Trinuclear Ruthenium Clusters for Electrochemical Biosensor Development

Hsiao-Tieh Hsu
Faculty Advisor: Thomas J. Meade
Postdoctoral Mentor: Amanda L. Eckermann
Graduate Mentor: Daniel J. Feld

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Abstract

Electrochemical biosensors have become a popular diagnostic tool in the past few decades. These non-invasive devices detect biomolecular analytes associated with diseases by utilizing changes of electrochemical properties. Although they have many advantages, they often suffer from low sensitivity. To further the development of more sensitive electrochemical biosensors, this study investigated weak interactions that govern ligand-receptor binding.

Weak interactions between molecules, such as van der Waals forces and hydrogen bonding, are crucial in biochemical processes but intrinsically difficult to measure. The Marcus Theory of Electron Transfer relates weak interactions to electron transfer rate ($k_{ET}$) and reorganization energy ($\lambda$). Therefore, information on these weak interactions can be obtained through electrochemistry. In the work presented here, weak interactions between proteins and small molecules were investigated by using transition metal-modified binding ligands; binding of these redox-modified binding ligands was studied using electrochemistry. The biotin/avidin system was chosen for the initial study; bovine serum albumin (BSA) was used as a negative control. Utilizing the avidin binding ligands 4-BMP (biotin aminomethyl pyridine) and 4-DMP ($d$-desthiobiotin aminomethyl pyridine), the monovalent and bivalent trinuclear ruthenium clusters $[\text{Ru}_3\text{O(OAc)}_6\text{L(py)}(4\text{-BMP})]^+$, $[\text{Ru}_3\text{O(OAc)}_6\text{L(4-DMP)}]^+$, and $[\text{Ru}_3\text{O(OAc)}_6\text{L(4-BMP)}_2]^+$, $[\text{Ru}_3\text{O(OAc)}_6\text{L(4-DMP)}_2]^+$, and $[\text{Ru}_3\text{O(OAc)}_6\text{L(py)}_2]^+$ (OAc = acetate, py = pyridine, L = pyC$_{16}$SH) were synthesized. These clusters were designed to maintain coupling with the electrode upon protein binding. Clusters were incorporated into self-assembled monolayers to facilitate electrochemical experiments.

Cyclic voltammetry (CV) was performed before and after protein binding. The
peak potential, peak current, and total amount of charge transferred in a redox event of the binding ligand were measured, and $\lambda$ and $k_{ET}$ were derived. The $i_p$ vs. scan rate plot of the CV data was used to evaluate how well the monolayers were packed. Alternating current voltammetry (ACV) was used to determine if there is a distribution of electron transfer rates on the monolayer. In the bivalent 4-BMP system, a shift of -43 mV in $E_{1/2}$ was observed upon avidin binding to the transition metal probe. In the monovalent 4-BMP, monovalent 4-DMP, and bivalent 4-DMP systems, the shift in potential was negligible, which indicates that there is no observable change in electron transfer rate and reorganization energy upon protein binding in these systems. The bivalent pyridine system was used to show the CV peak current decrease in these trinuclear ruthenium clusters before and after avidin addition was caused by nonspecific binding between avidin and the monolayer instead of binding between avidin and biotin. The biotin/BSA system verified that the interaction between biotin and avidin is specific, and the shift in $E_{1/2}$ is due to avidin binding. Finally, changing the dielectric constant in the bivalent 4-BMP system by using mixtures of DMF in water in various ratios resulted in a similar shift in $E_{1/2}$ to avidin binding. This study showed that the bivalent BMP tri-nuclear ruthenium cluster has the potential for detecting biological molecules such as avidin. In the future, this system can be further studied for better sensitivity or extended to biomolecular analytes that are associated with diseases.
Chapter 1. Introduction and background

1.1. Biosensors

A biosensor is a non-invasive device that can detect biomolecular analytes associated with diseases, and it has become a popular diagnostic tool in the past few decades (Figure 1). Electrochemical biosensors, where electrodes serve as either electron donors or electron acceptors, are useful because of their low cost, ease of use, and remarkable reproducibility. Previous studies show that electron transfer between electrodes and surface-confined redox molecules obeys the Marcus Theory of Electron Transfer. Therefore, changes in the system caused by interactions between redox molecules and the analytes they are sensing could translate into measurable changes of electrochemical signals such as electron transfer rate and overpotential.

In 1982, Gray and Winkler first demonstrated electron transfer through Ru-modified cytochrome c. Their later work showed that electronic coupling and hydrogen bonding in folded polypeptide structures could change the rate of electron transfer between redox sites in Ru-modified proteins. This suggests that electron transfer rate can be used for protein biosensor design because the detection of analytes involves electronic coupling and bonding between the sensor molecule and the analytes.

Additionally, Khan et al. studied the electrochemical properties of ferrocene-amino acid conjugates before and after they reacted with chemical warfare agent (CWA) mimics. Their results showed that the electrochemical measurements such as cyclic voltammetry (CV) and alternating current voltammetry (ACV) of these ferrocene-modified CWA mimics that catalyze phosphorylation changed dramatically.
after they reacted with each other.\textsuperscript{9} These promising results indicated that electrochemical measurements such as CV and ACV can be used as analytical tools for protein sensing.

Based on previous studies, redox-modified protein binding ligands were used to bind to proteins in the work presented here, and CV and ACV were utilized to measure the changes of electrochemical properties before and after protein binding.

**Figure 1:** Principle of biosensors\textsuperscript{2}. Biosensors can detect biomolecular analytes associated with diseases and transfer the chemical or electrochemical changes into signals.

1.2. Theoretical Background

In spite of their various advantages, electrochemical biosensors often suffer from low sensitivity.\textsuperscript{10} To aid the development of electrochemical biosensors the work presented here studied the weak interactions that govern ligand-receptor binding using trinuclear ruthenium clusters.\textsuperscript{11} Weak interactions between proteins and small molecules are important to biological processes such as photosynthesis and DNA
damage and repair, but they are difficult to measure directly. Weak interactions affect the static dielectric constant ($\varepsilon_s$) of a system, which is a part of reorganization energy ($\lambda$).\textsuperscript{12} The Marcus Equation of Electron Transfer relates $\lambda$ to electron transfer rate ($k_{ET}$).\textsuperscript{13} Therefore, the changes in weak interactions can be inferred by the changes in $\lambda$ by measuring $k_{ET}$ using electrochemistry.\textsuperscript{14} When a protein binds to a binding ligand, it displaces the H$_2$O molecules in the environment of the binding ligand, decreasing the static dielectric constant. This process lowers the outer sphere reorganization energy and increases the electron transfer rate (Figure 2).

![Figure 2: A scheme of how protein binding changes electrochemical parameters.](image)

Upon protein binding, water molecules are displaced, and $\varepsilon_s$ and $\lambda_o$ decreases, and $k_{ET}$ increases.

Reorganization energy ($\lambda$) is the energy required to force the reactants from their equilibrium configuration into the equilibrium configuration of the products before electron transfer actually occurs (Figure 3).\textsuperscript{12} According to The Marcus Theory of Electron Transfer, reorganization energy is the sum of outer sphere ($\lambda_o$) and inner sphere reorganization energy ($\lambda_i$) (Equation 1).\textsuperscript{13} $\lambda_o$ depends on several variables: the
radii of the electron donor and acceptor \((r_A \text{ and } r_D)\), the distance between the donor and acceptor \(d_{AD}\), the charge transferred \((e)\), the optical dielectric constant \(\varepsilon_{op}\) and the static dielectric constant \(\varepsilon_s\) (Equation 2).\(^{12,15,16}\) \(\varepsilon_{op}\) is approximately constant between different systems while \(\varepsilon_s\) differs and gives information on weak interactions. For example, \(\varepsilon_s\) of protein is smaller than that of water, and based on Equation 2, \(\lambda_o\) of protein is expected to be smaller than that of water, too. According to the Marcus Equation, \(k_{ET}\) depends on \(\lambda\) (Equation 3).\(^{12}\)

\[
\lambda = \lambda_t + \lambda_o \quad (1)
\]

\[
\lambda_o = N_A e^2 \left[ \frac{1}{2r_A} + \frac{1}{2r_D} - \frac{1}{d_{AD}} \right] \left( \frac{1}{\varepsilon_{op}} - \frac{1}{\varepsilon_s} \right) \quad (2)
\]

\[
k_{ET} = \frac{4\pi^2}{h} H^2_{AB} \frac{1}{\sqrt{4\pi\lambda k_B T}} \exp \left[ -\frac{(\Delta G + \lambda)^2}{4\lambda k_B T} \right] \quad (3)
\]

**Figure 3:** The Marcus parabolas and reorganization energy \((\lambda)\). The Marcus parabolas are generated by plotting Gibbs free energy \((G)\) against reaction coordinate \((X)\). The reactant parabola is on the left and the product parabola is on the right. \(\Delta G\) is the difference in free energy between the reactant and the product, and \(\Delta G^f\) is the activation energy of the reaction. \(\lambda\) is the energy required to force the reactant from its equilibrium configuration into the equilibrium configuration of the product before electron transfer occurs.\(^{12}\)
If the protein binding ligand is modified with a redox center, the potential, current and total amount of charge transferred in a redox event can be measured with electrochemical experiments, such as cyclic voltammetry (CV) and alternating current voltammetry (ACV). By fitting and simulating the CV and ACV data, \( \lambda \) and \( k_{\text{ET}} \) can be derived.\(^{17}\) Upon protein binding, potential (\( E_{1/2} \), the average of anodic and cathodic peak potential), \( \lambda \), and \( k_{\text{ET}} \) are expected to shift.

To investigate the change in electrochemical parameters upon protein binding, the biotin/avidin system was used in the work presented here. This system was chosen because biotin and avidin form specific, strong non-covalent interactions, with a dissociation constant of \( 10^{-15} \) M.\(^{18}\) Avidin is a tetrameric glycoprotein that is stable and widely used in biotechnological and medical research. It can bind to multiple ligands, including biotin, and its derivatives, such as \( d \)-desthiobiotin, which allows systematic studies for ligand-receptor binding. Further, the known crystal structure of avidin aids theoretical calculations (Figure 4).\(^{19}\)

**Figure 4:** Structure of avidin binding to a redox-modified biotin, \( \text{Fe}[(BMB)(CN)_4]^2^- \).
1.3. Experimental Design

In previous work, biotinylated iron- and ruthenium-modified complexes, such as \([\text{Fe(BMB)}(\text{CN})_4]^2-\) and \([\text{(4-BMP)}_n\text{Ru(NH}_3)_5]^2+\)\(^{14,20}\) were used for electrochemical detection of avidin binding. However, the current signal in the CV was lost (Figure 5).\(^{14}\) Due to the small size of the metal complex, the protein decreased the coupling between the metal center and the electrode, and therefore interfered with the electron transfer process and no current was observed. Additionally, these CV experiments were done in solution, so the slow diffusion of the protein to the electrode contributed to the signal loss. To solve these problems, trinuclear ruthenium clusters were used to modify the avidin-binding ligands biotin and desthiobiotin. Because these clusters are larger, protein binding is less likely to interfere with the coupling between the metal centers and the electrode. The target molecules were incorporated into self-assembled monolayers to overcome the poor diffusion of avidin in solution.

![Figure 5](image-url)

**Figure 5:** The current signal in CV was lost after avidin was added to the biotinylated iron complex \([\text{Fe(BMB)}(\text{CN})_4]^2-\).\(^{14}\)
CV and ACV were performed to analyze the species on the monolayer. CV is an electrochemical technique that scans the potential and measures the current in a redox event.\textsuperscript{17,20,22} The $i_p$ vs. scan rate plot of the CV data can be used to determine whether the monolayer is well-packed. $\lambda$ and $k_{\text{ET}}$ can be derived from plotting the CV data into a Tafel plot. The curvature is indicative of $\lambda$, and the y-intercept of the plot is $k_{\text{ET}}$. (Figure 6.)\textsuperscript{17,23} A modified Marcus Equation, which applies to species on monolayers, can be used to simulate the CV data to give electronic coupling ($H_{\text{AB}}$), $\lambda$ and $k_{\text{ET}}$. (Equation 4).\textsuperscript{17,21,22,24,25}

$$k = \frac{2\pi}{h} |H_{\text{AB}}|^2 \cdot \rho_{\text{AB}} \cdot (4\lambda k_B T)^{-1/2} \int_{-\infty}^{\infty} \left[ 1 - f(\epsilon) \right] \exp \left( \frac{(\lambda + \epsilon - e_0 n)^2}{4\lambda k_B T} \right) d\epsilon$$  \hspace{1cm} (4)

\textbf{Figure 6:} The curvature of the Tafel plot indicates $\lambda$ and the intercept with the y-axis shows $k_{\text{ET}}$.\textsuperscript{23}

In ACV, an alternating potential is applied, which generates an alternating current.\textsuperscript{17,21} When the frequency is low, electrons have ample time to transfer so the
peak current \((i_p)\) is at its maximum. As the frequency increases, fewer and fewer electrons are able to transfer. The peak current decreases and will eventually go to zero with respect to the background current \((i_b)\) (Figure 7).\(^{17,26}\) The data can be fit to a Randles circuit to get information about electron transfer rate and see whether there is a distribution of rates on the monolayer due to variations in the molecular environment of the redox centers caused by the defects of the monolayer (Figure 8).\(^{17,23,26}\)

\[\text{Figure 7:} \text{ (a) In ACV, an alternating potential is applied and the differences between the maximum and minimum of the current are measured. } i_p \text{ is the peak current and } i_b \text{ is the background current. (b) Peak current decreases as frequency increases. The peak current to background current ratio } (i_p/i_b) \text{ decreases and eventually goes to 1.}^{17}\]
Figure 8: A plot of $i_p/i_b$ vs. ACV frequency. The different line shapes represent different electron transfer rates in the system caused by monolayer defects.$^{17,23,26}$

CV and ACV experiments were performed on trinuclear ruthenium cluster systems (Table 1) before and after avidin addition to measure the changes in electrochemical properties of the systems upon protein binding. Ru(py)$_2$(C$_{16}$SH) was used as a control system because it does not have a ligand that can bind to avidin. Bovine Serum Albumin (BSA), a protein that does not bind to biotin and its derivatives, was also added to the trinuclear ruthenium cluster systems as a negative control to verify the changes in electrochemical properties were caused by biotin-avidin interactions. Finally, the solvent polarity of CV experiments was altered to simulate the environment after protein binding; this was done by titrating DMF into the electrochemical cell.
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**Table 1:** Trinuclear ruthenium clusters used in this study and their abbreviations
Chapter 2. Experimental Section

2.1. The 4-BMP system

2.1.1. Synthesis of 4-BMP

684.1 mg (2.800 mmol) of biotin and 1019.5 mg (3.386 mmol) of TSTU were dissolved in 10 mL of DMF while stirring. 1 mL of Et₃N was added to the stirring solution, and the resulting solution was stirred for 1 hour, during which time the color of the solution turned from orange to dark brown. 288 µL (2.798 mmole) of distilled 4-(aminomethyl)pyridine (4-AMP) was added and the solution was stirred overnight at room temperature under argon. When the reaction was completed, DMF was removed using rotary evaporation. The product was purified with column chromatography on silica in 10% MeOH/CHCl₃ and modified by TLC and Pt stain. The first band that stained dark brown was collected. The final product appeared to be a light yellow, oily solid. (Scheme 1).¹⁴ Yield: 61 %. ¹H NMR (500 MHz, CDCl₃) δ 8.44 (t, 2H), 7.28 (d, 1H), 4.48-4.42 (m, 1H), 4.38 (s, 2H), 4.26-4.22 (m, 1H), 3.31-3.23 (m, 2H), 2.88 (dt, 1H), 2.28 (dq, 2H), 1.79-1.51 (m, 4H), 1.38-1.48 (m, 2H). MS (ESI⁺) m/z calculated for 4-BMP + H⁺ is 335.15, found 335.1. 4-BMP + Na⁺ is 357.14, found 357.0.

Scheme 1: Synthesis of 4-BMP
2.1.2. Synthesis of Ru(H₂O)₃

991.0 mg (4.78 mmole) of RuCl₃•xH₂O, 2.206 g (24.7 mmole) of sodium acetate and 25 mL of glacial acetic acid were dissolved in 25 mL of absolute ethanol and refluxed overnight in the dark, during which time the color of the solution turned from dark brown to dark green. The mixture was cooled to room temperature and centrifuged at 2500 rpm for 20 minutes to remove the excess salt. The supernatant was filtered and EtOH was removed using rotary evaporation. The obtained solid was dissolved in 50 mL MeOH and filtered. MeOH was then removed using rotary evaporation and Ru(H₂O)₃, a dark green solid, was obtained (Scheme 2).²⁷,²⁸ Yield: 67 %. UV-visible (MeOH) λₘₐₓ = 699 nm.

![Scheme 2: Synthesis of Ru(H₂O)₃](image)

2.1.3. Synthesis of Ru(CO)(MeOH)₂

1.0 g (1.32 mmole) of Ru(H₂O)₃ was dissolved in 50 mL MeOH and the solution was degassed for 30 minutes. A few pieces of Zn/Hg (~8 g) were added to the solution with stirring. The solution was stirred for 2.5 hours under Ar, during which time the color of the solution turned more yellow. The air-sensitive solution was then cannulated to an argon-purged Schlenk flask to remove the Zn/Hg amalgam.
CO was bubbled into the cannulated solution for 45 minutes. The solution was then stirred under a CO atmosphere at room temperature in the dark overnight, during which time [Ru\textsubscript{3}O(OAc)\textsubscript{6}(CO)(MeOH)\textsubscript{2}]\textsuperscript{0} was formed and the color of the solution turned from dark green to dark purple. The product was purified with column chromatography on silica in 10% MeOH/CHCl\textsubscript{3} and appeared to be a purple solid upon rotary evaporation.\textsuperscript{27,28} Yield: 57%. UV-visible (MeOH) $\lambda_{\text{max}} = \sim 560$ nm (Scheme 3).

![Scheme 3: Synthesis of Ru(CO)(MeOH)\textsubscript{2}](image)

2.1.4. Synthesis of Ru(CO)(BMP)(MeOH)

154.7 mg (0.463 mmole, 0.8 equivalents) of 4-BMP was dissolved in 100 mL 1:1 MeOH/CH\textsubscript{2}Cl\textsubscript{2}. Once dissolved, 440.9 mg (0.580 mmole) of Ru(CO)(MeOH)\textsubscript{2} was added to the stirring solution. The reaction was stirred at room temperature in the dark for 3 days to form Ru(CO)(BMP)(MeOH). The product was purified with column chromatography on silica in 10% MeOH/CHCl\textsubscript{3} and appeared to be a purplish blue solid (Scheme 4). Yield: 47%. $^1$H NMR (500 MHz, MeOD) $\delta$ 8.95 (d, 2H), 7.98 (d, 2H), 4.82 (s, 2H), 4.38 (m, 1H), 4.20 (m, 1H), 3.13 (q, 2H), 2.82 (dt, 1H), 2.55 (dq, 1H) 2.35 (t, 4H), 1.85 (s, 6H), 1.80 (s, 6H), 1.75-1.50 (m, 10H), 1.50-1.35 (m, 2H). UV-visible (MeOH) $\lambda_{\text{max}} = 568$ nm.
2.1.5. Synthesis of (4-AMP)CO(CH$_2$)$_{15}$SH

98.2 mg (0.340 mmole) of HS(CH$_2$)$_{15}$COOH (16-mercaptohexadecanoic acid), 75.8 mg (0.367 mmole) of dicyclohexylcarbodiimide (DCC), and 38 µL (0.369 mmole) of distilled 4-AMP were dissolved in 10 mL 4:1 anhydrous, degassed CH$_2$Cl$_2$/acetone. The solution was stirred under a blanket of argon for 72 hours, during which time a white dicyclohexylurea precipitate and the product, (4-AMP)CO(CH$_2$)$_{15}$SH, were formed. The product was purified by filtration followed by column chromatography on silica in 5% MeOH/CHCl$_3$ and monitored by TLC and Pt stain. The first band that stained dark brown was collected. The product appeared to be a white solid (Scheme 5). Yield: 61%. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.52 (d, 2H), 7.17 (d, 2H), 5.91 (s, 1H), 4.45 (d, 2H), 2.57-2.51 (m, 2H), 2.29-2.25 (m, 2H), 1.85-1.58 (m, 8H), 1.50-1.42 (m, 8H), 1.40-1.32 (m, 22H). MS (ESI$^+$) m/z calculated for (4-AMP)CO(CH$_2$)$_{15}$SH + H$^+$ is 379.62, found 379.2, (4-AMP)CO(CH$_2$)$_{15}$SH + Na$^+$ is 401.60, found 401.2.
2.1.6. Synthesis of Ru(CO)(BMP)(C₁₆SH)

312.0 mg (0.293 mmole) of Ru(CO)(BMP)(MeOH) and 116.9 mg (0.309 mmole) of (4-AMP)CO(CH₂)₁₅SH were dissolved in 100 mL of degassed 1:1 MeOH/CHCl₃ solution. The resulting solution was stirred under a blanket of Ar at room temperature in the dark overnight, during which time the color turned more blue. The final product, Ru(CO)(BMP)(C₁₆SH), was purified by column chromatography on silica in 5% MeOH/CHCl₃ and appeared to be a blue solid (Scheme 6). Yield: 79%.

^1^H NMR (500 MHz, CDCl₃) δ 8.93 (d, 2H), 7.98 (s, 2H), 4.85 (s, 1H), 4.38 (m, 1H), 4.22 (m, 1H), 3.13 (q, 1H), 2.82 (dd, 1H), 2.55-2.50 (m, 3H), 2.38-2.23 (m, 4H), 1.86 (s, 12H), 1.75-1.48 (m, 16H), 1.48-1.38 (m, 4H), 1.36-1.10 (m, 22H). UV-visible (MeOH) λ_max = 585 nm
2.1.7. Electrochemical formation of Ru(BMP)$_2$(C$_{16}$SH) and Ru(BMP)(py)(C$_{16}$SH)

Gold ball electrodes were made from 7 cm pieces of 0.127 mm diameter, 99% gold wire (Alfa Aesar). The first 2 cm of each 7 cm piece was melted in a Bunsen burner flame to form a gold ball with radius of 0.04 cm. The gold ball electrodes were cleaned in 1 M H$_2$SO$_4$(aq) with CV using a CH Instruments 660A electrochemical workstation to remove contamination on the surface of the gold ball. The potential was cycled between 0V and 1.6V until a constant current was observed. After being cleaned. After cleaning, electrodes were rinsed with H$_2$O and ethanol. The electrodes were soaked in a 20:1 HO(CH$_2$)$_{11}$SH: Ru(CO)(BMP)(C$_{16}$SH) solution (2 mM total thiol in EtOH) overnight so that monolayers could be formed. CV was performed using the CH Instruments 660A electrochemical workstation in pH 7 PBS buffer with
100 mM NaCl under room temperature to oxidize the cluster electrochemically. The buffer was degassed with N₂ before all experiments. The reference electrode was Ag/AgCl and the counter electrode was a platinum wire. The potential was scanned between -0.6 V and 0.8 V for 20 minutes until the current of newly formed species no longer increased. The CO ligand becomes labile after oxidation and is replaced by a H₂O molecule (Scheme 7). The gold ball electrodes were then soaked in either 1 mM 4-BMP or 1 mM pyridine/ H₂O solution to form Ru(BMP)₂(C₁₀SH) or Ru(BMP)(py)(C₁₀SH), respectively (Scheme 8).

Scheme 7: Electrochemical oxidation of Ru(CO)(BMP)(C₁₀SH)
2.2. The 4-DMP System

2.2.1. Synthesis of 4-DMP

599.4 mg (2.798 mmole) of d-desthiobiotin and 1019.5 mg (3.386 mmole) of TSTU were dissolved in 10 mL of DMF with stirring. 1 mL of Et$_3$N was added to the stirring solution, and the resulting solution was stirred for 1 hour, during which time the color of the solution turned from orange to dark brown. 288 µL (2.798 mmole) of 4-AMP was added and the solution was stirred overnight at room temperature under argon. When the reaction was complete, DMF was removed with using rotary evaporation. The product was purified with column chromatography on silica in 10%
MeOH/CHCl₃. The final product appeared to be a light yellow, oily solid (Scheme 9).

Yield: 83%. ¹H NMR (500 MHz, CDCl₃) δ 8.44 (t, 2H), 7.18 (d, 1H), 4.49-4.39 (m, 2H), 3.82 (dt, 1H), 3.60 (dq, 1H), 2.23 (t, 2H), 1.48-1.20 (m, 8H), 1.04 (t, 3H). MS (ESI⁺) m/z calcd for 4-DMP + H⁺ is 305.39, found 305.1. 4-DMP + Na⁺ is 327.38, found 327.1.

Scheme 9: Synthesis of 4-DMP

2.2.2. Synthesis of Ru(CO)(DMP)(MeOH)

104.7 mg (0.344 mmole, 0.8 equivalents) of 4-DMP was dissolved in 150 mL 1:1 MeOH/CH₂Cl₂. Once dissolved, 326.1 mg (0.429 mmole) of Ru(CO)(MeOH)₂ was added to the stirring solution. The reaction was stirred at room temperature in the dark for 3 days to form Ru(CO)(DMP)(MeOH). The product was purified with column chromatography on silica in 10% MeOH/CHCl₃ and appeared to be a purplish blue solid. (Scheme 10). Yield: 42%. ¹H NMR (500 MHz, MeOD) δ 9.08 (d, 2H), 8.05 (d, 2H), 4.95 (d, 2H), 3.82 (dt, 1H), 3.74 (dq, 1H), 2.48-2.42 (t, 2H), 2.03-2.01 (m, 6H), 1.99-1.97 (m, 6H), 1.85-1.75 (m, 7H), 1.58-1.45 (m, 6H), 1.24 (t, 3H). UV-visible (MeOH) λₘₐₓ = 575 nm.
2.2.3. Synthesis of Ru(CO)(DMP)(C\textsubscript{16}SH)

94.45 mg (0.091 mmole) of Ru(CO)(DMP)(MeOH) and 37.6 mg (0.099 mmole) of (4-AMP)CO(CH\textsubscript{2})\textsubscript{15}SH were dissolved in 20 mL of degassed 1:1 MeOH/CHCl\textsubscript{3} solution. The resulting solution was stirred under a blanket of argon at room temperature in the dark overnight, during which time the color turned more blue. The final product, Ru(CO)(DMP)(C\textsubscript{16}SH), was purified by column chromatography on silica in 5% MeOH/CHCl\textsubscript{3} and appeared to be a blue solid after solvent removal (Scheme 11). Yield: 54 %. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta \) 8.96 (d, 4H), 7.99 (d, 4H), 4.91 (d, 4H), 3.79 (dt, 1H), 3.68 (dq, 1H), 2.42-2.36 (m, 4H), 2.05 (s, 6H), 2.01 (s, 6H), 1.78 (s, 6H), 1.71 (m, 4H), 1.70 (m, 7H), 1.40 (m, 41H), 1.07 (t, 3H).
2.2.4. Electrochemical formation of Ru(DMP)$_2$(C$_{16}$SH) and Ru(DMP)(py)(C$_{16}$SH)

Gold ball electrodes were made using the same method as the 4-BMP system. After being cleaned, the electrodes were soaked in a 20:1 HO(CH$_2$)$_2$:SH:Ru(CO)(DMP)(C$_{16}$SH) solution (2 mM total thiol in EtOH) overnight so that monolayers could be formed on the surface of the electrodes. The clusters on the monolayer were electrochemically oxidized with CV using the CH Instrument 660A electrochemical workstation in pH 7 PBS buffer with 100 mM NaCl degassed with N$_2$ at room temperature. The potential was scanned between -0.6 V and 0.8 V for 20 minutes until the current of newly formed species no longer increased. The CO ligand
becomes labile after oxidation and is replaced by a H$_2$O molecule (Scheme 12). The gold ball electrodes were then soaked in either 1 mM 4-DMP or 1 mM pyridine/ H$_2$O solution to form Ru(DMP)$_2$(C$_{16}$SH) or Ru(DMP)(py)(C$_{16}$SH), respectively (Scheme 13.)

**Scheme 12:** Electrochemical oxidation of Ru(CO)(DMP)(C$_{16}$SH)
Scheme 13: Formation of Ru(DMP)$_2$(C$_{16}$SH) and Ru(DMP)(py)(C$_{16}$SH)

2.3. The Pyridine System

2.3.1. Synthesis of Ru(CO)(py)(MeOH)

12.5 µL (0.154 mmole, 0.8 equivalents) of pyridine was dissolved in 150 mL 1:1 MeOH/CH$_2$Cl$_2$. Once dissolved, 146.6 mg (0.193 mmole) of [Ru$_3$O(OAc)$_6$(CO)(MeOH)$_2$]$^0$ was added to the stirring solution. The reaction was stirred at room temperature in the dark for 3 days to form [Ru$_3$O(OAc)$_6$(CO)(py)(MeOH)]$^0$. The product was purified with column chromatography on silica in 10% MeOH/CHCl$_3$ and appeared to be a purplish blue
solid (Scheme 14).\textsuperscript{27,28} Yield: 52.3 %. \textsuperscript{1}H NMR (500 MHz, MeOD) \(\delta\) 9.05 (d, 2H), 8.28 (t, 2H), 8.08 (dd, 2H), 1.98 (m, 6H), 1.92 (m, 6H), 1.64 (m, 6H), 1.51 (m, 6H).

\textbf{Scheme 14: Synthesis of Ru(CO)(py)(MeOH)}

\section*{2.3.2. Synthesis of Ru(CO)(py)(C\textsubscript{16}SH)}

92 mg (0.11 mmole) of Ru(CO)(py)(MeOH) and 92 mg (0.24 mmole, 2.2 equivalents) of (4-AMP)CO(CH\textsubscript{2})\textsubscript{15}SH were dissolved in 10 mL of degassed 1:1 MeOH/CHCl\textsubscript{3} solution. The resulting solution was stirred under a blanket of argon at room temperature in the dark overnight, during which time the color of the solution turned more blue. The final product, [Ru(CO)(py)(C\textsubscript{16}SH)]\textsuperscript{0}, was purified by column chromatography on silica in 5\% MeOH/CHCl\textsubscript{3} and appeared to be a blue solid (Scheme 15). Yield: 45 \%. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 9.10 (d, 2H), 9.00 (d, 2H), 8.19 (t, 1H), 8.04 (t, 2H), 7.92 (d, 2H), 4.91 (s, 2H), 2.49 (t, 2H), 2.38 (t, 2H), 2.09 (s, 12H), 1.81 (s, 6H), 1.75 (m, 2H), 1.63 (m, br, 4H), 1.4-1.1 (m 20H). UV-visible (MeOH) \(\lambda_{\text{max}}\) = 582 nm.
2.3.3. *Electrochemical formation of Ru(py)$_2$(C$_{16}$SH)*

Gold ball electrodes were made using the same method as the 4-BMP system. After being cleaned, the electrodes were soaked in a 20:1 HO(CH$_2$)$_{11}$SH:Ru(CO)(py)(C$_{16}$SH) solution (2mM total thiol in EtOH) overnight so that monolayers could be formed on the surface of the electrodes. The clusters on the monolayer were electrochemically oxidized with CV using the CH Instruments 660A electrochemical workstation in pH 7 PBS buffer with 100 mM NaCl degassed with N$_2$ at room temperature. The potential was scanned between -0.6 V and 0.8 V for 20 minutes until the current of newly formed species no longer increased. The CO ligand becomes labile after oxidation and is replaced by a H$_2$O molecule (Scheme 16). The gold ball electrodes were then soaked in 1 mM pyridine/ H$_2$O solution overnight to form Ru(py)$_2$(C$_{16}$SH) (Scheme 17).
Scheme 16: Electrochemical oxidation of Ru(CO)(py)(C$_{10}$SH)
Scheme 17: Formation of Ru(py)$_2$(C$_{16}$SH)

2.4. Electrochemical Experiments

2.4.1. CV and ACV measurements

CV and ACV experiments were performed on Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), Ru(DMP)(py)(C$_{16}$SH), and Ru(py)$_2$(C$_{16}$SH) in pH 7 PBS buffer with 100 mM NaCl at room temperature on the CH Instruments 660A workstation. The working electrode was the gold ball electrode with the monolayer, the reference electrode was Ag/AgCl, and the counter electrode was a platinum wire. In CV experiments, scan rates ranging from 0.01 V/s to 2500 V/s were applied, and the anodic and cathodic peak potential, peak current, and total
amount of charge transferred were measured. In ACV experiments, frequencies ranging from 1Hz to 1000 Hz were applied, and the peak potential and peak current were measured.

After the initial electrochemical measurements, all the gold electrodes were soaked in 30 µM avidin solution at 37°C overnight. For BSA studies, the Ru(BMP)$_2$(C$_{16}$SH) system was soaked in 30 µM BSA solution at 37°C overnight. Afterward, the same set of electrochemical experiments was performed on the gold electrodes to see if the electrochemical parameters changed after avidin binding.

2.4.2 DMF study

CV experiments of Ru(BMP)$_2$(C$_{16}$SH) were performed in 10%, 20%, 30%, 40%, and 50% DMF in pH 7 PBS buffer solution with 100 mM NaCl at room temperature using the CH Instruments 660A electrochemical workstation. Different scan rates, ranging from 0.01 V/s to 2500 V/s were applied, and the anodic and cathodic peak potential, peak current, and total amount of charge transferred were measured.
Chapter 3. Results and Discussion

3.1. Syntheses

Trinuclear ruthenium clusters, Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), Ru(DMP)(py)(C$_{16}$SH), and Ru(py)$_2$(C$_{16}$SH) were successfully synthesized and characterized. The precursor Ru(H$_2$O)$_3$ was reduced to Ru(CO)(MeOH)$_2$; the final products were oxidized back to +1 oxidation state. Ru(CO)(MeOH)$_2$ was made because the intermediate with a neutral oxidation state and a CO ligand is more air-stable and simplifies the purification. Compared to the previous [Fe(BMB)(CN)$_4$]$^{2-}$ system, the trinuclear ruthenium clusters allows easier study of monovalent vs. bivalent biotinylated systems because they have three ligand sites that are easily modified with biotin derivatives or alkane thiol chains.

3.2. CV: peak current

In the CV traces of Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), and Ru(DMP)(py)(C$_{16}$SH), the peak currents were still observable after avidin addition. This result suggests that the current loss observed in the single-centered biotinylated iron complex, [Fe(BMB)(CN)$_4$]$^{2-}$, in Figure 4 was resolved by using trinuclear ruthenium clusters incorporated into monolayers. Since the clusters are significantly larger than the mononuclear iron and ruthenium complexes, avidin binding is likely to have less effect on coupling between the cluster metal centers and the electrode. In addition, it is known that, in these clusters, the electron density is delocalized over all three metal centers. Therefore, electronic coupling and electron transfer were maintained, and current signals could still be observed after avidin addition. Further, since the clusters were incorporated into monolayers on the electrode, the avidin-cluster complexes would not have to diffuse...
to the electrode for electron transfer. Hence, the slow diffusion problem observed in previous study was solved. Additionally, attaching the ruthenium clusters to a monolayer facilitates analysis of the electrochemical properties of the system. By using this new system, current signals in CV were maintained after avidin bound to the binding ligand, which allowed further experiments and analyses that were not possible with the previous, mononuclear systems using the various trinuclear ruthenium clusters.

3.3. CV: \( i_p \) vs. scan rate plot

In a monolayer system, ideal reversible electrochemical behavior requires the monolayer to be homogeneous and the reduced and oxidized form of the redox species to be strongly attached to the monolayer on the electrode. There is a linear relation between peak current (\( i_p \)) and scan rate for ideal monolayers. For the CV experiments, \( i_p \) was first plotted against scan rate to see if the monolayers were well-packed. The plots for all systems showed a linear relationship with \( R^2 > 0.99 \), which indicates that the monolayers were well-packed (Figure 9).
Figure 9: $i_p$ vs. scan rate plots for (a) Ru(BMP)$_2$(C$_{16}$SH), (b) Ru(BMP)(py)(C$_{16}$SH), (c) Ru(DMP)$_2$(C$_{16}$SH), and (d) Ru(DMP)(py)(C$_{16}$SH). The linear relationship suggested that the monolayers were well-packed.
### 3.4. CV: Overpotencial \( E_{1/2} \)

The following is a table of \( E_{1/2} \) values for the 4-BMP and 4-DMP system before and after avidin binding:

<table>
<thead>
<tr>
<th>( E_{1/2} ) (V)</th>
<th>4-BMP system</th>
<th>4-DMP system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before avidin</td>
<td>Ru(BMP)(<em>2)(C(</em>{16})SH)</td>
<td>-0.066</td>
</tr>
<tr>
<td></td>
<td>Ru(DMP)(<em>2)(C(</em>{16})SH)</td>
<td>0.014</td>
</tr>
<tr>
<td>After avidin</td>
<td>-0.109</td>
<td>-0.052</td>
</tr>
<tr>
<td>( \Delta E_{1/2} ) (V)</td>
<td>-0.043</td>
<td>-0.006</td>
</tr>
</tbody>
</table>

**Table 2:** \( E_{1/2} \) values for 4-BMP and 4-DMP systems before and after avidin binding

In the Ru(BMP)\(_2\)(C\(_{16}\)SH) system, there was an observable change in \( E_{1/2} \) upon avidin addition of -43 mV. This shift is similar in magnitude to the shifts observed in ferrocene-amino acid conjugates on a monolayer presented in the work of Khan et al.\(^9\). The negative shift in \( E_{1/2} \) indicated that the oxidized form of the cluster is more stable in the environment of avidin. In the other three trinuclear ruthenium cluster systems, the changes in \( E_{1/2} \) were negligible compared to the experimental error range of approximately 10 mV. The changes in CV peak potential for the four systems can be seen in Figure 10:
Figure 10: The CV traces at 250 mV/s before (black) and after (red) avidin binding for (a) Ru(BMP)$_2$(C$_{16}$SH), where the anodic and cathodic peak potentials shifted upon avidin binding and gave rise to a change of \(-43\) mV in $E_{1/2}$, (b) Ru(BMP)(py)(C$_{16}$SH), (c)Ru(DMP)$_2$(C$_{16}$SH), and (d)Ru(DMP)(py)(C$_{16}$SH), where the peak potentials and $E_{1/2}$ did not change significantly upon avidin binding.
In the CV traces for 4-BMP and 4-DMP systems, a decrease in peak current was observed upon avidin addition. In order to investigate the cause of this current decrease and to verify that the changes in $E_{1/2}$ observed in the Ru(BMP)$_2$(C$_{16}$SH) system was caused by biotin-avidin interactions, the same set of CV experiments were conducted on Ru(py)$_2$(C$_{16}$SH), a trinuclear ruthenium cluster with no ligand that could bind to avidin. After the first set of CV measurements, electrodes with Ru(py)$_2$(C$_{16}$SH) monolayer were soaked in avidin solution and water separately overnight, and the CV experiments were repeated. In the CV data of Ru(py)$_2$(C$_{16}$SH) soaked in avidin solution, the peak potential did not shift. This was expected, since there was no binding between avidin and Ru(py)$_2$(C$_{16}$SH). This result confirmed that the shift of $-43$ mV in $E_{1/2}$ observed in the Ru(BMP)$_2$(C$_{16}$SH) system was caused by specific binding between avidin and biotin rather than non-specific binding or other changes in the monolayer, such as being heated at 37 °C overnight. Similar to what was observed in the 4-BMP and 4-DMP systems, the peak current decreased after avidin addition. In the CV data for Ru(py)$_2$(C$_{16}$SH) soaked in water, the peak potential did not shift, and the peak current remained the same (Figure 11). Since Ru(py)$_2$(C$_{16}$SH) cannot bind to avidin and the current remained the same at the absence of avidin, this observation suggested that the current decrease in all trinuclear ruthenium cluster systems was caused by nonspecific binding between avidin and the monolayer, rather than interactions between biotin and avidin.
Figure 11: CV traces for (a) Ru(py)$_2$(C$_{16}$SH) before (black) and after (red) avidin addition, where the peak current decreased, and (b) Ru(py)$_2$(C$_{16}$SH) before (black) and after (red) being soaked in water, where the peak current remained the same.

3.5. CV: Tafel plots

The Tafel plots of Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), and Ru(DMP)(py)(C$_{16}$SH) before and after protein binding were overlaid on each other (Figure 12). The curvature and the y-intercept of the Tafel plot give information on $\lambda$ and $k_{ET}$, respectively.$^{16,21}$ The overlapping plots showed unobservable changes in curvature and y-intercept, which suggested that there was little change in $\lambda$ and $k_{ET}$ for all four systems after avidin binding. These results were different from the expectation that $\lambda$ and $k_{ET}$ would change upon avidin binding. A possible explanation is the metal-centered nature of the +1/0 redox pair of the trinuclear ruthenium cluster. It is known that in these clusters, electrons are delocalized over all three metal centers but not to the binding ligands. Since there is little electron density on the binding ligands, protein binding is less likely to affect electron transfer,
$\lambda$ and $k_{\text{ET}}$, compared to a system where electron delocalization is extended to the binding ligands.

![Tafel plots](image)

**Figure 12:** Tafel plots for (a) Ru(BMP)$_2$(C$_{16}$SH), (b) Ru(BMP)(py)(C$_{16}$SH), (c) Ru(DMP)$_2$(C$_{16}$SH), and (d) Ru(DMP)(py)(C$_{16}$SH). No significant change in curvature or y-intercept was observed in the plots, which suggested that there is no detectable change in $\lambda$ and $k_{\text{ET}}$ after avidin bound to the trinuclear ruthenium clusters.
3.6. ACV: $i_p/i_b$ vs. frequency plot

Plots of $i_p/i_b$ vs. frequency for Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH) and Ru(DMP)(py)(C$_{16}$SH) were made from the ACV experimental results (Figure 13). In all four plots, there was no shift in the curve along the frequency axis after avidin addition, which indicated that any change in $k_{ET}$ upon avidin binding was unobservable. This observation supported the results from the Tafel plots. A decrease in $i_p/i_b$ at low frequency, caused by decreasing surface coverage, was observed. This result is likely due to the nonspecific binding between avidin.
Figure 13: $i_p/i_b$ vs. ACV frequency plots for (a) Ru(BMP)$_2$(C$_{16}$SH), (b) Ru(BMP)(py)(C$_{16}$SH), (c) Ru(DMP)$_2$(C$_{16}$SH), and (d) Ru(DMP)(py)(C$_{16}$SH). No shift along the frequency axis was observed upon avidin binding, which suggested that the change in $k_{ET}$ for all four systems after avidin binding is negligible.
Upon avidin binding, $E_{1/2}$, $\lambda$, and $k_{ET}$ were expected to change. However, only $E_{1/2}$ for Ru(BMP)$_2$(C$_{16}$SH) had an observable change after avidin addition. $E_{1/2}$ did not change significantly for the other three systems, and $\lambda$ and $k_{ET}$ did not change significantly for all four systems. The possible reason is the metal-centered nature of the $+1/0$ electron transfer for the ruthenium cluster system. The electrons are only delocalized over the metal centers but not to the binding ligands. Hence, protein binding is less likely to affect $E_{1/2}$, $\lambda$, and $k_{ET}$ during a redox event of the metal centers. Another possible reason for this observation is that, since the charge is delocalized over three Ru centers, the formal charge difference of the reduced and oxidized forms is $1/3$ for each Ru center. This change could be too small for the electrochemical techniques to measure.

3.6. BSA control

The CV trace for Ru(BMP)$_2$(C$_{16}$SH) remained almost identical before and after the addition of BSA (Figure 14). Since BSA does not bind to biotin, this experiment verified that the change in $E_{1/2}$ observed in the previous experiment was caused by the interactions between biotin and avidin. No current decrease was observed after BSA addition, which indicated that there was no nonspecific binding between BSA and the monolayer.
CV: Ru(BMP)$_2$(C$_{16}$SH) -- BSA

**Figure 14:** CV traces for Ru(BMP)$_2$(C$_{16}$SH) before (black) and after (red) BSA addition. No change in peak potentials or current decrease was observed.

3.7. DMF study

In the DMF study, $E_{1/2}$ decreased as DMF concentration increased (Figure 15). A shift of -43 mV in $E_{1/2}$ was observed from 0% DMF to 50% DMF at scan rate 250 mV/s, and the shift was consistent between scan rate 50 mV/s and 1000 mV/s (Figure 16). As DMF concentration increases, $E_{1/2}$ was expected to shift positively. As the polarity of the environment decreases, the neutral, reduced form of this cluster will become more favored than the charged, oxidized form of the cluster. As a result, the redox potential was expected to increase. However, the experimental results showed the opposite. A possible explanation for this observation is that, when DMF concentration is low, solubility of the neutral cluster is not favored in the polar solvent, water, and, hence, it interacts more with the hydrophobic monolayer. As the DMF concentration increases, the polarity of the solvent decreases and the neutral cluster no
longer interacts with the monolayer as strongly. Overall, the solvent is expected to be more polar than the monolayer even after DMF addition. As a result, as DMF concentration increases, the polarity of the environment around the cluster actually increases due to decreasing interactions between the cluster and the hydrophobic monolayer, which makes $E_{1/2}$ shift negatively. Further, this experiment showed that it is possible to simulate protein binding by changing the polarity of the solvent. The results showed that avidin binding is similar to 50% DMF because under both conditions, $E_{1/2}$ decreased by 43 mV.

Figure 15: $E_{1/2}$ vs. DMF concentration plot for Ru(BMP)$_2$(C$_{16}$SH) at 250 mV/s. $E_{1/2}$ decreased linearly as DMF concentration increased from 0% to 50%.
Figure 16: $E_{1/2}$ vs. DMF concentration plot for Ru(BMP)$_2$(C$_{16}$SH) at different scan rates. The change in $E_{1/2}$ is consistent between different scan rates.
Chapter 4. Conclusion and Future Work

In the work presented here, tri-nuclear ruthenium clusters, Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), Ru(DMP)(py)(C$_{16}$SH), and Ru(py)$_2$(C$_{16}$SH) were successfully synthesized and incorporated into self-assembled monolayers on gold ball electrodes. These clusters successfully overcame the current signal loss in CV observed previously in the biotinylated iron complex [Fe(BMB)(CN)$_4$]$^{2-}$. Since the tri-nuclear ruthenium clusters are larger than the iron complex, the coupling between the metal center and the electrode was maintained after avidin bound to the binding ligand. Because the clusters were incorporated into monolayers on the electrode, the avidin-cluster complexes did not need to diffuse to the electrode, which helped maintain the current signal in CV and facilitated measurement of electron transfer parameters.

The linear relationship between CV peak current and scan rate for Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), and Ru(DMP)(py)(C$_{16}$SH) suggested that the monolayers were well-packed on the electrode. The CV traces for Ru(BMP)$_2$(C$_{16}$SH) after avidin binding showed a change of -43 mV in $E_{1/2}$. However, in the other three systems, the CV traces shows no observable change in $E_{1/2}$ upon avidin addition. In addition to the four tri-nuclear ruthenium clusters, CV experiments were performed on Ru(py)$_2$(C$_{16}$SH), a cluster that cannot bind to avidin. A decrease in peak current was observed after Ru(py)$_2$(C$_{16}$SH) was soaked in avidin overnight, but the current remained the same after Ru(py)$_2$(C$_{16}$SH) was soaked in water. This result showed that the decrease in peak current was caused by the nonspecific binding between avidin and the monolayer rather than the interactions between avidin and biotin. The lack of shift in
$E_{1/2}$ indicated that the shift observed for the Ru(BMP)$_2$(C$_{16}$SH) system is due to avidin binding.

The CV data were used to generate Tafel plots for Ru(BMP)$_2$(C$_{16}$SH), Ru(BMP)(py)(C$_{16}$SH), Ru(DMP)$_2$(C$_{16}$SH), and Ru(DMP)(py)(C$_{16}$SH). The Tafel plots for all four clusters before and after avidin addition overlapped. No significant change in the curvature or the y-intercept of the plots was observed, which indicated that, upon avidin binding, there was no change in $\lambda$ and $k_{ET}$, respectively. For all four clusters, $i_p/i_b$ vs. frequency plots were generated from ACV data. All plots showed no change along the frequency axis after avidin addition. This supports the finding that there is no significant change in $k_{ET}$ upon avidin binding.

In order to confirm that the change in $E_{1/2}$ in the Ru(BMP)$_2$(C$_{16}$SH) was caused by the interactions between avidin and biotin, CV experiments were performed on Ru(BMP)$_2$(C$_{16}$SH) before and after the clusters were soaked in BSA, a protein that does not bind to biotin. The CV traces remained the same after BSA addition, which verifies that the change in $E_{1/2}$ was caused by binding interaction. Additionally, the peak current did not decrease, which suggested that there is no nonspecific binding between BSA and the monolayer.

To simulate the conditions of protein binding, CV experiments were performed on Ru(BMP)$_2$(C$_{16}$SH) in various DMF concentrations. The results show that $E_{1/2}$ decreased linearly as DMF concentration increased between 0% and 50%. In 50% DMF, a shift of -43 mV in $E_{1/2}$ was observed (as compared to 0% DMF), which was similar to avidin binding. The linear change in $E_{1/2}$ was consistent between different scan rates. $E_{1/2}$ was expected to increase with increasing DMF concentration (and decreasing solvent polarity), but the experimental result showed the opposite.
This could be caused by decreasing interaction of the cluster with the hydrophobic monolayer as DMF concentration increases.

In the future the sensitivity of the Ru(BMP)$_2$(C$_{16}$SH) system, will be improved by tuning electrochemical parameters of the system to enhance the changes in electrochemical signals upon avidin binding. The greater the changes are, the more sensitive the system is, and the more likely it can be applied to aid the development of electrochemical biosensors. Ru(NO)(4-BMP)$_2^{0/-}$, which is isoeletronic to Ru(BMP)$_2$(C$_{16}$SH)$^{+/0}$, can be used as well, because its electrons are known to be delocalized over the entire molecule. Finally, the biotin-avidin model system can be extended to molecules that are more biologically relevant, such as the Crizotinib-anaplastic lymphoma kinase (ALK) system. Anaplastic lymphoma kinase (ALK) is an oncological biomarker because overexpression of ALK can cause lung cancer. Crizotinib is a commercially available ALK inhibitor, and the binding between Crizotinib and ALK is well studied. Therefore, the Crizotinib-ALK ligand-receptor system can be utilized in the trinuclear ruthenium cluster system for biosensor design.
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