography (CT), optical molecular imaging (OMI), also known as fluorescence imaging) ultrasound imaging, and x-ray imaging. The integration or fusion of two imaging methods together can lead to a more effective imaging method. Instruments are currently available for two modes of imaging. For example, a combined PET/CT scanner is one of the most
frequently used instruments in the field of oncology.\textsuperscript{14} In a PET/CT, images are obtained separately and combined together using digital processing. This combination of instrumentation provides more information than one image and can provide higher image quality since there are two complimentary imaging methods being fused together.

Precise imaging is essential in determining the best method of treating the cancer and molecular imaging methods allow radiologists to precisely locate cancerous cells and determine how far cancer has spread. Although imaging methods can be accurate in their imaging of the tissues inside the body, it is sometimes difficult to distinguish healthy tissue from cancerous tissue cells. For cancer detection to be accurate, it is important that molecular imaging methods are reliable and can consistently provide an accurate picture of the cancer in the body.

\textbf{1.3. Targeted Molecular Imaging Agents}

In order for cells to be imaged with a high signal-to-background (signal-to-noise) ratio, targeted imaging agents can be used to increase the visibility of the diseased cells.\textsuperscript{15} This allows cancerous cells to be more easily differentiated from healthy cells, even when there is a small amount of diseased cells. Targeted molecular imaging agents (TMIAs) are the best means of providing better contrast of the disease to improve the overall quality of the image.\textsuperscript{9,16}

Targeting peptides have been previously coupled with imaging agents (e.g. radiometals and fluorophores) using different chemical techniques in order to create TMIAs.\textsuperscript{15} These imaging peptides, when coupled with separate cancer-targeting moieties, proved to be extremely effective in the early detection and diagnosis of cancer. This is due to the fact that cancer targeting compounds are able to target cancer cells selectively
and with high affinity.\textsuperscript{17} Since the imaging peptides are connected to the cancer-targeting moiety, the imaging agents will be in a high concentration due to specific localization in the cancerous cells only. When appropriate molecular imaging techniques are used to detect the imaging agents, only the cancerous cells become illuminated and are thus more accurately distinguished compared to the background, neighboring tissues, or other artifacts in the image.

Figure 1 shows a schematic of how a TMIA is able to selectively target a receptor on a tumor cell membrane using the receptor targeting biomolecule. Since the receptor targeting biomolecule is attached to an imaging agent (the radioactive $^{64}\text{Cu}$ atom in this case), the cancer can be detected using PET. This image with illuminated cancer cells is seen in the top left of Figure 1.

\begin{center}
\includegraphics[width=\textwidth]{image1.png}
\end{center}

\textit{Figure 1. Schematic of a TMIA being used in PET showing a targeting biomolecule attached to a PET imaging agent binding to a tumor receptor on the membrane of a cancer\textsuperscript{18}}

1.4. Cancer Targeting Groups

TMIAs would not possibly be able to target cancer without having affinity for cancer cells. In the early years of molecular imaging, scientists found that there are certain peptides that target biomarkers found in cancerous cells. The most widely used peptide for this purpose for many years was the cyclic peptide c(RGDyK). This peptide contains the RGD peptide sequence, known to have a strong affinity for the $\alpha_v\beta_3$ integrin that is overexpressed in many different cases of cancer, such as melanoma, ovarian, lung, and breast cancer. Integrin proteins are critical for self-adhesion of cells in cancerous tissue and it is also a biomarker in plaques that form in blood vessels due to atherosclerosis. The chemical structure of c(RGDyK) is shown in Figure 2.

![Chemical structure of c(RGDyK)](image)

*Figure 2. c(RGDyK), a well-known cancer targeting peptide*

Due to the preferential binding of the RGD peptide to cancerous cells, it proves to be a promising method for the delivery of anticancer drugs and contrast agents for cancer therapy and diagnosis. This peptide was the first targeting agent that was used by the Schmitthenner Molecular Imaging Lab (MIL) at Rochester Institute of Technology (Figure 3), where c(RGDyK) in red was directly bound to the Cy 5.5 dye (in green) and used for confocal fluorescence microscopy (CFM) of A549 lung cancer cells.
There are about ten cells shown in this image, with the heart shaped cell in the middle as an example. The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis.

A second, more recently discovered cancer targeting compound of interest in our molecular imaging laboratory is the peptidomimetic inhibitor N[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-(S)-lysine, which is abbreviated as DCL. DCL targets the prostate-specific membrane antigen (PSMA) receptor which is overly expressed in aggressive forms of prostate cancer (PCa). DCL was designed after the three dimensional X-ray structure of was determined, as an inhibitor of the active site. This makes DCL extremely effective in the targeting PCa and in wide use currently.\textsuperscript{20,21} A method for the synthesis of this urea-based moiety shown in Figure 4, to be used in targeting PCa has been developed in our group as well.\textsuperscript{22}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{c(RGDyK)-Cy 5.5 TMIA (left) and CFM image of lung cancer cells targeted by the TMIA (right). There are about ten cells shown in this image, with the heart shaped cell in the middle as an example. The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{DCL urea-based PSMA inhibitor}
\end{figure}
Prior to the breakthrough of small-molecule TMIAs in 2008, the main targeting agent used in clinical diagnosis of PCa by PET was the antibody based ProstaScint®.23 ProstaScint® presented challenges and was unreliable due to low plasma clearance, long circulation times which resulted in high-background signal, limited tissue penetration, and restriction of molecular binding to the intracellular domain of cancer cells.22,24

When creating TMIAs, it is important to consider the wide variety of different cancer targeting agents that can be used in the molecule that is being synthesized. Our group currently uses a modular synthetic approach, where the imaging agents (dyes, radioactive metals, etc.) are created first and the cancer agent is added to the molecule in the final steps of the synthesis. This provides an enormous advantage as it makes it possible to combine a given modular imaging system with a variety of different targeting agents to be used for a wide variety of different cancer types.

1.5. Relevant Imaging Agents

There are many different imaging methods that are currently utilized in the detection and examination of cancer cells including MRI, CT, PET, and CFM. Each method visualizes cancer cells with varying levels of effectiveness (e.g. contrast of the cells). The imaging agents discussed in this chapter are not a comprehensive review of all imaging agents, but are a summary of those types of agents that are most prominent in our research. Each imaging method uses different physical parameters to obtain an image and each has unique advantages or disadvantages over other methods. By combining imaging modalities, we can often combine aspects that are advantageous. By combining agents for each of those modalities into one molecule we can provide further improvements to molecular imaging methods.
1.5.1. MRI Imaging Agents

MRI functions by detecting energy released from protons in the body as they align with the magnetic field of the instrument then relax to their ground states. MRI imaging (contrast) agents are either ferromagnetic or paramagnetic agents. These agents work by shortening the relaxation times of the nuclei within the body. MRI is sensitive to different relaxation times ($T_1$ and $T_2$). The $T_1$ relaxation time (spin-lattice relaxation time) is a measure of how quickly the net magnetization vector recovers back into the field of the MRI magnet. The $T_2$ relaxation time (spin-spin relaxation time) refers to the time required for the decay of the magnetization that is transverse to the field of the MRI magnet. These relaxation times are the basis for which an MRI instrument makes measurements. Gadolinium(III) ($\text{Gd}^{3+}$) may enhance those relaxation times and is therefore one of the most common and widely used paramagnetic contrast agents used in clinical and pre-clinical (animal studies) MRI.

While gadolinium has proved to be an extremely effective contrast agent in MRI, the metallic form of gadolinium can have toxic effects if it is released in the body. It is therefore necessary to use gadolinium, which is chelated to an organic structure which can facilitate its bioavailability in circulation and excretion. This can be achieved by using chelating groups such as dodecane tetraacetic acid (DOTA), a well-known complexing agent that has medical applications as a contrast agent and in cancer treatment. DOTA is highly effective as chelating gadolinium ions and other metals used in radioactive molecular imaging modalities such as PET. The structure of DOTA is shown in Figure 5.
In addition to chelation, a second means of increasing safety is to reduce the concentration of gadolinium per injection. The use of a targeted imaging agent enables the contrast agent to concentrate only in the area of interest. This allows for the use of far less gadolinium-based agent, leading to much lower concentrations in the body.

Furthermore, there have been studies showing that gadolinium is not removed at wastewater treatment plants, allowing the toxic metal to be released into the aquatic environment. Through the use of targeted imaging agents, far less gadolinium will be required resulting in far less gadolinium in the waste stream.27

Thus, in addition to increased brightness and quality of the image provided by a targeted DOTA-chelated gadolinium contrast agent, the safety of patients is enhanced along with the quality of downstream aquatic environments.

As alternatives to the use of gadolinium, different metals or inorganic nanoparticles compatible with MRI can be used to increase the sensitivity of the image. These have been reviewed by Hyon Bin Na et al. who summarizes the progress of inorganic MRI contrast agents.28 However, gadolinium contrasts agents continue as the overwhelming majority of agents used in the clinical setting.27

Figure 5. DOTA, a well-known complexing agent used in cancer imaging
1.5.2. PET Imaging Agents

PET detects gamma rays emitted by a positron-emitting radionuclide to create images. Unlike MRI, which uses a contrast agent to enhance an image, PET is dependent on an exogenous agent and requires the injection of a radioactive molecule into the patient in order to obtain an image. Although an imaging agent is required, in PET an extremely small amount of material is needed (on the nmol to pmol scale), which causes a minimal pharmacological effect on the subject.

PET uses radiopharmaceuticals labelled with positron emitting radionuclides (such as $^{11}$C, $^{13}$N, $^{15}$O, and $^{18}$F) to obtain the image. Radionuclides, which are atoms with an unstable nucleus, can easily be incorporated into organic molecules and used in the body for PET imaging. In the past, $^{18}$F has been widely used as a radionuclide for PET in oncology in the form of fluoro-2-deoxy-glucose ($^{18}$F-FDG). However, difficulties in the synthesis and the short lifespan of the isotope has encouraged imaging scientists to find alternative radionuclides.

A radioactive isotope of copper, $^{64}$Cu, has been found to have longer lasting decay characteristics that allow for PET imaging and targeted radiotherapy of cancer. The well-known coordination chemistry of copper allows for it to be used in a wide range of systems, such as DOTA chelates, that can be linked to peptides and other biologically relevant compounds.

Radioactive gallium, $^{68}$Ga is another radionucleotide used in PET imaging. Since radioactive metals are not used in our lab for safety purposes, we use the stable isotopes of each respective metal in our work. Since the metals’ size is not greatly affected
between the radionucleotide and stable isotopes, it is assumed that the reactions with the radioactive isotopes would be similar.

The radioactive half-life of common radionucleotides that can be used in PET imaging are given in Table 1. The radioactive half-life is defined as the time for half of the radioactive nuclei to undergo radioactive decay. Consequently, after one half-life, there will be one half of the original sample remaining. For this reason, longer half-lives are desired as they will give imaging agents more time to accumulate in the desired area in the body leading to a more resolved image.

*Table 1. Radioactive half-lives of common radionucleotides used in PET*

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{11}$C</td>
<td>20.364 minutes</td>
</tr>
<tr>
<td>$^{13}$N</td>
<td>9.965 minutes</td>
</tr>
<tr>
<td>$^{15}$O</td>
<td>122.24 seconds</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>109.77 minutes</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>12.701 hours</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>67.71 hours</td>
</tr>
</tbody>
</table>

**1.5.3. OMI and CFM Imaging Agents**

Optical molecular imaging is a broad term that encompasses many techniques which are based on the fluorescence of dyes. In molecular imaging these are most often in the near infrared (NIR) region of the electromagnetic spectrum. In our research, we rely on confocal fluorescence microscopy (CFM) in order to prove that a TMIA binds specifically to the particular cancer cell lines that we are targeting.
CFM is a microscopic technique that provides 3D optical resolution of the subject. In CFM, white light is used to image all cells and cell components within those such as the mitochondria and cell walls which may be visualized with great clarity. Along with white light, a powerful light beam from a laser is used to excite a fluorescent dye to produce an image of the cell.\textsuperscript{32} In molecular imaging the dyes used are generally absorb and emit light in the near infrared (NIR) region of the spectrum. One disadvantage to this technique compared to the other techniques mentioned (MRI and PET) is that it can only be used on the surface of the skin or with invasive surgery, where cells are removed from the body.

Cyanine 5.5 NHS ester (Cy 5.5) is a reactive dye that has previously been used in the labeling of amino-groups in peptides, proteins, and oligonucleotides.\textsuperscript{33} Cy 5.5 is a far-red and near-infrared emitting dye which is ideal for fluorescence measurements. It can be used in CFM as to produce an enhanced image in a different wavelength channel than the white light. When overlaid, thee two channels will show the entire cell (generally in black and white) with a fused image of those cells and regions of cells to which the TMIA has targeted and bound. The chemical structure of a tri-sulfonated form of Cy 5.5 3S, synthesized by our published methods, is shown in Figure 6.\textsuperscript{34}
1.5.4. Dual-modal Imaging Agents

Imaging agents are generally utilized in single modal imaging using their respective imaging techniques such as ferromagnetic and paramagnetic metals for MRI, radionuclides for PET, and dyes for CFM. To improve imaging, these imaging agents can also be combined to make dual-modal imaging agents. Dual-modal imaging agents combine two different imaging agents into one single molecule. This allows two different imaging methods to be used simultaneously using specialized instrumentation. Dual-modal imaging is a very powerful method that allows for two imaging techniques to complement each other and provide much more information than one single method.

Pairing modalities such as in PET/CT and PET/MRI in current clinical applications allow for improved diagnostic sensitivity and specificity in the detection of diseased regions in the body.\textsuperscript{32} Single photon emission computed tomography (SPECT)/CT and MRI/Optical (fluorescence) are two other dual-modal imaging methods that are used clinically.\textsuperscript{9} In addition to clinical applications, the largest use in current
research for dual modal agents is in preclinical research in which animals such as mice are utilized to test and develop imaging methods and imaging agents.

While it is possible to use two separate imaging agents and achieve enhancements in each by using dual techniques, there is a distinct advantage of using a single targeted probe containing imaging agents for each modality. When using two different single modal agents there can be questions regarding their equivalence of reaching the binding site. For example, a metal containing agent may have a different uptake, circulation half-life and biodistribution than a dye containing agent. Likewise, metabolism and clearance are a challenge for imaging agents just as they are for drugs.

By combining these into a single agent, such ambiguity and uncertainly is avoided and this assures that the circulation, targeting kinetics and pharmacodynamics for each motif is identical. This is a main advantage of dual-modal imaging agents. A general schematic of a peptide-based dual-modal TMIA is given in Figure 7.

![Figure 7. General schematic of a dual-modal TMIA](image)

In Figure 7, the imaging agents are connected together through a peptide by attaching a chelated metal or a dye to the side chain of lysine modules, then connecting
those modules using our patented method. Theoretically, these imaging agents would provide two different imaging methods. For example, one imaging agent may contain a paramagnetic gadolinium atom that would be suitable as a MRI and one may contain a radioactive copper-64 atom suitable for PET. This would allow the molecule to be used for PET/MRI in the detection of cancer, assuming that an appropriate cancer targeting agent was used.

The images that are created in dual-modal imaging are typically more enhanced and provide more information than the single modal imaging methods. Because of this, the imaging methods are said to be complimentary to each other. Figure 8 shows an image of a 60-year-old patient’s brain with glioblastoma multiforme. The image was obtained using simultaneous PET/MRI acquisition.

![Figure 8. PET/MRI scan of patient with glioblastoma multiforme](https://link.springer.com/article/10.1007%2Fs10334-012-0357-0)

Reprinted with permission from PET/MRI in cancer patients: first experiences and vision from Copenhagen | SpringerLink

In the Figure 8, the MRI contrast agent is not targeted, but is able to provide detailed morphological (spatial) information to the radiologist. The PET agent is targeted and thus provides the functional information (what is it, or is it a tumor?) However, PET images are typically very blurry compared to MRI enables determination of exact
location (where is it, exactly?). Thus, PET and MRI are complementing each other in dual PET-MRI imaging. Because of the complementary benefit from both agents, dual-modal imaging agents for cancer detection has been the focus of many research programs with the ultimate goal of improving the sensitivity and contrast of molecular imaging.

1.5.5. **Comparison of MRI, PET, and OMI**

In molecular imaging methods, the combination of different imaging modalities is important since each modality has its own set of advantages and disadvantages.\(^{37}\) Complimenting imaging methods with the dual of dual-modal imaging agents adds value over single-modal imaging. While two different TMIAs could be used in some circumstances, there is uncertainty whether they would have the same bioavailability in circulation, or the same concentration at different time points based on the kinetics of their metabolism and excretion. By combining two different modalities into a single molecule, this uncertainty is removed.

While OMI utilizes NIR dyes to increase penetration through body tissue over visible dyes, the depth of imaging is only between 2-5 mm. The main use of OMI is in cellular research using *in vitro* studies which use microscopy and other commercial instruments. In preclinical research, OMI is often used in mice as the depth of penetration is suitable to study the binding of agents to grafted tumors that are actively growing. However, as a result of the depth of penetration, clinical use for detection is largely restricted to melanoma and other types of skin cancer.

Although there are issue with the depth of penetration, there has been a large surge in both research and translation to human patients in the area of *light directed surgery* including the use of NIR-based TMIAs for guiding the removal of cancer tissue.
during surgery. Beyond this, OMI is not useful for detecting cancer in internal organs and it is not possible to image the human brain using OMI due to the lack of penetration. Another drawback to OMI is non-microscopic animal images can be blurry, making OMI a low-resolution imaging method.

Unlike OMI, MRI has excellent depth of penetration and is considered a whole body imaging method. This includes its ability to image the brain, which is encased in bone, and other organs deep within the body. A second advantage is that MRI provides excellent spatial resolution, and it is the only technique that can differentiate soft tissues. It can provide contrast, for example, between a tumor and the organ in which the tumor is located. The main disadvantage is that MRI is not very sensitive compared to OMI and PET. A high concentration of gadolinium-based contrast agents is required. Targeting the agent can improve the contrast ability.

As a complement to both MRI and OMI, PET imaging (which uses targeted radioactive metals) has excellent depth penetration. Like MRI, PET can be a whole body technique (including imaging of the brain). PET also has excellent sensitivity as it only takes a small concentration of the imaging agent to be detected. One disadvantage of PET is that the resolution is considered low and, like OMI, yields blurry images on the macro-scale. It is therefore advantageous to combine PET, with its excellent sensitivity, with MRI, which has great resolution.

1.6. Previous Literature Research in Imaging Agents

In the past, DOTA has been used in the synthesis of imaging agents due to its strong chelating characteristics which make it beneficial in carrying and holding metals. Chelation of a metal, such as $^{64}$Cu has proved to be extremely useful in PET imaging. In
early, pioneering work, Zhang and colleagues demonstrated a method where DOTA was conjugated to the antibody TRC105 and labeled with $^{64}\text{Cu}$ for PET imaging. The scheme for this reaction is given in Figure 9.

Figure 9. DOTA conjugation with TRC105

Figure 9 shows how the TRC105 antibody can be coupled to DOTA, which would go on to chelate copper-64. In the reaction, the NHS ester acts as a leaving group and reacts with the primary amine of the TRC105. Since TRC105 is a specific monoclonal antibody, it can be used as a marker for tumor angiogenesis and thus the DOTA-TRC105 is a TMIA in PET for detection of cancerous tumors.

DOTA can be used in a different fashion, where multiple DOTA complexes are added to a single antibody. Cai and colleagues demonstrated a method where multiple DOTA complexes were added to a Cetuximab molecule, which is an antibody that targets cancer. This was done for PET imaging and copper-64 was used as the radioactive label. Although this method proved to be successful in the clinical evaluation of patients using PET, the molecule could only be used in PET imaging. The presence of multiple DOTA groups in this application did not allow for multiple imaging methods to be used since all the DOTA complexes were chelating copper-64 atoms. The current goal of our group reaches beyond the work of Cai et al., into the realm of multimodal imaging.
1.6.1. Previous Literature Research in Dual-modal Imaging Agents

As mentioned, imaging methods can be used simultaneously on specialized instrumentation by incorporating another type of imaging. Multi-modal imaging agents emerged in the 1990s, when dual modal imaging methods such as PET/CT came to clinical use.\textsuperscript{9,41} In 2013, Suchy and colleagues presented a method that used DOTA-derived heterometallic complexes as potential MRI/PET(SPECT) molecular probes.\textsuperscript{42} This method used Gd\textsuperscript{3+} as an MRI agent and the cold (non-radioactive) isotopes of Cu\textsuperscript{2+}, Ga\textsuperscript{3+}, and In\textsuperscript{3+} as potential PET or SPECT agents. The molecules that were made in this project were synthesized using the well-known “click” chemistry method.\textsuperscript{43} The structure of one of the bimodal MRI/PET(SPECT) molecular imaging agents that was synthesized in this work shown in Figure 10. It is noted that this agent is not targeted.

![Figure 10. Structure of a DOTA-derived chelator coupled with a porphyrin moiety via a peptide bond to form a non-targeted molecular imaging agent\textsuperscript{42}](https://doi.org/10.1039/C3RA23260C)

In Figure 10, it can be seen that the DOTA moiety is chelating a gadolinium atom (for MRI) and the porphyrin moiety contains a copper atom (for PET). The use of both of these metals in the same molecule yields a bimodal imaging agent. Although the authors did not use a radioactive isotope of copper, which is the only form that would work with PET, it is suggested that their synthetic method would be similar with the radioactive isotope.

Similar to our modular approach, both the MRI and PET chelating complexes are built separately and are combined in a later step the synthetic method used by Suchy et al. Each metal containing framework built before the metal is added to the complex. This is due to the fact that once the metal is added, it serves as a protecting group for the complex and the complex then becomes unreactive. Although this presented a useful methodology to be used in potential MRI/PET(SPECT) molecular probes, the authors did not utilize a cancer-targeting group in their synthesis. Thus, these agents are not targeted molecular imaging agents.

In a related project, Gros et al. present a method to create bimodal contrast agents for medical imaging, mostly notably in PET/MRI. Their methodology was relatively similar to the method that Suchy et al. used. However, instead of incorporating the metal atoms in the imaging moieties prior to their combination, metalation was done in the last steps of the synthesis. Figure 11 shows the addition into the bimodal contrast agent of the atoms Gd for MRI and Cu for PET.
In this method, the synthesis of the desired PET/MRI imaging agent is based on the selectivity and affinity of the metal chelating groups. Gros et al. demonstrated that the gadolinium atom has a higher affinity for the DOTA cavity (as it is shown in the figure) whereas copper matched better for the tetravalent pyrrole based chelator. As in the prior dual modal method, there is no targeting in this scheme.

In 2008, Overoye-Chan et al. presented a method in which a fibrin specific gadolinium-based MRI contrast agent was made for the detection of thrombus (EP-2104R). The synthetic approach for creating this multi-Gd containing molecule is shown in Figure 12.
As it can be seen, the molecule in Figure 12 is not a bimodal imaging agent due to the fact that the only imaging moieties contained within the molecule are the DOTA-chelated gadolinium atoms, which are useful in MRI. The goal of the group at Harvard was to image thrombus using both MRI and PET simultaneously. To achieve this, the MRI agent EP-2104R was improved upon by partially exchanging the gadolinium metal atoms from copper-64, which is a suitable PET radionucleotide. Figure 13 shows the synthetic approach that was used.
The PET-MRI probe did enhance the images of thrombus in rats. However, in terms of synthesis, the approach is flawed in that the partial metal exchange method that was used in this approach is not selective and leads to a mixture of dual metal species with an average reported ration of 5% radiolabel. The result is not a single compound as there is no way to exactly control the percentage of the gadolinium atoms that are exchanged.

In the pharmaceutical industry, when a drug is translated to the clinic and approved by the Food and Drug Administration (FDA), the process is faster when the drug is a single molecule that can be purified and characterized completely by spectroscopy. Likewise, in the development of targeted imaging agents it is always preferable to produce a single, purified and well characterized compound. This presents a challenge that our group has worked to improve.
In 2015, Kumar and coworkers at the University of Texas Southwestern Medical Center (Dallas, Texas) reported a versatile dendritic molecule that can be used for developing dual-modality imaging agents. The molecules created are dendritic structures with a NOTA core and multiple copies of DOTA groups branching out (Figure 14).

![Figure 14. Dual-modal molecular imaging agent design by Kumar et al.](image)


In an elegant solution to dual modal imaging, this molecule is capable of selective chelation to gallium and gadolinium based on the different sizes of the NOTA and DOTA cores. As outlined in Figure 14, the NOTA core is selective for $^{67,68}$Ga$^{3+}$, which is useful for SPECT and PET, and the DOTA moieties are selective for Gd$^{3+}$, which is useful for MRI. Out of the many imaging agents cited in literature, this dual-modal imaging approach is possibly the most similar to the molecules synthesized in our lab at RIT.
Functionally and structurally, the molecule has similar characteristics. However, our group’s molecules are both smaller and have a simpler synthetic approach.

1.7. Dual-Modal Imaging Instrumentation

In order for dual-modal molecular imaging agents to be most useful, appropriate dual-modal imaging instrumentation must be available. MILabs currently markets an imaging module for pre-clinical imaging (of animals) called the U-OI that couples fluorescence imaging with PET, SPECT, and/or CT for preclinical imaging. Although all of these imaging platforms are not housed in the same instrument, the imaging module is designed to have a single animal holder that ensures its position does not change in order to facilitate the exact fusion of images. Animals are typically anesthetized and immobilized during imaging procedures so this is entirely possible. A picture of the instrument from the manufacturer’s website is shown in Figure 15.

![Figure 15. Duet Optical Imaging module, U-OI from MILabs](image)

The first MILabs imager is a standalone instrument that is capable of bioluminescence, fluorescence, and Cherenkov imaging (optical imaging of radiotracers), and this was then integrated with additional imaging platforms such as PET, SPECT, for
dual modal imaging (OMI-PET or CT) with an optional CT built into the latter to provide true tri-modal imaging (OMI/PET-SPECT/CT).

There are no current dual or tri-modal imaging systems involving fluorescence or optical molecular imaging as this modality is now largely viewed as mostly useful for directing real time surgical procedures such as light-directed surgery (LDS) in human patients as a single modal process.

There are, however, instruments that combine PET and MRI for simultaneous acquisition. Dual PET instrumentation was first introduced into the clinic in 2008 and since that time has been reviewed as a significant investment opportunity.\textsuperscript{47–50} To date, a number of companies have designed and manufactured preclinical and clinical hybrid imaging systems for PET-MRI. Some of these instruments are also offered with CT to provide tri-modal imaging. Bruker manufactures a system that combines MRI and PET into an instrument for preclinical imaging of mice, the PET/MR 3T.\textsuperscript{51} This instrument allows for scientists to test their dual-modal imaging agents in the early stages of development before moving onto clinical trials. MedisoUSA also makes a preclinical imaging platform known as the nanoScan Family.\textsuperscript{52} This instrument combines PET and SPECT with CT and MRI to allow for nine different imaging combinations.

A number of additional companies including Siemens are ramping up the design and manufacturing phase or PET-MRI instrumentation, clearly showing a robust growth rate in preclinical and clinical dual modal imaging systems. However, it is also clearly apparent that the development of dual modal targeted probes, in particular TMIAs for PET-MRI, lags the development in instrumentation. This represents a driving force for the research presented here. In particular, there are no general methods for the synthesis
of dual modal probes in which a given dual-modal imaging system can be tethered to a wide variety of targeting agents. It is anticipated that more dual-modal, and possibly tri-modal, imaging instrumentation will be developed which will give rise to a pressing need for dual- and tri-modal TMIAs for a wide variety of cancer types.
2. Modular Method for Multi-Modal Agents

Our group has developed a modular method to synthesize peptide-based imaging agents that is based on the principles of peptide chemistry. DOTA-chelated metals and imaging dyes are synthesized separately from each other and separate from the cancer-targeting module. These various imaging groups, used in imaging methods such as MRI, PET, CFM, etc., are placed on the side chains of amino acids early in the synthesis. Using amino acids allows the use of well-known peptide coupling methods to connect different imaging moieties to cancer-targeting agents. In this approach, lysine, shown in Figure 16, is the amino acid that was used as the basis for each module due to the primary amine on its side chain. The primary amine can be easily reacted with imaging modules, to produce stable imaging modules.

![Figure 16](image.png)

*Figure 16. Structure of L-lysine, the amino acid commonly used in our targeted molecular imaging agents*

Due to the nature of the well-known features and reactions of peptides, the design of our imaging agents is simple and the synthesis is practical. There are many advantages to using peptides over other organic molecules. Peptides are small and compact, typically ranging from 1,000 to 3,000 g/mol, which is relatively small for an imaging agent. The intermediate imaging peptides and final TMIAs can be readily purified by LC and characterized by LC-Mass Spectroscopy. This enables our group to focus effort on synthesizing new, improved dyes, developing new TMIAs and less time on developing and troubleshooting new chemical reactions.
2.1. Modular Synthetic Approach

Our group’s approach utilizes preformed amino acid-based imaging moieties that are eventually combined with a cancer-targeting agent in the final steps. The amino acid moieties are connected to each other using peptide coupling chemistry which forms an amide bond (peptide bond) from the amine terminus (on the left of each amino acid) to carbon terminus of the in-coming amino acid. A generalized schematic of our group’s modular peptide synthetic approach is outlined (Figure 17).

![Figure 17. General schematic of the modular peptide synthesis of TMIAs](image)

The two imaging agents being connected to each other in this scheme are: a NIR dye and chelated-metal. These imaging modules (which can be seen as reactants on the left side of the arrow) are typically synthesized and purified in-house using different methodologies, depending on the component. It can be seen that both of the imaging modules in this reaction contain a lysine-based backbone, which allows for the different components to be connected using peptide coupling methods.

Also shown in Figure 17 is an example of a *linker* that is used in the synthesis. Linkers are commonly used in our modular synthetic method to connect the cancer-targeting component of the molecule to the imaging component of the molecule. This allows the targeting group to be put on during the last step in the synthesis.
A color-coded example of a molecule created in this project using this modular synthetic methodology is shown in Figure 18. The colors in this figure match the colors of the components of the TMIA shown in Figure 17.

![Color-coded structure of final TMIA](image)

*Figure 18. Color-coded structure of final TMIA, c(RGDyK)-SMCC-dK(Cy5.5)-K(DOTA-Gd)-NH₂*

A key aspect of the modular synthesis is to apply a given imaging system to a variety of targeting systems. To exemplify this, a similar color-coded example of a TMIA created previously in our group using similar modular synthetic methodology, but employing the PCa PSMA-targeting group DCL as described earlier, with a linker derived from di-succinyl suberate, DSS, is shown in Figure 19.
In Figure 19, from the left, the prostate cancer-targeting DCL group is connected to a lysine module which carries a NIR imaging dye through a DSS linker. This is, in turn, connected to a second lysine module carrying a DOTA-chelated gadolinium atom. Each of these components serve a purpose: the DCL targets the cancer, the linker connects the targeting agent to the imaging modules. The NIRF dye is useful in optical imaging, and the gadolinium is useful in MRI. When connected together, these four components combine their separate purposes to form a powerful targeted imaging agent.

There are many different methods that can be used to combine amino acids and form larger molecules, including linear and convergent synthetic methods. These methods each have different orders in which the molecule is built. Our group typically uses linear synthetic method to build peptides, although convergent methods sometimes lead to a better yield.

2.1.1. Linear Synthetic Approach

In a linear synthetic method, the desired molecule is synthesized through a sequence where the product of one reaction is the reactant in the next reaction. In terms
of the imaging agents that we work with, this method builds the peptide based modules from the right to left. Polypeptides are created by forming peptide bonds, which involves the nitrogen (N-terminus) of the first amino acid being bonded to the carbon (C-terminus) of the second. A schematic of the linear synthetic method of the TMIA\texteuro s created in our group is given in Figure 20.

![Figure 20. General schematic showing linear synthetic method of TMIA\texteuro s, linker added in a separate linker step](image)

### 2.1.2. Convergent Synthetic Approach

In a convergent synthetic method, fragments of the desired molecule are synthesized separately and brought together at a later stage in the synthesis to form the target molecule. A schematic for convergent synthesis involving the TMIA\texteuro s created in our imaging group is given in Figure 21.
In our syntheses of TMIA s, each method (convergent and linear) has shown advantages and disadvantages depending on the chemistry involved for each particular TMIA that has been synthesized. This will be described in further detail.

2.2. Identification and Analysis of TMIA s and Intermediates

In this study, reactions were monitored via HPLC-MS instrumentation (Waters) or UPLC -MS (Shimadzu). This allows for confirmation of the molecule when the calculated m/z = [M+H]+, [M+2H]2+ and/or [M+3H]3+ peak(s) are present in the positive electrospray (ES+) spectra. In some instances, for example when sulfonated dyes are present, these are seen in negative electrospray (ES-) as m/z = [M-H]−, [M-2H]2−, and/or [M-3H]3−. Key intermediates and final products that were first characterized in our lab were sent to the Mass Spectrometry Laboratory at University of Illinois School of
Chemical Sciences for high resolution mass spectroscopy (ESI-HRMS). The masses in these HRMS are typically accurate to four or five decimal places and can be used to confidently report that the desired molecule was synthesized.

In most cases, important intermediates and final products were purified by preparative reverse phase chromatography and assaying collected eluant fractions by analytical HPLC-MS. Fractions containing pure products (90-100% purity in single wavelength chromatograms at 264 nm for compounds containing Fmoc group, and 90% + purity in total ion chromatograms for compounds with no chromophore groups) were pooled, concentrated by rotary evaporation under a vacuum, then lyophilized (freeze-dried) and yields determined from the resultant mass obtained.

TMIA’s synthesized by these methods have been tested for their binding and selectivity by confocal microscopy in the past, including imaging the TMIA shown in Figure 18. However, this testing has not taken place (due to the unfortunate passing of our collaborator in Biology, Dr. Irene Evans) on any of the compounds in this new work. However, our group has begun a collaboration with Dr. Maureen Ferran from RIT’s GSOLS and we also collaborate with Roswell Park Cancer Center, and the University of Rochester. Several of the TMIAs described have been sent to these new collaborators. These collaborations allow us to test our imaging agents both in vivo and in vitro.

An example of the targeting that is achieved by a TMIA synthesized earlier by students in our lab is shown in Figure 22. In this study A549 lung cancer cells were cultured, stained with the dual modal TMIA shown in Figure 18, (prepared earlier by students in our lab) and imaged by confocal fluorescence microscopy. As described earlier, the targeting agent c(RGDyK) targets the integrin receptors. The targeting agent
clearly binds well to the cancer cells as shown by the red imaging. The blue imaging is created by a stain called NucBlu that specifically stains the nuclei of cells.

In addition, the process of endocytosis can be clearly seen. This is a mechanism where by xenophobic molecules such as the TMIA, after binding to the receptor, can be engulfed into the cytoplasm of the cells. The receptor site is regenerated very rapidly, and addition agents may enter the cell. It is our speculation that this can act as an amplification scheme to increase the signal. It can be easily deduced then, that since this is a dual modal agent, that the gadolinium moiety is also contained within the cell and, as it is covalently linked through the modular system to the dye, that the gadolinium must therefore also be present in the cells.

![Figure 22](image)

Figure 22. Confocal fluorescence microscopy (CFM) of three A549 lung cancer cells targeted by the TMIA, c(RGDyK)-SMCC-dK(Cy5.5)-K(DOTA-Gd)-NH₂ shown in Figure 18. There are about three cells shown in this image. The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis. The blue color is a result of staining of the cells by NucBlu, a widely used dye that stains only cell nuclei.

Similar to the targeting of A549 cancer cells by c(RGDyK) assayed by CFM, our collaborators in GSOLS under the direction of Dr. Evans have achieved many images of prostate cancer (PCa) cells including an aggressive type called C4-2 which express PSMA (PSMA+) and compared these with atype of PCa cells that are indolent called
PC3 cells that express low levels of PSMA (PSMA-). An example of a TMIA for this is B2, synthesized earlier in the Molecular Imaging Laboratory at RIT. The structure is shown in Figure 23, followed by images of the B2-targeted C4-2 cells in Figure 24 (on the left and middle) and the control cells, PC3 (on the right).

Figure 23. Structure of B2 showing the single modal DCL-DSS-Lys(Cy5.5)-NH₂

In Figure 24 as noted, the TMIA binds to the (PSMA+) prostate cancer cells and is engulfed by a process known as endocytosis, illuminating the cells in red. The middle image are the same cells with a blue channel turned on that shows staining by NucBlu which specifically illuminates the cell nuclei. On the right are the control PC-3 (PSMA-)

Figure 24. Left: Image of about ten C42 (PSMA+) prostate cancer cells illuminated by the TMIA B-2, DCL-DSS-Lys(Cy5.5). The red color is a result of targeting by the TMIA, which is engulfed and accumulates in each cell by the process of endocytosis. Middle: Same image of cells in which the nuclei are lit up with NucBlue. Right: About 20 PC3 (PSMA-) cells in a control experiment which were treated with the same concentration of TMIA, showing weak binding.
cells which show very little binding as the expression of PSMA is not elevated in that cell line. A similar structure was previously reported by the Pomper group at Johns Hopkins who similarly showed successful targeting by several similar NIR dye-based TMIAs using DCL-DSS as the targeting motif.\textsuperscript{20,53}

It is important to note that while we have not tested the PCa targeted imaging agents prepared in this work by CFM, based on the successful targeting of the dual modal agent using c(RGDyK) that matched earlier successful targeting of single modal agents both in our lab and in the literature\textsuperscript{53}, it may be predicted that dual modal agents containing both the dye used in Figures 23 and 24 with the same cell lines will also produce successful targeting and imaging.
3. Results and Discussion

3.1. Synthetic Approach to Metal-Containing Imaging Modules

This project focused largely on metal-containing modules that were designed to be used in MRI and PET. The reaction method that is used to synthesize these metal-containing modules was carried out using a one-pot synthetic approach, which was optimized by a fellow lab member, Dana Murphy Soika. The method used to create these modules is similar regardless of the metal used. Scheme 1 is the reaction scheme outlining a “one-pot” synthetic method that was used to create the metal-containing puzzle pieces used in this research project.\(^\text{22}\)

![Scheme 1. General one-pot synthesis for F-K(M\textsubscript{1}-DOTA)-R imaging modules, R=NH\textsubscript{2} or OH and M\textsubscript{1}=Gd or La](image)

There are several conditions that were important to optimize yield. When running this reaction, DOTA was first dissolved in a dilute solution of DMF and heated to 70°C. This starting solution should be dilute to discourage any possible di- and tri-substituted DOTA groups, since mono-substitution is desired. It was found that heating the reaction mixture aids with solvation of the DOTA, which also maximizes yield of the desired
Once the DOTA is dissolved in the reaction mixture, NMM is added to create a basic reaction mixture that allows activation of DOTA by TBTU. TBTU, a peptide coupling reagent, is added at a rate of 0.3 mL/min to also favor mono-substitution on the DOTA. The lysine is then also added at this slow rate to favor the preferred product. The metal (Gd or La) is added in the final step and is chelated by the DOTA molecules. This method is extremely versatile, as it can be used to make many different imaging modules. In Scheme 1, the R-group is either -OH or -NH₂ and M₁ is either Gd or La in the scope of this project. This makes the method useful for both MRI and PET imaging modules. However, this method could theoretically be used for a countless number of different metal and amino acid combinations.

### 3.2. Fmoc Deprotections

The imaging modules discussed in Section 3.1 are made with an amino acid (lysine) that can be reacted with from the N-terminus or the C-terminus.²² A fluorenylmethyloxy carbonyl (Fmoc) group is the most commonly used protecting group in peptide synthesis. It is used to protect the N-terminus of an amino acid from any unwanted side reactions during other synthetic steps. A deprotection step to remove the Fmoc group must be carried out in order to further couple other molecules to the N-terminus amine. Scheme 2 is a reaction scheme showing a Fmoc deprotection reaction of a metal-containing imaging module introduced in Section 3.1.
Scheme 2. General schematic showing simple Fmoc deprotection procedure, F-K(M₁-DOTA)-NH₂ to H-K(M₁-DOTA)-NH₂

As can be seen in Scheme 2, treatment of the Fmoc group with diethylamine in an organic solvent successfully removes the group with yields nearing 100%. After the Fmoc group is removed, the N-terminus of the molecule is ready to further react with another molecule. Figure 25 shows the mechanism for the Fmoc deprotection.

Figure 25. Mechanism for Fmoc Deprotection
3.3. NHS Ester Synthesis

N- Hydroxysuccinimide (NHS) is a reagent that is commonly used for activating acids to prepare them for coupling to amines such as the alpha N-terminus amine when elongating a peptide chain. The structure of NHS is given (Figure 26).

![NHS Structure](image)

Figure 26. NHS Structure

NHS is frequently used in biological chemistry to activate carboxylic acids so they are able to react with amines to form an amide. In this project, the NHS ester was commonly utilized to couple two different modules together. This includes coupling dyes to peptide side chains, coupling peptide modules together, and coupling imaging modules to targeting modules and/or linkers to targeting modules. Scheme 3 is a reaction scheme showing creation of an NHS ester using TSTU and the reaction with an amine in a subsequent reaction as outlined in a paper by Wilchek et al.\textsuperscript{54}.

![Scheme 3](image)

Scheme 3. General reaction scheme showing formation of NHS ester in first reaction and coupling in second reaction (R and R’ are different modules)

3.4. Synthesis of Dual-Modal TMIA for Prostate Cancer Using OMI-MRI

While single modal agents based on targeting by DCL have been synthesized, and one dual modal agent for OMI-PET\textsuperscript{55}, we have found no dual modal agents for OMI-MRI or PET-MRI that utilize DCL. Thus, it seems appropriate to demonstrate our modular method to synthesize both of these dual TMIA s. In the first set of examples, a dual-
modal TMIA was created for the purpose of imaging prostate cancer using both OMI and MRI. This was achieved by pairing a Cy 5.5 dye module (for OMI) with a gadolinium-based imaging module (for MRI) to the prostate cancer targeting agent DCL. Following the protocol outlined in Section 3.2, the Fmoc group was removed from the F-K(Gd-DOTA)-NH$_2$ (2) module to create H-K(Gd-DOTA)-NH$_2$ (5), which would be ready to react with the NHS ester of the Cy 5.5 dye module. Cy 5.5 3S was synthesized using the procedure published by previous group members Damien Dobson et al.$^{34}$ The NHS ester of the Cy 5.5 3S dye was created using the methodology outlined in Section 3.3. The specific reaction scheme showing the formation of the Cy 5.5 NHS ester can be seen in Scheme 4. Scheme 4 also shows the Cy 5.5 NHS ester being coupled to the Fmoc protected lysine to form the desired dye-based imaging module.

\[ \text{Scheme 4. Synthesis of peptide-based dye module, } F-dK(Cy5.5 3S)-OH (4) \]
Using the same methodology presented Section 3.3, the NHS ester of the F-dK(Cy5.5 3S)-OH (4) molecule was created, then reacted with a second module. The NHS ester, also referred to as the oxysuccinimidyl (OSu) group, F-dK(Cy5.5 3S)-Osu, was reacted with deprotected gadolinium module for MRI, H-K(Gd-DOTA)-NH₂ (5), as outlined in Scheme 5 to create the dual-modal imaging module for OMI-MRI.

Scheme 5. Synthesis of Dual-Modal Imaging Module, F-dK(Cy5.5 3S)-K(Gd-DOTA)-NH₂ (6)

The Fmoc group of this dual-modal molecule was removed using the method outlined in Section 3.2 to create a reactive form of the peptide. From here, the molecule could be reacted with a cancer targeting group in order to create the desired TMIA.

In order to complete the synthesis of the dual modal TMIA for PCa, a convergent method, developed by Damien Dobson and published earlier by former group members, was utilized.²²

In this convergent method, the DCL was first connected to the linker DSS. This targeting module was then coupled to the imaging module consisting of the dual modal
imaging system for OMI-MRI. The reaction in which the targeting module containing the linker was connected to the dual-modal imaging module is shown in Scheme 6.

**Scheme 6. Synthesis of Final Prostate Cancer Targeting Dual-Modal TMIA, DCL-DSS-dK(Cy5.5 3S)-K(Gd-DOTA)-NH₂ (8)**

Since the linker *and* targeting group were added as one module in this step, this presents a convergent synthetic approach. Our group has found that using the convergent approach most often increases the yield of the TMIA in the final synthetic steps.

The product shown in Scheme 6 has been dubbed as G3 in our Molecular Imaging Lab and a sample has been supplied to Dr. Ferran in RIT’s GSOLS and to Dr. Nastiuk of Roswell Park for evaluation in both the CFM imaging and MRI of prostate cancer.

All of the intermediates and final products (4-8) described in Section 3.4 were purified and assayed as described in Section 2.2. The details of their synthesis, purification and identification by LC-MS and HRMS are in in Appendix I.
3.5. Synthesis of Dual-Modal TMIAs for PET-MRI

Two pseudo PET-MRI agents were next created using a similar methodology as the OMI-PET agent described in Section 3.4. In these examples, a lanthanum atom was used as a placeholder for radioactive atoms that could be used in PET. As we are unable to use radioactive metals in research at RIT (for safety concerns), the nonradioactive form of these atoms were used in our lab. It is expected that the radioactive forms of these metals would behave chemically similar—and allow for PET functionality. In order to make a dual-modal imaging agent for PET-MRI, the challenge was to couple two different metal containing modules together, and then to differentiate these based on the stability of one metal over the other metal in dilute acid.

Using the same method outlined in Section 3.1, F-dK(La-DOTA)-OH (1) and F-K(Gd-DOTA)-NH$_2$ (2) were synthesized. These modules were coupled together after removing the Fmoc group from the gadolinium-containing molecule and creating the NHS ester of the lanthanum-containing molecule. It is important to note that the lanthanum-containing module contained an alpha carboxylic acid functional group for the specific purpose of coupling this to the gadolinium module. The order of these molecules could have been flipped if the lanthanum piece had an amide functional group at the end and the gadolinium piece had a carboxylic acid group at the end. As the DOTA-Gd chelate is a very robust complex we typically used that as our C-terminus starting module. The dipeptide imaging agent was created as shown in Scheme 7.
After the product in Scheme 7 was synthesized, the Fmoc group was removed using the deprotection reaction outlined in Section 3.2 in order to prepare it for conjugation to the cancer targeting group. In this case, instead of the DCL-urea based targeting group used for the TMIA in Section 3.4, this the cancer-targeting c(RGDyK) was employed. Although a different cancer-targeting group was used, the same DSS linker was used for this TMIA as it was discovered to be easier to react than SMCC, a linker used previously in our lab. As in the case of the targeting module DCL-DSS, the targeting group was connected to c(RGDyK) before it was added to the dual-modal imaging molecule. As with the novelty of DCL-DSS, we believe this to be the first instance of c(RGDyK)-DSS as a targeting module as shown in Scheme 8.
In order to discourage the di-substituted product in Scheme 8, the c(RGDyK) was added dropwise to the reaction mixture. This technique was found to sufficiently produce the mono-substituted product. Once this module was created, it was ready for reaction with the dual-modal imaging molecule previously described and shown in Scheme 7.

After the dual-modal imaging module (in Scheme 7) and the c(RGDyK)-DSS (11) (in Scheme 8) pieces were synthesized and appropriately purified—and the Fmoc group was removed from the dual-modal imaging module—the two groups could be combined to form the final scaffold for the desired TMIA. The term “scaffold” is used since the only difference between the desired TMIA and the scaffold is replacing the lanthanum metal placeholder atom with a gallium or copper atom. This convergent synthesis of the penultimate final TMIA is shown in Scheme 9. Kelsea Jones, a former group member, was able to successfully synthesize the compound in Scheme 9. However, Kelsea was unable to successfully complete the transmetalation step (Scheme 10).\textsuperscript{56}
The transmetalation procedure shown in Scheme 10 takes advantage of previous studies in our laboratory where we learned that lanthanum is labile in dilute TFA (0.2 M), whereas gadolinium robust and is not labile under these conditions. This provides a convenient method which is based on the stability of Gd-DOTA coordination bonds which remain intact while the La-DOTA coordination bonds are labile and broken, allowing for the lanthanum to be transmetalated. This can occur in a one-step method, but we find it more effective to use a two-step method that is further described in the experimental section. The transmetalation step is depicted in Scheme 10.
Scheme 10. Transmetalation of lanthanum atom with potential PET imaging atoms to create final TMIA, c(RGDyK)-DSS-dK(M-DOTA)-K(Gd-DOTA)-NH$_2$, M=Cu (13) or Ga (14)

All of the intermediates and final products (9-14) described in Section 3.5 were purified and assayed as described in Section 2.2. The details of their synthesis, purification and identification by LC-MS and HRMS are in Appendix I.

The use of the placeholder metal and the synthetic feature involving a final transmetalation to metal atom useful for PET imaging provides an important advantage of the modular method. It is important to consider that the radioactive forms of the metals copper and gallium, may be avoided in the entire synthesis and that this step could be performed in the clinic or animal research lab. As the non-radioactive placeholder metal would not be vulnerable to radioactive decay with short half-lives, it could be stored for long periods of time and a portion of it be exchanged to the radioactive metal when needed. In addition, it could be transported safely to remote testing sites with no concerns. The transmetalation would be performed easily by the radiologist or their team right before the TMIA was about to be used in the healthcare setting.
3.6. Synthesis of Dual-Gadolinium Targeted Contrast Agent for MRI

The contrast of an MRI imaging can be increased with increasing molarity contrast agent. A widely used method of achieving this is to place two or more gadoliniums in a single agent. Among many reports in the literature is a study involving similar DCL-DSS targeted agents by the Pomper group at Johns Hopkins. Apart from this study, there have been few reports of contrast agents for MRI which rely on DCL or other PSMA inhibitors as targeting moieties. We therefore wished to demonstrate that the modular method developed in the MIL lab would be a useful means of creating dual or even multi-Gd TMIAs.\textsuperscript{22}

A dual-gadolinium MRI contrast agent was therefore created by coupling two Gd imaging molecules, F-dK(Gd-DOTA)-OH (3) and F-K(Gd-DOTA)-NH\textsubscript{2} (2), which were synthesized using the same method outlined in Section 3.1. These modules were coupled together after removing the Fmoc group from the amide molecule and creating the NHS ester of the carboxylic acid molecule, as outlined in Scheme 11.

\textit{Scheme 11. Synthesis of Dual-Modal Imaging Molecule for MRI, F-dK(Gd-DOTA)-K(Gd-DOTA)-NH\textsubscript{2} (15)}
After the dual-modal imaging agent in Scheme 11 was synthesized, the Fmoc group was removed using the deprotection reaction outlined in Section 3.2. At this point, the molecule could be reacted with a cancer-targeting group. Similar to the synthesis presented in Section 3.5, c(RGDyK)-DSS (11) was made in a coupling reaction (identically to Scheme 8) and was attempted to be added to the dual-gadolinium imaging module. After monitoring the reaction it was determined that the c(RGDyK)-DSS was not reacting with the dual-gadolinium module. This may have been due to the fact that the NHS ester of the DSS had been hydrolyzed from residual water. To overcome this, a linear synthesis was attempted in which the DSS and c(RGDyK) were added separately. Using this method, which is outlined in Scheme 12, the final dual-gadolinium contrast agent was created.
In Scheme 12, it can be seen that a linear synthetic method was used in the synthesis of this TMIA. Although the convergent approach described earlier was attempted the c(RGDyK)-DSS did not effectively couple to the dual-gadolinium module using the convergent synthetic method. This was attributed to the low solubility of the di-gadolinium product which required heating, which led to degradation of the sensitive NHS ester on the DSS moiety. In contrast, the two-step linear method of adding the DSS first was more effective, as the DSS itself added solubility to the otherwise sparingly soluble di-gadolinium complex.
All of the intermediates and final products (15-17) described in Section 3.6 were purified and assayed as described in Section 2.2. The details of their synthesis, purification and identification by LC-MS and HRMS are in Appendix I.

3.7. Work Towards a Novel Tri-Modal Imaging Agent for OMI-PET-MRI

There are few references of tri-modal imaging agents in literature. This may be due to the fact that current medical instrumentation does not allow for the simultaneous acquisition of data using three different methods. However, one of the goals of this research project was to create a novel tri-modal imaging agent that could potentially be used in OMI-PET-MRI when technology allows for it. To achieve this, the same dual-modal imaging agent that was synthesized in Scheme 7 (for PET-MRI) was to be combined with the NHS of the dye created in Scheme 4 (for OMI). This would contain three different imaging moieties, each useful for a different application, making it a tri-modal imaging agent. The reaction in which the previously described PET-MRI imaging module (after removing the Fmoc group) and the NHS ester of the Cy 5.5 3S dye were combined is given in Scheme 13.

In this final reaction, the crude reaction mixture was carried out, and quenched two ways, with water and with butyl amine solution, then and assayed by LC-MS. Although the un-optimized reaction mixture contained five products, all of which contained the Cy5.5 dye as shown by the UV-Vis trace of each and a single wavelength chromatogram at 684 nm, one of these products, with a retention time of 4.89 minutes, was determined to be the product. The positive and negative ion mass spectra showing half mass [M-2H]^{2-}/2 in negative ion (ESI⁻) and third mass [M+3H]^{3+}/3 in positive ion (ESI⁺), along with an expanded spectra showing the gadolinium isotope pattern, and along
with the expected wavelength for Cy5.5 clearly prove that the structure had been confirmed. This data is shown in the experimental section under the synthesis of Compound 18 as shown in Scheme 13.

Scheme 13. Synthesis of Tri-Modal Imaging Molecule for OMI-PET-MRI, F-dK(Cy 5.5 3S)-dK(Gd-DOTA)-K(Gd-DOTA)-NH$_2$ (18)

Due to time constraints, and notably the inability to return to lab in March 2020 due to the dire global health situation which required social distancing, this crude reaction mixture was precipitated and remained in the freezer for the duration of the semester.

However, the project was taken to the point where a future student could carry on this synthesis to completion. The crude reaction mixture could be purified by preparative HPLC, then the Fmoc group would be deprotected followed by convergent addition of the either with a targeting group and linker used in this research: c(RGDyK)-DSS and DCL-DSS in schemes analogous to Schemes 6 and 9 to form a tri-modal imaging agent.
4. Conclusion

The modular synthetic method used in this project has proven to be useful and practical in the synthesis of multi-modal TMIAs. This research project demonstrated the complete synthesis of four (4) novel TMIAs that could be used in dual-modal molecular imaging. Studies towards tri-modal and other multi-modal imaging agents are still ongoing, but current results show much promise for the feasibility of these compounds.

The synthesis of the compounds in which lanthanum is used as a placeholder for a radioactive metal, such as copper-64 or gallium-68, has proved to be a sufficient method. It is important that the final transmetalation step was worked out in this project, as this is the step in which the compound becomes useful for PET imaging. This has been an ongoing project in the molecular imaging laboratory and it is encouraging that the full synthesis of these molecules was finally worked out.

Although these compounds have been successfully synthesized and characterized using HPLC-MS and HRMS, their effectiveness as imaging agents still needs to be tested. Collaborations and plans for both \textit{in vivo} and \textit{in vitro} testing have been set up and will take place in the future. This will allow our group to determine if it is feasible to use these compounds as TMIAs.

The results and data from this project not only show the synthesis of many novel TMIAs, but also presents the framework for synthesizing other multi-modal imaging agents using a modular synthetic approach. Using amino acids with imaging agents attached to their side chains is a method that has proven to be effective through this research project and this method could be used in a variety of different imaging applications.
5. Experimental Procedures

Chemicals were purchased from Acros Organics (Morris Plains, NY), Alfa Aesar (Ward Hill, MA), Sigma Aldrich (St. Louis, MO), TCI (Tokyo, Japan), and VWR (Randnor, PA) and were used as received unless stated otherwise. All chemicals were either American Chemical Society (ACS) or HPLC grade. Amino acid starting materials were purchased from Bachem (Bubendorf, Switzerland) and Chem-Impex International Inc. (Wood Dale, IL). DOTA was purchased from Macroyclics (Houston, TX).

The HPLC instrument used was an Agilent 1100 with Diode Array Detector and for LC-MS a Waters 2695 Alliance HPLC with a Waters 2998 Diode Array Detector and a Waters 3100 SQ Mass Spectrometer was used. For HPLC the columns used were: an Agilent XDB C18 column, with dimensions of 3 mm x 100 mm or a Waters XBridge C18 column 50 mm x 3 mm and 3µ particle size. Mass spectra from this instrument were recorded at unit resolution with positive and negative switching mode at 35 or 50 V cone voltages. The flow rate for HPLC-MS was 0.5 mL/min. All aqueous mobile phases for HPLC are 0.01M ammonium acetate unless otherwise noted. Preparative HPLC (prep-HPLC) was carried out with a Waters 600E system controller, and Waters 600 multi-solvent delivery system using a 30 mL/min flow rate.

For SPE purification, a 20 g C-18 Sep-pack Varian Mega Bond Elut (20CC/5GRM) SPE cartridge was utilized for DCL (7) and Gd/La DOTA compounds, and the DOTA transmetalations utilized a Varian Bond Elut (C18, 12CC/2GRM) SPE cartridge.

Transmetalation studies were monitored using a Shimadzu UHPLC-MS with a Nexera X2 UHPLC, and LCMS-2020 Mass Spectrometer. A Phenomenex Kinetex EVO C18 150 x 2.1mm column with a 1.7µ particle size was used to assay all transmetalation reactions and purifications. The flow rate for UHPLC-MS was 0.4 mL/min. The aqueous mobile phase for UHPLC was 0.01M ammonium acetate and organic phase was methanol with the gradient described below and in each experimental section.

Aqueous mobile phases for SPE are not buffered unless otherwise noted. The SPE cartridges were conditioned with their respective organic solvent, then pure DI H2O, then equilibrated with the initial gradient concentration. Gradients were performed in 5% increments with 3-10 mL fractions each unless otherwise noted.

High resolution mass spectra (HRMS) were obtained on a Waters Synapt G2Si (School of Chemical Sciences, University of Illinois at Urbana-Champaign) using the following parameters: Flow injection at flow rate of 0.1 ml/min, H2O/ACN/0.1% Formic Acid, positive and negative mode ESI, Cone voltage = 25, capillary voltage = 3.0, ion source temperature = -100°C, desolation temperature =180°C, nebulizing gas (N2) flow = 200 L/h, cone gas (N2) flow = 5L/h.
F-dK(La-DOTA)-OH (1) DOTA (493.6 mg, 0.96 mmol, Macrocyclics) was stirred into suspension in a solution of NMM (1124.1 mg, 11.1 mmol) in anhydrous DMF (50 mL). This solution was heated briefly to promote solvation, then allowed to cool back to room temperature. A solution of TBTU (356.9 mg, 1.11 mmol) in DMF (10 mL) was added to the reaction flask over 30 minutes with the use of a syringe pump, followed by the addition of a solution of Fmoc-dLys(H)-OH (300.0 mg, 0.74 mmol) in the same manner. Immediately following the dropwise addition of this reagent, La(NO₃)₃ (481.5 mg, 1.48 mmol) was added to the reaction flask as a crystalline salt. The reaction was stirred another 25 minutes. Crude product was precipitated from the solution by the addition of ethyl ether and isolated by decanting the organic solvent after centrifugation. Purification by SPE (ACN/H₂O, 10-60%) yielded the desired product. Yield: 47.4 mg (7%). MS (HR, ESI) calc. for C₃₇H₄₇LaN₆O₁₁ 890.23663, found 891.2431 [M+H]+.

F-K(Gd-DOTA)-NH₂ (2) Commercially-available DOTA (692.3 mg, 1.35 mmol) was stirred into suspension in a solution of NMM (1576.6 mg, 15.6 mmol) in anhydrous DMF (50 mL). This solution was heated briefly to promote solvation, then allowed to cool back to room temperature. A solution of TBTU (667.32 mg, 2.08 mmol) in DMF (10 mL) was added to the reaction flask over 30 minutes with the use of a syringe pump, followed by the addition of a solution of Fmoc-Lys(H)-NH₂ (500.0 mg, 1.04 mmol) in the same manner. Immediately following the dropwise addition of this reagent, Gd(OAc)₃ (844.6 mg, 2.08 mmol) was added to the reaction flask as a crystalline salt. The reaction was stirred another 25 minutes. Crude product was precipitated from the solution by the addition of ethyl ether and isolated by decanting the organic solvent after centrifugation. Purification by SPE (ACN/H₂O, 10-60%) yielded the desired product. Yield: 105.2 mg (11%). MS (HR, ESI) calc. for C₃₇H₄₈GdN₇O₁₀ 908.27037, found 909.2765 [M+H]+.

F-dK(Gd-DOTA)-OH (3) Commercially-available DOTA (493.8 mg, 0.964 mmol) was stirred into suspension in a solution of NMM (1124.5 mg, 11.1 mmol) in anhydrous DMF (50 mL). This solution was heated briefly to promote solvation, then allowed to cool back to room temperature. A solution of TBTU (357.0 mg, 1.11 mmol) in DMF (10 mL) was added to the reaction flask over 30 minutes with the use of a syringe pump, followed by the addition of a solution of Fmoc-dLys(H)-OH (300.1 mg, 0.741 mmol) in the same manner. Immediately following the dropwise addition of this reagent, Gd(OAc)₃ (495.7 mg, 1.48 mmol) was added to the reaction flask as a crystalline salt. The reaction was stirred another 25 minutes. Crude product was precipitated from the solution by the addition of ethyl ether and isolated by decanting the organic solvent after centrifugation. Purification by SPE (ACN/H₂O, 10-60%) yielded the desired product. Yield: 146.8 mg (22%). MS (HR, ESI) calc. for C₃₇H₄₇GdN₆O₁₁ 909.25439, found 910.2621 [M+H]+.

F-dK(Cy5.5 3S)-OH (4) Commercially available Fmoc-dLys(H)-OH (28.0 mg, 0.0692 mmol) was dissolved in NMP (3 mL) the solution was cooled to 0°C. To this solution was added DIPA (89.37 mg, 0.692 mmol) followed by the Cy 5.5 3S-NHS ester. The reaction was brought to room temperature and was stirred for 1 hour. Crude product was precipitated by the addition of diethyl ether, and the organic layer was decanted after centrifugation. Pure product was obtained by preparatory HPLC (ACN/0.01% AmAc, 5-
Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 14.8 mg, 35%.

H-K(Gd-DOTA)-NH$_2$ (5) Compound 2 (31.1 mg, 0.034 mmol) was dissolved in anhydrous DMF (5 mL). DEA (50.1 mg, 0.69 mmol) was added to this solution. The reaction stirred under inert atmosphere one hour. Crude product was precipitated by addition of ethyl ether to the reaction solution, and organic solvent was decanted after centrifugation. This process was repeated with ethyl acetate. Product was dried under vacuum. Yield: 23.5 mg (100%). MS (HR, ESI) calc. for C$_{22}$H$_{37}$GdN$_7$O$_8$ 686.20229, found 687.2101 [M+H]$^+$.

F-dK(Cy5.5 3S)-K(Gd-DOTA)-NH$_2$ (6) Compound 4 (10.0 mg, 8.23x10$^{-3}$ mmol) was dissolved in DMF (10 mL) and to this solution was added DIPA (10.63 mg 0.0823 mmol) and TSTU (4.95 mg, 0.0165 mmol). This reaction stirred under argon 1 hour and was monitored by HPLC-MS after treating a sample with 0.1% aqueous butylamine. Upon completion of this portion of the reaction, crude intermediate product was precipitated by the addition of EtOAc and diethyl ether, and after centrifugation the organic layer was decanted and the crude solid dried under argon. Compound 5 (7.34 mg, 0.0107 mmol) was dissolved in DMSO (1 mL). To this solution was added DIPA (10.63 mg, 0.0823 mmol), followed by the previously isolated product which had been reconstituted in DMF (5 mL). The reaction ran for 2.5 hours. Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation. Pure product was obtained by SPE (MeOH/H$_2$O, 10-65%). Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 15.5 mg, 100%. MS (HR, ESI) calc. for C$_{86}$H$_{106}$GdN$_{11}$O$_{21}$S$_3$ 1882.59624, found 942.3064 [M+2H]$^2^+$/2.

H-dK(Cy5.5 3S)-K(Gd-DOTA)-NH$_2$ (7) Compound 6 (15.0 mg , 7.96x10$^{-3}$ mmol) was dissolved in DMF (3 mL) and to this solution was added diethylamine (11.7 mg, 0.159 mmol). Reaction ran for 3 hours. Crude product was precipitated by the addition of diethyl ether. This was centrifuged and the organic layer decanted; the crude solid was triturated with EtOAc to remove Fmoc byproduct, and was again centrifuged and the organic layer, decanted. Yield: 13.2 mg, 100%. MS (HR, ESI) calc. for C$_{71}$H$_{96}$GdN$_{11}$O$_{19}$S$_3$ 1660.52816, found 831.2723 [M+2H]$^2^+$/2.

DCL-DSS-dK(Cy5.5 3S)-K(Gd-DOTA)-NH$_2$ (8) Compound 7 (13.2 mg, 7.95x10$^{-3}$ mmol) was dissolved in DMF (2 mL) and to this solution was added TEA (20.1 mg, 0.199 mmol). DCL-DSS (9.09 mg, 0.0159 mmol) was dissolved in DMSO (0.2 mL) and added to the reaction. Reaction was left to stir. After 2.5 hours, TEA (12.1 mg, 0.119 mmol) was added. After 4 hours, additional TEA was added (8.0 mg, 0.0796 mmol). The reaction was left to react for 12 hours. DCL-DSS (18.18 mg, 0.0318 mmol) was added after being dissolved in DMSO (0.462 mL). Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation. Pure product was obtained by preparatory HPLC (ACN/H$_2$O, 5-100%). Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 11.3 mg, 67%. MS (HR, ESI) calc. for C$_{91}$H$_{127}$GdN$_{14}$O$_{28}$S$_3$ 2117.73419, found 1059.8750 [M+2H]$^2^+$/2.
F-dK(La-DOTA)-K(Gd-DOTA)-NH₂ (9) Compound 1 (32.6 mg, 3.66x10⁻² mmol) was dissolved in NMP (2.5 mL) and to this solution was added DIPA (47.3 mg, 0.366 mmol) and TSTU (22.0 mg, 7.32 x10⁻² mmol). This reaction stirred under argon for 1 hour and was monitored by HPLC-MS after treating a sample with 0.1% aqueous butylamine. Upon completion of this portion of the reaction, crude intermediate product was precipitated by the addition of EtOAc and diethyl ether, and after centrifugation the organic layer was decanted and the crude solid dried briefly under argon. Compound 5 (41.4 mg, 6.04x10⁻² mmol) was dissolved in 1.5 mL DMSO. To this solution was added triethylamine (74.04 mg, 0.732 mmol), followed by the previously isolated crude product which had been reconstituted in NMP (2 mL). This reaction ran for 1 hour. Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation. Pure product was obtained by SPE (ACN/H₂O, 10-60%). Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 18.8 mg, 33%. MS (HR, ESI) calc. for C₅₉H₈₃GdLaN₁₃O₁₈ 1558.42836, found 780.223 [M+2H]²⁺/2.

H-dK(La-DOTA)-K(Gd-DOTA)-NH₂ (10) Compound 9 (18.8 mg, 1.21x10⁻² mmol) was dissolved in DMF (3 mL) followed by the addition of diethylamine (17.6 mg, 0.24 mmol). Reaction ran for 1 hour. Crude product was precipitated by the addition of diethyl ether. This was centrifuged and the organic layer decanted; the crude solid was triturated with EtOAc to remove Fmoc byproduct, and was again centrifuged and the organic layer, decanted. Yield: 14.8 mg, 92%. MS (HR, ESI) calc. for C₄₄H₇₃GdLaN₁₃O₁₆ 1336.36028, found 1337.369 [M+H]+.

c(RGDyK)-DSS (11) Commercially purchased c(RGDyK) (6.97 mg, 0.0112 mmol) was dissolved into DMF (1 mL) and to this solution was added TEA (5.69 mg, 0.0562 mmol). This solution was added dropwise to a separate solution of DSS (4.56 mg, 0.0124 mmol) that was previously dissolved in DMF (1 mL). Yield: 9.82 mg 100%. MS (HR, ESI) calc. for C₃₉H₅₆N₁₀O₁₃ 872.40283, found 873.4103 [M+H]+.

c(RGDyK)-DSS-dK(La-DOTA)-K(Gd-DOTA)-NH₂ (12) Compound 10 (5.00 mg, 3.74x10⁻³ mmol) was dissolved in DMF (2 mL) and to this solution was added TEA (9.47 mg, 0.0935 mmol). Compound 11 (6.53 mg, 7.4x10⁻³ mmol) was dissolved in DMSO (1 mL) and added to the reaction. Reaction was left to stir. After 1 hour, TEA (3.79 mg, 0.0374 mmol) was added. After 3 hours, additional TEA (3.79 mg, 0.0374 mmol) was added. The reaction was left to reaction for 12 hours following the addition of DMSO (1 mL). Butylamine (2.19 mg, 0.0299 mmol) was added to the reaction to quench remaining compound 11. Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation. Yield: 4.1 mg, 52%. MS (HR, ESI) calc. for C₇₉H₁₂₄GdLaN₂₂O₂₆ 2093.736169, found 1047.8777 [M+2H]²⁺/2.

c(RGDyK)-DSS-dK(Cu-DOTA)-K(Gd-DOTA)-NH₂ (13) Compound 12 (0.91 mg, 4.35x10⁻⁴ mmol) was dissolved in aqueous TFA (0.2 M, 1 mL). This solution was stirred and monitored via UPLC-MS until all of the lanthanum was displaced. After 4 days, CuSO₄ (0.69 mg, 4.35x10⁻⁶ mmol) was dissolved in H₂O (0.2 mL) and added to the solution. The reaction was stirred for 30 min. Additional CuSO₄ (1.4 mg, 8.70x10⁻⁶
61 mmol) was added to the reaction in H₂O (0.2 mL). Reaction was stirred for an additional 30 min. Sodium bicarbonate (0.1 M) was added dropwise until the solution was neutral. Pure product was obtained by SPE (ACN/0.05% aq. TFA, 2-25%). Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 0.68 mg, 77%. MS (HR, ESI) calc. for C₇₉H₁₂₄CuGdN₂₂O₂₆ 2017.75832, found 673.9305 [M+3H]+/3.

c(RGDyK)-DSS-dK(Ga-DOTA)-K(Gd-DOTA)-NH₂ (14) Compound 12 (0.88 mg, 4.20x10⁻⁴ mmol) was dissolved in aqueous TFA (0.2 M, 1 mL). This solution was stirred and monitored via UPLC-MS until all of the lanthanum was displaced. After 24 hours, this compound was concentrated by rotary evaporation. The compound was dissolved in distilled H₂O (1 mL). Ga(NO₃)₃•H₂O (3.21 mg, 0.0126 mmol) was dissolved in H₂O (0.2 mL) and added to the solution. The reaction was stirred for 1 hour. Sodium bicarbonate (0.1 M) was added dropwise until the solution was neutral. Pure product was obtained by SPE (ACN/0.05% aq. TFA, 4-25%). Fractions containing pure product were collected, concentrated by rotary evaporation and freeze dried. Yield: 0.85 mg, 100%. MS (HR, ESI) calc. for C₇₉H₁₂₄GaGdN₂₂O₂₆ 2023.75429, found 675.5933 [M+3H]+/3.

F-dK(Gd-DOTA)-K(Gd-DOTA)-NH₂ (15) Compound 3 (41.7 mg, 0.0459 mmol) was dissolved in NMP (5 mL) and to this solution was added DIPA (118.6 mg, 0.917 mmol) and TSTU (27.6 mg, 0.0917 mmol). This reaction stirred under argon 1 hour and was monitored by HPLC-MS after treating a sample with 0.1% aqueous butylamine. Upon completion of this portion of the reaction, crude intermediate product was precipitated by the addition of EtOAc and diethyl ether, and after centrifugation the organic layer was decanted and the crude solid dried briefly under argon. Compound 5 (31.47 mg, 0.0459 mmol) was dissolved in NMP (1 mL). To this solution was added DIPA (118.59 mg, 0.918 mmol), followed by the previously isolated product which had been reconstituted in NMP (1 mL). The reaction ran for 3 hours. Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation. Pure product was obtained by SPE (ACN/H₂O, 10-60%). Fractions containing pure product were collected, concentrated by rotary evaporation, and freeze dried. Yield: 23.6 mg, 33%. MS (HR, ESI) calc. for C₅₉H₈₃Gd₂N₁₃O₁₈ 1577.44501, found 789.7323 [M+2H]²⁺/2.

H-dK(Gd-DOTA)-K(Gd-DOTA)-NH₂ (16) Compound 15 (23.6 mg, 0.015 mmol) was dissolved in NMP (2 mL) followed by the addition of diethylamine (32.8 mg, 0.449 mmol). Reaction ran one hour. Crude product was precipitated by the addition of diethyl ether. This was centrifuged and the organic layer decanted; the crude solid was triturated with EtOAc to remove Fmoc byproduct, and was again centrifuged and the organic layer, decanted. Yield: 20.3 mg, 100%. MS (HR, ESI) calc. for C₄₄H₇₃Gd₂N₁₃O₁₈ 1355.37693, found 1356.3833 [M+H]+.

c(RGDyK)-DSS-dK(Gd-DOTA)-K(Gd-DOTA)-NH₂ (17) Compound 16 (1.27 mg, 9.35x10⁻⁴ mmol) was dissolved in DMF (2 mL) and to this solution was added TEA (2.36 mg, 0.0234 mmol). Compound 11 (1.63 mg, 1.87x10⁻³ mmol) was dissolved in DMSO (0.4 mL) and added to the reaction. The reaction was set to stir. After 1 hour, TEA (4.36 mg, 0.043 mmol) was added to increase the basicity of the solution. Additional 16 (1.27
mg, 9.35x10^{-4} \text{ mmol}) was added. It was determined via HPLC-MS that the coupling reaction was not progressing. DSS (4.19 mg, 0.0114 mmol) was added to the reaction mixture. The reaction was heated to 40°C and stirred for 24 hours. Remaining DSS was removed after precipitating solid by the addition of ethyl ether, and the organic layer was decanted after centrifugation. This process was repeated with cold ethyl acetate, followed by warm ethyl acetate. The solid was dried under argon. The following day, the solid was dissolved in a 1:1 solution of DMSO and DMF (2 mL). c(RGDyK) (6.41 mg, 0.0103 mmol) was added along with TEA (26.15 mg, 0.258 mmol). The solution was stirred for 3 hours. Upon completion of the reaction, crude product was precipitated by the addition of EtOAc and diethyl ether, and after centrifugation the organic layer was decanted and the crude solid dried under argon. Pure product was obtained by SPE (ACN/0.05% aq. TFA, 4-25%). Fractions containing pure product were collected, concentrated by rotary evaporation and freeze dried. Yield: 14.1 mg, 65%. MS (HR, ESI) calc. for C_{79}H_{124}CuGdN_{22}O_{26} 2017.75832, found 673.9305 [M+3H]^3+/3.

F-dK(Cy5.5 3S)-dK(La-DOTA)-K(Gd-DOTA)-NH_{2} (18) Compound 4 (14.8 mg, 1.22x10^{-2} \text{ mmol}) was dissolved in DMF (10 mL) and to this solution was added DIPA (15.74 mg 0.122 mmol) and TSTU (7.33 mg, 0.0244 mmol). This reaction stirred under argon 1 hour and was monitored by HPLC-MS after treating a sample with 0.1% aqueous butylamine. Upon completion of this portion of the reaction, crude intermediate product was precipitated by the addition of EtOAc and diethyl ether, and after centrifugation the organic layer was decanted and the crude solid dried briefly under argon. Compound 10 (4.79 mg, 3.58x10^{-3} \text{ mmol}) was dissolved in DMSO (0.5 mL). To this solution was added TEA (7.25 mg, 0.0717 mmol), followed by the previously isolated product which had been reconstituted in NMP (2 mL). The reaction ran for 1 hour. Crude product was precipitated by the addition of ethyl ether, and the organic layer was decanted after centrifugation.
References


(49) Medical Hybrid Imaging System Market Research on Medical Hybrid Imaging System Market 2019 and Analysis to 2025 – Monroe Scoop.


Appendix I. HPLC-MS and HRMS Results

**Compound 1 F-dK(La-DOTA)-OH**

Single wavelength chromatogram of 1 at 263 nm for crude reaction mixture

UV-Vis of 1 spectrum at 1.6 min with characteristic peak at 263 nm for Fmoc protecting group

Mass spectrum of 1 at 1.6 min, 891.66 m/z [M+H]^+
HRMS of pure 1 product, 891.2431 m/z [M+H]^+
Compound 2 F-K(Gd-DOTA)-NH₂

Single wavelength chromatogram of 2 at 263 nm for crude reaction mixture

UV-Vis spectrum of 2 at 3.6 min with characteristic peak at 263 nm for Fmoc protecting group

Mass spectrum of 2 at 3.6 min, 909.96 m/z [M+H]⁺
Expanded parent ion from spectrum above, displaying Gd isotope pattern of 2

HRMS of pure 2 product displaying Gd isotope pattern, 909.2765 m/z [M+H]^+
Compound 3 F-dK(Gd-DOTA)-OH

Single wavelength chromatogram of 3 at 263 nm for crude reaction mixture

UV-Vis spectrum of 3 at 1.6 min with characteristic peak at 263 nm for Fmoc protecting group

Mass spectrum of 3 at 1.6 min, 909.51 m/z [M+H]^+
Expanded parent ion from spectrum above, demonstrating Gd isotope pattern of 3

HRMS of pure 3 product displaying Gd isotope pattern, 910.2621 m/z [M+H]^+
Compound 4 F-dK(Cy5.5 3S)-OH

Single wavelength chromatogram of 4 at 680 nm for pure product

UV-Vis spectrum of 4 at 5.3 min with characteristic peak around 684 nm for Cy 5.5 dye

Mass spectrum of 4 at 5.3 min, 1214.65 m/z [M-H]+, 606.57 m/z [M-2H]²/²
Compound 5 H-K(Gd-DOTA)-NH$_2$

UV-Vis spectrum of 5 at 1.1 min, demonstrating lack of characteristic Fmoc absorption

XIC for 5 of mass range 685 to 687 m/z

Mass spectrum of 5 at 1.1 min, 685.4 m/z [M+H]$^+$
Expanded parent ion of 5 from spectrum above, displaying Gd isotope pattern.

HRMS of pure 5 product displaying Gd isotope pattern, 687.2101 m/z [M+H]^+.
**Compound 6 F-dK(Cy5.5 3S)-K(DOTA)-NH₂**

Single wavelength chromatogram of 6 at 680 nm for purified product

**UV-Vis spectrum of 6 at 3.4 min with characteristic peak around 683 nm for Cy 5.5 dye**

**Mass spectrum of 6 at 3.4 min, 943.32 m/z [M+2H]^{2+}/2**
Expanded parent ion of 6 from spectrum above, displaying Gd isotope pattern

HRMS of pure 6 product, 942.3064 m/z [M+2H]^2+/2
Compound 7 H-dK(Cy5.5 3S)-K(Gd-DOTA)-NH2

Single wavelength chromatogram of 7 at 680 nm showing product

UV-Vis spectrum of 7 at 1.8 min with characteristic peak around 683 nm for Cy 5.5 dye

Mass spectrum of 7 at 1.8 min, 831.52 m/z [M+2H]^{2+}/2
Expanded parent ion of 7 from spectrum above, demonstrating Gd isotope pattern

HRMS of pure 7 product displaying Gd isotope pattern, 831.2723 m/z [M+2H]^2+/2
Compound 8 DCL-DSS-dK(Cy5.5 3S)-K(Gd-DOTA)-NH₂

Single wavelength chromatogram of 8 at 680 nm for pure product

UV-Vis spectrum of 8 at 2.9 min with characteristic peak around 683 nm for Cy 5.5 dye
Mass spectrum of 8 at 2.9 min, 1058 m/z [M-2H]²⁻, 705 m/z [M-3H]³⁻/3

HRMS of pure 8 product showing Gd isotope pattern, 1059.8750 m/z [M+2H]²⁻/2
Compound 9 F-dK(La-DOTA)-K(Gd-DOTA)-NH₂

Single wavelength chromatogram of 9 on UPLC-MS at 263 nm for pure product

UV-Vis spectrum of 9 at 3.3 min with characteristic peak at 265 nm for Fmoc protecting group
Mass spectrum of 9 at 3.3 min, 1560 m/z [M+H]^+, 780 m/z [M+2H]^{2+}/2

HRMS of pure 9 product showing Gd isotope pattern 780.2230 m/z [M+2H]^{2+}/2
Compound 10 H-dK(La-DOTA)-K(Gd-DOTA)-NH₂

UV-Vis spectrum of 10 at 0.5 min, demonstrating lack of characteristic Fmoc absorption

XIC of crude reaction mixture for 10 showing conversion of starting material (780 m/z) to product (669 m/z)
Mass spectrum of 10 at 0.5 min, 669 m/z [M+2H]^2+/2

HRMS of pure 10 product displaying Gd isotope pattern 1337.3690 m/z [M+H]^+
Compound 11 c(RGDyK)-DSS

XIC of mass range 832 to 833 m/z for compound 11

Mass spectrum of 11 in 0.1% aq. butylamine at 3.0 min, 832.19 m/z butylamine adduct of product, 416.83 half mass of butylamine adduct
HRMS of pure 11 product, 873.4103 m/z [M+H]^+
Compound 12 c(RGDyK)-DSS-dK(La-DOTA)-K(Gd-DOTA)-NH₂

XIC of mass range 1047 to 1050 m/z for compound 12

Mass spectrum of 12 at 3.8 min, 1046.96 m/z [M+2H]^{2+}, 698.63 m/z [M+3H]^{3+}/3

Expanded parent ion of 12 from spectrum above, displaying Gd isotope pattern
HRMS of pure 12 product displaying Gd isotope pattern, 1047.8730 m/z [M+2H]^+/2
Compound 13 c(RG\text{DyK})-DSS-dK(Cu-DOTA)-K(Gd-DOTA)-NH$_2$

XIC of mass 674 m/z for compound 13

Mass spectrum of 13 at 1.4 min, 1011 m/z [M+2H]$^{2+}$, 674 m/z [M+3H]$^{3+}$/3
HRMS of pure 13 product displaying Gd isotope pattern, 673.5975 m/z [M+3H]^{3+}/3
Compound 14 c(RGDiK)-dK(Ga-DOTA)-K(Gd-DOTA)-NH$_2$

XIC of mass 676 m/z for compound 14

Mass spectrum of 14 at 0.9 min, 1014 m/z [M+2H]$^{2+}$, 676 m/z [M+3H]$^{3+}$/3
HRMS of pure product 14, 675.5933 [M+3H]^{3+}/3
Compound 15 F-dK(Gd-DOTA)-K(Gd-DOTA)-NH₂

Single wavelength chromatogram of 15 at 263 nm for crude reaction mixture

UV-Vis spectrum of 15 at 2.9 min with characteristic peak around 263 nm for Fmoc protecting group

Mass spectrum of 15 at 2.9 min, 1577.97 m/z [M+H]⁺, 789.71 m/z [M+2H]²⁺/2
Expanded parent ion of 15 from spectrum above, displaying Gd isotope pattern

HRMS of pure 15 product displaying Gd isotope pattern, 789.7323 m/z [M+2H]^2+
UV-Vis spectrum of 16 at 1.5 min, demonstrating lack of characteristic Fmoc absorption

XIC of mass range 678 to 680 m/z for compound 16

Mass spectrum of 16 at 1.5 min, 678.19 m/z [M+2H]^2+/2
Expanded parent ion from spectrum above of 16, displaying Gd isotope pattern

HRMS of pure 16 product displaying Gd isotope pattern, 1356.3833 m/z [M+H]⁺
Compound 17 c(RGDyK)-DSS-dK(Gd-DOTA)-K(Gd-DOTA)-NH₂

XIC of mass range 1057 to 1059 m/z for compound 17

Mass spectrum of 17 at 1.0 min, 1057.46 m/z [M+2H]^{2+}, 705.03 m/z [M+3H]^{3+}/3

Expanded parent ion of 17 from spectrum above, displaying Gd isotope pattern
HRMS of pure 17 product displaying Gd isotope pattern, 1057.3828 m/z \([\text{M+2H}]^{2+}\)/2
Compound 18 F-dK(Cy5.5 3S)-dK(La-DOTA)-K(Gd-DOTA)-NH₂

Single wavelength chromatogram of 18 at 684 nm showing product in crude reaction mixture. The product 18 is the peak at 4.89 minutes.

UV-Vis spectrum of 18 at 4.9 min, showing absorption peak for Cy5.5 dye

XIC of compound 18, the product is the peak at 4.88 min
ESI mass spectra of product peak at 4.9 min at showing product 18 at 1265.73, [M+2H]^{2+}/2

Expanded region of ESI mass spectra of 18 showing Gd isotope pattern

Mass spectrum of 18 at 4.8 min, 1268.22 m/z [M+2H]^{2+} displaying Gd isotope pattern
Mass spectrum of 18 at 4.8 min, 845.86 m/z [M+3H]^3+ displaying Gd isotope pattern. This result taken with the result from [M+2H]^2+ is evidence that the compound 18 was indeed formed in the final coupling reaction.