

Preparation and Electrochemical Analysis of Several Solvent Reorganization Energy Probes

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Abstract

Weak interactions in ligand-receptor pairs are important in biological systems. However, the energetics of these interactions are often difficult to quantify for ligand-receptor pairs. Weak interactions are a part of reorganization energy, which plays a role in electron transfer reactions. Under certain conditions the rate of electron transfer can be measured using electrochemistry (cyclic voltammetry). Therefore, electron transfer can be used to study binding. The premise is that when protein binds to a ligand, it changes the dielectric constant of the medium surrounding the metal complex, resulting in a shift in electrochemical potential. For the work presented here, the biotin-avidin system was chosen because it has been extensively studied and can be easily modified. Two different types of these solvent reorganization energy probes are being investigated in this system — solution probes and solid-state probes — which differ mainly in the method by which electron transfer is measured. Synthetic methods for solution probes to modify biotin with iron are discussed — specifically, 5-BMB and 5-BPB. The syntheses of the ligands were carried out. The synthesis of the iron

complex is under way. For the solid-state probes, a synthetic method is described for a biotinylated thiol. This thiol is combined with a ruthenium complex and functionalized alkane-thiols, namely 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, and octadecanethiol, to form mixed monolayers on a gold electrode. The analysis of these monolayers in the presence and absence of avidin using cyclic voltammetry is discussed. For the monolayer incorporating 11-mercapto-1-undecanol, a potential shift of 21 mV was observed in the absence of biotin upon addition of avidin, and a potential shift of 29 mV was observed in the presence of biotin upon addition of avidin. For the monolayers incorporating 11-mercaptoundecanoic acid and octadecanethiol, no significant shift in potential was observed upon avidin addition.

Introduction

Weak interactions, particularly hydrogen bonding and van der Waals forces, have important functions in biological events. Examples include signal transduction and the interactions of drugs with biological macromolecules. Signal transduction is a biochemical event by which a cell converts a particular stimulus into another. In other words, sensing at the cellular level in all organisms is governed by signal transduction. For example, a hormone such as adrenaline's binding to an adrenoceptor in the heart causes the heart to beat more quickly. For drugs to be designed more efficiently, an understanding of the noncovalent bonding between the binding sites of macromolecules and proposed drug molecules is vital. These biochemical events are governed by the energetics of each reaction. It is crucial, therefore, to understand the energetics of these noncovalent bonds. However, the energetics

of biological ligand-receptor pairs have been difficult to quantify. It is the goal of this research to use electrochemistry to study the energetics of weak interactions of ligand receptor pairs.

According to electron transfer theory, several factors influence the electrochemical potential of a metal ion and changes in the rate of electron transfer (Figure 1). These include the change in Gibb's free energy (ΔG), the electronic coupling matrix (H_{AB}), and reorganization energy (λ). Gibb's free energy changes and the electronic coupling matrix have been studied previously.¹⁻⁴ The focus of this study is on reorganization energy, defined as the energy required to distort a system from the most probable configuration of the reactant state to the most probable configuration of the reactants. Reorganization energy has both an outer sphere component (λ_o) and an inner sphere component (λ_i) (Figure 1). The inner sphere component includes intramolecular changes, namely, changes in bond lengths and geometries. The outer sphere component includes weak interactions such as van der Waal's forces and hydrogen bonding. Metal complexes were chosen for this study that have a negligible λ_i and small polar ligands to maximize λ_o .

Because reorganization energy plays a role in electron transfer reactions, this study focused on these reactions. Using a portion of electron transfer theory, the semiclassical Marcus theory of donor-acceptor pairs, an equation emerges relating reorganization energy and the rate of electron transfer (Figure 1). The rate of electron transfer can be measured experimentally using cyclic voltammetry. In solution, the rate can be determined by fitting simulations to data.

Electron transfer rate can also be measured electrochemically by attaching probes to an electrode surface. The equation relating heterogeneous peak potential (E_p) to the rate of electron transfer is shown in Figure 1. Electrochemical methods can be used to determine the reorganization energy of a metal complex attached to an electrode.⁵ Using cyclic voltammetry as a method for the determination of reorganization energy of ligand-receptor pairs involving proteins has never been done, however.

The chosen ligand-receptor pair for this study was that of biotin and avidin. Avidin binds to biotin with one of the strongest noncovalent interactions known ($K_D \sim 10^{-15}$ M), and it is extremely resistant to denaturation over a wide range of pH and temperatures.⁶ Biotin (vitamin H) is involved in important

metabolic pathways such as fatty acid synthesis and amino acid catabolism, functioning as a cofactor that aids in the transfer of CO₂ groups. Small quantities are found in egg yolk, milk, barley, and some dietary supplements. Avidin is a 66 kDa tetrameric glycoprotein found in the whites of chicken eggs. Other than sequestering biotin, the function of avidin is unknown.⁷

Background

Most of the theory behind modern electron transfer research was developed by Rudolph A. Marcus.^{8,9} He derived a number of equations critical to research in this field. One of these, the Marcus Equation, uses Gibbs free energy (ΔG), reorganization energy (λ), temperature (T), and electronic coupling between

the donor and the acceptor (H_{AB}) to determine the rate of electron transfer (k^0) (Figure 1).

The avidin-biotin system is unusual in that the noncovalent binding is so strong. This characteristic has made it widely applicable in biotechnology for separations and labeling of biochemical systems. Numerous kinetic and thermodynamic studies have been carried out on the system. Kinetics studies have been done specifically investigating the binding of avidin to biotin.¹⁰⁻¹³ Other studies have been conducted that show the binding affinity of both immobilized and solubilized avidin is not affected over the duration of the electrochemical experiments, an important fact for this study.^{14,15} The solid-state structure of avidin has been determined for a number of forms by x-ray crystallography.¹⁶

Reorganization Energy

$$\lambda = \lambda_i + \lambda_o$$

$$\lambda_o = N_A e^2 \left[\frac{1}{2r_A} + \frac{1}{2r_D} - \frac{1}{d_{AD}} \right] \left(\frac{1}{n^2} - \frac{1}{\epsilon_g} \right)$$

Marcus Equation

$$k^0 = \frac{4\pi^2 H_{AB}^2}{h\sqrt{4\pi\lambda RT}} \exp \left[-(\Delta G^0 + \lambda)^2 / 4\lambda k_B T \right]$$

Peak Potential

$$E_p = E^{0'} + \frac{RT}{\alpha F} \ln \left(\frac{RTk^0}{\alpha F v} \right)$$

Figure 1: Marcus equation for rate of electron transfer and equations for reorganization energy and peak potential. These equations show the relationship between the peak potential (E_p) for surface-based systems, electron transfer rate (k^0), and reorganization energy (λ). In the reorganization energy equation r_A is the radius of the electron acceptor, r_D is the radius of the donor, d_{AD} is the distance between the donor and acceptor, n is the refractive index, and ϵ_g is the dielectric constant. In the Marcus equation H_{AB} is the electronic coupling matrix and ΔG^0 is the driving force. In the equation for peak potential, v is scan rate. By altering the dielectric atmosphere of a metal, reorganization energy changes, which changes the rate of electron transfer, thus shifting the peak potential. This shift in peak potential can be used to characterize the energetics of weak interactions.

Research has been done using solution-based probes of iron and ruthenium complexed with modified biotin ligands. Binding studies were conducted showing that the modified complexes bound to avidin in a similar fashion as to biotin and desthiobiotin. It was found that upon addition of avidin to the solvent reorganization energy probes, the current signals decreased dramatically. Because of this, it was found that mediators had to be used in order to determine the rate of electron transfer.¹⁷

Approach

One reason the biotin-avidin system was chosen for this study is that it has been widely investigated. Other major reasons include its low dissociation constant and the fact that biotin can be easily modified using standard peptide coupling methods. To use electron transfer to probe weak interactions between ligands and receptors, it is necessary to incorporate a metal into the ligand. Two different metals, iron(II) and ruthenium(II), were chosen for comparison. The basic premise behind these electrochemical experiments is that the electrochemical signal (cyclic voltammetry) of biotinylated metal ions should change upon binding to avidin. This change occurs because the presence of the protein alters the dielectric atmosphere around the metal. Water, the solvent used in the electrochemical experiments, has a high dielectric constant ($\epsilon = 80$). The dielectric constant has been estimated to be much lower within the protein ($\epsilon \sim 4$ -20).^{18,19} A change in the dielectric constant (ϵ_s) changes reorganization energy (λ), which in turn changes the

rate of electron transfer (k^0), which can be seen via electrochemistry as a shift in the peak potential (E_p) (Figure 1).

Both solution-based and solid-state electrochemical probes have been designed. Solution-based probes, as the name implies, are freely diffusing in solution during the cyclic voltammetry (CV) experiments with a mediator. Electron transfer rate can be determined by fitting the CV data to simulations based on homogeneous (mediator to protein-bound) and heterogeneous (mediator to electrode) kinetic reactions.

The solid-state ruthenium probes are incorporated into mixed monolayers containing a binding ligand. The ruthenium is anchored to the gold electrode used in the CV experiments. For these probes, electron transfer rate can be found using the equation for peak potential (Figure 1). The transfer coefficient, α , can also be determined experimentally.

Solution Probes

All ligands synthesized were modifications of biotin and were for use as probes containing iron. In the future, ligands incorporating desthiobiotin will be synthesized and analyzed as well (Figure 3). The main difference between biotin and desthiobiotin is the strength of the interaction with avidin ($K_D \sim 10^{-15}$ for biotin versus $K_D \sim 10^{-13}$ for desthiobiotin).

A set of biotinylated iron complexes — one containing a short chain linker, and one containing a long chain linker between the bipyridine and biotin moieties — was designed to investigate the effect of the distance of the avidin binding site from the metal. The short chain was chosen to be 1 methylene and the long chain 5 methylene in length.

A shorter chain would result in the metal being shielded more by avidin than it would be with a longer chain. A different dielectric environment would result in different reorganization energy, and thus potential changes. For iron-complexed ligands, past experiments have concentrated on using 4,4'-disubstituted-2,2'-bipyridine.¹⁵ In order to give the biotin ligands a slightly different orientation when bound to avidin, 5,5'-disubstituted-2,2'-bipyridine compounds were synthesized. The alternate orientation is especially important for surface work because, for the 4,4'-disubstituted-2,2'-bipyridine complexes, the iron complex was directed toward the surface instead of away from it as was desired.

All ligands were synthesized using common organic synthetic methods and were based upon modifications of literature procedures²⁰⁻²² (Figure 4).

5-Bromomethyl-5'-methyl-2,2'-bipyridine

The procedure was carried out using a modification of a literature method.²⁰ A solution of 5,5'-dimethyl-2,2'-bipyridine (1.0142 g, 6.000 mmol), N-bromosuccinimide (1.0870 g, 6.000 mmol) and a catalytic quantity of AIBN in 50 mL CCl_4 was refluxed under N_2 for 5 h. Reaction progress was measured by TLC (silica gel, 10% MeOH in CH_2Cl_2). The solution was filtered hot, and the solvent was removed under reduced pressure. The dibromomethyl derivative was precipitated from CH_2Cl_2 by placing the solution in the freezer overnight. The solid was filtered off, and the filtrate was purified by column chromatography (10% MeOH in CH_2Cl_2). The solvent was evaporated under reduced pressure to give white crystals. Yield: 40%. ¹H NMR (400 MHz, CDCl_3) δ 8.68

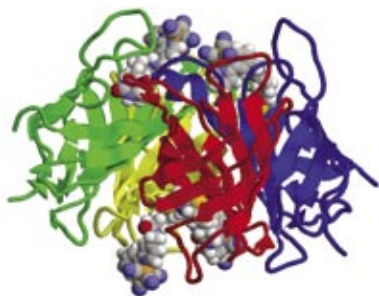


Figure 2: Model by Matt Hartings of avidin tetramer (ribbon) bound to iron-modified biotin complexes (spacefill). Note the binding is at the outer edge of each monomer. Avidin changes the dielectric constant of the metal in the ligands (spacefill), altering reorganization energy.

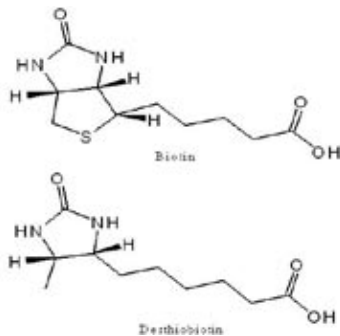


Figure 3: The chemical structures of biotin and desthiobiotin. Biotin binds to avidin with $K_D \sim 10^{-15}$ M. Desthiobiotin binds to avidin with $K_D \sim 10^{-13}$ M.

(s, 1H), 8.51 (s, 1H), 8.38 (d, 1H), 8.30 (d, 1H), 7.85 (d, 1H), 7.62 (d, 1H), 4.54 (s, 2H), 2.39 (s, 3H).

5-Azidomethyl-5'-methyl-2,2'-bipyridine

The procedure was carried out using a modification of a literature method.²⁰ A solution of 5-bromomethyl-5'-methyl-2,2'-bipyridine (0.5420 g, 2.27 mmol) in 5 mL DMSO was added dropwise to a solution of six equivalents of NaN_3 (0.8955 g) in 10 mL DMSO. The solution was heated to 70°C and stirred under N_2 for ~17 h. The reaction was monitored using TLC (2% MeOH in CH_2Cl_2). The solution was cooled to room temperature, and 22 mL H_2O was added to quench the reaction. The product was extracted with toluene (5 x 10 mL), and the organic layer was dried with MgSO_4 . The solvent was evaporated under reduced pressure, and the residue was redissolved in a minimum of CH_2Cl_2 . The product was purified by column chromatography (silica gel, 2% MeOH in CH_2Cl_2). The solvent was evaporated under reduced pressure to give white crystals. Yield: 80%. ^1H NMR (400 MHz, CDCl_3) δ 8.56 (s, 1H), 8.46 (s, 1H), 8.36 (d, 1H), 8.25 (d, 1H), 7.75 (d, 1H), 7.58 (d, 1H), 4.37 (s, 2H), 2.32 (s, 3H). ^{13}C NMR (400 MHz, CDCl_3) δ 156.5, 153.2, 149.7, 148.9, 137.6, 136.9, 133.9, 130.8, 120.9, 120.5, 52.3, 18.6.

5-Aminomethyl-5'-methyl-2,2'-bipyridine

The procedure was carried out using a modification of a literature method.²⁰ 5-Azidomethyl-5'-methyl-2,2'-bipyridine (0.4016 g, 1.78 mmol) was dissolved in 25 mL MeOH. A thin layer of 10% Pd/C (0.0988 g) was placed in a thin layer at the bottom of a reaction bomb. The MeOH solution was slowly added and slowly diluted to ~75 mL. The bomb was connected to a Parr Hydrogenation

Apparatus, and the compound was reacted under 3 atm H_2 for 24 h. The solution was filtered through Celite on a sintered glass funnel and rinsed with MeOH and 1:1 EtOH/ CH_2Cl_2 . The solvent was removed under reduced pressure. The solid was washed with ether and collected on a sintered glass funnel. Yield: 45%. ^1H NMR (400 MHz, CD_3OD) δ 8.75 (s, 1H), 8.62 (s, 1H), 8.36 (d, 1H), 8.25 (d, 1H), 8.00 (d, 1H), 7.79 (d, 1H), 4.24 (s, 2H), 2.43 (s, 3H), 2.15 (s, NH_2).

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid (5'-methyl-[2,2']-bipyridinyl-5-ylmethyl)-amide (5-BMB)

A solution of biotin (0.671 g, 0.275 mmol) in 5 mL DMF was added to a solution of TSTU (0.0853 g, 0.275 mmol) in 0.5 mL NEt_3 with stirring under N_2 . This solution was stirred for an hour, after which 5-aminomethyl-5'-methyl-2,2'-bipyridine (0.0437 g, 0.219 mmol) was added. The reaction was stirred under N_2 for 72 h. Reaction progress was monitored using TLC (10% MeOH in CH_2Cl_2). The solvent was evaporated under reduced pressure to give crude 5-BMB as red crystals. Yield (crude product): 98%. MS (ESI+) m/z calcd. for $\text{C}_{22}\text{H}_{28}\text{N}_5\text{O}_2\text{S}$: 426.6 found: 426.3, for $\text{C}_{22}\text{H}_{27}\text{N}_5\text{O}_2\text{SNa}^+$: 448.6 found 448.3, (ESI-) m/z calcd. for $\text{C}_{22}\text{H}_{26}\text{N}_5\text{O}_2\text{S}$: 424.6 found 424.1, for $\text{C}_{22}\text{H}_{27}\text{ClN}_5\text{O}_2\text{S}$: 461.0 found: 460.2.

5-(5-Bromopentyl)-5'-methyl-2,2'-bipyridine

The procedure was carried out using a modification of a literature method.²¹ To a -40°C (dry ice and CH_3CN) solution of 1.4 mL diisopropylamine in 3 mL dry THF, 6.25 mL 1.6 M solution of n-butyllithium in hexane was added dropwise. The mixture was stirred at 0°C for

15 min under N_2 and was subsequently cooled again to $-40^\circ C$. A solution of 5,5'-dimethyl-2,2'-bipyridine (1.7030 g, 9.24 mmol) in 50 mL dry THF was added dropwise into the reaction mixture, which turned dark brown. The mixture was stirred for 2 h at $-40^\circ C$ under N_2 . To the reaction mixture a mixture of 11 mL 1,4-dibromobutane and 10 mL dry THF was added dropwise. The mixture was stirred for 30 min and was slowly brought up to room temperature, after which it was stirred for 48 h. The reaction was quenched with 1 mL water, 80 mL ether was added, and the solution was placed in the freezer for several days. The solution was removed from the freezer, warmed to room temperature, and filtered. The filtrate was evaporated under reduced pressure to give a yellow oil. The crude product was purified by column chromatography (silica gel, 73:24:3 pentane/ethyl acetate/triethylamine). The solvent was evaporated under reduced pressure to give a yellow oil. Yield: 70%. 1H NMR (400 MHz, $CDCl_3$) δ 8.48 (s, 2H), 8.27 (d, 2H), 7.59 (d, 2H), 3.39 (t, 2H), 2.66 (t, 2H), 2.37 (s, 3H), 1.90 (m, 2H), 1.67 (m, 2H), 1.50 (m, 2H).

5-(5-Azidopentyl)-5'-methyl-2,2'-bipyridine
 The procedure followed was the same as that for 5-azidomethyl-5'-methyl-2,2'-bipyridine with the following modifications: A solution of 5-(5-bromopentyl)-5'-methyl-2,2'-bipyridine (1.2327 g, 4.36 mmol) in 10 mL DMSO was added dropwise to a solution of 6 equivalents of NaN_3 (1.8012 g) in 20 mL DMSO. The solution was heated to $70^\circ C$ and stirred under N_2 for ~17 h. The reaction was monitored using TLC (2% MeOH in CH_2Cl_2). The solution was cooled to room temperature, and 40 mL H_2O was added to quench the reaction. The product was extracted with

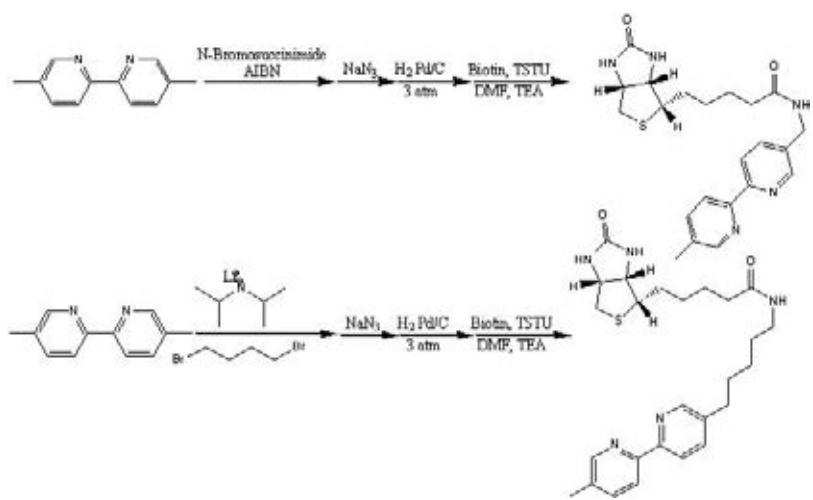


Figure 4: Reaction scheme for 5-BMB and 5-BPB ligands to be complexed to Fe.

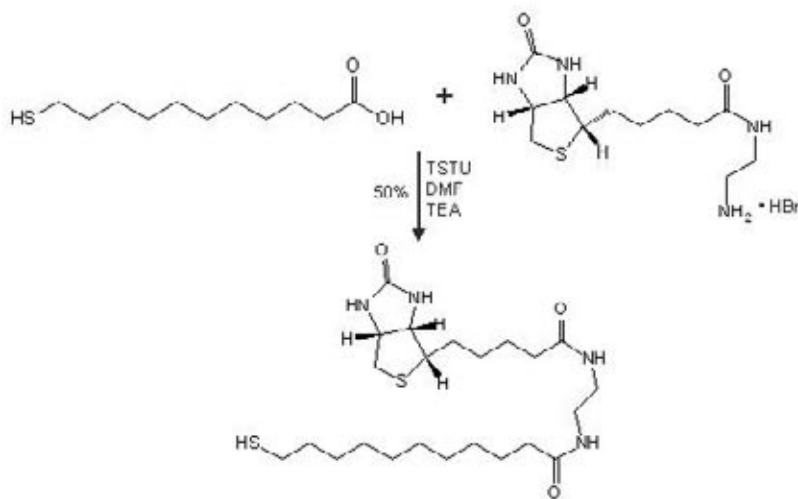


Figure 5: Reaction scheme for the biotinylated thiol used in the formation of monolayers.

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toluene (5 x 20 mL), and the organic layer was dried with MgSO₄. The solvent was evaporated under reduced pressure, and the residue was redissolved in a minimum of CH₂Cl₂. The product was purified by column chromatography (silica gel, 2% MeOH in CH₂Cl₂). The solvent was evaporated under reduced pressure to give white crystals. Yield: 70%. ¹H NMR (400 MHz, CDCl₃) δ 8.47 (s, 2H), 8.24 (d, 2H), 7.59 (d, 2H), 3.24 (t, 2H), 2.65 (t, 2H), 2.36 (s, 3H), 1.65-1.59 (m, 4H), 1.42 (m, 2H). ¹³C NMR (400 MHz, CDCl₃) δ = 154.3, 153.8, 149.7, 149.3, 137.6, 136.9, 133.3, 120.6, 51.6, 32.9, 30.9, 29.0, 26.5, 18.6.

5-(5-Aminopentyl)-5'-methyl-2,2'-bipyridine

The procedure followed was the same as that for 5-aminomethyl-5'-methyl-2,2'-bipyridine with the following modifications: 5-(5-azidomethyl)-5'-methyl-2,2'-bipyridine (0.4427 g, 1.57 mmol) was dissolved in 25 mL MeOH. A thin layer of 10% Pd/C (0.1158 g) was placed in a thin layer at the bottom of a reaction bomb. The MeOH solution

was slowly added and was subsequently diluted to ~75 mL. The bomb was connected to a Parr Hydrogenation Apparatus, and the compound was reacted under 3 atm H₂ for 24 h. The solution was filtered through Celite on a sintered glass funnel, rinsing with MeOH and 1:1 EtOH/CH₂Cl₂. The solvent was removed under reduced pressure. The solid was washed with ether and collected on a sintered glass funnel. Yield: 60%. ¹H NMR (500 MHz, CD₃OD) δ = 8.60 (s, 2H), 8.30 (d, 2H), 7.80 (d, 2H), 5.30-5.10 (s, NH₂), 2.95 (t, 2H), 2.65 (t, 2H), 2.38 (s, 3H), 1.78 (m, 2H), 1.60 (m, 2H), 1.38 (m, 2H). MS (ESI+) m/z calcd. for C₁₆H₂₂N₃⁺: 256.4 found: 256.1.

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [5-(5'-methyl-[2,2']-bipyridinyl-5-yl)-pentyl]-amide (5-BPB)

The procedure followed was the same as that for 5-BMB with the following modifications: A solution of biotin (0.1220 g, 0.500 mmol) in 8 mL DMF was added to a solution of TSTU

(0.1505 g, 0.500 mmol) in 0.5 mL NEt₃ with stirring under N₂. This solution was stirred for an hour, after which 5-(5-aminopentyl)-5'-methyl-2,2'-bipyridine (0.0437 g, 0.219 mmol) was added. The solution was stirred under N₂ for 72 h. Reaction progress was monitored using TLC (10% MeOH in CH₂Cl₂). The solvent was evaporated under reduced pressure to give crude 5-BPB as red crystals. Yield: 95%. MS (ESI+) m/z calcd. for C₂₆H₃₆N₅O₂S⁺: 482.7 found: 482.3.

K₂[(5-BPB)Fe(CN)₄]

The procedure was carried out using a modification of a literature method.²² FeCl₂ • 4 H₂O (42.5 mg, 0.2076 mmol) was dissolved in 75 mL H₂O with heating. The solution was heated to 95°C, and 5-BPB (100.8 mg, 0.2076 mmol) in 10 mL DMF was added dropwise, with stirring over the course of 1 hr, during which the heating solution turned red. The solution was heated (95°C) with stirring for another hour. A solution of KCN (54.1 mg, 65.12 mmol) in 10 mL H₂O was added dropwise over the course of

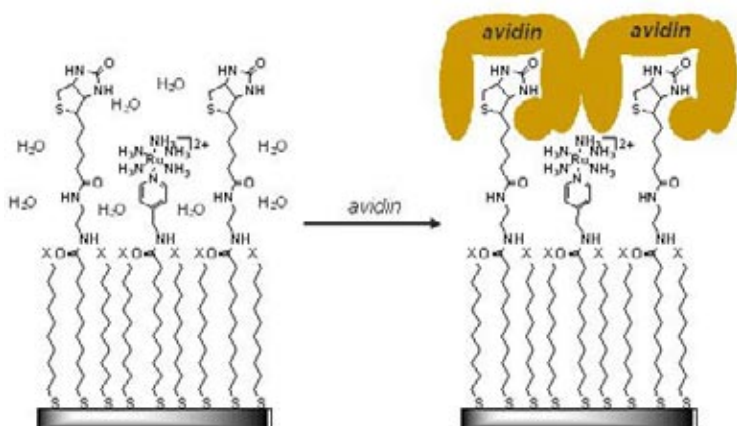


Figure 6: Cartoon depicting the effect of avidin when bound to biotin on the environment of the ruthenium complex. Upon addition of avidin, the atmosphere around the metal complex is changed due to the exclusion of water molecules. This change in dielectric constant results in a change in reorganization energy, altering the rate of electron transfer and shifting peak potential. Each monolayer consisted of the ruthenium-thiol complex as well as a functionalized alkane-thiol, where X was chosen to be -CH₂OH, -C₈H₁₇, and -CO₂H. Monolayers were formed both with and without the biotinylated thiol for each functionalized alkane-thiol used.

15 min to the hot solution, which turned purple. The solution was heated at 95°C for 1.5 h. The solvent was evaporated under reduced pressure to give a crude product of purple crystals. Yield (crude): 98%. Purification and characterization have yet to be performed.

Solid-State Probes

Mixed monolayers containing ruthenium-thiol complex combined with a functionalized alkane-thiol and a biotinylated thiol were designed. This biotin ligand was synthesized using standard peptide coupling methods (the same as for 5-BMB and 5-BPB) (Figure 5).

The idea was that the biotin-thiols bind close enough to the ruthenium complexes that avidin is able to shield the ruthenium complexes from the solvent during the CV experiments (water) (Figure 6). Due to their sensitivity to air and to light, the ruthenium complexes were synthesized and handled by postdoctoral mentor Amanda Eckermann. Monolayers were formed both with and without the

biotinylated thiol. Monolayers without biotin were examined as controls in the electrochemical experiments.

Monolayers were formed on gold electrodes from thiol solutions of 0.1-1.0 mM in CH₃CN by soaking the electrodes overnight in air-free flasks containing the solutions. A variety of functionalized alkane-thiols were used when forming the monolayers: 11-mercapto-1-undecanol, 11-mercaptoundecanoic acid, and octadecanethiol. This modification was done to analyze the effect of the functionalized group on the interaction of avidin with the monolayer in order to optimize the conditions for future solid-state experiments.

Electrochemical analyses were performed using a pH 7.57 phosphate buffer for the monolayers containing 11-mercapto-1-undecanol. For 11-mercaptoundecanoic acid and octadecanethiol at pH 4.0, 1 M Na₂SO₄ buffer was used. The acidic solution was chosen, especially for the monolayer containing the acid, so that the carboxylic acid was fully protonated when interacting with the avidin. Cyclic

voltammograms (CVs) were obtained at decreasing scan rates starting at 50V/s and ending at 0.05 V/s in order to acquire a wide range of data points for analysis.

Results

Successful syntheses of 5-BMB and 5-BPB were developed. Progress has been made on the syntheses of iron complexes starting with K₂[(5-BPB)Fe(CN)₄]. Purification is under way.

Formation of a variety of mixed monolayers containing Ru was successful. Monolayer formation was confirmed using and examining the *i* vs v relationship. For a solution-based system, the current is proportional to the square root of the scan rate ($i \propto v^{1/2}$), and for monolayers, $i \propto v$. The correlation coefficient for all plots of *i* vs v was above 0.99. Also, as the scan rate increased, the separation between peak potentials also increased (Figure 7). The relationship of peak potential to scan rate will be used to calculate the rate of electron transfer.

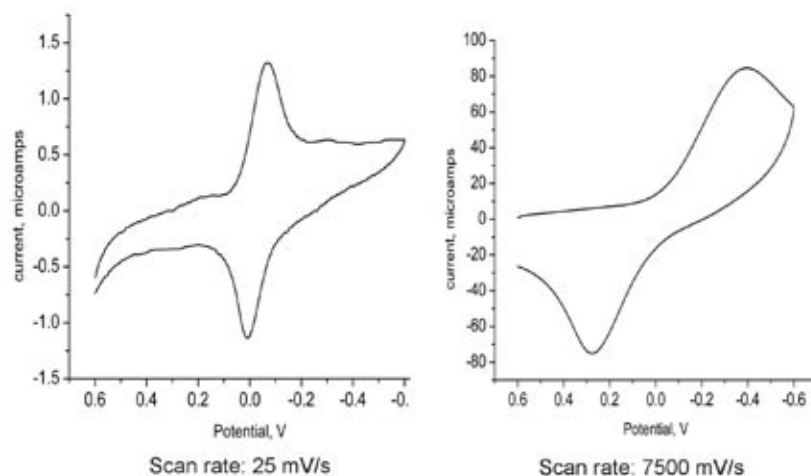


Figure 7: Cyclic voltammograms of a monolayer with the functionalized alkane-thiol 11-mercapto-1-undecanol. Notice that as the scan rate increases, the peak potentials get farther apart. The changes in peak potentials are used in the determination of the rate of electron transfer. For surface-based systems (monolayers), the current, *i*, is proportional to the scan rate, v , and this relationship was observed. This relationship was used to confirm monolayer formation.

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| Average of Scan Rates | E 1/2 (mV) |
|------------------------------------|------------|
| X = CH ₂ OH | |
| Rua 5 + OH | -39 |
| Rua 5 + OH + avidin | -60 |
| Rua 5 + OH + biotin | -46 |
| Rua 5 + OH + biotin + avidin | -75 |
| X = C ₈ H ₁₇ | |
| Rua 5 + H | -2 |
| Rua 5 + H + avidin | 7 |
| Rua 5 + H + biotin | -5 |
| Rua 5 + H + biotin + avidin | -2 |
| X = CO ₂ H | |
| Rua 5 + COOH | -7 |
| Rua 5 + COOH + avidin | -8 |
| Rua 5 + COOH + biotin | -7 |
| Rua 5 + COOH + biotin + avidin | -7 |

Table 1 summarizes the results of the electrochemical experiments. When the functionalized group on the alkane-thiol was -CH₂OH, the addition of avidin to the monolayer containing no biotin ligand resulted in a change in potential of 21 mV. The monolayer with the biotin ligand resulted in a change in potential of 29 mV. When the functionalized group on the alkane-thiol was -C₈H₁₇, the potential change was 9 mV without the presence of biotin; with it, the change in potential was 3 mV. For the monolayer incorporating the carboxylic acid, the change in potential without biotin was found to be 1 mV; with biotin in the monolayer, the potential change was found to be less than 1 mV.

Discussion

Progress has been made on solution probes incorporating iron complexes to 5,5'-disubstituted-2,2'-bipyridine. Once iron complexation is completed, these

molecules can be used in solution-based electrochemistry experiments. The synthetic procedures used to make 5-BMB and 5-BPB can also be modified to prepare compounds for use in solid-state probes.

Based on the linear dependence of i on v , among the cyclic voltammetry experiments it appears that the best monolayer was formed using 11-mercapto-1-undecanol as the functionalized alkane-thiol, and 11-mercaptoundecanoic acid and octadecanethiol also gave good monolayers. Cyclic voltammograms were obtained, and the relationship between the scan rate and the current was linear, also confirming monolayer formation. However, this relationship does not rule out the possibility of pinhole defects.

The addition of avidin to the system including biotin resulted in a potential shift of 29 mV when the functionalized group of the alkane thiol was -CH₂OH; without biotin, the shift was 21 mV

Table 1: Summary of electrochemical data of monolayers. A significant change in potential was seen for the alcohol-functionalized monolayers. This was true both with biotinylated thiol in the monolayer (29 mV change) and without it (21 mV change). As expected, the potential change upon avidin addition for the monolayer containing biotin-thiol was greater than that for the monolayer without it. The 21 mV change without biotin-thiol indicates nonspecific binding between the monolayer and the avidin. For the long chain alkane, no significant potential change was observed. This is because the hydrophobic functionalized alkane-thiols were long enough to engulf the ruthenium complex, shielding it from the water in the solution, even without the presence of avidin. For the acid functionalized alkane-thiol, the likely cause for the lack of potential change was a repulsion between the positively charged avidin and the overall positively charged monolayer (the experiment was done at pH 4.0).

(Table 1). The addition of avidin does alter the environment around the metal complex significantly, and, as one would expect, the potential shift was larger when the monolayer contained biotin. Therefore, this method for investigating weak interactions seems very promising. The shift of 21 mV in the absence of biotin is most likely due to nonspecific interactions between the protein and the surface of the monolayer. Nonspecific binding can be eliminated using PEG functionalized groups.²³

For the carboxylic acid functionalized alkane-thiol monolayer, which was fully protonated due to the use of a buffer at pH 4.0, the likely cause for the lack of potential change was repulsion between the avidin and the acid functional groups. At this low pH, not only was the acid monolayer fully protonated, but the avidin was protonated as well (isoelectric point of avidin = 10.5). It is likely that the positively charged avidin was repelled by

the overall positively charged monolayer resulting in poor shielding of the Ru complex from the solvent, water.

For the octadecanethiol monolayer, the length of the carbon chain was the likely cause for the small potential change upon addition of avidin. An 18-carbon chain would extend beyond the Ru complexes (an 11-carbon chain plus the ruthenium pentamine) (Figure 6). These octadecanethiols are already very hydrophobic or “greasy.” Thus, they are both long enough and hydrophobic enough to shield the Ru complexes in the monolayer from water even without the presence of avidin, resulting in a small potential change upon its addition. It is also possible that the use of a different buffer played an even larger role than expected, resulting in the lack of a potential shift in the acid and alkane functionalized monolayers.

Conclusions

Successful procedures for the synthesis of the solution probe ligands 5-BMB and 5-BPB were carried out. The synthesis of $K_2[(5-BPB)Fe(CN)_4]$ was also carried out, but it has not been purified or fully characterized. In the future, more iron solution probes will be synthesized, particularly those containing desthiobiotin instead of biotin. It is also necessary to complex the remaining biotin and the desthiobiotin ligands to an $Fe(CN)_4$ center so they can be analyzed as solution probes using cyclic voltammetry experiments. Future work includes modifying the syntheses of these iron complexes for use in a surface-based system.

A successful procedure for the biotinylation of a thiol for use in monolayer formation was developed. Several mixed monolayers were formed for use in cyclic voltammetry experiments. A significant change in potential — 21 and 29 mV, respectively, for the absence and presence

of biotin — was observed upon the addition of avidin for the monolayer containing 11-mercapto-1-undecanol. For the acid and alkane functionalized monolayers, only a small change in potential was observed upon the addition of avidin. It appears that pH can affect the interaction of avidin with monolayers. It also seems that there is nonspecific binding that plays a role in the interaction between avidin and monolayers. In the future, variations in the carbon chain length between the biotin and the Ru complex (and the surface of the electrode) need to be carried out. Also, incorporating the ruthenium complex and the biotin into one molecule should eliminate much of the nonspecific binding effect.

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