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DEVELOPMENT OF LUMINESCENT RUTHERNIUM COMPLEXES FOR IN-VITRO FLUORESCENCE IMAGING OF ANGIgenesis WITH THE RGD PEPTIDE.

by

ROSMERY VICTORIA

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biology in the College of Sciences and in The Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Charles R. Hinkle
ABSTRACT

Herein we report the synthesis of an RGD-ruthenium bipyridine [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$ complex aimed at the detection of angiogenesis. Angiogenesis plays a critical role in many pathophysiological processes, such as tumor growth. The $\alpha_v$-integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) are currently used as molecular targeting sites for anti-angiogenic therapies. The [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$ complex is an organometallic luminescent probe, which enables noninvasive, in vitro imaging of $\alpha_v\beta_3$ expression. Peptides containing the arginine-glycine-aspartic acid (RGD) sequence have been shown to bind strongly to the $\alpha_v\beta_3$ integrin. The RuBpy probes are soluble in water, display long lifetimes, and are photochemically stable. These properties enable the Ru(tris-bpy) complexes to be useful in numerous applications in biophysical and cell biology. The [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$ complex was synthesized by combining the succinimidyl ester on the RuBpy complex with the lysine of the c(RGDfK) peptide. The results of the one-photon fluorescence bioimaging showed selective binding of the cyclic RGD to $\alpha_v\beta_3$ integrin, which supports previous literature. The high luminescence intensity, long lifetimes, and low cell toxicity levels of dye [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$, illustrates the potential usage of this probe for future biological applications.
DEDICATION

To all of those who have been affected by cancer.
ACKNOWLEDGEMENTS

An undertaking of this magnitude is not possible without the assistance of several important individuals. I would like to express my deepest appreciation to my advisors Dr. Kevin D. Belfield, Dr. Andy Frazer, and Dr. Alma Morales. Each of you has provided me with a tremendous amount of guidance and support.

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CHAPTER 1: BACKGROUND

Cancer is a disease that may lead to death due to uncontrolled cell proliferation that creates masses inside the body known as tumors. Not all tumors are malignant, but given time these abnormal growths can metastasize, spread to other organs, and kill nearby healthy tissue. These characteristics differentiate cancers from benign tumors. When tumors are detected early the chances of survival are tremendously increased (Figure 1). However, most cancer patients are non-symptomatic, and by the time a diagnosis is reached the tumor may have become cancerous.

Figure 1. Graph shows the correlation between survival rate and diagnosis at different cancer stages.⁹
In the early 1970s, it was suggested that tumor development and metastasis are dependent on angiogenesis, the process of forming new capillaries from pre-existing blood vessels. It has since been demonstrated that angiogenesis plays a critical role in many pathophysiological processes, for example: tumor growth, metastasis, rheumatoid arthritis, embryonic development, atherosclerosis, and post-ischemic vascularization of the myocardium. Therefore, detection of new pro-angiogenic, as well as anti-angiogenic molecules is vital for biomedical research.

Integrins are transmembrane receptor proteins that interconnect the extracellular matrix and the cytoskeleton. Integrins are typically made of $\alpha$ and $\alpha\beta$ subunits. The $\alpha_R\beta_3$-integrins ($\alpha_R\beta_3, \alpha_R\beta_5$) are currently used as molecular targeting sites for anti-angiogenic therapies.

Detection of $\alpha_R\beta_3$ integrin that regulates angiogenesis is a major concern facing cancer researchers. A short peptide sequence arginine-glycine-aspartic acid (RGD) has been shown to selectively bind to endothelial tumor cells overexpressing $\alpha_R\beta_3$ integrin. Attachment of a luminescent probe to an RGD containing peptide will enable noninvasive, in vitro, and perhaps, in vivo, imaging of $\alpha_R\beta_3$ expression.

One compound that has been explored as such a probe is ruthenium (II), the most stable oxidative state of ruthenium. Ruthenium is an uncommon transition metal. Similar to most metals, ruthenium is largely inert. Ruthenium complexes have unique characteristics which make them useful in numerous applications in biophysical chemistry, clinical chemistry, and DNA diagnostics. Advantages of these metal probes are their display of high chemical and photochemical stability, as well as its high luminescence, long lifetimes, and reasonable solubility in water.
Ruthenium complexes are generally luminescent. Luminescence is the release of light (photons) by a substance.\textsuperscript{6} It occurs during the return of electrons to its ground state from the first singlet excited state.\textsuperscript{6} Fluorescence is the term used for emission of light for an excited singlet state while phosphorescence refers to emission of light from a triplet state, the ground state singlet state in both cases.\textsuperscript{6} In order for photons to be released the substance must first absorb a photon to reach an excited state.\textsuperscript{6}

A fluorophore refers to the chromoporic unit in a molecule that leads to light absorption and emission.\textsuperscript{6} Different fluorophores absorb photons at different wavelengths (\(\lambda\)). A molecule’s absorption is indicated by the wavelength of the molecule in a particular solvent. On average, the absorption of a photon takes roughly \(10^{-15}\) s.\textsuperscript{11} Conversely, a molecule’s emission is affected by its excited state energy, solvent, and is indicated by the wavelength in which photons are emitted by the molecule. In order for emission to occur, the molecule needs to be excited at a specific wavelength, which is characterized by the maximum wavelength (\(\lambda_{\text{max}}\)) of absorption.

A Jablonski diagram (Figure 2) is a diagram that shows the electronic states of a molecule and the intermediate vibrational and rotational states of each electronic state.\textsuperscript{11} Electrons aspire to get to the ground level, \(S_0\), where they are stable. When a substance is excited there are different energy levels to which the electrons can jump to, known as electronic transitions, \(S_1, S_2,\ldots S_n\).\textsuperscript{11} Rotational and vibrational levels are found between the electronic transitions levels. The excited electrons can lose their energy through vibrational relaxation (\(10^{-12}\) - \(10^{-10}\) s), a non-radiative process in which an electron loses energy to the surroundings or through internal conversion between vibrational levels. Internal conversion is another type of
non-radiative process, where an excited molecule loses its energy from one electronic state to another. Internal conversion takes about $10^{-14} - 10^{-10}$ s.\textsuperscript{11}

\begin{center}
\textbf{Figure 2.} A Jablonski diagram.\textsuperscript{11}
\end{center}

Once a photon is absorbed by a molecule, an electron is promoted from the ground state to an excited state. Soon after excitation, electrons proceed down to their lowest closest electronic transition level by internal conversion through molecular rotations or vibrations.\textsuperscript{11} The desire for an electron to move down to its ground state gives it potential energy. The emission of a photon can occur as the electron drops back to its ground state. This is known as fluorescence if the transition is from an excited singlet state to the ground state singlet state.\textsuperscript{11}

When an electron undergoes internal conversion to the first electronic excited state, $S_1$, it can behave in two different ways. The first possibility is for the electron to lose energy, returning
from $S_1$ to the ground state ($S_0$) via a radiative (fluorescence) and/or non-radiative mechanism. Second, the electron has the ability to transfer to a triplet excited state.\textsuperscript{11} The conversion of an electron from $S_1$ to the triplet state and visa-versa is called intersystem crossing.\textsuperscript{11} During intersystem crossing the electron spin changes from antiparallel to parallel. In the triplet state, the electron is excited, but is slightly lower in energy compared to $S_1$.\textsuperscript{6} From the triplet state, the electron can either return to $S_1$ directly or fall to the ground state in a non-radiative or radiative fashion. This radiative energy loss is called Phosphorescence.\textsuperscript{6} Phosphorescence has a longer lifetime (10$^{-3}$ to 10$^2$s) as compared to fluorescence (10$^{-9}$ – 10$^{-7}$s) since changing the multiplicity of states (spin) forbidden process.\textsuperscript{11} If the electron goes back to $S_1$ and then falls to the ground state releasing photons, this is called delayed fluorescence.\textsuperscript{6} The lifetime of delayed fluorescence is also longer than fluorescence, again since changing the multiplicity of states (spin) forbidden process.\textsuperscript{6} Figure 3 shows a diagram of what happens to the electron during fluorescence, phosphorescence, and delayed fluorescence. Metal-ligand probes' have the ability to exhibit both phosphorescence and delayed fluorescence, due to its ability to experience metal to ligand charge transfer (MLCT) and, as a result, display long lifetimes.\textsuperscript{13} MLCT is an electronic transition that occurs between the metal to the ligand due to excitation.\textsuperscript{13} These long lifetimes allow the use of metal-ligand probes in time-gated detection.\textsuperscript{10} Additionally, metal-ligand probes can be used to suppress interfering autofluorescence, natural emission of light from biological samples; therefore, providing increased sensitivity.\textsuperscript{14}
Figure 3. (A) Fluorescence, (B) phosphorescence, and (C) delayed fluorescence diagrams.¹

Ruthenium tris(bipyridine), Ru(tris-bpy), is a well-known polypyridine complex. Polypyridine complexes are compounds in which a polypyridine attaches to a metal ion, like ruthenium. The photophysical properties of ruthenium (II) polypyridine complexes are very well characterized.¹³ Ru(tris-bpy) complexes display decay times ranging from 100 ns to 10 μs. Ru(tris-bpy) absorbs ultraviolet and visible light. In an aqueous solution it absorbs at 452 nm with an extinction coefficient of 11,500 M⁻¹cm⁻¹.⁵ Solutions of the resulting excited complex have comparatively long lifetimes.⁵ The excited state relaxes to the ground state by emission of a photon at a wavelength of ca. 600 nm.⁵

The specificity of a RGD-ruthenium bipyridine probe will enable the fluorescent labeling of sites where higher concentrations of the integrin are localized within cells, which could aid in the detection of small cancer tumors and, importantly, serve as markers of disease progression and the efficiency of anti-angiogenic therapy.²
Herein, is reported the synthesis of a long luminescent lifetime ruthenium complex, Ru(_tris-_bpy), and its subsequent conjugation to the cyclic peptide RGD, followed by linear characterization and cell bioimaging.
CHAPTER 2: EXPERIMENTAL SECTION

2.1 Materials

The following were purchased from ACROS or Sigma Aldrich: 4,4'-dimethyl-2,2'-bipyridyl, selenium(IV) oxide, 1,4-dioxane, ethyl acetate, sodium carbonate (Na$_2$CO$_3$), sodium metabisulfite anhydrous (Na$_2$S$_2$O$_3$), dichloromethane (CH$_2$Cl$_2$), sodium sulfate (Na$_2$SO$_4$), ethanol (95%), silver nitrate (AgNO$_3$), ultra-pure water, hydrochloric acid, acetic acid, sodium hydroxide pellets (NaOH), cis-bis(2,2'-bipyridine)dichlororuthenium(II) hydrate (Ru[(bpy)$_2$]Cl$_2$), ethanol (70%), hexafluorophosphoric acid (HPF$_6$), 60 wt.% solution in water, ammonium hexafluorophosphate (NH$_4$PF$_6$), N-methylmorpholine (NMM), methyl sulfoxide-d$_6$, for NMR, packaged in 1.00 mL ampoules, 99.9 atom % D, (CD$_3$)$_2$SO. N,N'-dicyclohexylcarbodiimide, DCC was distilled. N-Hydroxysulfosuccinimide sodium salt, (NHS) was dried en vacuo. Acetonitrile, ACN, was distilled and dried over calcium (II) hydride. 2-propanol (99.5+) was dried. Cyclo (Arg-Ala-Asp-D-Phe-Lys), RGD, was purchased from Peptides International, Louisville, Kentucky, U.S.A.).

Cell Lines. U87MG cells and MCF7 cells were purchased from America Type Culture Collection, ATCC, Manassas, VA, U.S.A. The incubation was done in Minimal Essential Medium (MEM) purchased from Life Technologies (Grand Island, New York, U.S.A.), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, U.S.A.) and 1% penicillin- streptomycin (Atlanta, GA, U.S.A.), and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO$_2$.

2.2 Measurements

Varian 500 MHz nuclear magnetic resonance (NMR) and a Bruker Avance III 400 MHz NMR spectrometers were used to measure $^1$H and $^{13}$C NMR spectra. The different solvents used
were CDCl₃, (CD₃)₂CO, (CD₃)₂SO, CD₃CN. The internal reference was tetramethysilane, TMS, at δ = 0.0 ppm. In order to predict and analyze the ¹H and ¹³C NMR spectra, CS ChemDraw Ultra version 11.0 and Mestrec software were utilized. High-resolution mass spectrometry (HRMS) analysis was performed at the University of Florida’s Department of Chemistry.

2.3 Synthesis

Details of the synthesis and characterization measurements of the intermediates and dye 1 can be found in the following section, Results and Discussion.

Synthesis of Intermediates

The intermediates (B, C, D, and E) have all been synthesized in the past. Slight modifications were done to previously published literature in order to produce compound E.

Synthesis of 4’-methyl-2,2’-bipyridine-4-carboxyaldehyde (B)

4’-Methyl-2,2’-bipyridine (A) was used for the synthesis of B. A mixture of A (10.54 g, 57.21 mmol) and SeO₂ (6.96 g, 62.73 mmol) in 1,4-dioxane (260 mL) was refluxed under nitrogen at slightly above 101 °C for 72 h. The reaction mixture was filtered hot and concentrated. The remaining solid was dissolved in ethyl acetate and then filtered. The filtrate was extracted with 1M Na₂CO₃ (4 x 100 mL) followed by 0.3M Na₂S₂O₅ (6 x 100 mL). The product was determined to be in the aqueous phase. Additional Na₂CO₃ was added to the combined aqueous extracts until a pH of 10 was achieved and then extracted with CH₂Cl₂ (for every 350 mL of aqueous extract use 5 x 400 mL). The organic phase was dried with Na₂SO₄ and purified by recrystallization using hexane to reveal a white powder (5.57 g, 49%): melting point 130-131 °C. ¹H NMR (500 MHz, (CD₃)₂SO) δ: 10.21 (s, 1H), 8.96 (d, J = 5 Hz, 1H), 8.80 (d, J = 4 Hz, 1H), 8.60 (d, J = 5 Hz, 1H), 8.28 (d, J = 20 Hz, 1H), 7.87 (d, J = 10 Hz, 1H), 7.36
(d, \( J = 10 \text{ Hz}, 1\text{H} \)) 2.47 (s, 3H). ¹³C NMR (500 MHz, (CD₃)₂SO) \( \delta \): 193.87, 157.39, 154.52, 151.11, 149.68, 148.70, 143.05, 125.95, 122.31, 121.80, 119.88, 21.15.

**Synthesis of 4’-Methyl-2,2’-bipyridine-4-carboxylic acid (C)**

A mixture of B (0.805 g, 4.06 mmol) with 95% ethanol (34.5 mL), AgNO₃ (0.72 g, 4.24 mmol), and H₂O (7.5 mL) was degassed under nitrogen for 15 min. 1M NaOH was then added dropwise. The reaction mixture was left stirring under nitrogen overnight. The ethanol was evaporated off and then AgNO₃ was filtered off and washed with 1.3 M NaOH (2 x 10 mL) and H₂O (10 mL). The filtrate was extracted with CH₂Cl₂ (2 x 100 mL). The aqueous phase was collected and acidified to a pH 3.5 using 1:1 (v/v) 4N HCl:AcOH. The acidic solution was placed in a freezer at -10°C overnight. The frozen solution was then left at room temperature, and the precipitate was filtered off and collected.¹⁵ Purified by recrystallization using ethyl acetate to reveal a white fluffy solid (0.444 g, 51%): melting point 279-280°C. ¹H NMR (400 MHz, (CD₃)₂SO) \( \delta \): 13.74 (broad s, 1H), 8.88 (d, \( J = 4 \text{ Hz}, 1\text{H} \)), 8.83 (d, \( J = 4 \text{ Hz}, 1\text{H} \)), 8.58 (d, \( J = 4 \text{ Hz}, 1\text{H} \)), 8.27 (d, \( J = 4 \text{ Hz}, 1\text{H} \)), 7.87 (d, \( J = 8 \text{ Hz}, 1\text{H} \)), 7.33 (d, \( J = 4 \text{ Hz}, 1\text{H} \)), 2.44 (s, 3H). ¹³C NMR (400 MHz, (CD₃)₂SO) \( \delta \): 166.03, 156.44, 154.10, 150.16, 149.19, 148.06, 139.22, 125.37, 122.63, 121.23, 119.42, 20.58.

**Synthesis of Ruthenium(II)-bis(2,2’-bypiridine)-(4’-Methyl-2,2’-bipyridine-4-carboxylic acid)-bis(hexafluorophosphate) (D)**

A mixture of Refluxed C (0.15 g, 0.70 mmol) and cis-dichlorobis(2,2-bipyridyl) ruthenium, Ru[(bpy)₂]Cl₂, (0.303g, 0.582 mmol) in 70% ethanol (30 mL) heated under nitrogen at slightly above 100 °C overnight. The reaction mixture was cooled to room temperature and filtered. The ethanol was evaporated off. The rest of the reaction mixture was left at room temperature overnight. Solid was removed by filtration. HPF₆ (60%) was added dropwise to the
filtrate to acidify to a pH 1 and form precipitate. A saturated solution of NH$_4$PF$_6$ until no more precipitation formed. Left at room temperature for 3 hours. Collected precipitate and washed with water (2 x 1 mL). The product is a dark red solid (0.417g, 95%). $^1$H NMR (500 MHz, CD$_3$CN) δ: 8.90 (s, 1H), 8.55-8.50 (m, 5 H), 8.06- 8.09 (m, 4H), 7.92-7.91 (d, $J = 5$ Hz, 1H), 7.78-7.71 (d, $J = 5$ Hz, 5H), 7.58-7.57 (d, $J = 5$ Hz, 1H), 7.43-7.38 (m, 4H), 7.29-7.28 (d, $J = 5$ Hz, 1H), 3.25 (very broad s, 1H), 2.56 (s, 3H). $^{13}$C NMR (500 MHz, CD$_3$CN) δ: 164.10, 155.78, 152.85, 151.78, 150.76, 137.99, 127.66, 125.74, 124.33, 123.10, 20.26.

**Synthesis of Ruthenium(II)-bis(2,2'-bypiridine)-(4'-Methyl-2,2'-bipyridine-4-carboxamido-methane)-bis(hexafluorophosphate) (E)**

Compound D (0.05 g, 0.07966 mmol) and N-hydroxysuccinimide (NHS; 0.09168 g, 0.07966 mmol) were dissolved in dry acetonitrile (ACN, 0.11 mL) under nitrogen. Then dicyclohexylcarbodiimide (DCC; 0.0214 g, 0.1036 mmol) was added. The reaction was left stirring under nitrogen overnight. The reaction mixture was filtered into a stirring solution of dry 2-propanol (5mL). The mixture was left in the freezer at -10 °C overnight. The precipitate was then collected and washed with minimal amount of ultra-pure water. The product was a dark red solid (0.046g, 80%). $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ: 9.03 (1H), 8.91 (1 H), 8.84 (4H), 8.22 (4 H), 8.06 (4H), 7.87 (3H), 7.59 (4H), 7.44 (1H), 2.83 (4H), 2.63 (3H). IR ν 1732 – 1706 (C=O), 1207 (C-O) cm$^{-1}$.

**Synthesis of the RGD-ruthenium bipyridine [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$ complex (1)**

Compound E (12.7 mg, 0.0176 mmol) and RGD (10 mg, 0.016 mmol) were dissolved in (CD$_3$)$_2$SO (800 µL) in an NMR tube with a drop of N-methylmorpholine (NMM). The reaction was left stirring using a stir bar for 36 h. MALDI-TOF-MS [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$: m/z 1226.03 [M - (CD$_3$)$_2$SO)]. Theoretical m/z 1,229.31 [M - (CD$_3$)$_2$SO)].
2.4 Linear Photophysical Properties

An Agilent (Model 8453) diode-array spectrometer was utilized for Ultraviolet–visible (UV-vis) absorption spectroscopy. Concentrations of $1 \times 10^{-5}$ M of the precursor E and dye 1 were prepared in ultra-pure water and acetonitrile at room temperature in 1 cm quartz cuvettes. Fluorescence emission measurements were obtained using a PTI International, Model MD-5020 fluorometer. The quantum yields were calculated using a comparative method against 9,10-diphenylanthracene, DPA (QY = 0.95).12

2.5 Cytotoxicity (MTS) Assay

To evaluate the cytotoxicity of dye, 1, $5 \times 10^3$ cells/well of U87MG cells in 96-well plates were incubated in 100 µL of MEM, supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin for 48 h. Then the cells were incubated with several concentrations of dye 1 (0.5, 1.0, 1.5, 3.0, 5.0, 10.0, 15.0, 20.0 in µM), respectively, for an additional 22 h. Next, 20 µL of CellTiter 96 AQueous One Solution reagent was added into each well, followed by further incubation for 2 h at 37 °C. The relative viability of the MCF-7 and U87MG cells incubated with probe 1 to unaltered cells was determined by subtracting the absorbance of the cell-free medium blank (volume at 490 nm) by the absorbance of the MTS-formazan on a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, U.S.A.) at 490 nm. Three individual experiments were prepared and averaged.3

2.6 Cell Culture and Incubation

U87MG cells or MCF-7 cells were placed onto poly-D-lysine coated glass coverslips in 24-well plates (60,000 cells per well), and the cells were incubated for 48 h before incubating with the dye 1. A 500µM stock solution of dye 1 was prepared in water. The solution was diluted to 5 µM with MEM, and then incubated for a period of an hour. Following incubation, the cells
were washed with PBS (x 3) and fixed using 3.7% formaldehyde solution for 10 min at 37 °C. Then freshly prepared NaBH₄ (1 mg/mL, prepared by adding few drops of 6 N NaOH solution into PBS (pH = 7.2)) solution in PBS (pH = 8.0) was added to each well (0.5 mL/well) for 10 min (x 2) (done to decrease autofluorescence). Afterwards the plates were washed with PBS (x 2) and water. Lastly, the coverslips were mounted using Prolong Gold mounting media (Life Technologies) for microscopy.³

2.7 Blocking Experiment

A blocking experiment was implemented in order to test the selectivity of the RGD-containing ruthenium probe 1 to the αᵥβ₃ integrin. U87MG cells were placed onto poly-D-lysine coated coverslips (12 mm) in 24-well plates (60,000 cells per well), and the cells were incubated for 48h. After, the cells were incubated with unlabeled cRGDfK (2 mg/mL of MEM) for 1 h. Then, 5 μM solution of dye 1 in MEM was added over the cells and incubated for a 1 h period. The cells were washed, treated, and mounted once incubation was completed.³

2.8 One-Photon Fluorescence Imaging

Conventional one-photon fluorescence (1PF) images were obtained using an inverted microscope (Olympus IX-81) DSU microscope equipped with Hamamatsu EM-CCD C9100 digital camera and mercury lamp (100 W). A custom designed filter cube (Ex 377/50, DM 409, Em 525/40) was used for the one-photon fluorescence microscopy imaging.³
CHAPTER 3: RESULTS & DISCUSSION

3.1 Synthesis

Figure 4 shows the synthetic steps to prepare RGD-ruthenium bipyridine, 1. The intermediates (B, C, D, and E) were reported previously. Slight modifications were done to previously published literature in order to produce compounds B, C, D, and E.

Figure 4. Synthesis of the RGD-Ruthenium Bipyridine Probe [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$. 
Intermediate B was synthesized by the oxidation of a methyl group on 4’-methyl-2,2’-bipyridine by the mild oxidant SeO₂, which is being reduced. The reaction was monitored by thin layer chromatography, TLC. Probable side products are the oxidation to an aldehyde of both methyl groups on the 4’-methyl-2,2’-bipyridine reagent or a complete oxidation to a carboxylic acid of one or both methyl groups. These side products and excess reagents were removed through the steps discussed in the experimental section, such as filtration and various extractions. In order to remove the carboxylic acid the filtrate was extracted with Na₂CO₃. To protect the aldehyde an aldehyde bisulfate was formed by further extraction with Na₂S₂O₃. The change of pH reformed the aldehyde. For the best results the aqueous phase was extracted with CH₂Cl₂ in sections. The best way to check if all of the aldehyde was extracted was by TLC and staining with 2,4-Dinitrophenylhydrazine, 2,4-DNPH stain. Ketones and aldehydes are detected by a yellow precipitate on the TLC plate. For purification hexane gave the best results for recrystallization. ¹H and ¹³C NMR, as well as melting point were used to characterize the product. In the ¹H NMR the aldehyde proton peak was found downfield at 10.21 ppm and the methyl protons were found upfield at 10.21 ppm. In the ¹³C NMR the carbonyl carbon peak was found downfield at 193.87 ppm and the methyl carbon was found upfield at 21.15 ppm. The melting point matched precious literature.⁷
Intermediate C was oxidized by AgNO₃ in a redox reaction. The reaction was monitored using TLC and Bromocresol Green, BCG, stain, which detects different functional groups that have a pKa that is less than five. Carboxylic acids are stained green. The side products of this reaction are Ag₂O and metallic silver, which are removed by filtration. To remove excess B, the mixture was extracted with CH₂Cl₂. In order to purify the compound, NaOH was used to turn the acid into a salt, making it water soluble. The acidic solution changed the salt back into a carboxylic acid inducing the precipitation. For purification ethyl acetate gave the best results for recrystallization. ¹H and ¹³C NMR, as well as melting point were used to characterize the product. In the ¹H NMR the carboxylic acid proton peak was found more downfield to the aldehydic proton peak from intermediate B at 13.74 ppm. This downfield shift was expected due to the increase in resonance and the electronegativity effect of the oxygen. The melting point matched precious literature.⁷

Compound D was synthesized by using the reagent Ru[(bpy)₂]Cl₂, which is easily bound to each nitrogen on C under reflux. The reaction was monitored by TLC and the BCG stain, as well as UV-vis in ultra-pure water. The side products and excess reagents were removed by filtration. The pH was dropped dramatically in order to decrease the oxygen’s attractiveness to the Ru(II) metal. The product was precipitated out with the addition of saturated aqueous NH₄PF₆. Previous literature reviews had suggested washing with both water and ether; however, ether caused the product to change texture and became more of a paste.¹⁵ ¹H and ¹³C NMR, as well as UV-vis were used to characterize the product. The carboxylic acid proton peak in the ¹H NMR was found to be an extremely broad peak from 2.75-4.00 ppm. The absorption λₘₐₓ was at 455 nm in ACN and 457 nm in ultra-pure water.
Compound E was synthesized by the activation of the acid by DCC, followed by the NHS coupling to the acid. Succinimido esters are easily hydrolyzed by water; therefore, extra precautions were done for this reaction. Intermediate D was left under vacuum and heated (≥40°C) for a few hours to insure dryness before use. The Schlenk tubes, stir bars, and solvents were also dried. The reaction was monitored by UV-vis in ACN. This reaction resulted in multiple side products such as dicyclohexylurea, confirmed with 1H NMR, and excess NHS, confirmed by TLC. In order to reduce the amount of side product once the reaction was complete, the reaction mixture was filtered two times. 1H NMR and IR were used to characterize the product. In the IR spectra the two ester peaks were found at 1732 – 1706 (C=O) and 1207 (C-O) cm⁻¹.

Product 1 was synthesized by the conjugation of the succinimidyl functionality with the amine component of the c(RGDfK) peptide. The cyclic RGD peptide was used to target αvβ3 integrin. The αv- integrins are currently used as molecular targeting sites for anti-angiogenic therapies and RGD has been shown to mediate cell adhesion via the αvβ3 integrins. RGD has been shown to selectively bind to endothelial tumor cells over expressing αvβ3 integrins. The reaction was monitored by 1H NMR. Mass spectrometry was used to characterize the product.
3.2 Linear Photophysical Properties

The linear photophysical properties, UV-vis absorption, steady-state fluorescence, and fluorescence quantum yield (measure of emission efficiency) of E (Table 1) were carefully characterized in ultra-pure water and acetonitrile (Figure 5). DPA was used as the reference. In ultra-pure water the absorption $\lambda_{\text{max}}$ was equal to 457 nm and emission $\lambda_{\text{max}}$ was equal to 640 nm. The stoke shift was equal to 183 nm and the quantum yield ($\Phi$) of E was found to be $0.05 \pm 0.01$. In acetonitrile the absorption $\lambda_{\text{max}}$ is equal to 455 nm and emission $\lambda_{\text{max}}$ is equal to 664 nm. The stoke shift was equal to 209 nm and the quantum yield of E was found to be $0.02 \pm 5 \times 10^{-3}$. The quantum yield was calculated by the equation shown in Figure 6.

Table 1. Summary of normalized UV-visible absorbance, fluorescence emission spectra and stokes shift of precursor ([Ru(Bpy)$_2$(BpyOH)]$^{2+}$), E, and dye ([Ru(Bpy)$_2$(BpyRGD)]$^{2+}$), 1, in ultra-pure water and ACN.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Absorption $\lambda_{\text{max}}$ (nm)</th>
<th>Emission $\lambda_{\text{max}}$ (nm)</th>
<th>Stokes shift (nm)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Ultra-pure water</td>
<td>457</td>
<td>640</td>
<td>183</td>
<td>$0.05 \pm 0.015$</td>
</tr>
<tr>
<td>E</td>
<td>Acetonitrile</td>
<td>455</td>
<td>664</td>
<td>209</td>
<td>$0.02 \pm 5 \times 10^{-3}$</td>
</tr>
<tr>
<td>1</td>
<td>Ultra-pure water</td>
<td>457</td>
<td>641</td>
<td>184</td>
<td>$0.04 \pm 0.01$</td>
</tr>
<tr>
<td>1</td>
<td>Acetonitrile</td>
<td>455</td>
<td>661</td>
<td>206</td>
<td>$0.02 \pm 5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
Figure 5. Normalized UV-visible absorbance and fluorescence emission spectra of precursor \([\text{Ru(Bpy)}_2(\text{BpyOH})]^{2+}\) in (A) ultra-pure water and (B) acetonitrile, respectively.

\[
Q = Q_R \frac{I \ OD_R \ n^2 \ RP_R}{I_R \ OD \ n_R^2 \ RP}
\]

I = Integrated Data  
OD = Optical Density Concentration  
n = Refractive Index  
RP = Relative Power  
Subscript R = Reference

Figure 6. Quantum Yield Equation

The linear photophysical properties, UV-vis absorption, steady-state fluorescence, and fluorescence quantum yield, of 1 (Table 1) was carefully characterized in ultra-pure water and acetonitrile (Figure 7). DPA was used as the reference.\(^{12}\) In ultra-pure water the absorption \(\lambda_{\text{max}}\) is equal to 457 nm and emission \(\lambda_{\text{max}}\) is equal to 641 nm. The stoke shift was equal to 184 nm and the quantum yield of E was found to be 0.04 ± 0.01. In acetonitrile the absorption \(\lambda_{\text{max}}\) is
equal to 455 nm and emission $\lambda_{\text{max}}$ is equal to 661. The stoke shift was equal to 184 nm and the quantum yield of E was found to be $0.02 \pm 5 \times 10^{-3}$.

![Normalized UV-visible absorbance and fluorescence emission spectra](image)

**Figure 7.** Normalized UV-visible absorbance and fluorescence emission spectra of dye ([Ru(Bpy)$_2$(BpyRGD)]$^{2+}$) in (A) ultra-pure water and (B) acetonitrile, respectively.

Ultra-pure water was used as a solvent since the dye was dissolved in ultra-pure water for bioimaging. Acetonitrile was used as a solvent in order to have a reference. The absorption $\lambda_{\text{max}}$ for both E and 1 are equal in their respective solvents, since the cyclic RGD does not change the conjugation of E. The quantum yields were low in both solvents for both compounds; however, supporting data will show that dye 1 was still useful for bioimaging.

### 3.3 Cell Viability Study

Cytotoxicity of dye 1 was tested using an MTS assay, shown in Figure 8 at various concentrations were calculated. The concentrations utilized were 0.5, 1.0, 1.5, 3.0, 5.0, 10.0, 15.0, 20.0 in $\mu$M. The MCF-7 cells showed excellent results of 100% viability after 24 h incubation time. These results also support the selectivity of the cyclic RGD to the $\alpha_v\beta_3$ integrin. The U87-MG cells results after 24 h of incubation were not as notable as MCF-7; however, the cell viability was high. From 0.5- 5.0 $\mu$M the cell viability was above 90%. From 10- 20 $\mu$M the
cell viability dropped to around 80%. These results indicate that the cytotoxicity of dye 1 is low, especially to the MCF-7 cell line.

![Graph showing viability percentage among different µM concentrations of dye \([\text{Ru(Bpy)}_2(\text{BpyRGD})]^2\) in both U87MG and MCF-7 cells.]

**Figure 8.** Graph shows the percentage of viability among different µM concentrations of dye \([\text{Ru(Bpy)}_2(\text{BpyRGD})]^2\) in both U87MG and MCF-7 cells.

### 3.4 One-Photon Fluorescence Bioimaging

The U87MG human glioblastoma cells and MCF-7 human breast cancer cells were used in order to test the selectivity of dye 1. One positive control was tested using the elevated integrin \(\alpha_v\beta_3\) expressing U87-MG cells (Figure 9). Different concentrations and incubation times with dye 1 were tested; ranging from 1 µM to 10 µM and one to three hours of incubation time. The best results were seen at a concentration of 5 µM and an incubation time of one hour; there was high intensity fluorescence.
Three negative control experiments were done. The blocking experiment was done in order to assure that dye 1 was only binding to the $\alpha_{\text{v}}\beta_{3}$ integrin by incubating the U87MG cells with the cyclic RGD peptide before the addition of dye 1. The fluorescence shown on the images (Figure 10) is autofluorescence from the U87MG cells.
The second U87MG negative control was incubating the U87MG cell line with the precursor E to see if there was any binding of any kind. Once again the fluorescence shown in Figure 11 is autofluorescence.

![Figure 11. 1PF images of U87MG Cells labeled with precursor E.](image)

The third negative control was using the MCF-7 cell line, which resulted in virtually no fluorescence (Figure 12). When comparing all three negative controls to the positive control there is a clear difference, the intensity of luminescence of the positive control U87MG cells are stronger compared to the negative controls. For the MCF-7 negative control the results are excellent due to little observable fluorescence after incubation.
Figure 12. 1PF images of negative control MCF7 cells.
CHAPTER 4: CONCLUSION

This study reports the successful synthesis and characterization of a ruthenium bipyridine complex and its successfully conjugation to the cyclic RGD peptide. The complex in solution had low fluorescence quantum yield. In contrast cell images showed fluorescence enhancement when the bioconjugate was introduced into the cells. The images indicate the ([Ru(Bpy)2(BpyRGD)]2+) bioconjugate showed strong recognition of the high integrin αvβ3 expressing U87MG cells and not the low integrin αvβ3 expressing MCF-7 cells. The cell viability tests indicated that the toxicity of complex is low, confirming a potential use of the bioconjugate for future studies in early tumor detection.

Future work will entail the calculation of the probes lifetime, cell fluorescence lifetime imaging (FLIM), and the use of time-gated detection in order to reduce the effects of the interfering autofluorescence. Determining the binding site of the probe 1 on the cell will also be explored.
APPENDIX A:

METHOD 2: SYNTHESIS OF [Ru(Bpy)$_2$(BpyRGD)]$^{2+}$
The scheme above is an in situ reaction, which was another method that was attempted in order to synthesize $[\text{Ru(Bpy)}_2(\text{BpyRGD})]^2+$. The reason this method was not carried out to completion was due to the unsuccessful purification of C, as well as the impracticality of isolating D. The scheme below (part of the above scheme) was successfully synthesized.$^{16}$
APPENDIX B:
$^1$H-NMR AND $^{13}$C-NMR OF 4'-METHYL-2,2'-BIPYRIDINE-4-
CARBALDEHYDE
APPENDIX C:
$^1$H-NMR AND $^{13}$C-NMR OF 4'-METHYL-2,2'-BIPYRIDINE-4-CARBOXYLIC ACID
APPENDIX D:
$^1$H-NMR OF 2,5-DIOXOPYRROLIDIN-1-YL 4'-METHYL-2,2'-BIPYRIDINE-4-CARBOXYLATE
APPENDIX E:

$^1$H-NMR AND $^{13}$C-NMR OF RUTHENIUM (II)-BIS(2,2’-BYPYRIDINE)-(4’-METHYL-2,2’-BIPYRIDINE-4-CARBOXYLIC ACID)-BIS(HEXAFLUOROPHOSPHATE)
APPENDIX F:

$^1$H-NMR OF RUTHENIUM(II)-BIS(2,2’-BYPYRIDINE)-(4’-METHYL-2,2’-
BIPYRIDINE-4-CARBOXAMIDO-METHANE)-
BIS(HEXAFLUOROPHOSPHATE)
APPENDIX G:
IR OF RUTHENIUM(II)-BIS(2,2’-BYPYRIDINE)-(4’-METHYL-2,2’-
BIPYRIDINE-4-CARBOXAMIDO-METHANE)-
BIS(HEXAFLUOROPHOSPHATE)
APPENDIX H:
MASS SPECTROMETRY ([RU(BPY)₂(BPYRGD)]²⁺)
-Reaction solvent: Deuterated DMSO ((CD$_3$)$_2$SO)

-Anion: PF$_6$
REFERENCES


