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Electrogenerated Chemiluminescence Of Luminophores And Enhancement With Melatonin

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ELECTROGENERATED CHEMILUMINESCENCE OF LUMINOPHORES

AND ENHANCEMENT WITH MELATONIN

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Chemistry

By

Sarah Michelle Roughton

July 2016

ELECTROGENERATED CHEMILUMINESCENCE OF LUMINOPHORES AND ENHANCEMENT WITH MELATONIN

Chemistry

Missouri State University, July 2016

Master of Science

Sarah Michelle Roughton

ABSTRACT

The photoluminescent (PL), electrochemical, and electrogenerated chemiluminescent (ECL) properties of 1,5-I-Aedans (*N*-(iodoacetylaminotheyl)-1-naphthylamine-5-sulfonic acid) in aqueous buffered (KH$_2$PO$_4$) and 50:50 (*v/v*) acetonitrile:KH$_2$PO$_4$ solutions were obtained. Tri-*n*-propylamine (TPrA) was used as the oxidative-reductive coreactant. The PL efficiencies (ϕ_{em}) were 1.83 in KH$_2$PO$_4$ and 4.81 in mixed solvent compared to Ru(bpy)$_3{}^{2+}$ standard solutions (ϕ_{em} = 1.0). 1,5-I-Aedans displayed quasi-reversible oxidative electrochemistry in aqueous solutions and irreversible to quasi-reversible oxidation in mixed solvent. ECL efficiencies (ϕ_{ecl}) were obtained by comparison to a Ru(bpy)$_3{}^{2+}$/TPrA (bpy = 2,2'-bipyridine) standard (ϕ_{ecl} = 1) and were higher in KH$_2$PO$_4$ (ϕ_{ecl} = 6.8 x 10^{-4}) than in the mixed solvent system (ϕ_{ecl} = 0.072 x 10^{-4}). A second project studied the ECL of the Ru(bpy)$_3{}^{2+}$/TPrA system when melatonin (*N*-acetyl-5-methoxytryptamine; MLT) is present in aqueous buffered solution. MLT displays weak ECL when TPrA is used as the oxidative-reductive coreactant, but when micromolar concentrations of MLT were added to the system, up to 2.5-fold enhancement was seen. PL efficiencies did not change in the presence of MLT unless the solution underwent electrochemical bulk oxidation. Spectroscopic, electrochemical and spectro-electrochemical studies indicate that the mechanism involves oxygen scavenging by MLT oxidation products. This scavenging prevents quenching of the *Ru(bpy)$_3{}^{2+}$ excited states.

KEYWORDS: enhanced electrogenerated chemiluminescence (ECL), coreactants, melatonin, 1,5-I-Aedans, Ru(bpy)$_3{}^{2+}$

This abstract is approved as to form and content

_____
Dr. Mark M. Richter
Chairperson, Advisory Committee
Missouri State University

ELECTROGENERATED CHEMILUMINESCENCE OF LUMINOPHORES

AND ENHANCEMENT WITH MELATONIN

By

Sarah Michelle Roughton

A Masters Thesis
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July 2016

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iv

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ACKNOWLEDGEMENTS

I would like to thank the following people for their support during the course of my graduate studies. Thanks to the Missouri State University Graduate College and the Department of Chemistry for funding this work. Thanks to Dr. Matthew Siebert, Dr. Kevin Evans, and Dr. Adam Wanekaya for being on my committee and for the guidance and advice. Thanks to my family and friends for encouragement, patience, and understanding during this journey. Finally, thanks to my mentor and friend, Dr. Mark Richter. He was the person who sparked my interest in chemistry and over the years his guidance, assistance, unending patience, encouragement, and somewhat snarky sense of humor have been invaluable. Words cannot express my gratitude.

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CHAPTER 1: INTRODUCTION

Electrogenerated chemiluminescence (ECL) is a means of transforming electrical energy into radiative energy via electron transfer reactions. ECL systems can be used as markers to detect organic, inorganic, and bioactive molecules. Because of this, it has found use in the areas of analytical, inorganic, organic, environmental, and biochemistry.[1-3]

ECL can be initiated through several different routes, including annihilation and coreactant ECL. Annihilation ECL involves electron transfer reactions between two species (one oxidized and one reduced), generated at an electrode by alternate pulsing of the potential. The potential of the working electrode is quickly changed between two values to produce the oxidized and reduced radicals (Equations 1 and 2, below) that will then react (Equation 3) to emit light (Equation 4). Annihilation ECL has found uses in the rapid screening of potential organic light-emitting diodes (OLEDs), but it has some drawbacks. Cells, electrodes, and procedures have to be modified to allow for the generation of two reactants. Stray light must be blocked out and organic solvents must be extremely pure since water and oxygen tend to quench ECL in this method. A generic mechanism is presented in the following equations:[3]

$$A - e^- \rightarrow A^{\bullet+} \text{ (oxidation at electrode)} \tag{1}$$

$$A + e^- \rightarrow A^{\bullet-} \text{ (reduction at electrode)} \tag{2}$$

$$A^{\bullet-} + A^{\bullet+} \rightarrow A^* + A \text{ (excited-state transformation)} \tag{3}$$

$$A^* \rightarrow A + h\nu \tag{4}$$
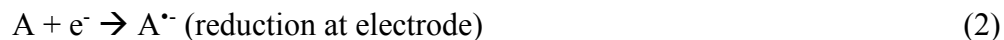
In coreactant ECL, the electrode either oxidizes or reduces the reagents in a single potential step (versus the double potential step of annihilation ECL). When a potential is applied to a solution through an electrode, an excited state is formed that is capable of light emission. Utilizing the commercially available $Ru(bpy)_3^{2+}$/TPrA (bpy = 2,2'-bipyridine; TPrA = tri-n-propylamine) system as an example, an electric potential is applied to a solution containing both the luminophore ($Ru(bpy)_3^{2+}$) and the coreactant (TPrA). This potential results in the oxidation of both species (Equations 5 and 6, below), with the TPrA forming a strong reducing agent (probably TprA$^•$, where TPrA$^•$ = TPrA radical), which can then react with the oxidized $Ru(bpy)_3^{3+}$ to produce an excited state molecule (i.e., $Ru(bpy)_3^{*2+}$) (Equation 7). $Ru(bpy)_3^{*2+}$ can then decay to the ground state, producing luminescence (Equation 8).[4,5]

This system has found use in DNA quantification; clinical diagnostic immunoassays; in the measurement of biological and pharmacological compounds including opiates, antibiotics, and antihistamines; and in environmental applications, allowing detection of concentrations down to $\leq 10^{-18}$ M.[1-3] In most clinical applications, $Ru(bpy)_3^{2+}$ acts as an ECL marker. For example, in a study of human C-reactive protein (CRP), liposomes containing $Ru(bpy)_3^{2+}$ were prepared as an ECL marker for a sandwich-type immunoassay. Human CRP antibodies were introduced onto liposomes and magnetic beads, allowing the antigen-antiody conjugates to be separated from unreacted species magnetically. Addition of 0.1M tri-n-propylamine and 0.1M phosphate buffer with Triton X-100 liberated the $Ru(bpy)_3^{2+}$ from the liposome, allowing ECL detection down to 100ng/mL.[6] $Ru(bpy)_3^{2+}$-containing microspheres have also been used to detect and quantify 2,4,6-trinitrotoluene (TNT) in soil and water down to ≤ 0.1 ppt or

0.1 pg. TNT is a toxic explosive chemical used in military shells, bombs, grenades, and underwater blasting. It is so dangerous, the United States Environmental Protection Agency has issued a lifetime health advisory level of 2 ppb in drinking water.[7] The most commonly proposed mechanism for the $Ru(bpy)_3^{2+}$/TPrA system is presented in the equations below and in Figure 1 with a representative photo in Figure 2:[3]

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+} \tag{5}$$

$$TPrA - e^- \rightarrow [TPrA^{\bullet}]^+ \rightarrow TPrA^{\bullet} + H^+ \tag{6}$$

$$Ru(bpy)_3^{3+} + TPrA^{\bullet} \rightarrow Ru(bpy)_3^{*2+} + products \tag{7}$$

$$Ru(bpy)_3^{*2+} \rightarrow Ru(bpy)_3^{2+} + hv \tag{8}$$
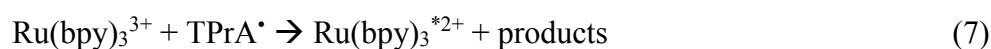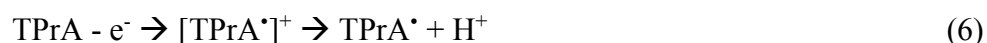
While the $Ru(bpy)_3^{2+}$/TPrA system is very useful, there is still a search for methods to improve detection limits. These methods may include the use of additives, utilizing different coreactants, synthesizing dual- or multi-core ruthenium complexes, and the study of complexes other than ruthenium to name a few.[1] Structures of some of the compounds utilized in this study can be seen in Figure 3. In this work, two systems will be discussed. The first investigates the photoluminescent, electrochemical, and ECL properties of 1,5-I-Aedans (*N*-(iodoacetylaminoetheyl)-1-naphthylamine-5-sulfonic acid) in aqueous buffered (KH_2PO_4) and 50:50 (*v/v*) acetonitrile:aqueous buffered (CH_3CN:KH_2PO_4) solutions. The second investigates the ECL of the $Ru(bpy)_3^{2+}$/TPrA system when melatonin (*N*-acetyl-5-methoxytryptamine; MLT) is present in aqueous buffered solution.
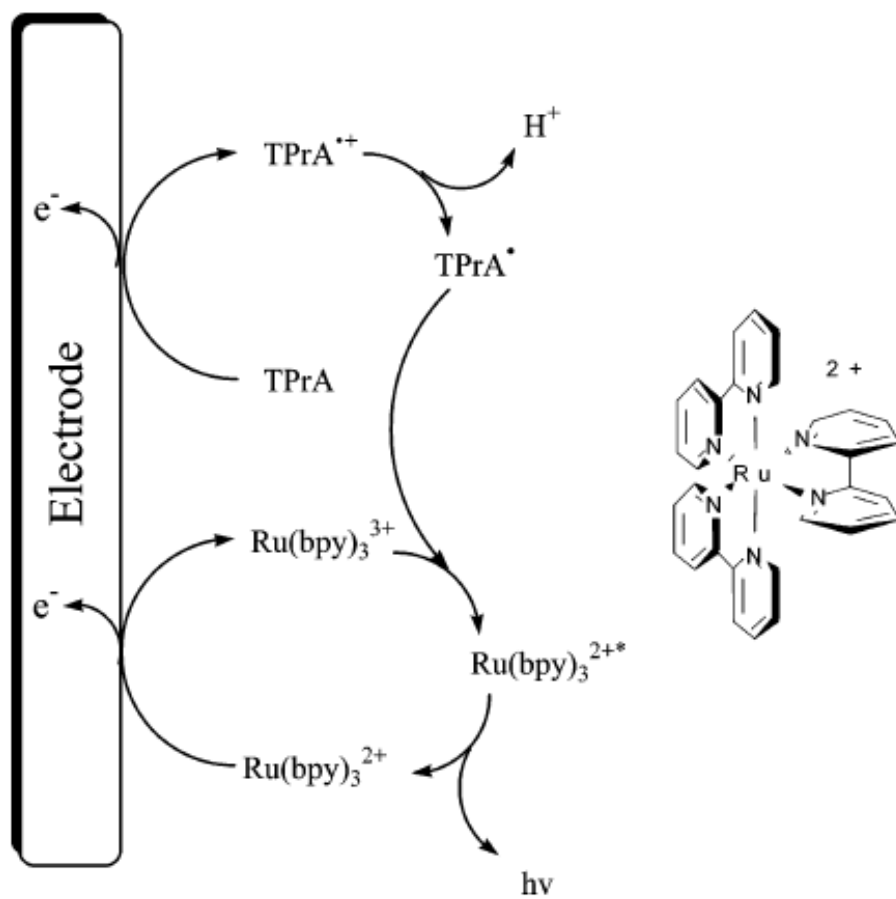
Figure 1. ECL reaction of $[Ru(bpy)_3]^{2+}$ and TPrA. TPrA is tri-*n*-propylamine $(Pr_2NCH_2CH_2CH_3)$

Figure 2. ECL emission of [Ru(bpy)₃]²⁺ at a platinum electrode. Stirred solution of 0.15 M [Ru(bpy)₃]²⁺ and 40 mM TPrA in 0.18 M potassium phosphate buffer (KPB) pH 8.0. Working electrode held at +2.0 V vs. Ag/AgCl. Photograph by D.J. Vinyard. 27 Jan. 2008.

Ru(bpy)$_3^{2+}$

Triton X-100

Ir(ppy)$_3$

1,5-I-Aedans

(bpy)$_2$Ru(DM-bpy)$^{2+}$

(bpy)$_2$Ru(DC-bpy)$^{2+}$
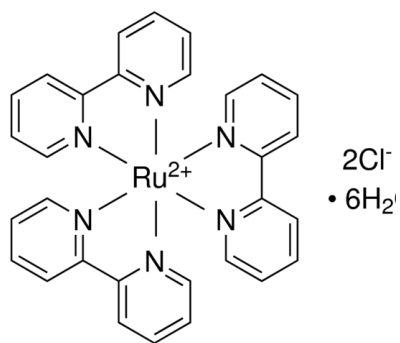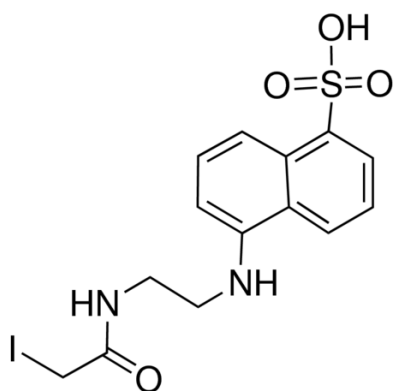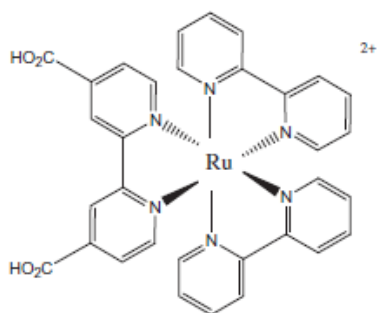
Figure 3. Structures of Compounds: Ru(bpy)$_3^{2+}$, 1,5-I-Aedans, (bpy)$_2$Ru(DC-bpy)$^{2+}$, Triton X-100, Ir(ppy)$_3$, and (bpy)$_2$Ru(DM-bpy)$^{2+}$.

CHAPTER 2: EXPERIMENTAL METHODS

Materials

For Chapter 3. Potassium phosphate monobasic (KH_2PO_4, Spectrum Chemical Mfg. Corp., Gardena, CA), tris(2,2'-bipyridyl)ruthenium (II) chloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$; Strem Chemicals, Newburyport, MA), acetonitrile (CH_3CN; Sigma-Aldrich Co., St. Louis, MO), tri-*n*-propylamine (TPrA; 98%, Sigma-Aldrich), were used as received. *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonic acid (1,5-I-Aedans; Sigma-Aldrich) was purified by literature methods via recrystallization from hot deionized water.[8] Purity was tested using thin layer chromatography with ethanol as the eluent following published procedures. Also, a molar absorptivity (A) of 5650 $M^{-1}cm^{-1}$ was obtained in deionized water, with a reported value[8] of 5700 $M^{-1}cm^{-1}$ indicating greater than 99% purity after recrystallization. Aqueous experiments (including 50:50 (v/v) CH_3CN: KH_2PO_4) were performed in ~0.18 M potassium phosphate (Spectrum Chemical Manufacturing Corp., Gardena, CA) buffered solution. pH was adjusted using either sodium hydroxide or concentrated sulfuric acid. Deionized water was filtered using a Barnstead/Thermolyne triple filtration system.

For Chapter 4. $Ru(bpy)_3Cl_2 \cdot 6H_2O$ (98%, Strem Chemical, Newburyport, MA), melatonin (Sigma-Aldrich, St. Louis, MO), serotonin (Sigma-Aldrich), 7-azatryptophan (Sigma-Aldrich), tryptophan (Sigma-Aldrich), and tri-*n*-propylamine (98%, Sigma-Aldrich) were used without further purification. $Ir(ppy)_3$, and $(DC-bpy)Ru(bpy)_2^{2+}$ and $(DM-bpy)Ru(bpy)_2^{2+}$ [9] were available from previous studies. Aqueous experiments were performed in either 0.18 M potassium phosphate (Spectrum Chemical Manufacturing

Corp., Gardena, CA) buffered solution or Bioveris® assay buffer (approximate composition: 0.18M TPrA, 0.02M potassium phosphate buffer, 0.05 M TPrA, 0.1% by weight Triton X-100 at pH = 7.5). Deionized water was purified using a Barnstead E-Pure filtration system.

Methods

Electrochemistry. Electrochemical experiments with no photon detection used a CH Instruments electrochemical analyzer (Austin, TX) with a glassy carbon working electrode, a platinum auxiliary electrode and a Ag/AgCl electrode (0.20 V vs. the Normal Hydrogen Electrode (NHE)).[10] The working electrode was cleaned after each run by polishing with 0.05 μm alumina, followed by rinsing with purified and filtered water.

ECL. ECL instrumentation and experimental methods incorporated a conventional three-electrode system. A CH Instruments electrochemical analyzer was paired with a Hamamatsu HC 135 Photomultiplier Tube (PMT) contained in a "light-tight" box. The working electrode was a platinum mesh electrode, with a platinum wire auxiliary electrode and a Ag/AgCl quasi-reference electrode (0.20 V vs. NHE).[10] This same system was also used in conjunction with yellow, green and blue plastic absorption filters to obtain the wavelength ranges of ECL emission. The working electrode was cleaned before each run by repeated cycling (+2.0 to -2.0 V) in 6.0 M sulfuric acid followed by rinsing with deionized water. Luminophore concentrations were between 1×10^{-3} M and 1×10^{-7} M with 50mM TPrA in 0.18 M potassium phosphate at pH 8.0 ± 0.1, and typically contained millimolar concentrations of analyte (e.g., MLT). ECL

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efficiencies (ϕ_{ecl} = photons generated per redox event) were obtained using the following equations using $Ru(bpy)_3^{2+}$ ($\phi_{ecl} = 1$) as the standard[11]:

$$\phi_{ecl} = \phi^o_{ecl}\ \frac{IQ^o}{I^oQ} \qquad (9)$$

where ϕ^o_{ecl} is the ECL efficiency of the standard, I and I^o are the integrated intensities of the luminophore and the standard, and Q and Q^o are the charges passed in Coulombs for 1,5-I-Aedans and $Ru(bpy)_3^{2+}$, respectively. This equation is useful when the same electrode is used consistently, when luminophore and electrolyte concentrations are similar, and when electron transfers are equivalent between the target molecules. Reported values are the average of at least three scans with a relative standard deviation of ± 5%.

Cyclic Voltammetry. Cyclic voltammetry studies without photon detection utilized a CH instruments electrochemical analyzer (Austin, TX) with a glassy carbon working electrode (to prevent adsorption of compound on the surface of the electrode), a platinum wire auxiliary electrode and a Ag/AgCl quasi-reference electrode (0.20 V vs the Normal Hydrogen Electrode (NHE)).[11] The glassy carbon electrode was cleaned after each run by polishing with 0.1 μm alumina, followed by rinsing with water.

UV-Vis and Photoluminescence. UV-Vis spectroscopy was performed using an Agilent Technologies Cary-60 UV-Visible Spectrophotometer. Photoluminescence spectra were obtained with a Shimadzu RF-5301 Spectrofluorophotometer (Shimadzu Corporation, Japan) with detection between 500 and 700 nm. Slit widths for PL spectroscopy were 4 nm for 1,5-I-Aedans. Slit widths for PL spectroscopy (for MLT) were 3nm and for ECL spectroscopy were 20nm. Excitation for photoluminescence was at the lowest energy absorption wavelength maximum. Photoluminescence efficiencies

(ϕ_{em}; photons emitted per photons absorbed) were obtained relative to Ru(bpy)$_3{}^{2+}$ in each solvent mixture using the following equation:

$$\phi_{em} = \phi^o{}_{em} \frac{(I)}{I^o} (10)$$

where $\phi^o{}_{em}$ is the PL efficiency of the Ru(bpy)$_3{}^{2+}$ while I and I° are the emission intensities of 1,5-I-Aedans and Ru(bpy)$_3{}^{2+}$, respectively. The PL efficiency of Ru(bpy)$_3{}^{2+}$ was set to 1.0 to allow direct comparisons between the different solvent mixtures. For the MLT studies, I represented the intensities of solutions containing MLT and I° represented the standard solution (Ru(bpy)$_3{}^{2+}$/TPrA/KH$_2$PO$_4$). UV-Vis absorption and PL were run in these solvent systems to allow direct comparisons with ECL data.

CHAPTER 3: ECL OF *N*-(IODOACETYLAMINOETHYL)-1-NAPHTHYLAMINE-5-SULFONIC ACID (1,5-I-AEDANS)

Introduction

8-amino-1-naphthalenesulfonic acid (ANS) and its analogues such as *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonic acid (1,5-I-Aedans; Figure 3) have been used as photoluminescent probes of biomolecular interactions.[8,12,13,14] For example, they are in widespread use for the characterization of protein aggregates, fibrils, conformational changes, and to locate hydrophobic moieties in native proteins.[15,16] 1,5-I-Aedans, in particular, has been used to determine conformational changes in skeletal muscle G-actin upon addition of Mg^{2+} and Ca^{2+} to aqueous solutions[13] and to characterize the interactions between troponin units via fluorescence lifetime and quenching of the 1,5-I-Aedans excited state.[14] In addition, they have been incorporated into high throughput screening assays to develop therapeutic protein formulations.[17] The emission spectra of these compounds are also sensitive to the polarity of the environment, showing blue shifts in emission maxima and increased emission quantum efficiencies in less polar solvents due to decreased non-radiative decay.[18,19] This makes them ideal probes for fundamental and applied luminescent studies such as electrogenerated chemiluminescence (ECL). In this study detailed spectroscopic, electrochemical and ECL properties of 1,5-I-Aedans are reported to better understand its properties and evaluate its potential usefulness as an ECL sensor.

Results and Discussion

Absorption and Photoluminescence. UV/Vis and PL data are presented in Table 1 and Figure 4. 1,5-I- Aedans displays an absorption maximum, λ_{max}, around 337 nm in aqueous buffered solution and 340 nm in mixed (50:50 v/v) KH_2PO_4:CH_3CN. The slight red shift of λ_{max} in the mixed solvent has been observed numerous times for naphthalene sulfonic acids as the hydrophobicity of the solvent increases.[8,11,15] An absorption maximum is also observed at around 270 nm in both solvent systems, deriving from the aniline substructure of the molecules while the lower energy absorptions are from the fully delocalized naphthalene moiety.[20] Absorption spectra were taken on a single sample in 10-minute intervals for a period of 120 minutes to test the stability of the complex to laboratory room light since 1,5-I-Aedans is sensitive to light exposure over extended periods of time. Consistent spectra (intensity, peak shape, etc.) were obtained in each experiment suggesting that no photodegradation was observed in the timeframe of normal spectroscopic, electrochemical or ECL experiments.

Table 1. Spectroscopic UV/Vis and PL data for 0.1 mM 1,5-I-Aedans.

Solvent	λ_{abs} (nm)	λ_{em} (nm)	ϕ_{em} [a]	ϕ_{ecl} [b]
KH_2PO_4	337	496	1.83	6.8×10^{-4}
50:50 KH_2PO_4: CH_3CN	340	500	4.81	0.072×10^{-4}

[a]ϕ_{em} measured by comparison of 1,5-I-Aedans (0.01 mM) to 0.01 mM $Ru(bpy)_3^{2+}$ ($\phi_{em}=1$)in each solvent.
[b]ϕ_{ecl} measured relative to $Ru(bpy)_3^{2+}$ ($\phi_{ecl} = 1$) in each solvent.
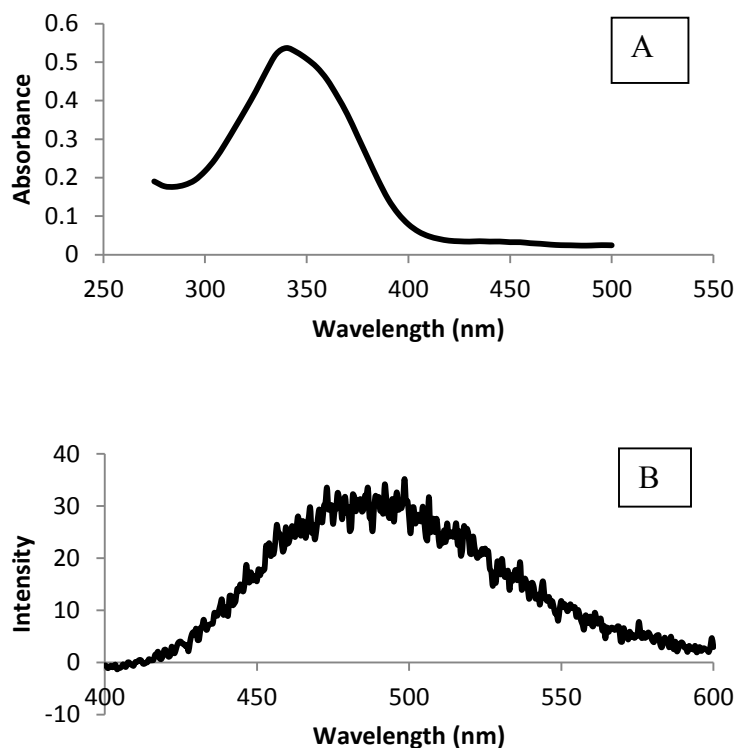
Figure 4: (A) UV-Vis, [1,5-I-Aedans = 1 mM], and (B) PL, [1,5-I-Aedans] = 0.1 mM, spectra of M 1,5-I-Aedans in 50:50 KH_2PO_4:CH_3CN.

Excitation into the broad visible absorbance band around 340 nm produces room temperature photoluminescence around 500nm for 1,5-I-Aedans in both solvents (Table 1) with a band width at half height of ~75 nm (Figure 4). The emission maxima are also solvent dependent, following the same trend observed in the absorbance studies. Photoluminescence efficiencies (ϕ_{em}; photons emitted per photons absorbed) for 1,5-I-Aedans are reported in Table 1, are solvent dependent and are higher than $Ru(bpy)_3^{2+}$. The photoluminescence efficiencies also increased with lower solvent polarity (the relative polarity of water is 1.000 and acetonitrile is 0.460).[21] This was also observed for $Ru(bpy)_3^{2+}$ in a series of alcohols[22] and was traced to increased non-radiative decay as the polarity of the solvent increases.

Several studies have probed conformational changes in biomolecules using 1,5-I-Aedans. Metal cations such as Mg^{2+}, K^+ and Ca^{2+} were shown to enhance or attenuate.[13,14] 1,5-I-Aedans luminescence, presumably due to binding events occurring within the biomolecule itself. However, it is possible that the PL of 1,5-I-Aedans itself was impacted directly by the metal ions and since metal ions are often part of PL and ECL assays the effect of Fe^{+3}, Al^{+3}, Mg^{+2}, Zn^{+2}, and Cu^{+2} on 1,5-I-Aedans PL was included as part of this work. In summary, Al^{+3} had no effect upon 1,5-I-Aedans luminescence at $[Al^{+3}]$ between 1×10^{-5} M and 1×10^{-3} M and produced a slight attenuation (~20%) at 5×10^{-3}M. Mg^{+2}, Zn^{+2}, and Cu^{+2} all behaved similarly with no effect at lower concentrations (between 1×10^{-5}M and 1×10^{-4}M), ~20% attenuation at 1×10^{-3}M and ~50% attenuation of the PL signal at 5×10^{-3} M. Fe^{+3} quenched at all concentrations, with >90% at $[Fe^{+3}] = 5 \times 10^{-3}$ M. Overall, the effect of metal ions on 1,5-I-Aedans is insignificant within experimental error at the micromolar concentrations routinely found in PL and ECL assays.

Electrochemistry. Representative cyclic voltammetric data is presented in Table 2 and cyclic voltammograms of 1,5-I-Aedans provided in Figure 5. The voltammograms were obtained for 1 mM 1,5-I-Aedans solutions by sweeping oxidatively starting at 0.0 V in both air saturated and deoxygenated solutions. No reductions were observed within the solvent windows (~-1.6 V vs Ag/AgCl).

In air saturated KH_2PO_4 an irreversible to quasi reversible peak occurs at +0.42 V, with a smaller electrochemically irreversible peak at ~+0.72 V. In deoxygenated solution the peak at +0.42 V disappears, while the peak at +0.72 V becomes quasi reversible (i_a/i_c

= 2.1 where i_a is the anodic peak current, i_c is the cathodic peak current and a value of 1.0

is predicted for an electrochemically reversible system).

Table 2. Electrochemical data for 0.1mM 1,5-I-Aedans.

Solvent	E_a (V)	E_c (V)	$E_{1/2}$ (V)	i_a/i_c
KH$_2$PO$_4$ – deoxygenated	0.71	0.61	0.66	2.1
KH$_2$PO$_4$ – air saturated	0.42	---	---	---
50:50 (v/v) KH$_2$PO$_4$:CH$_3$CN – deoxygenated	0.49	---	---	---

However, in the deoxygenated solution a shoulder appears at slightly more positive

potentials on the anodic wave suggesting that an electrochemically active species is being

formed upon oxidation of 1,5-I-Aedans followed by a chemical reaction/decomposition

or the system undergoes two closely spaced electrochemical oxidation events. This

overlap of peaks might explain the less than ideal behavior of this system (i_a/i_c = 2.1). In

mixed 50:50 KH$_2$PO$_4$:CH$_3$CN the complex displays an irreversible to quasi-reversible

oxidation under both air saturated and deoxygenated conditions.

Electrogenerated Chemiluminescence. ECL was observed for 1,5-I-Aedans in

aqueous buffered and 50:50 (v/v) KH$_2$PO$_4$:CH$_3$CN at a Pt interface by sweeping to

positive potentials in the presence of 0.05M TPrA. Above pH 10 luminophores such as
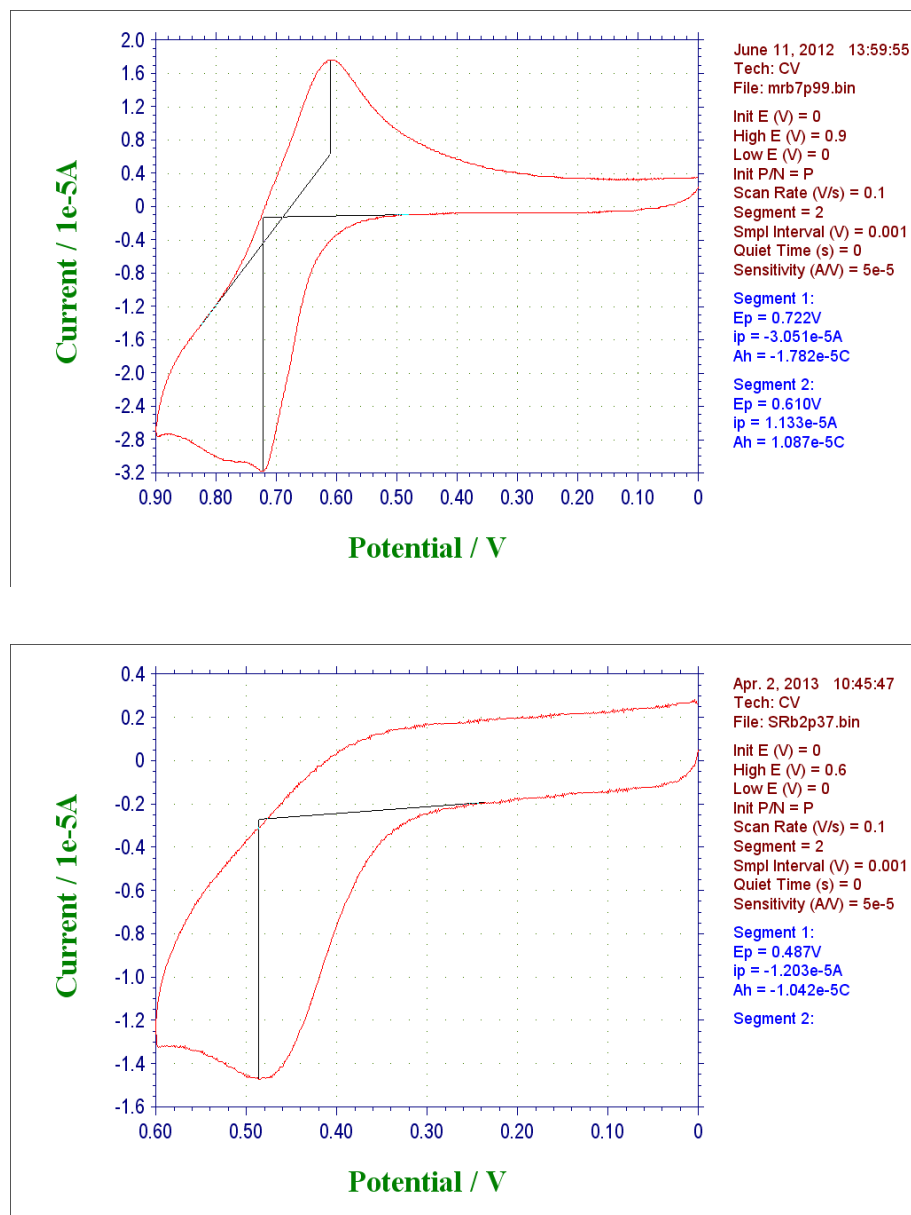
Figure 5. Electrochemical data for 1×10^{-3} M 1,5-I-Aedans in aqueous buffered solution (top) and 50:50 KH_2PO_4: CH_3CN (bottom).

Ru(bpy)$_3^{2+}$ can react with hydroxyl groups from solvent oxidation to produce ECL.[23] To be certain that the ECL emission was from the reaction of 1,5-I-Aedans with TPrA all experiments were run at pH = 8.0 ± 0.2 (this pH also resulted in very efficient ECL compared to lower pHs, *vide infra*). Background studies also confirmed that very

little ECL was emitted at this pH from 1,5-I-Aedans when TPrA was absent from solution and no ECL was observed above background in the absence of 1,5-I-Aedans.

Intensity vs. potential. The ECL intensity peaks at potentials around +1.0 V (Figure 6) in aqueous buffered solution and around +1.5 V in 50:50 (v/v) $KH_2PO_4:CH_3CN$, indicating oxidation of both TPrA ($E_a \sim$ +0.9 V vs. Ag/AgCl) and 1,5-I-Aedans. The 500 mV difference in peak potential when acetonitrile is present in solution is surprising, and probably reflects the greater electrochemical irreversibility of the oxidative electrochemistry in 50:50 $KH_2PO_4:CH_3CN$.

Intensity vs. time. 1,5-I-Aedans ECL is diffusion controlled in KH_2PO_4 (Figure 7) and similar to that observed for other ECL luminophores such as $Ru(bpy)_3^{2+}$. In the Intensity vs. time transient the potential of the working electrode was poised at +2.0 V for the duration of the experiment (typically 15 minutes), and the ECL intensity measured. As expected, there was a sharp increase in light intensity due to the higher concentrations of coreactant and luminophore near the electrode surface that then decreased over time due to the formation of a diffusion layer of electrochemical and ECL products near the electrode surface. Surprisingly, ECL in mixed solvent increases over time. This phenomenon has been observed before[3] and suggests that either the reactants are being regenerated at the electrode surface (seen as unlikely due to the irreversible nature of both TPrA and 1,5-I-Aedans) or decomposition products are taking part in ECL.

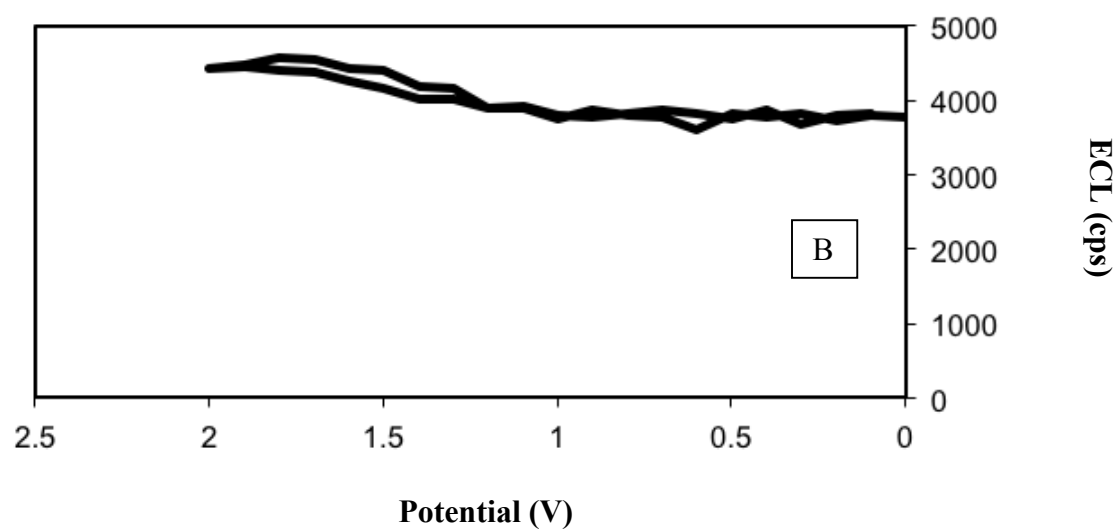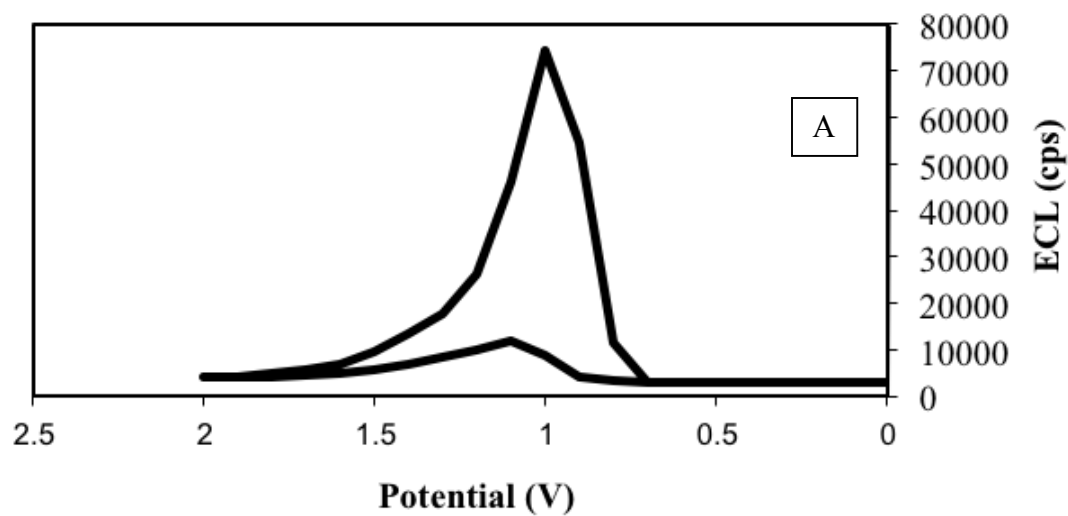Figure 6: ECL Intensity versus potential for (A) 1×10^{-3} M 1,5-I-Aedans in aqueous buffered solution. Scan rate 0.1 mV/sec. (B) ECL Intensity versus potential for 1×10^{-3} M 1,5-I-Aedans in 50:50 (v/v) $KH_2PO_4:CH_3CN$. Scan rate 0.1 mV/sec.
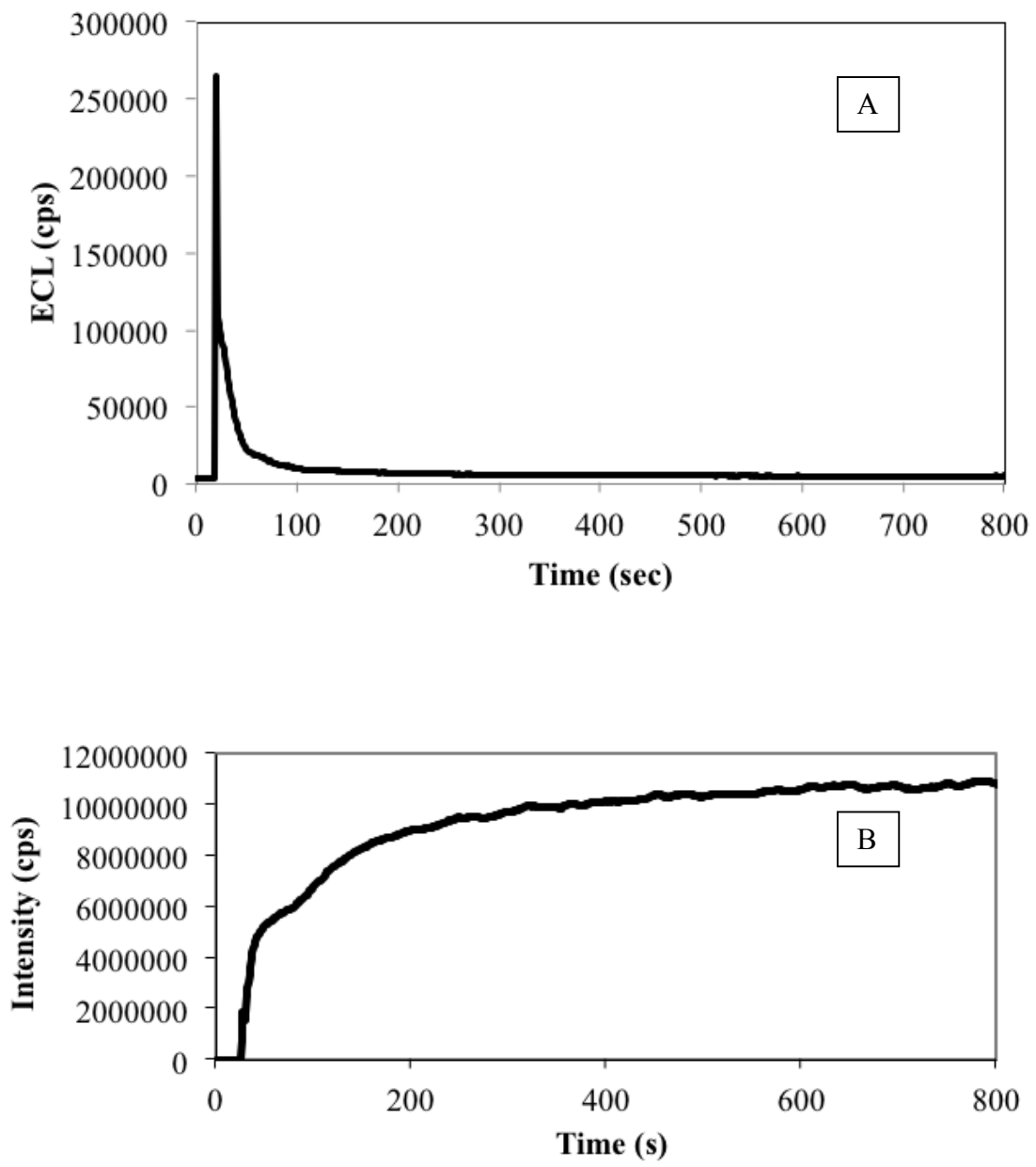
Figure 7: ECL Intensity versus time for 1×10^{-3} M 1,5-I-Aedans in (A) aqueous buffered solution and (B) 50:50 (v/v) $KH_2PO_4:CH_3CN$ ([TPrA] = 50 mM).

Intensity vs. pH. The ECL increases fairly linearly as a function of pH between

pHs 3.0 and 8.0, mirroring other luminophores such as $Ru(bpy)_3^{2+}$ when TPrA is the

coreactant.[5] This suggests that deprotonation of the TPrA radical cation is important to

the generation of ECL in both 1,5-I-Aedans and ruthenium systems. The solubility of

TPrA decreases at higher pHs with a clearly visible material suspended in solution above

pH 9.3. Therefore, a pH of 8.0 was used in all experiments.

ECL. ECL efficiencies (photons emitted per redox event) of 1,5-I-Aedans were

6.8×10^{-4} and 0.072×10^{-4} in aqueous and 50:50, respectively (Table 1), much lower than

the $Ru(bpy)_3^{2+}$ standard ($\phi_{ecl} = 1$). Also, the efficiency is much lower in mixed solvent

than aqueous buffer, opposite to the trend for other luminophores such as $Ru(bpy)_3^{2+}$ and

opposite to the trend in ϕ_{em}. This is not surprising, considering the irreversible to quasi-

reversible oxidative electrochemistry.

Attempts to directly measure ECL spectra were unsuccessful, presumably due to

the low ϕ_{ecl}. Therefore, yellow, green and blue plastic absorption filters were used to

obtain the wavelength ranges of ECL in aqueous buffered solution containing TPrA. 1,5-

I-Aedans displays blue-green emission with a maximum around 500 nm, indicating that

the same excited states are formed via ECL and photoluminescence. However, the

qualitative nature of this data makes definitive assignments impossible.

On the basis of photoluminescence emission wavelengths, the energy needed to

generate the excited state of the complexes is in the range of 2.48 to 2.50 eV (e.g., if the

wavelength is 496 nm (or 0.496 μm), the frequency is 6.04 GHz and the energy is 2.48

eV using $E = hc/\lambda$ where c is the speed of light and h is Planck's constant). Therefore, for

direct generation of the excited state the energy of the electron or energy-transfer events

between the electrogenerated coreactant and luminophore molecules must exceed these values. However, studies on a series of polyaromatic hydrocarbons have indicated that while TPrA[•] is a good reductant, it does not have a sufficiently negative potential to generate excited states above 2.32 eV.[24] Since qualitative ECL spectra suggest emission in the same region as photoluminescence emission, excited states in 1,5-I-Aedans are not populated by the direct reaction of oxidized 1,5-I-Aedans with TPrA[•] but by a more complicated mechanism. The exact mechanism of ECL in 1,5-I-Aedans is beyond the scope of the present work.

1,5-I-Aedans as a Ru(bpy)$_3^{2+}$ Coreactant. It is well known that amines can act as ECL coreactants,[1,5] with tertiary amines such as TPrA generating the most intense light emission.[1-3] Since 1,5-I-Aedans contains two secondary amines it might be capable of acting as an ECL coreactant with Ru(bpy)$_3^{2+}$ as the luminophore when no TPrA is present. These experiments used aqueous 0.18M KH$_2$PO$_4$ as the solvent and electrolyte, with 0.01 mM Ru(bpy)$_3^{2+}$ and 1 mM 1,5-I-Aedans at pH = 8.0 ± 0.1. Blank solutions of Ru(bpy)$_3^{2+}$ or 1,5-I-Aedans produced 1.4 x 10^4 and 0.034 x 10^4 counts per second (cps), respectively, while a solution containing both Ru(bpy)$_3^{2+}$ and 1,5-I-Aedans produced 0.24 x 10^4 cps. Clearly, 1,5-I-Aedans does not act as an ECL coreactant with Ru(bpy)$_3^{2+}$ and, in fact appears to be quite efficient at attenuating the ECL signal of Ru(bpy)$_3^{2+}$.

Mixed 1,5-I-Aedans and Ru(bpy)$_3^{2+}$ with TPrA Coreactant. One goal of research in ECL is to discover new light emitting systems.[1-3] Not only to find new molecules that emit light, but to find more efficient ECL luminophores (so that better detection limits and sensitivity than Ru(bpy)$_3^{2+}$ might be obtained) and to find luminophores whose signal can be determined in the same sample solution as Ru(bpy)$_3^{2+}$ (thus allowing so called

multianlyate detection). For example, $Ir(ppy)_3$ (ppy = 2-phenylpyridine) emits with an ECL maximum of approximately 500 nm and this allowed for the determination of both $Ir(ppy)_3$ and $Ru(bpy)_3^{2+}$ in the same sample solution when TPrA was present.[25] Therefore, to test whether both 1,5-I-Aedans and $Ru(bpy)_3^{2+}$ can emit ECL while in the same solution a series of experiments were conducted varying solution composition and using TPrA as the coreactant. A standard solution containing 0.1mM $Ru(bpy)_3^{2+}$ and 0.40 mM TPrA in phosphate buffer produced 5.2×10^4 cps, a solution of 1mM 1,5-I-Aedans and 0.40 mM TPrA in phosphate buffer produced 0.46×10^4 cps, while a solution containing both 1,5-I-Aedans and $Ru(bpy)_3^{2+}$ with TPrA produced 1.6×10^4 cps. Unfortunately, even with TPrA present in solution 1,5-I-Aedans attenuates the ECL signal of $Ru(bpy)_3^{2+}$.

Conclusions

This study illustrates that 1,5-I-Aedans exhibits electrogenerated chemiluminescence in aqueous and 50:50 KH_2PO_4 solutions when TPrA is a coreactant. Although the ECL emission quantum efficiency is weaker than $Ru(bpy)_3^{2+}$ under similar conditions, the excited state formed via ECL appears to be the same as that formed via PL suggesting the possibility of using 1,5-I-Aedans ECL as a probe of biomolecular interactions.

CHAPTER 4: ENHANCED ECL VIA OXYGEN QUENCHING USING MELATONIN

Introduction

Understanding the ECL properties of melatonin (*N*-acetyl-5-methoxytryptamine), Figure 8, is of interest for several reasons. Melatonin is a pineal hormone known for its role in numerous physiological processes such as metabolism, reproduction, circadian sleep-wake cycles, appetite, and muscular coordination. It also plays a role in psychiatric processes (such as anxiety and seasonal depression), in the immune system, has anti-oxidant properties[26,27] and is a free radical scavenger.[26,28,29]
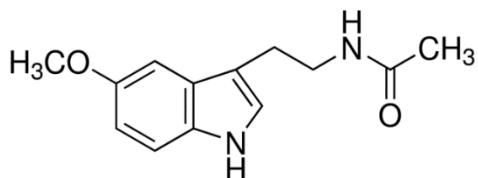
Figure 8. Structure of melatonin.

Analytical methods for the detection of melatonin include voltammetric,[30] photoluminescence[31] (PL) and chemiluminescence[32] spectroscopy, liquid[33] and gas[34] chromatography, capillary electrophoresis,[35] liquid chromatography coupled with mass spectroscopy,[36] and electrogenerated chemiluminescence.[37-40] ECL offers some advantages over other methods of analyses. For example, compared to fluorescent methods those involving ECL do not require an excitation source and tend to be less sensitive to interferences from luminescent impurities and scattered light.[29] Several

reports have appeared on the ECL properties of structurally similar compounds.[37-39] Although $Ru(bpy)_3^{2+}$ is the most widely used luminophore in both fundamental and applied studies, a number of other transition metal chelates also display ECL. For example, ortho-metalated complexes of Ir(III) display ECL that, like their Ru(II) counterparts, make them of interest in fundamental and applied studies.[25,41-49] Also, the ruthenium chelates $(bpy)_2Ru(DC-bpy)^{2+}$ (bpy = 2,2'-bipyridine and DC-bpy = (4,4'-dicarboxy-2,2'-bipyridine) and $(bpy)_2Ru(DM-bpy)^{2+}$ (DM-bpy = 4,4'-dimethyl-2,2'-bipyridine), much like $Ru(bpy)_3^{2+}$, show enhanced ECL in the presence of the nonionic surfactant Triton X-100 making it possible that they, too, will show sensitivity to the presence or absence of oxygen in solution.[9] In the case of MLT, ECL has focused on MLT quenching of the luminol/H_2O_2 system. However, to our knowledge, the ECL of melatonin itself or its effect on the ECL luminophore $Ru(bpy)_3^{2+}$ have not been reported.

Therefore, this work focuses on the ECL of Ruthenium[9] and Iridium[47] complexes in the presence and absence of melatonin. In particular, a method for the enhancement of the commercially important $Ru(bpy)_3^{2+}$/TPrA reaction system via a unique mechanism is presented. The effect on the coreactant ECL of $(bpy)_2Ru(DC-bpy)^{2+}$ (bpy = 2,2'-bipyridine and DC-bpy = 4,4'-dicarboxy-2,2'-bipyridine), $(bpy)_2Ru(DM-bpy)^{2+}$ (DM-bpy = 4,4'-dimethyl-2,2'-bipyridine), and $Ir(ppy)_3$ (ppy = 2-phenylpyridine) when MLT is present in aqueous buffered solution with tri-*n*-propylamine as the coreactant is also reported.

Results and Discussion

        UV-Vis and PL. MLT displays strong absorptions between 250 and 325 nm characteristic of π-π^* and n-π^* events while $Ru(bpy)_3^{2+}$ displays a series of ligand based transitions in the UV and Metal-To-Ligand Charge Transfer (MLCT) bands in the visible ($\lambda_{abs} \sim 450$ nm). The presence of MLT does not affect the wavelength or absorbance of the MLCT transitions in any of the luminophores within experimental error.

        Excitation into the lowest energy absorption band for each complex (λ_{abs}) produces room temperature photoluminescence (PL) with emission bands (λ_{em}) centered at 612 nm for $Ru(bpy)_3^{2+}$, 507 nm for $Ir(ppy)_3$, 605 nm for $((bpy)_2Ru(DM\text{-}bpy)^{2+}$, and 629 nm for $((bpy)_2Ru(DC\text{-}bpy)^{2+})$ as shown in Table 3. Since MLT enhanced $Ru(bpy)_3^{2+}$/TPrA ECL, the PL of MLT was studied in detail. Excitation into the ultraviolet band of MLT ($\lambda_{exc} = 300$nm) produces PL between 325 and 450 nm, with a wavelength maximum of 375 nm. The photoluminescence efficiency of MLT (ϕ_{em}; photons emitted per photons absorbed) is 0.002 (or 0.2%) relative to $Ru(bpy)_3^{2+}$ at 0.042 (or 4.2%). The presence of melatonin does not affect the shape, intensity or ϕ_{em} of the MLCT emission bands when excited at their lowest energy absorption. This suggests that a product of melatonin oxidation, and not the MLT itself, is responsible for the observed increases in ECL.

Table 3. Spectroscopic UV/Vis, PL, and ECL data for MLT.[a]

Luminophore	λ_{abs}(nm)	λ_{em}(nm)	ϕ_{em}[b]	ϕ_{ECL}[c]	ϕ_{ECL}-MLT[d]
$Ru(bpy)_3^{2+}$	454	596	0.042	1.0	2.5
$(bpy)_2Ru(DC\text{-}bpy)^{2+}$	458	629	0.020	0.73	0.99
$(bpy)_2Ru(DM\text{-}bpy)^{2+}$	455	605	0.030	0.84	1.2
$Ir(ppy)_3$	380	507,532(sh)	0.050	0.0044	0.0073

[a][KH_2PO_4] = 0.18 M (aqueous buffered solution) at pH 8.0 ±0.1. [Luminophore] = 0.01 mM. [TPrA] = 0.05 M.
[b]Relative photoluminescence emission efficiencies using $Ru(bpy)_3^{2+}$ (H_2O) as a standard.
[c]Relative ECL efficiencies (photons emitted per ejected) using $Ru(bpy)_3^{2+}$/TPrA/KH_2PO_4 as a standard (ϕ_{ECL} = 1). Values are the averages of at least 3-replicates with a standard deviation of ±5%.
[d][MLT] = 0.05M. Relative efficiencies using the luminophore/TPrA/KH_2PO_4 solution with no MLT as a standard. Values are the averages of at least 3-replicates with a standard deviation of ±5%.

Several PL experiments were run in both air saturated and deoxygenated solution. The results for $Ru(bpy)_3^{2+}$ are reported in Table 4 and Figure 9. Simply deoxygenating a solution results in an approximately 20% increase in PL intensity, consistent with ECL experiments. Bulk electrolyzing the MLT prior to mixing with the other reagents generates results consistent with deoxygenating with Argon. Also, when the MLT solution is degassed prior to bulk electrolysis the increase in PL is approximately the same as the air saturated bulk-electrolyzed solution. The results for all three compounds are consistent and also correlate well with the $Ru(bpy)_3^{2+}$/TPrA system suggesting that oxygen scavenging is an integral part of the enhancement mechanism.

Table 4. PL data with MLT in air or deoxygenated solution.

Solution Composition	Air Saturated (Air) or Deoxygenated (Deox)[a]	PL Intensity (au)[b]
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$	Air	1.795
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$	Deox	2.216
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$, MLT	Air	1.630
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$, MLT	Deox	2.222
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$, MLT Oxidation products [c]	Air	2.102
KH_2PO_4, TPrA, $(DC)Ru(bpy)_2^{2+}$	Air	15.775
KH_2PO_4, TPrA$(DC)Ru(bpy)_2^{2+}$	Deox	20.476
KH_2PO_4, TPrA, $(DC)Ru(bpy)_2^{2+}$, MLT	Air	13.549
KH_2PO_4, TPrA, $(DC)Ru(bpy)_2^{2+}$, MLT	Deox	19.333
KH_2PO_4, TPrA, $(DC)Ru(bpy)_2^{2+}$, MLT Oxidation products [c]	Air	20.391
KH_2PO_4, TPrA, $(DC)Ru(bpy)_2^{2+}$, MLT Oxidation products [c]	Deox	20.495

[a]Deoxygenated with Argon for ~ 5 minutes prior to each run.
[b]λ_{exc} = 450 nm, Slits 5 nm, value is the average of three runs with freshly mixed solutions.
[c]The MLT was bulk electrolyzed for 40 minutes prior to mixing with the other reagents.

Electrochemistry. The electrochemistry of the luminophores has been extensively studied.[10] They display electrochemically reversible oxidation around +1.2 V for $Ru(bpy)_3^{2+}$, +0.452 V for $Ir(ppy)_3$, +0.982 V for $(DM-bpy)Ru(bpy)_2^{2+}$ and +1.08 V for $(DC-bpy)Ru(bpy)_2^{2+}$ and a series of three sequential ligand based reductions. MLT displays one oxidation with a peak potential (E_a) of 0.58 V vs Ag/AgCl in aqueous buffered solution.
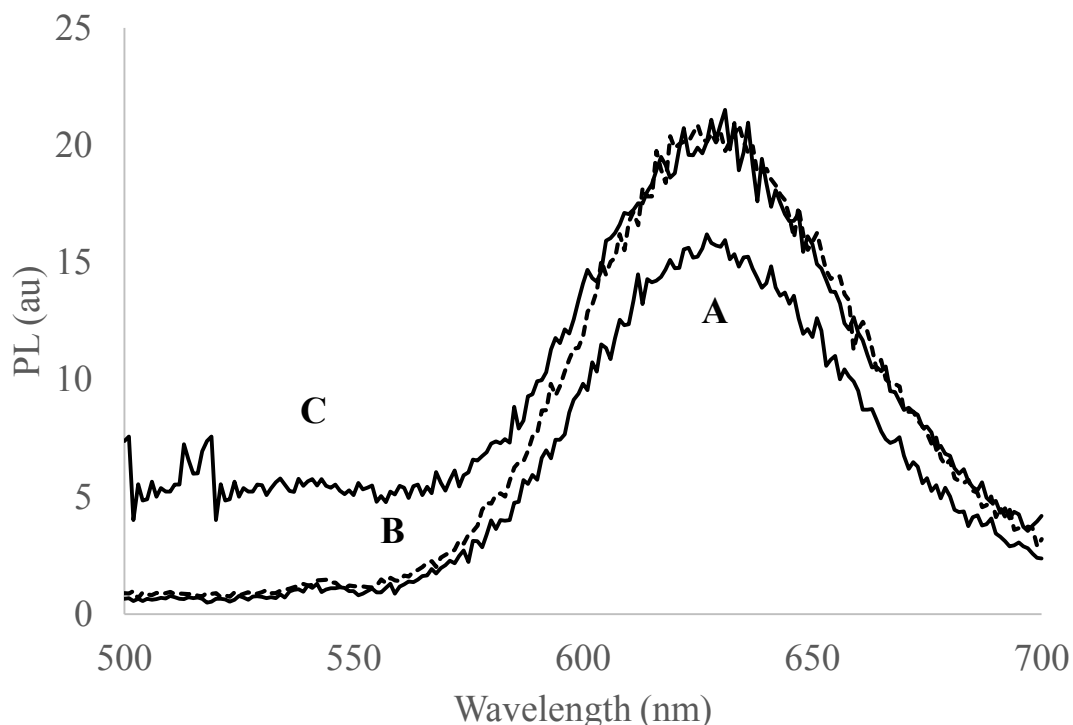
Figure 9. Photoluminescence coupled with bulk electrolysis scans of solutions containing different combinations of 1 x 10^{-5}M (DC-bpy)Ru(bpy)$_2^{2+}$, 0.05M TPrA, and 5mM MLT in aqueous 0.18M KH$_2$PO$_4$ (pH = 8.0). (A, solid line) (DC-bpy)Ru(bpy)$_2^{2+}$/TPrA in air saturated solution with no bulk electrolysis, [MLT] = 0; (B, dashed line) (DC-bpy)Ru(bpy)$_2^{2+}$/TPrA in deoxygenated solution with no bulk electrolysis, [MLT] = 0; and (C, solid line) MLT/TPrA bulk electrolyzed for 40 minutes at a potential of +2.0 V and added to an air saturated solution containing (DC-bpy)Ru(bpy)$_2^{2+}$/TPrA.

The process is electrochemically irreversible on the time scale of the experiment (i.e., 0.1 V/sec) and at scan rates up to 5 V/s. Dissolved oxygen in solution does not affect the MLT oxidation potential since identical results are observed in air saturated solution and solutions degassed for 10 minutes with Argon.

Electrogenerated Chemiluminescence. ECL data is shown in Table 3 (above). MLT is a poor coreactant compared to TPrA when Ru(bpy)$_3^{2+}$ is the luminophore (ϕ_{ECL} (Ru(bpy)$_3^{2+}$/TPrA) = 1) with an ECL efficiency of 0.0053 when TPrA is absent from

solution. This is not surprising since MLT contains secondary amines, which are weaker

ECL coreactants than tertiary amines like TPrA.[1-3] A representative example of ECL for

a solution of (DC-bpy)Ru(bpy)$_2^{2+}$ containing MLT and TPrA is also shown in Figure 10.

All luminophores display ECL at potentials corresponding to oxidation of both

luminophore and coreactant ($E_a = 0.90V$) with ECL efficiencies between 4.4 x 10^{-3} and

0.84 when MLT is absent from solution. In all four luminophore/TPrA systems, the shape

of the emission and the potentials at which emission occurs are similar to solutions

without MLT. This suggests that the presence of MLT or MLT oxidation products do not

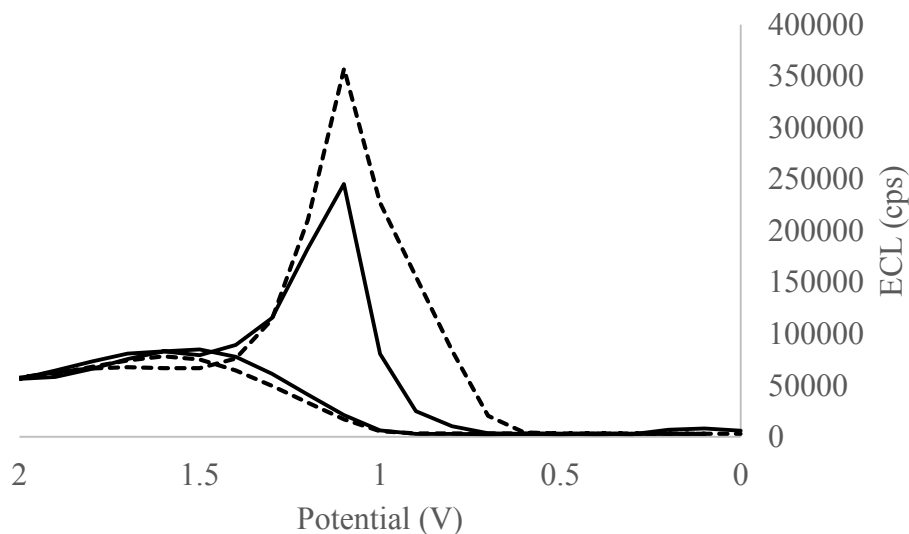interfere with excited state formation or emission.[29,49]

Figure 10. ECL Intensity versus potential for 1x10^{-6}M (DC-bpy)Ru(bpy)$_2^{2+}$ and 0.05M TPrA in 0.18M KH$_2$PO$_4$ with (——) no MLT and (- - -) 1x10^{-5} M MLT. Scan rate 0.1 mV/sec.

A representative ECL emission intensity versus time profile is shown in Figure 11. In these experiments, the potential of the working electrode was stepped to +2.0V in a quiescent solution while light emission was measured. All systems showed the characteristic sharp increase in light intensity upon application of the electrochemical potential due to the high concentrations of co-reactant and luminophore near the electrode surface.[29,49] Within approximately 1 second, the ECL emission becomes diffusion controlled in the presence or absence of MLT. As in the $Ru(bpy)_3^{2+}$/TPrA system, the presence of melatonin does not appear to interact directly with the ECL reaction sequence to enhance ECL efficiencies.[49] Furthermore, the formation of two possible oxidation products,[27] N^1-acetyl-N^2-formyl-5-methoxykynurenine (AFMK), and hydroxymelatonin (HO-MLT), depend on the presence of molecular oxygen in solution (Figure 12).[29,49] The most likely mechanism is electrochemical production of ortho- and benzo-quinones followed by energy or electron transfer to nonemissive states.[50] The 2.5-fold enhancement with melatonin was unexpected given the structural similarities to other complexes and the experimental observation that melatonin is an efficient free radical scavenger that might interact with TPrA•.[29]

The ECL of MLT/$Ru(bpy)_3^{2+}$/TPrA is linear between $1x10^{-8}$ and $1x10^{-4}$ M with an r^2 value of 0.9851 (experimental range tested was from 1×10^{-3} to 1×10^{-12} M) for six data points with a theoretical detection limit (blank signal plus three times the standard deviation of the blank) of $1x10^{-12}$ M and a practical detection limit of $1x10^{-8}$ M using our experimental system. At mM concentrations of MLT the ECL intensity plateaus, indicating that the enhanced ECL is dependent on melatonin concentration. The observation that the presence of melatonin does not affect the PL of $Ru(bpy)_3^{2+}$/TPrA
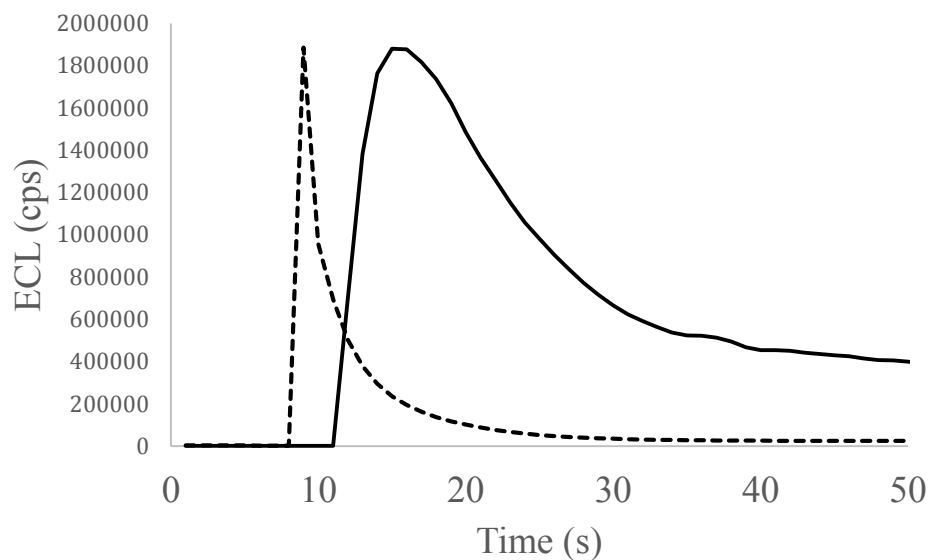
Figure 11. ECL Intensity versus time for $1x10^{-6}$M (DC-bpy)Ru(bpy)$_2^{2+}$ and 0.05M TPrA in 0.18M KH$_2$PO$_4$ with (—) no MLT and (- - -) $1x10^{-5}$ M MLT. The scans have been normalized to the same scale and offset for clarity. Potential was poised at +2.0V for the duration of the experiment.
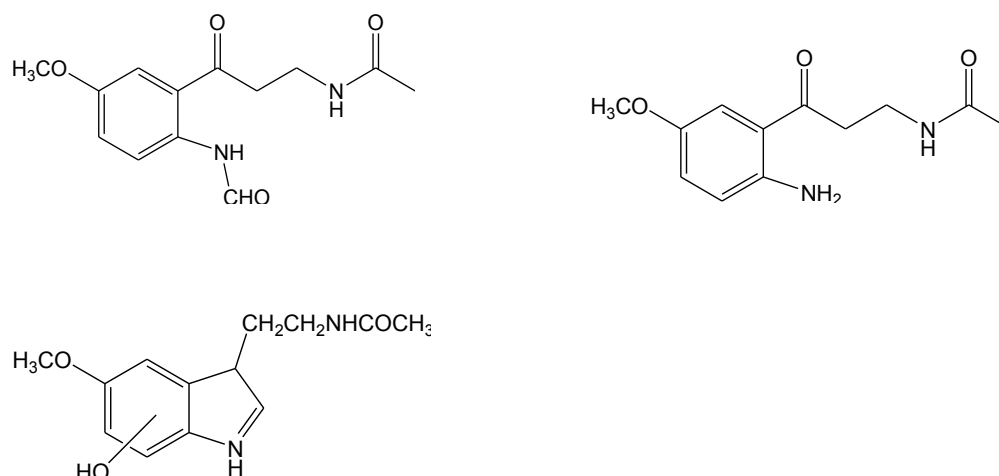
Figure 12. Structures of AFMK (top), HO-MLT (bottom), and AMK (right).

also suggests that a product of melatonin oxidation is responsible for the observed ECL increases.[29]

A representative example of ECL for a solution of $Ru(bpy)_3^{2+}$ containing MLT and TPrA is shown in Figure 13. Emission occurs at potentials corresponding to oxidation of both $Ru(bpy)_3^{2+}$ ($E_a = 1.20$ V) and TPrA ($E_a = 0.90$V) and are similar in shape and potential of emission to solutions without MLT present. ECL emission spectra (Figure 11) are nearly identical to photoluminescence spectra, indicating the same MLCT excited state is formed in both ECL and PL and that the presence of MLT or oxidation products does not interfere with excited state formation or emission.[29]
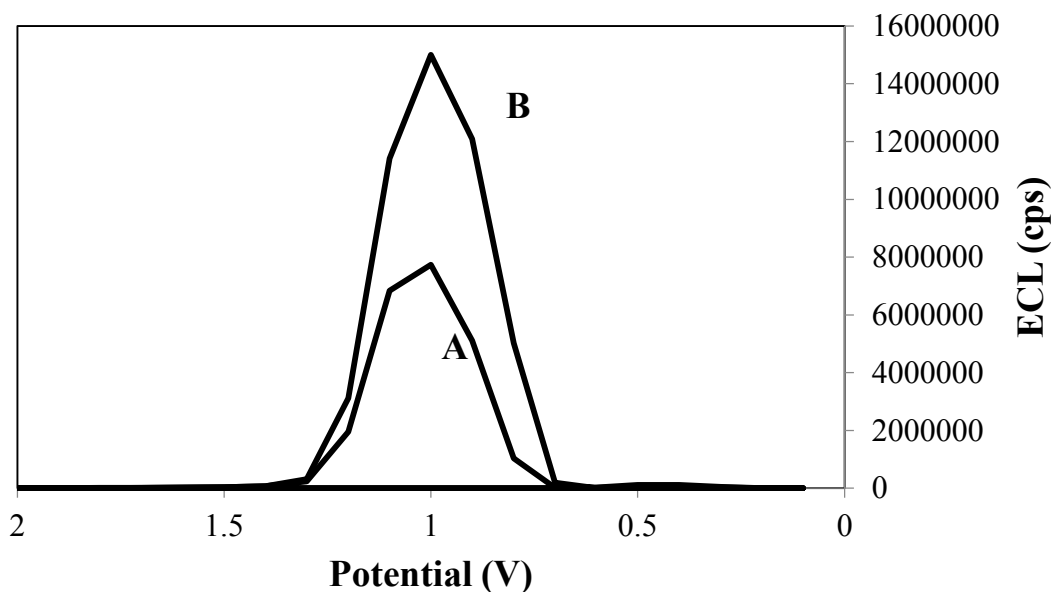
Figure 13. ECL Intensity Versus Potential plots of 1 x 10^{-5}M $Ru(bpy)_3^{2+}$ and 0.05M TPrA in aqueous 0.18M KH_2PO_4 (pH = 8.0) with (A) [MLT] = 0 and (B) [MLT] = 1 x 10^{-5}M.

The pH was varied from 4 to 9 with maximum ECL intensities observed between pHs 8 and 9 (Figure 14). Similar trends are observed for $Ru(bpy)_3^{2+}$ under a variety of

conditions using TPrA as a coreactant. This indicates that the proposed mechanism of

TPrA oxidation/deprotonation to form a highly reducing intermediate is operating when

MLT is present in solution.[29]
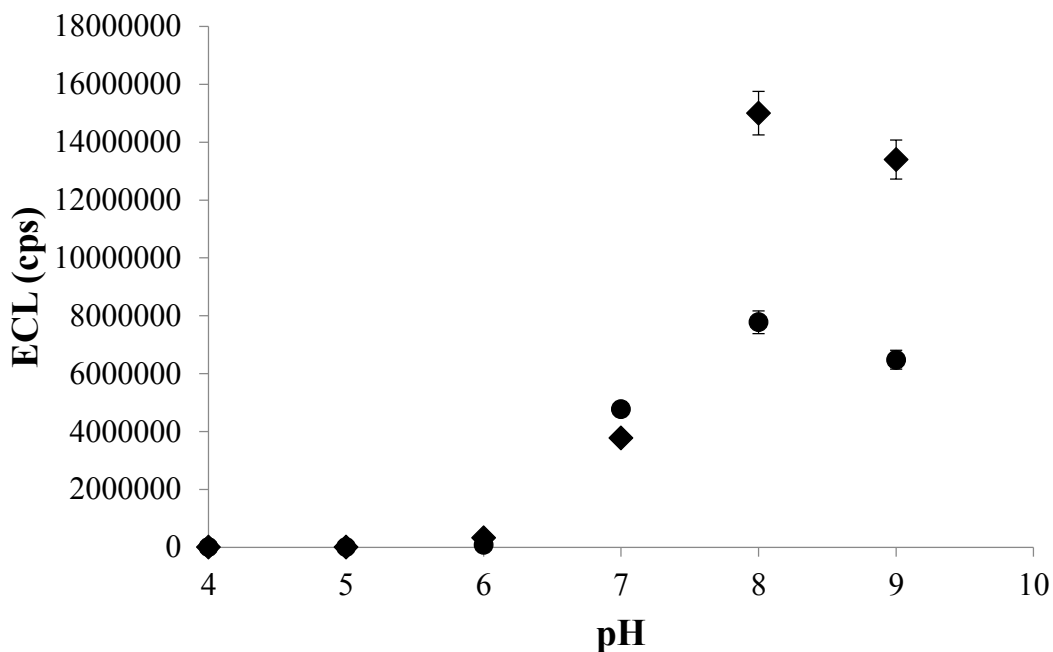
Figure 14. ECL intensity versus pH of 1 x 10^{-5}M Ru(bpy)$_3$$^{2+}$ and 0.05M TPrA in aqueous 0.18M KH$_2$PO$_4$ solution with (●●●) [MLT] = 0 and (♦ ♦ ♦)[MLT] = 1x 10^{-5}M. Each point is the average of at least three scans with error bars at ±5%.

An ECL emission intensity versus time profile displays diffusion controlled

characteristics (Figure 15). The potential of the working electrode was stepped to +2.0V,

and the ECL intensity measured. As expected, there was a sharp increase in light intensity

due to the high concentrations of co-reactant and Ru(bpy)$_3$$^{2+}$ near the electrode surface.

Under these conditions, the time taken to reach peak intensity was approximately 1s. At

longer times, the ECL emission becomes diffusion controlled. Again, the presence of

melatonin does not appear to interact directly with the $Ru(bpy)_3^{2+}$/TPrA system to change ECL intensities.[29]
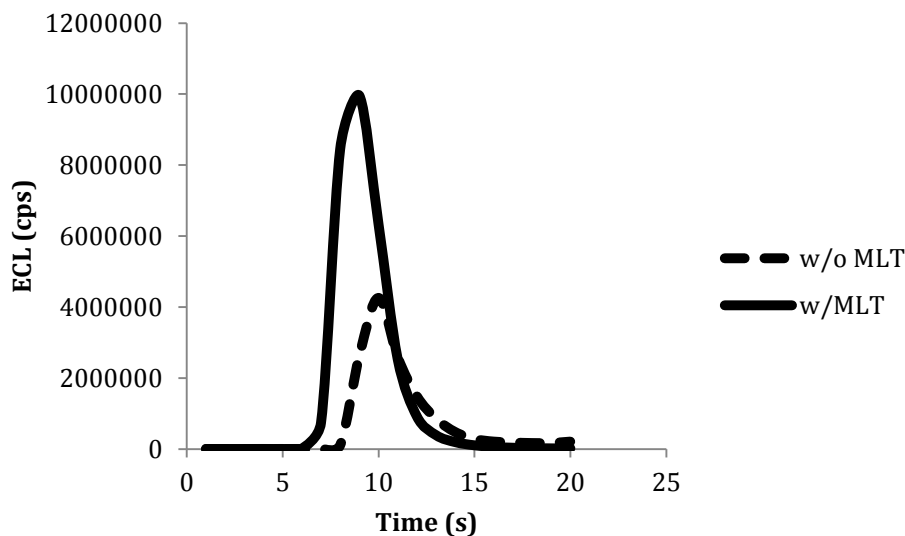
Figure 15. ECL intensity versus time for 1 x 10^{-5}M $Ru(bpy)_3^{2+}$ and 0.05M TPrA in aqueous 0.18M KH_2PO_4 solution with (- -) [MLT] = 0 and (-)[MLT] = 1x 10^{-5}M. Each point is the average of at least three scans with a standard deviation of ±5%.

Mechanism of Enhancement. The experimental observation that PL is not affected by the presence of MLT while ECL is enhanced suggests that a product or products of melatonin oxidation are responsible for the increases. To test this, bulk electrolysis was coupled with PL spectral analysis. During bulk electrolysis the potential of the working electrode was poised at +2.0 V vs Ag/AgCl while the solution was stirred, and aliquots tested every 10 minutes for a period of approximately 40-minutes (λ_{exc} = 450 nm).[29]

A background solution containing TPrA and MLT displayed no PL between 400 and 700 nm. Also, a solution containing TPrA and $Ru(bpy)_3^{2+}$ with no MLT displayed no

increases in PL intensity during bulk electrolysis. With a MLT/TPrA/Ru(bpy)$_3^{2+}$ solution, however, a 17% increase in PL was observed at 10 minutes that gradually changed to 23% at 40 minutes. No further increases were observed beyond 40 minutes. Another BE/PL experiment was conducted where only MLT in buffer underwent BE. Aliquots were then added to a cuvette containing Ru(bpy)$_3^{2+}$ and TPrA and PL measured, with nearly identical results (i.e., ~23% increase in PL after 40 minutes).[29]

To probe the phenomenon further a solution of MLT in phosphate buffer was bulk electrolyzed for 40 minutes and added to a solution of Ru(bpy)$_3^{2+}$/TPrA. The ECL of this solution was measured, with a 2.3-fold enhancement relative to a Ru(bpy)$_3^{2+}$/TPrA solution containing no MLT (Figure 16). For reference (Table 4), a Ru(bpy)$_3^{2+}$/TPrA/MLT solution that has not undergone BE prior to mixing displays a 2.5-fold enhancement. This clearly shows that the products of MLT oxidation are leading to increased ECL intensities and efficiencies.[29]

Several oxidation products have been identified in the literature,[27,29,49] including N^1-acetyl-N^2-formyl-5-methoxykynurenine (AFMK), N^1-acetyl-5-methoxykynurenine (AMK), and hydroxymelatonin (HO-MLT).[27,29,49] However, the exact nature of the products depends upon solution conditions (e.g., oxygenated versus deoxygenated solutions) and the means by which oxidation was induced (e.g., radiolysis of melatonin solutions, metabolism, or electrochemical). Numerous electrochemical studies [29,51] show that MLT undergoes a two-electron oxidation followed by a proton transfer with the formation of a cation at the 5-position of the indole ring.[29]
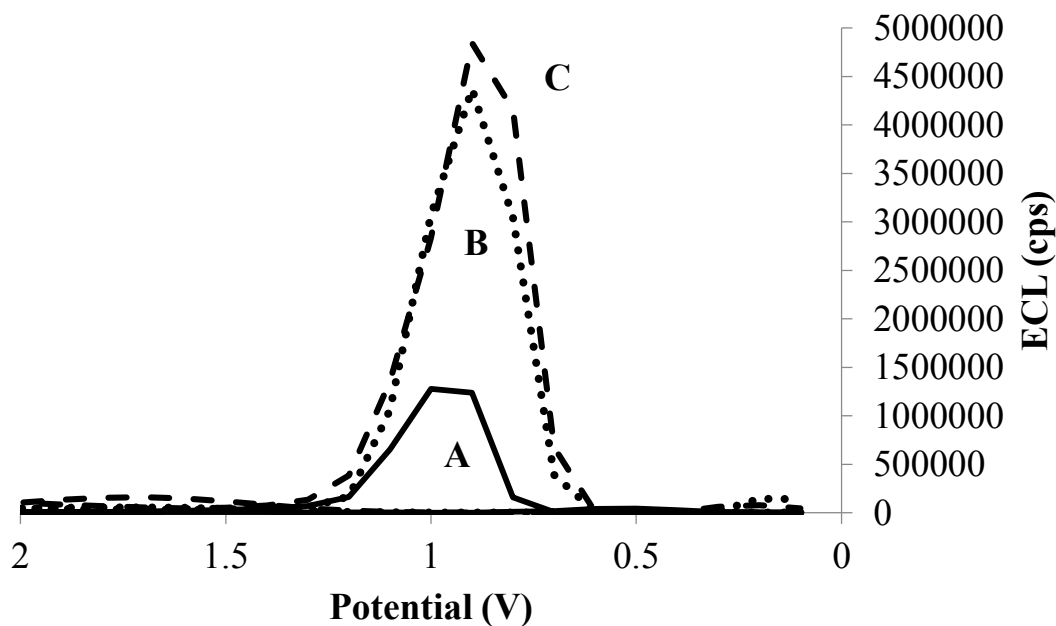
Figure 16. ECL coupled with bulk electrolysis scans of solutions containing different combinations of 1 x 10^{-7} M Ru(bpy)$_3^{2+}$, 0.05M TPrA, and 1 x 10^{-5}M MLT in aqueous 0.18M KH$_2$PO$_4$ (pH = 8.0). (A, solid line) Ru(bpy)$_3^{2+}$/TPrA in air saturated solution with no bulk electrolysis, [MLT] = 0; (B, dotted line) Ru(bpy)$_3^{2+}$/TPrA/MLT in air saturated solution with no bulk electrolysis; (C, dashed line) MLT/TPrA bulk electrolyzed for 40 minutes at a potential of +2.0 V and added to an air saturated solution containing Ru(bpy)$_3^{2+}$/TPrA.

Of particular interest to this study is that melatonin quenched H$_2$O$_2$/luminol ECL.[36] The quenching was traced to the annihilation of the oxygen free radicals by MLT or an oxidation product. These free radicals are an integral part of the light generating mechanism in H$_2$O$_2$/luminol. Also, the generation of AFMK and HO-MLT strongly depended on the presence of molecular oxygen in solution,[27] showing that MLT interacts directly with dissolved oxygen.[29]

Ruthenium polypyridyl excited states are sensitive to microenvironmental effects of the solvent media,[52] such as interactions of the excited state species with solvent,

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electrolyte and dissolved oxygen in solution. In particular, the $Ru(bpy)_3^{2+}$ excited state can be quenched by dissolved oxygen in solution. If a melatonin oxidation product is intercepting dissolved O_2 this might explain why the ECL increases when MLT undergoes electrochemical oxidation, leading to enhanced luminescence. To test this hypothesis, several PL experiments were run in both air saturated and deoxygenated solution. The results are reported in Table 4. Simply deoxygenating a solution results in an approximately 20% increase in PL intensity, consistent with the increases observed in the ECL experiments. Bulk electrolyzing the MLT prior to mixing with the other reagents generates results consistent with deoxygenating with Argon. Also, when the MLT solution is degassed prior to bulk electrolysis the increase in PL is approximately 2% compared to the air saturated bulk electrolyzed solution suggesting that oxygen scavenging is an integral part of the enhancement mechanism. Also, several ECL experiments were run in air saturated and deoxygenated solutions and compared to the ECL of a luminophore/TPrA/MLT solution (Figure 17). To summarize, an air saturated solution containing $(DC\text{-}bpy)Ru(bpy)_2^{2+}$ and TPrA with no MLT displayed ECL of approximately 1,100,000 counts per second (cps). A degassed solution containing $(DC\text{-}bpy)Ru(bpy)_2^{2+}$ and TPrA with no MLT displayed ECL of approximately 2,200,000 cps while an air saturated solution containing $(DC\text{-}bpy)Ru(bpy)_2^{2+}$ and TPrA with 5mM MLT displayed ECL of approximately 1,800,000 counts per second (cps). Clearly, the presence of dissolved oxygen influences the ECL in these systems.[29,49]
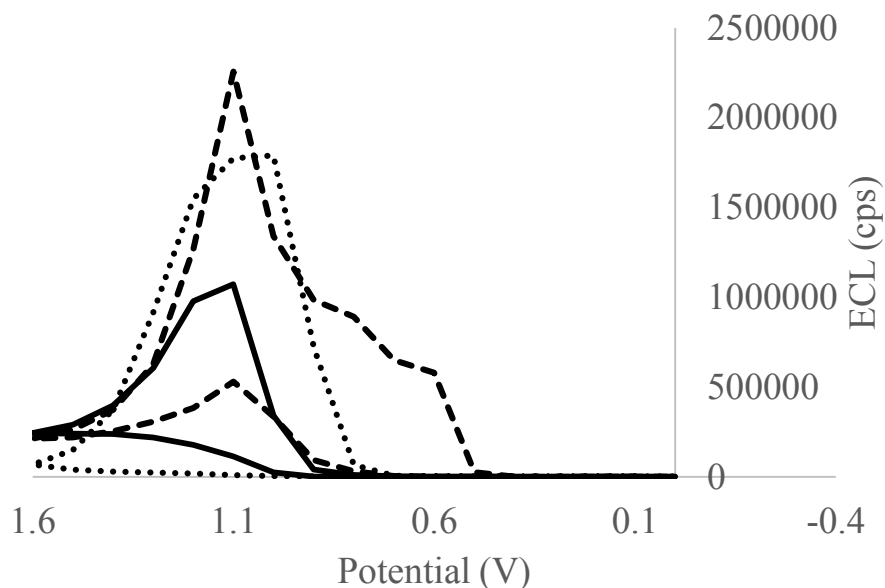
Figure 17. ECL intensity versus potential scans of solutions containing different combinations of 1 x 10^{-6} M (DC-bpy)Ru(bpy)$_2{}^{2+}$, 0.05M TPrA, and 5mM MLT in aqueous 0.18M KH$_2$PO$_4$ (pH = 8.0). (Solid line) (DC-bpy)Ru(bpy)$_2{}^{2+}$/TPrA in air saturated solution with [MLT] = 0; (Dotted line) (DC-bpy)Ru(bpy)$_2{}^{2+}$/TPrA/MLT in air saturated solution; (Dashed line) (DC-bpy)Ru(bpy)$_2{}^{2+}$/TPrA in a solution degassed for a minimum of 10 minutes with argon.

ECL in 2,2,2-trifluoroethanol (TFE) and Triton X-100 (TX100) Solutions.

Numerous methods have been reported for increasing ECL intensities, including the

addition of surfactants to solutions, changing the nature of luminophore and coreactants

and changing solution composition.[1] However, none to our knowledge have observed

ECL enhancement in the presence of oxygen scavengers. For example, dramatic

increases in ϕ_{ecl} ranging from 6- to 270-fold were reported when fluorinated

alcohol:water mixtures were combined with the Ru(bpy)$_3{}^{2+}$/TPrA system.[53] In

particular, 30:70 (v/v) 2,2,2-trifluoroethanol (TFE):H$_2$O results in a 270-fold increase in

Ru(bpy)$_3^{2+}$/TPrA ECL and potentially much lower detection limits compared to Ru(bpy)$_3^{2+}$/TPrA in water.[29]

Therefore, the effect of melatonin in Ru(bpy)$_3^{2+}$/TPrA ECL in 30:70 (v/v) TFE:H$_2$O was investigated. An approximately 273-fold increase in ECL was obtained in a solution containing Ru(bpy)$_3^{2+}$/TPrA/MLT 30:70 (v/v) TFE:H$_2$O, and an approximately 270-fold increase in a solution containing Ru(bpy)$_3^{2+}$/TPrA in 30:70 (v/v) TFE:H$_2$O compared to a Ru(bpy)$_3^{2+}$/TPrA standard in aqueous buffered solution ($\phi_{ECL} = 1$). Clearly, the ECL enhancement of MLT and TFE is additive in the Ru(bpy)$_3^{2+}$/TPrA system.[29]

Similarly, the effect of MLT on the surfactant effect with Ru(bpy)$_3^{2+}$/TPrA was investigated. This effect is well established[54] and has been traced to adsorption of a surfactant layer on the surface of an electrode. In the case of TX100 this renders gold and platinum surfaces more hydrophobic, facilitating pre-concentration of coreactant and Ru(bpy)$_3^{2+}$ near the surface of the electrode and more light emitting events. Ru(bpy)$_3^{2+}$/TPrA in potassium phosphate buffer produced an ECL signal of 9.9 x 10^5 cps (counts per second); Ru(bpy)$_3^{2+}$/TPrA with MLT 2.6 x 10^6 cps, Ru(bpy)$_3^{2+}$/TPrA with TX100 7.2 x 10^6 cps and Ru(bpy)$_3^{2+}$/TPrA with both MTL and TX100 1.3 x 10^7 cps showing that a combination of methods can be used to enhance Ru(bpy)$_3^{2+}$/TPrA ECL.[29]

Conclusions

Weak ECL has been observed from aqueous solutions of MLT using TPrA as an oxidative-reductive coreactant. When Ru(bpy)$_3^{2+}$ is present in solution, MLT enhances the ECL emission by approximately 2.5-fold (Table 5). The enhancement by MLT is

linear over a wide concentration range leading to its possible use in ECL applications and assays. Detailed spectroscopic, electrochemical and spectroelectrochemical studies indicate that products of MLT oxidation are responsible for the enhancement. Also, the mechanism appears to be solution phase oxygen "scavenging". These scavenging reactions prevent the quenching of the $^*\text{Ru(bpy)}_3^{2+}$ excited states by dissolved oxygen in solution opening up a new method for enhancing ECL in fundamental and applied studies. Melatonin can be coupled with 30% by volume 2,2,2-trifluorethanol (TFE) or with 3% (v/v) Triton X-100 for even greater enhancement of Ru(bpy)_3^{2+}/TPrA ECL. Furthermore, ECL enhancement has been observed in ruthenium chelates and iridium complexes when melatonin is present in solution.

Table 5. Melatonin electrogenerated chemiluminescence data.[a]

Solution Composition	Analyte/Coreactant[b]	ECL (cps)[c]	ϕ_{ECL}[d]
KH_2PO_4	————	2.3×10^3	3.4×10^{-4}
KH_2PO_4, TPrA	————	3.8×10^3	5.6×10^{-4}
KH_2PO_4, $Ru(bpy)_3^{2+}$	————	3.9×10^3	5.7×10^{-4}
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$	————	6.8×10^6	1.0
KH_2PO_4, $Ru(bpy)_3^{2+}$	MLT	3.6×10^4	0.0053
KH_2PO_4, TPrA, $Ru(bpy)_3^{2+}$	MLT	1.7×10^7	2.5
KH_2PO_4, TPrA, $(bpy)_2Ru(DC\text{-}bpy)$	————	1.3×10^7	1.0
KH_2PO_4, TPrA, $(bpy)_2Ru(DC\text{-}bpy)$	MLT	1.8×10^7	1.4
KH_2PO_4, TPrA, $(bpy)_2Ru(DM\text{-}bpy)$	————	6.5×10^6	1.0
KH_2PO_4, TPrA, $(bpy)_2Ru(DM\text{-}bpy)$	MLT	8.9×10^6	1.4
KH_2PO_4, TPrA, $Ir(ppy)_3$	————	9.5×10^3	1.0
KH_2PO_4, TPrA, $Ir(ppy)_3$	MLT	1.5×10^4	1.7

[a][KH_2PO_4] = 0.18 M (aqueous buffered solution) at pH 8.0 ±0.1. [$Ru(bpy)_3^{2+}$] = 0.01 mM. [TPrA] = 0.05 M.
[b]When TPrA is absent in the solution composition, then the species listed acts as a coreactant. If TPrA is present the species is an analyte. All species were present in solution at 0.005 M.
[c]CPS = counts per second.
[d]Relative ECL efficiency (photons emitted per ejected) using $Ru(bpy)_3^{2+}$/TPrA/KH_2PO_4 as a standard (ϕ_{ECL} = 1). All values are the averages of at least 2-replicates with a standard deviation of ±5% (or 0.05).

CHAPTER 5: FUTURE WORK

This project can be expanded in several ways. There are many other luminophore/coreactant combinations that could be studied with MLT to see if enhancement occurs (e.g., $Ru(bpy)_3^{2+}/S_2O_8^{2-}$) ECL in those as well. So far, the ECL enhancement MLT offers has only been studied in inorganic systems, but there are numerous inorganic and organic systems that could be utilized. Another possibility for further study is to bind the luminophore to a biological molecule and test its physical applicability in a bioassay with MLT in the buffer/assay solution. Finally, it would be interesting to test other molecules similar to melatonin that form products that are oxygen scavengers that might also prevent quenching.

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