Synthesis and Electrochemical Characterization of a Transition Metal–Modified Ligand-Receptor Pair

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Abstract

Weak, noncovalent interactions (dipole-dipole interactions, hydrogen bonding, and Van der Waals forces) in ligand-receptor pairs play a very important role in living systems. A new way to study these interactions is by using electron transfer theory. The rate of electron transfer between redox centers is dependent on several factors, including reorganization energy, which incorporates these weak interactions. Binding of a protein receptor to a redox-modified ligand will change the reorganization energy of a system. Thus, the rate of electron transfer can be measured using electrochemical techniques.

A molecule was designed to incorporate a ligand (desthiobiotin), a metal complex (ruthenium pentaammine), and an alkane thiol. The molecule was synthesized and characterized, and there was an attempt made to incorporate the redox-modified ligand into self-assembled monolayers on gold electrodes. The potentials and the rates of electron transfer of this system were measured in the presence and absence of avidin, a tetrameric protein. From the electrochemical data gathered thus far, it was impossible to conclude whether or not avidin affects the rate of electron transfer.

Introduction

Living organisms rely on chemical interactions to survive. Almost all biological processes involve the interaction of two or more entities. The size, shape, and function of the two linking bodies vary greatly. Some examples include the binding of hemoglobin to oxygen, anti-DNP antibodies to dinitrophenyllysine, and neurotransmitters to synapses. At the molecular level, the smaller entity is referred to as the ligand, while the larger is known as the receptor. The ligand-receptor pair can be held together by covalent bonds or noncovalent bonds, such as hydrogen binding and Van der Waals forces. Typically, when a ligand binds to a receptor, solvent molecules are excluded and new hydrogen bonds or hydrophobic contacts form between the ligand and the receptor. The energetics of this process is of interest to life science studies.[1]

Desthiobiotin and avidin were chosen for this work because this ligand-receptor pair exhibits a dissociation constant of 10^{-13} M.[2] There are several factors that contribute to the strong binding. The binding pocket of avidin contains polar and aromatic residues in an arrangement that fits biotin extremely well. The aromatic residues form a hydrophobic box that stabilizes bound biotin molecules. In addition, the avidin-biotin complex contains many strong hydrogen bonds that stabilize the system.[3] Structures of avidin and desthiobiotin are shown in Figure 1.

According to Marcus theory, the rate of electron transfer (k_{ET}) depends on the temperature (T), Gibbs free energy (ΔG),

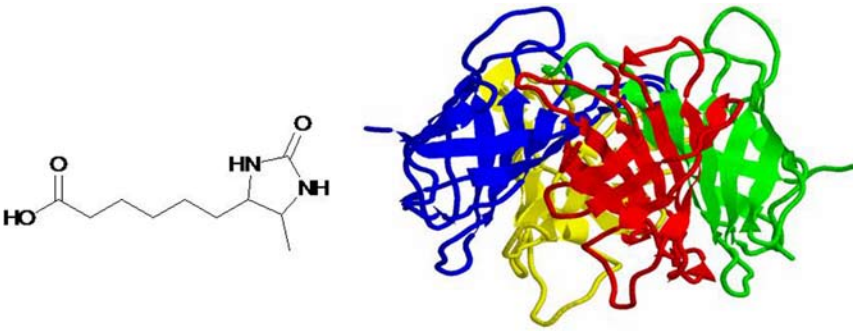


Figure 1: Ribbon structure of the binding ligand desthiobiotin (left) and avidin (right).

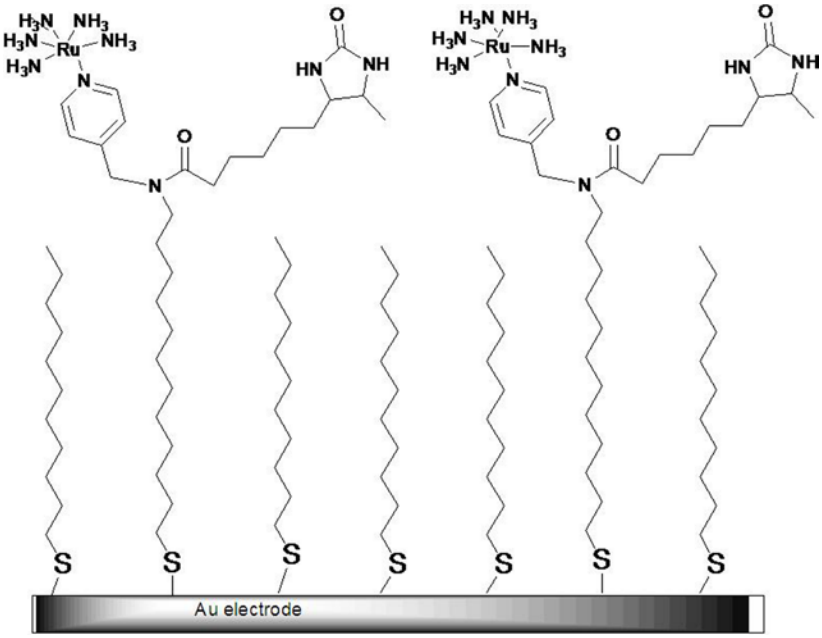


Figure 2: The target system incorporated into a monolayer on a gold electrode. Undecanethiols are placed in between the ligands to prevent other molecules in the solvent from transferring electrons to the electrode.

electronic coupling (H_{ab}), and reorganization energy (λ) (Equation 1). When a metal complex undergoes an electron transfer, the geometry of the redox center changes and surrounding solvent molecules rearrange to accommodate the new charge. The energy of this rearrangement is known as reorganization energy.[4]

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

Using electrochemical techniques, the rate of electron transfer can be readily obtained for metal complexes covalently bound to an electrode. For example, it has been previously shown that ruthenium complexes appended to alkane thiols have well-behaved surface electrochemistry.[5] Data from cyclic voltammograms obtained at several scan rates for which $\Delta Ep > 200$ mV can be used with the Laviron equation to determine k^o (Equation 2). The equation relates the change in peak potentials (ΔE_p) between the anodic and cathodic peaks to the rate of electron transfer (k).[6] In this equation, α is the transfer coefficient, R is the ideal gas constant, T is the temperature, n is the number of electrons transferred, F is Faraday's constant, ν is the scan rate, and ΔEp is the difference in peak potentials.

$$(2)\quad \log k = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\left(\frac{RT}{nF\nu}\right) - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3RT}$$

The goal of this work is to observe a change in k_{ET} due to the change in λ that occurs upon ligand-receptor binding. The environment of the redox-modified ligand will change when bound to the receptor, and that should also change the observed potential of the metal complex. For this study, a system was designed that keeps all of the k_{ET} factors identical when avidin binds to a ruthenium-modified desthiobiotin ligand except reorganiza-

tion energy; therefore, any observed change in k_{ET} can be attributed to changes in λ.

This work can be applied to real-life medical situations and has the potential to save many lives. One of the long-term goals of this research is to develop a biosensor that can detect certain disease proteins based on their electrochemical interactions with the body's molecules. This method will allow scientists and doctors to detect disease proteins in the body at a much smaller scale than with the use of normal assays. Being able to determine the presence of a disease at the nanoscale level will enable doctors to diagnose and treat patients before a disease has progressed too far.

Background

Electrochemical reactions are fundamental functions of all living systems. Electrons are transferred during many biological and chemical processes including cellular respiration, photosynthesis, and metabolism. Electron transfer has been studied in great detail since the 1940s. The first experiments were performed to study exchange reactions between isotopes. Over the past 50 years, new instruments were developed that allowed researchers to spectroscopically monitor the rate of electron transfer. The simplicity of electron transfer reactions has also advanced this research. The reactants and products of electron transfer reactions are identical. Therefore, thermodynamic stability does not affect the outcome of the reactions. In many electron transfer reactions, no chemical bonds are broken or formed.[4]

Over the past 15 years, cyclic voltammetry has become a very popular method for studying electrochemical reactions.

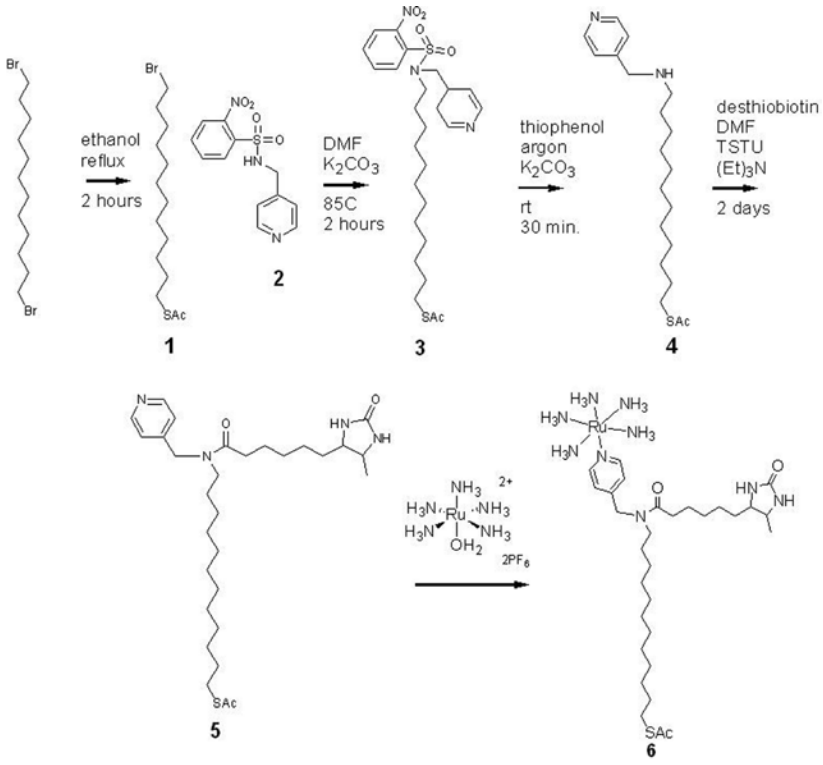
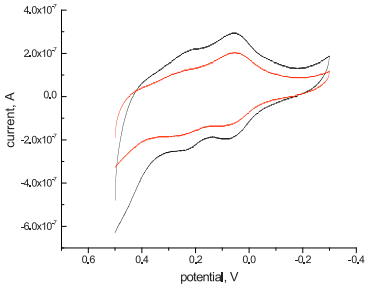
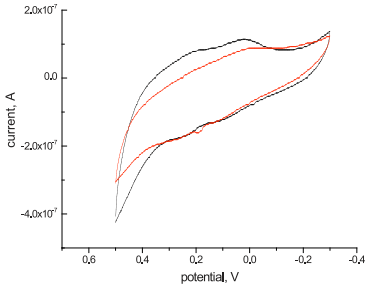


Figure 3: Overall synthesis of the target redox-modified ligand. Ruthenium pentaamine is the redox modification, and desthiobiotin is available for binding to avidin. The thioacetate in 6 can be deprotected to give a thiol to attach to the gold electrode.



Graph A
Black= w/o avidin
Red= w/ avidin

ΔE_p =.031
$E_{1/2}$ = .0705



Graph B
Black= w/o avidin
Red= w/ avidin

ΔE_p =.044
$E_{1/2}$= .074

Figure 4: Cyclic voltammograms of electrodes from Method 2. Graph A is of one electrode, with and without avidin. Graph B is the control electrode, with and without buffer.

This technique is used to observe the oxidation and reduction of metal complexes. In a typical experiment, the three electrodes involved in cyclic voltammetry are the working, reference, and counter electrodes. An analyte solution is used to ensure conductivity. Voltage is applied to the working electrode up to a determined negative potential and then is scanned backwards to the positive potential. The peaks on the graph of current versus potential indicate the potentials at which all the species have been reduced and oxidized.[8]

Another method commonly used to study electrochemical reactions is known as differential pulse voltammetry, or DPV. In this process, the applied potential is pulsed positive, held for a set amount of time, and then pulsed negative. The difference in pulses is the step height over a set amplitude. The pulses continue until a final determined potential has been reached. The current is measured at two points relative to the time of voltage application, i(1) and i(2), and the difference of these two currents is graphed against the applied potential. This method eliminates the background currents generated by the double layer of ions that typically form during cyclic voltammetric measurements and therefore increases the sensitivity of peak detection.[9]

In previous work, desthiobiotin was modified with ruthenium pentaammine and iron tetracyano complexes. Solution electrochemistry of this compound was normal, but the addition of avidin dramatically decreased the signal in the CV. Avidin therefore effectively blocked the electron transfer between the electrode and metal complexes in solution, preventing the measurement of changes in k_{ET} and λ.[6,7]

Approach

Alkane thiols with appended ruthenium complexes have been previously shown to have well-behaved surface electrochemistry.[7] Therefore, for this study, the ligand was designed to incorporate a desthiobiotin moiety for avidin binding, a ruthenium pentaammine complex, and an alkane thiol for bonding to the electrode surface as part of a monolayer. The new design incorporates both known systems. Directly attaching the metal-modified ligand complex to the electrode will allow currents and potentials to be observed (Figure 2).

Synthesis of the molecule is based on methods known to be successful. To incorporate the ruthenium complex, 4-aminomethylpyridine was chosen. The alkane thiol was incorporated as a thioacetate that can be deprotected, resulting in an alkane thiol for monolayer formation. Desthiobiotin has a carboxylic acid, and many methods are known to conjugate this group to amines to form amides.

Diluents, or unmodified alkane thiols, form the body of the monolayer and space out the redox-modifed ligands. The monolayer also prevents other molecules in the solution from reaching the electrode and interfering with electrochemical measurements. Diluents with various terminating

groups can be used to examine the interaction of the protein with the surface of the monolayer.

Two different methods can be used to form the monolayer. The first approach involves deprotecting the thiol group of the ligand and then forming the monolayer on the electrode. This step is followed by exposure of the modified electrode to a solution of ruthenium (aquo)pentaammine to form the final complex on the surface. In the second method, the ruthenium complex is bound to the acetate-protected ligand, which is then deprotected and used in the formation of the monolayer.

Electrochemical measurements (CV and DPV) can be used to determine whether the metal complex is bound to the electrode or not. If the metal complex is present, the potential of the redox couple can be determined. CV measurements over a number of scan rates yield data for electron transfer rate analysis using the Laviron method. The rates can be determined in the absence and presence of avidin for comparison. If protein binding affects the environment of the metal complex and therefore the reorganization energy, the rates of electron transfer are expected to be different.

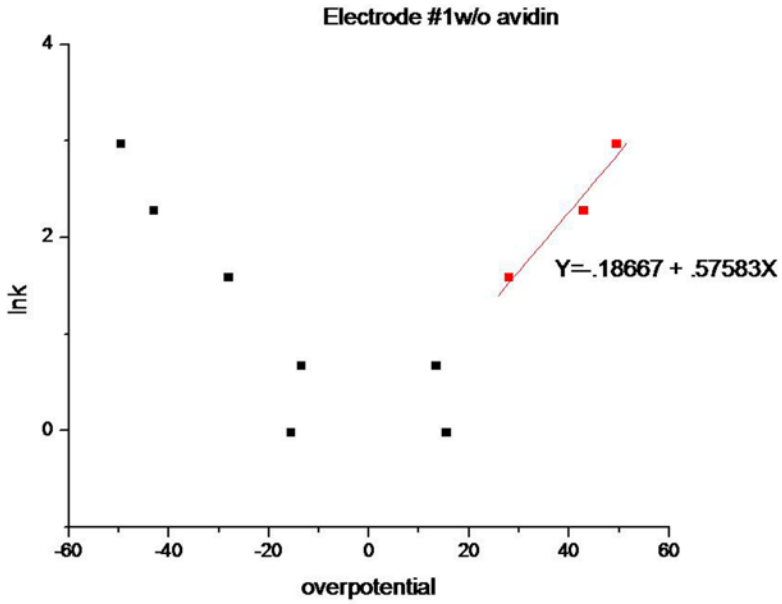


Figure 5: Tafel plot of electrode in 6.36 mM H_2SO_4 before attachment of avidin. In order to find the rate, the inverse log was taken of the x-intercept. Rate = 0.83 s^{-1}.

Results

The ligand incorporating desthiobiotin, a pyridine moiety for ruthenium complexation, and an alkane thiol for monolayer formation, was synthesized as follows (Figure 3). To form **1**, 12-bromododecane thioacetate, 1,12-dibromododecane was combined with 1 eq. potassium thioacetate. Flash column chromatography using 5% diethyl ether in hexane was used to purify

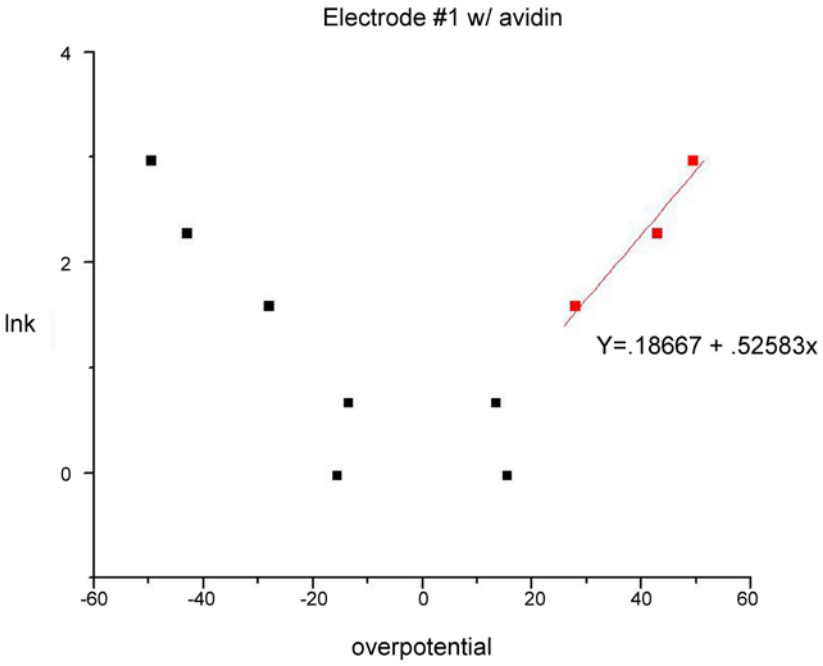


Figure 6: Tafel plot of electrode in 6.36 mM H_2SO_4 after addition of avidin. In order to find the rate, the inverse log was taken of the x-intercept. Rate = 0.83 s^{-1}.

the product which was obtained in a yield of 42%. Compound **3** was generated by alkylating **2** with **1** using potassium carbonate in DMF. The compound was purified by flash column chromatography using 2% MeOH in CH2Cl2. The yield of **3** was 28%. The nosyl protecting group in **3** was removed using thiophenol and potassium carbonate in DMF. Flash column chromatography was performed using 9:1 $CHCl_3$ to MeOH, and a 60% yield of **4** was obtained. At each step, ESI-MS and ^{1}H NMR spectroscopy confirmed the identity of the compound, and TLC indicated the compounds were pure.

The secondary amine in **4** was conjugated to the carboxylic acid in desthiobiotin using TSTU and NEt_3 in DMF to give **5**. The reaction was stopped after 48 hr although not complete by TLC. Flash column chromatography was used to purify the product using gradient of 1–4% MeOH in $CHCl_3$. The yield of **5** was 81%. Although TLCs in many solvent combinations showed only one spot, the ^{1}H NMR spectrum indicated an impurity was present, revealing small undefined peaks in the aromatic region. In addition, the ESI-MS showed peaks at m/z=649, the impurity, and 569, the desired product.

In order to test methods of removing the acetate-protecting group from the thiol functionality, a sample of the ligand was dissolved in MeOH and divided into four equal aliquots. Sodium methoxide, ammonium hydroxide, sulfuric acid, and hydrochloric acid were added to the samples, which were allowed to stir overnight. Small aliquots from each sample were taken at various intervals and examined using ESI-MS. After two days, the MS results indicated that sulfuric acid removed the acetate

group quickly and no starting material remained, whereas in each of the other samples a significant amount of acetate-protected ligand remained.

A procedure found in the literature was followed to generate the ruthenium aquo species used for the redox modification of the ligand.[10] $Ru(NH_3)_5Cl_3$ was reduced with Zn/Hg in water under argon for 30 min and then precipitated using NH_4PF_6 to give $[Ru(NH_3)_5OH_2](PF_6)_2$. This compound was used to add ruthenium pentaammine to the pyridine of the ligand.

To form the monolayer, two methods were used. In Method 1, the ligand was deprotected using sulfuric acid and then incorporated into a monolayer followed by Ru modification. Using a solution of the ligand that had been deprotected with sulfuric acid, two samples each of 1.0 mM, 0.5 mM, and 0.1 mM solutions in MeOH were prepared. 1-undecanethiol and 1-octadecanethiol (0.1mM) were added to each ligand concentration as diluents. A clean gold electrode was placed into each solution and left to stand overnight. These electrodes were washed briefly and then soaked overnight in a solution of $[Ru(NH_3)_5(OH_2)](PF_6)_2$ in THF that was prepared under argon.

Method 2 involved forming the ruthenium complex of the ligand first, followed by removal of the acetate group and monolayer formation. Under argon, the ligand was dissolved in MeOH with a slight excess of $[(Ru)(NH_3)_5(OH_2)](PF_6)_2$. Addition of the ruthenium complex appeared to be successful, given the appearance of a charge-transfer band in the UV-visible spectrum at 380 nm, typical for a pyridine ruthenium pentaammine complex.

In order to determine whether a lower concentration of sulfuric acid would remove the acetate group, a solution of the ruthenium complex **6** was divided into two aliquots (17 ⌈L of 6M H_2SO_4 were added to one sample, and 8⌈L 6M H_2SO_4 were added to another sample). After two days, the color of the solution changed from yellow to brown and a precipitate formed, indicating decomposition of the metal complex. Each sample was diluted with EtOH to 1 mM, and undecanethiol (1.0 mM) was added. Two electrodes were soaked in each solution overnight to form the monolayer.

Electrodes from each deprotection method were tested in the presence and absence of avidin. Avidin was added to the electrode by placing a 20 µL drop of a solution of 3.4 mg/mL avidin onto the electrode and waiting 10 min. Cyclic voltammetry was performed on all electrodes at the following scan rates: 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, 25, 50, and 100 V/s.

The results from Method 1 showed no redox couples present at any scan rate, indicating that no metal complex was present in the monolayer. However, DPVs for the undecanthiol monolayers were obtained and showed weak signals, indicating that metal complexes were present but in low coverage (Table 1).

In the results from Method 2, all but one electrode showed no distinguishable peaks at any of the scan rates. DPVs for the electrodes were obtained (Table 2). For each set of electrodes, one electrode was tested with and without avidin, and the other was tested with and without a 20 uL drop of buffer as a control. For this method, only one electrode (from the more concentrated acid test) showed peaks when CV was performed. The CVs

Solution	Ep (V)
1.0 mM	0.092
1.0 mM w/ avidin	0.080
0.5 mM	0.084
0.5 mM w/ avidin	0.076
0.1 mM	0.092
0.1 mM w/ avidin	0.108

Table 1: DPVs obtained from Method 1 electrodes soaked with undecanethiol.

for the electrodes in the 6.36 mM H_2SO_4 solution at scan rate 0.05 V/s is shown in Figure 4. Tafel plots (Lnk vs. overpotential) were graphed using data from the CVs obtained before and after the addition of avidin (Figures 5 and 6).

Discussion

The desired ligand containing desthiobiotin was successfully synthesized and characterized using ESI-MS and ^{1}H NMR spectroscopy. An impurity in the final product was identified in the ^{1}H NMR spectrum but could not be separated using silica gel chromatography. Based on the NMR spectrum, the impurity contains a pyridine, but otherwise the composition is unclear. It is unlikely that the impurity is an unreacted intermediate, due to the extensive purification at each preceding step of the synthesis. The impurity differs in mass from the desired compound by 79 amu, and it was not possible to propose reasonable structures based on the final coupling reaction. HPLC purification methods are currently being investigated. Although it is present in small amounts, it is possible that this species is on the monolayer as well as on the target redox-modified ligand.

Sulfuric acid was used to deprotect the thiol group of the ligand because it was experimentally shown to remove the acetate group very efficiently. However, it may be possible that this strong acid removes the ruthenium complex by protonating the pyridine. Because of this potential complication, it may be helpful to try other deprotection methods. Hydrochloric acid was found to deprotect the thioacetate as well, but not as efficiently, so this may be used in future work as an alternate deprotection method.

Despite extensive mechanical and electrochemical cleaning of the electrodes before soaking in the monolayer solutions, our methods for forming monolayers were not reproducible. Figure 4 shows CVs from two electrodes soaked in the same solution. As they did not give identical CVs, this method of monolayer formation may not be reliable.

The intermediate ruthenium aquo complex and the final complex **6** are air sensitive. Although Schlenk techniques were used to exclude oxygen, the solutions typically changed colors over the course of hours, which is indicative of decomposition. This instability may have prevented formation of the final target redox-modified molecule and therefore be one cause of irreproducible CVs.

The best fit lines of the points for which $\Delta Ep>200mV$ in Figures 5 and 6 were plotted. In order to find the rate of electron transfer (k°), the inverse log of the x-intercepts were calculated. Although the best fit line equations were slightly different, they resulted in the same k° for each experiment. Thus, the data gathered indicates that the rate of electron transfer with and without avidin is the same. It is possible that the nonpolar binding ligand is folding into the alkane monolayer, effectively hiding it from the avidin and preventing binding. The time allowed for binding may have not been long enough, given that the binding ligand is on a surface and not in solution.

Conclusions

Synthesis of a desthiobiotin ligand incorporating a protected alkane thiol was successful. Mass spectrometry and NMR indicate that the target molecule is present but with an impurity that we were unable to separate using traditional methods. Mass spectrometry indicates that H_2SO_4 efficiently deprotects the thioacetate of the ligand to yield the thiol. Addition of the ruthenium complex appeared to be successful, given the appearance of a charge-transfer band in the UV-visible spectrum at 380 nm, typical for a pyridine ruthenium pentaammine complex.

Better characterization of the ligand, in both the acetate-protected and the thiol form, is under way. Preliminary HPLC results indicate this method may be used to purify these compounds. In addition, less harsh deprotection methods may be useful.

Incorporation of the metal complex into the monolayer was not complete. Since signals in general were very weak or not present in most of the obtained DPVs and CVs, formation of monolayers with the redox-modified ligand was not ideal. Therefore, current methods must be perfected. An alternative is to attempt another sequence: the ligand may be deprotected, then reacted with the ruthenium species and purified before attaching to the gold electrode.

The results regarding the effect of avidin on electron transfer are inconclusive. The data collected indicates that the rate of electron transfer when avidin is present is equal to the rate of electron transfer when avidin is not present. However,

since the majority of the data obtained did not allow calculation of the kET, this conclusion is not definitive.

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Solution	Electrode w/ only monolayer (Ep)	Electrode w/ avidin (Ep)	Electrode w/ only monolayer (Ep)	Electrode w/ buffer (Ep) (.1 M Na_2SO_4 pH4) control
3.00 mM #1	0.078V	0.065V	_____	_____
3.00 mM #2	_____	_____	0.063V	0.066V
6.36 mM #1	0.092V	0.092V	_____	_____
6.36 mM #1	_____	_____	_____	0.140V

Table 2: DPVs obtained of electrodes from Method 2.