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## Derivatives of 3,4-ethylenedioxythiophene (EDOT) for Enzyme-Immobilized Conducting Polymer: Toward Development of Biofuel Cells and Biosensors

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**Derivatives of 3,4-Ethylenedioxythiophene (EDOT) for Enzyme-Immobilized  
Conducting Polymer: Toward Development of Biofuel Cells and Biosensors**

**Corinne Songer**

Department of Chemistry and Biochemistry

University of Arkansas

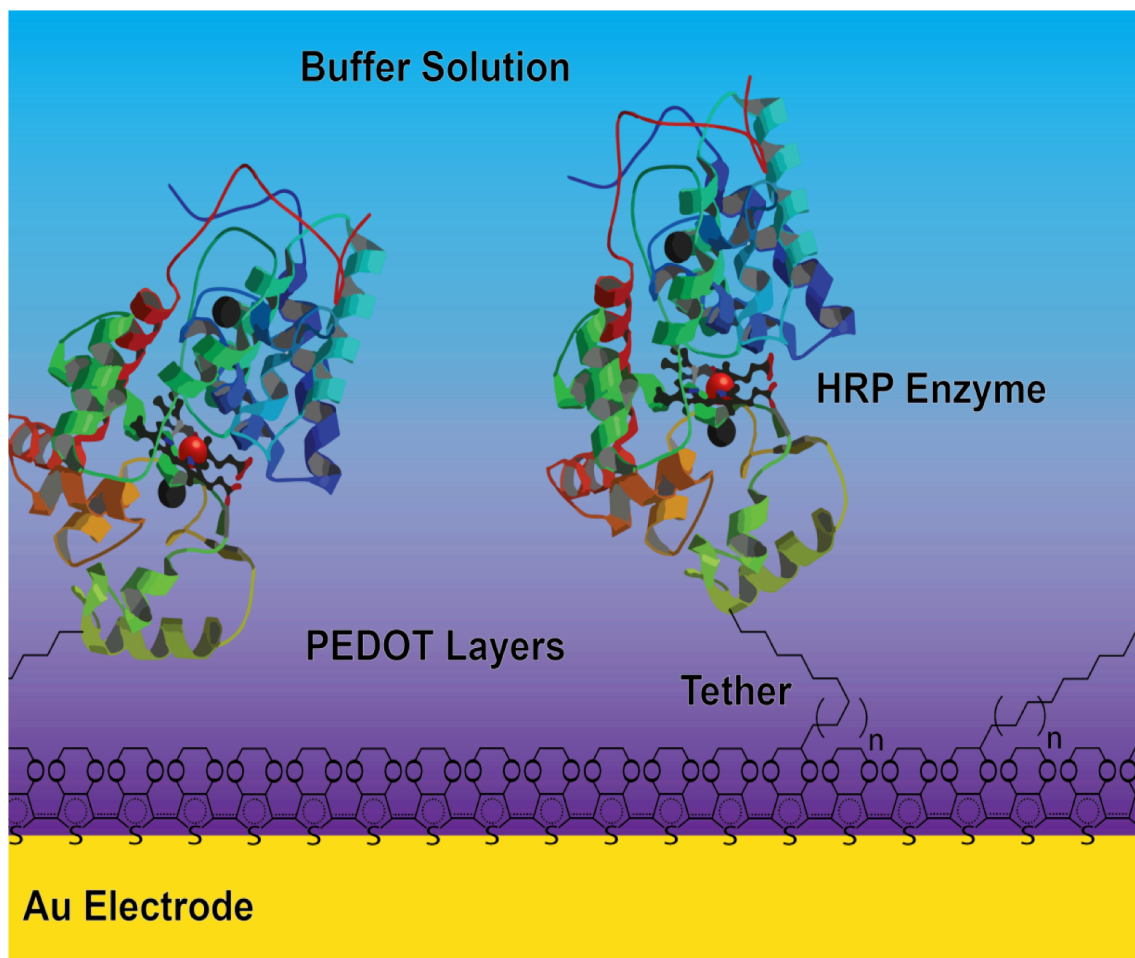
April 15, 2015

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## Specific Aims

Figure 1 illustrates the overall goal, which is to conjugate the horseradish peroxidase (HRP), which reduces hydrogen peroxide ( $H_2O_2$ ) to water ( $H_2O$ ), to a conducting poly(3,4-ethylenedioxythiophene) (PEDOT) film and shuttles electrons from the electrode surface through the conducting polymer to the heme group of HRP. The specific task of this project was to conjugate an enzyme to a conducting polymer for the purpose of immobilizing the enzyme with the ultimate intent of developing a miniature biofuel cell. In order to achieve successful, targeted enzyme immobilization with retained activity, three specific aims were addressed. **Specific Aim 1** focused on determining the conditions for electropolymerization of the monomer 3,4-ethylenedioxythiophene (EDOT) and one with a functional group to which an enzyme can ultimately be covalently attached, such as thieno[3,4-b]-1,4-dioxin-2-methanol (HM-EDOT). Stable, robust, and highly conductive electropolymerized films are desirable. **Specific Aim 2** involved conjugating the enzyme, horseradish peroxidase (HRP), to a carboxylic acid derivative of HM-EDOT, abbreviated COOH-EDOT, while enabling for future insertion of a longer tether. The HRP will then be conjugated to the COOH-EDOT following the electropolymerization of the synthesized product. **Specific Aim 3** was to develop a method to check the activity of the enzyme covalently bound to the polymer and determine the conditions for optimal enzymatic activity of the polymer-enzyme conjugate.



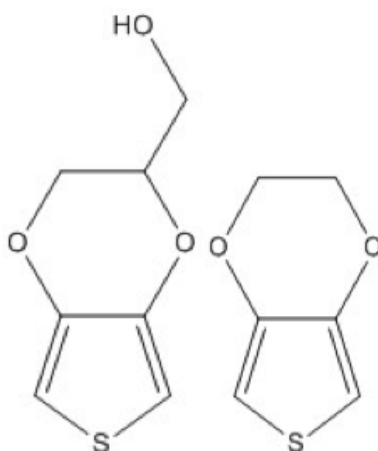
**Figure 1.** Illustrates the ultimate goal of the research. A conducting-polymer-modified electrode surface capable of transferring electrons from the electrode surface to the heme group of HRP that has been immobilized to the PEDOT film using a tether.

### Background and Significance

Biofuel cells use biological processes to convert chemical energy from chemical processes into an electrical current.<sup>1</sup> The hope is that biofuel cells, when paired with biosensors, will eventually be able to power small electronics, such as sensors, within the human body. Enzyme immobilization is being combined with biofuel cells because it is thought to improve the sustainability of the biofuel cell, as it allows the enzyme to remain covalently attached to the polymer, while also being able to maintain its catalytic

activity.<sup>2</sup> This will allow the biological processes to be carried out quicker than without the enzyme.

The conducting polymer used in this project is intended to serve as a means to immobilize enzymes onto an electrode surface and aid in shuttling electrons between the enzyme and the electrode. Poly (3,4-ethylenedioxythiophene), PEDOT, was chosen as the polymer because it can be produced in large quantities, has high conductivity, can be polymerized in aqueous conditions, and is a stable polymer.<sup>4</sup> The structure of the monomer, EDOT, and the derivative, HM-EDOT, used in this project are shown in Figure 2.



**Figure 2.** An illustration of EDOT and the Hydroxymethyl-EDOT derivative that were used for determining electropolymerization conditions. The HM-EDOT is used for to further synthesize another molecule that will be used for the enzyme conjugation.

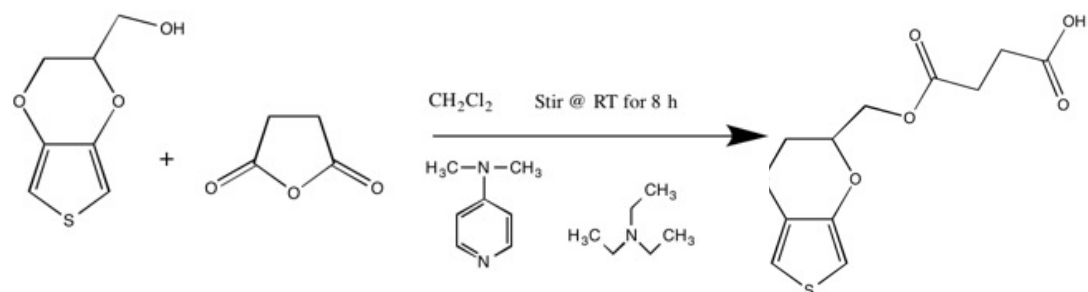
This polymer has also been shown to be a suitable choice for electropolymerization because it is stable in buffered solutions, which suggests that it would be compatible with pH sensitive biological components and a good candidate for biosensors within the body. In addition, it has a strong binding affinity for gold, which will help secure the electropolymerized film at the gold electrodes.<sup>3,4</sup> The derivative for this project,

hydroxymethyl-3,4-ethylenedioxythiophene, HM-EDOT, is used because it can be used to synthesize the carboxylic acid functionalized EDOT, which will be used to the enzyme conjugation. The additional functional group allows the monomer to be more soluble in water, which makes the formation of aqueous electropolymerization solutions easier.

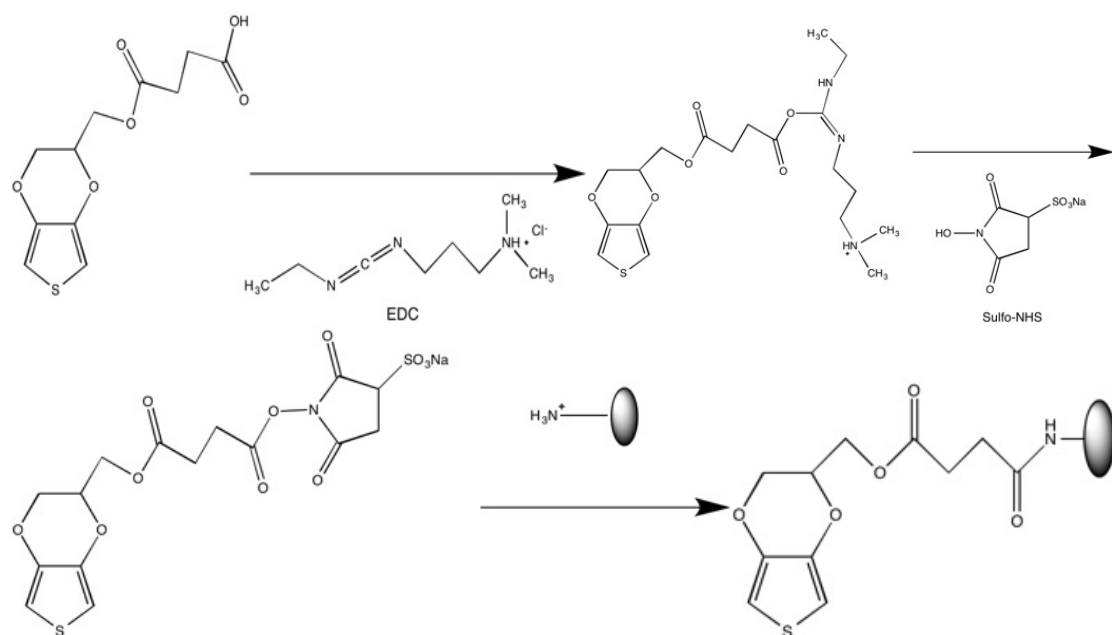
The enzyme used in this project is HRP because it is inexpensive, has been used previously in biofuel cells, and its activity can easily be measured using absorbance based enzyme activity assays. In solution, the HRP is first activated by hydrogen peroxide,  $\text{H}_2\text{O}_2$ , because the  $\text{H}_2\text{O}_2$  oxidizes the HRP, removing electrons. The HRP then removes electrons from 3,3',5,5'-Tetramethylbenzidine (TMB), oxidizing the TMB and retaining the electrons for itself. The oxidized form of TMB produces a diimine product that is yellow in color and absorbs light at 450 nm. HRP contains a heme metal center, which is an iron molecule surrounded by a porphyrin ring. The binding sites are above or below the plane of the heme group in HRP that allow for  $\text{H}_2\text{O}_2$  to bond during oxidation-reduction reactions. HRP also contains six lysine residues, which contain primary amines that can be used to form amide linkages with an activated carboxylic acid containing molecule.

The HM-EDOT was derivatized with a small organic tether that is terminated by a carboxylic acid that can be used to conjugate to enzymes. Enzyme conjugation is carried out using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS), allowing the lysine residues to form an amide bond with the COOH-EDOT. Figure 3 shows a reaction scheme of the synthesis reaction for the carboxylated EDOT derivative, and Figure 4 shows a general reaction scheme for enzyme conjugation with EDC/NHS. The COOH-EDOT is synthesized using the HM-

EDOT and succinic anhydride mixed with dimethyl-aminopyridine (DMAP), triethylamine, and dichloromethane. The enzyme conjugation is achieved by first using the EDC to activate the carboxylic acid group, and then adding the NHS to protect the activated ester, which allows the primary amine in the lysine to form an amide bond with the HRP.



**Figure 3.** Reaction scheme for the synthesis of COOH-EDOT using HM-EDOT, succinic anhydride, DMAP, and  $\text{Et}_3\text{N}$ .



**Figure 4.** General reaction scheme for the synthesis of the enzyme-EDOT conjugate using EDC and NHS. The final product is the enzyme, HRP, conjugated to the monomer. The EDC activates the carboxylic acid group, and the NHS protects the activated oxygen and allows the primary amine to form an amide bond with the HRP.



## Methods for Specific Aims

EDOT and HM-EDOT, (Figure 2) have been chosen as monomers for the initial studies that address **Specific Aim 1**. First EDOT was used to learn how to electropolymerize the monomer units to modify electrodes under conditions that have been demonstrated by the Fritsch lab to be successful. The conditions found to successfully electropolymerize EDOT on the electrode surface were used initially to electropolymerize HM-EDOT onto an electrode surface. The films were electrochemically characterized by measuring the capacitance of the electrode before and after modification in an electrolyte solution.

In order to complete **Specific Aim 2**, a method to conjugate the HRP to the COOH-EDOT derivative was developed. For this, 1-Ehtyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were used to couple the enzyme to the polymer film. It involved activating the carboxylic acid groups on the COOH-EDOT derivative that was synthesized with the NHS ester and then forming an amide bond with the lysine residues on the HRP. Confirmation of the conjugation was determined by enzyme activity assays, which determined the activity of the enzyme.

**Specific Aim 3** involved combining the enzyme-polymer conjugate with a mediator to determine if the electrons can be efficiently shuttled from the electrode surface to the HRP.

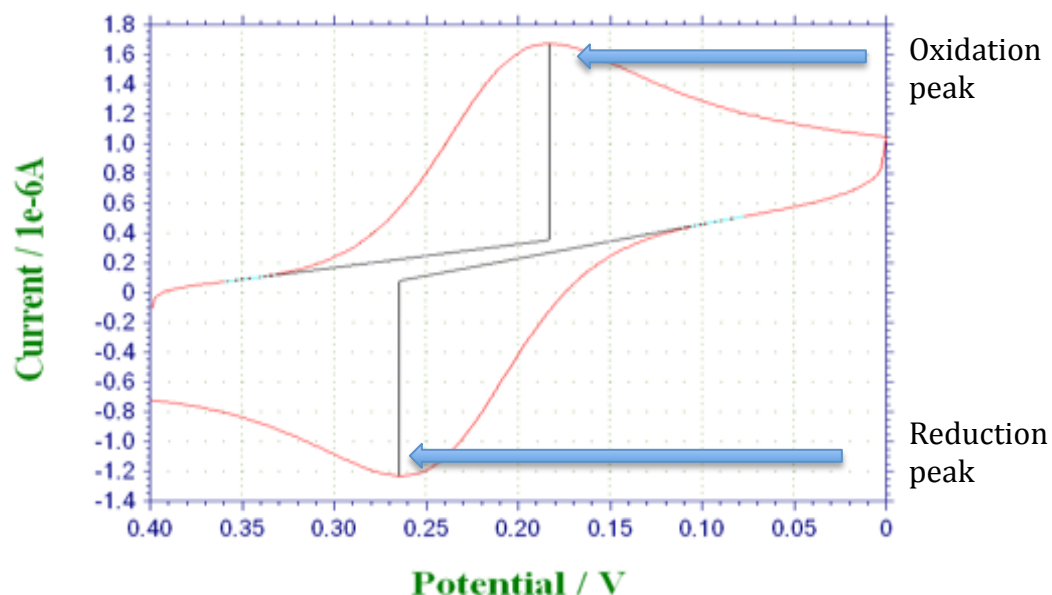
## Techniques

Cyclic voltammetry (CV) is the main technique used for this project. CV is a technique that allows the researcher to determine if the electrodes are electrochemically

active, which is a function of the concentration of redox species, electrode area, number of electrons transferred per molecule of redox species, mass transport, information about the double layer, and temperature. It measures the change in current as a function of potential of an electrochemical cell. CV can be useful when determining the elements within the solution in the cell. When a redox species is introduced to the electrochemical cell, the voltammogram shows peaks representing the reduction and oxidation of the species. The CV experiment involves three electrodes placed in a beaker, and a potential is applied to the working electrode relative to the reference electrode. Each CV starts at an initial potential, scans linearly across the redox potential of the species being examined, switches potential, and scans back to end at the initial potential. Figure 4 illustrates a typical CV response at a macroelectrode for a redox species, this case  $\text{K}_3\text{Fe}(\text{CN})_6$ , in 0.1 M KCl where the reduction and oxidation peaks can be seen. The CV response can be used to relate the peak current ( $i_p$ ) of a redox species to other parameters using:

$$i_p = 2.69 \times 10^5 n^{3/2} A C D^{1/2} v^{1/2} \quad (1)$$

where  $i_p$  is peak current,  $n$  is the number of electrons transferred,  $A$  is the area of the electrode,  $C$  is the concentration of redox species,  $D$  is the diffusion coefficient in centimeters squared per second, and  $v$  is the scan rate in volts per second.



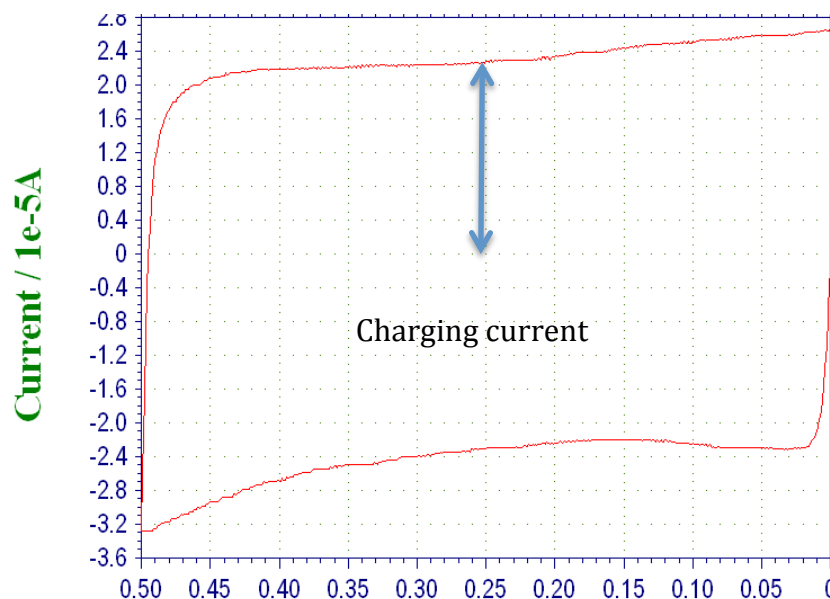
**Figure 4.** A typical cyclic voltammogram of a redox species. The upper peak represents the reduction of the species, in this case  $\text{Fe}(\text{CN})_6^{3-}$  and the lower peak is the oxidation. Conditions: area =  $0.080 \text{ cm}^2$ , scan rate =  $0.1 \text{ V/s}$ , solution =  $10 \text{ mM K}_3\text{Fe}(\text{CN})_6$  in  $0.1 \text{ M KCl}$ .

The CV response can also be used to calculate values, such as capacitance, using:

$$i_c = \nu C_{dl} A \quad (2)$$

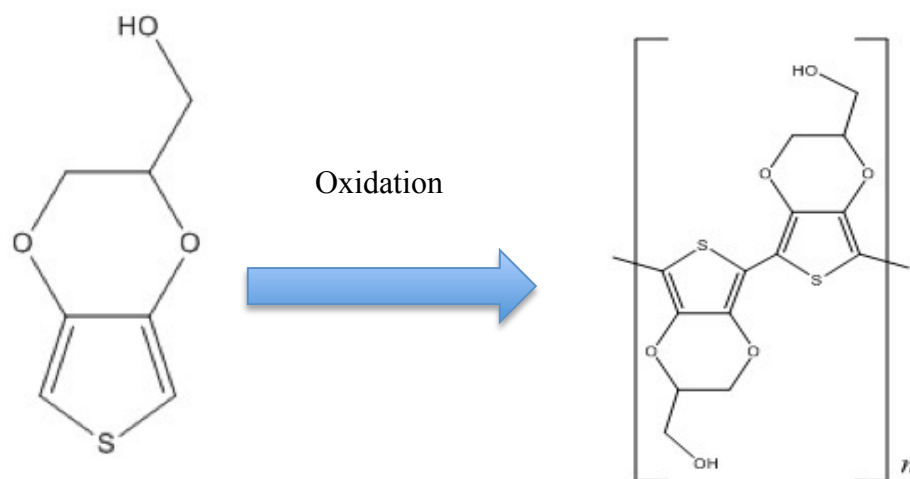
where  $i_c$  is the current,  $\nu$  is the scan rate in volts per second,  $A$  is the area, and  $C_{dl}$  is the double layer capacitance in farads per unit area. This experiment is done using three electrodes as before, and applying a voltage to the working electrode relative to the reference. The CV scans from the initial voltage to the switching potential and back to the initial voltage, and the response shows the charging current of the bare electrode in pure electrolyte. Capacitance calculations can allow for an understanding of the polymers present on the electrode surface because it indicates the amount of charge that can be stored at the modified electrode. The charging current of an electrode is represented by

Equation 2, and can be seen in Figure 5, when the current is relatively constant with changing potential.



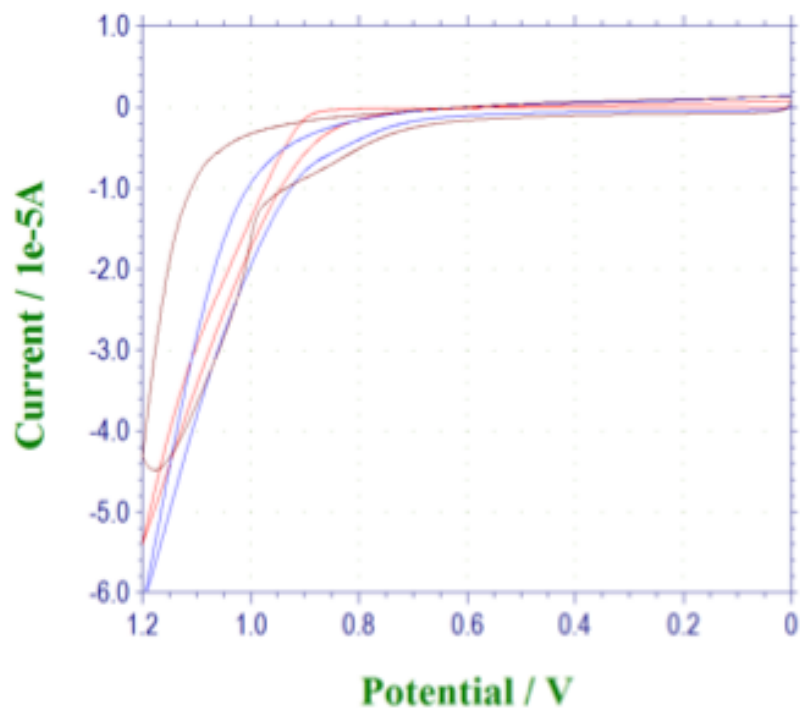
**Figure 5.** A representative CV response of a bare gold electrode immersed in electrolyte alone (electroactive redox species are not present), in this case 0.1 M KCl. The double headed arrow represents where the charging current of an electrode was measured, which can be measured by finding the current difference between the forward and return sweeps and dividing it by two. Conditions: area = 0.080 cm<sup>2</sup>, scan rate = 0.1 V/s, solution = 0.1 M KCl.

This project utilizes electropolymerization as a technique. Electropolymerization works by oxidizing a monomer, such as HM-EDOT, which then deposits on the electrode surface in a polymer form. It is an important technique because it allows for the deposition of a polymer to an electrode surface in a relatively short amount of time, and it results in stable, electrochemically active polymers. Figure 6 shows the general mechanism for the electropolymerization of HM-EDOT.



**Figure 6.** The general mechanism for the generation of the HM-PEDOT polymer. The monomer is oxidized and used to form a polymer that deposits on the electrode surface.

The electropolymerization is done in a beaker with three electrodes and a voltage is applied to the working electrode being polymerized. The voltage is swept from the initial potential, past the point of oxidation of the monomer, switched at the switching potential, and swept back to the initial potential. Figure 7 shows a representative CV response for the electropolymerization of HM-EDOT, where all three cycles are overlaid to show the oxidation of the monomer.



**Figure 7.** A representative cyclic voltammogram of the polymerization of HM-EDOT on the gold surface of an electrode, with the three cycles overlaid on the scan. The first cycle is represented by the brown scan, the second cycle is the red scan, and the third cycle is the blue scan. It illustrates the oxidation occurring at the electrode surface when the polymer is deposited. Conditions: area = 0.080 cm<sup>2</sup>, scan rate = 0.005 V/s, solution: 0.001 M  $\beta$ -cyclodextrin sulfated sodium, 0.01 M HM-EDOT in water.

## References

- <sup>1</sup> Bullen RA, Arnot TC, Lakeman JB, Walsh FC. Biofuel cells and their development. *Biosens Bioelectron* 2006;21(11):2015-45.
- <sup>2</sup> Yang X, Tian G, Jiang N, Su B. Immobilization technology: A sustainable solution for biofuel cell design. *Energy Environ Sci* 2012;5(2):5540-63.
- <sup>3</sup> Kros A, Sommerdijk NAJM, Nolte RJM. Poly(pyrrole) versus poly(3,4-ethylenedioxythiophene): Implications for biosensor applications. *Sensors Actuators B: Chem* 2005 /4/29/;106(1):289-95.
- <sup>4</sup> Groenendaal L", Jonas F, Freitag D, Pielartzik H, Reynolds JR. Poly(3,4-ethylenedioxythiophene) and its derivatives: Past, present, and future. *Adv Mater (Weinheim, Ger )* 2000;12(7):481-94.

## Chapter II: Electropolymerization and Characterization of Films Formed from HM-EDOT

### Introduction

Conducting polymers modify the surface of an electrode by expanding the surface area of an electrode, which allows increased current to flow with an increased capacitance and from additional faradaic processes. As seen in Equations 1 and 2, the current flowing through the electrode is directly proportional to the area of the electrode. The hydroxymethyl derivative form of EDOT is much more applicable to biological molecules because it has increased solubility in aqueous solutions, due to the added alcohol group.<sup>1</sup> Figure 1 shows the structure of the HM-EDOT molecule.

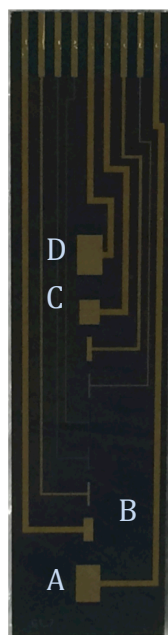


**Figure 1.** An illustration of HM-EDOT used in the electropolymerization studies.

The added functional group also allows for further derivatization of the monomer to synthesize a new molecule to which the enzyme can be conjugated. The selection of HM-EDOT was also based on the fact that the thiophene ring has a strong binding affinity for the gold electrodes that are used in these experiments.<sup>2</sup> The chips used for the



experiments were similar to the one shown in Figure 2, with gold electrode surfaces that were developed and made by members of the research group. The chip has an insulating layer of benzocyclo-butene (BCB) over the leads.



**Figure 2.** A schematic of a chip used for experiments and electrode modification. The electrodes are made of gold with an insulating layer of benzocyclo-butene (BCB) surrounding the entire chip except the electrode surfaces and contact pads. There are four main electrodes used for the polymerization and characterization experiments, and the area of each electrode is as follows: A:  $0.08 \text{ cm}^2$ , B:  $0.04 \text{ cm}^2$ , C:  $0.04 \text{ cm}^2$ , D:  $0.08 \text{ cm}^2$

A greater binding affinity between the monomer and electrode results in stable, robust polymer films. The results of experiments to determine polymerization conditions and how the polymer film affects the electrode are crucial for the next part of this project, which is to conjugate an enzyme to the COOH-EDOT and use it toward the development of a biofuel cell.

## Experimental

### *Materials*

The materials used for these experiments were hydroxymethyl-3,4-ethylenedioxythiophene (HM-EDOT), and  $\beta$ -cyclodextrin sulfated sodium ( $\beta$ -CDSS), which were obtained from Sigma US. The potentiostat models used were 650A and 760B from CH Instruments (Austin, Texas). All aqueous solutions were prepared in distilled deionized bottled water (Sigma), DDI.

All the experiments are performed with a platinum flag counter electrode and a Ag/AgCl (saturated KCl) reference electrode. The reference electrode has a standard potential, and allows the potentiostat to determine the voltage and current relative to the reference. The purpose of the counter electrode allows for current to pass through the system between the working electrode (the gold chip) and the counter electrode. The current is then measured with the potentiostat.

The chips used throughout these experiments had gold electrodes insulated with a benzo-cyclobutene (BCB) layer. The chips were fabricated by other members of the group, plasma cleaned, and stored in ultrapure 18.2 M $\Omega$ •cm water before use. The plasma cleaning was performed using a Harrick PDC-32G plasma cleaner, at a pressure of 60 mTorr, using oxygen gas to generate the plasma that cleans the chips. Each chip is plasma cleaned for fifteen minutes.

### *Characterization Chip #1 and Chip #2*

Two chips were used for the electropolymerization experiments: Chip #1 and Chip #2. Chip #1 was used to conduct a scan rate study on the bare gold electrodes before and after polymerization, and Chip #2 was used to determine the change in capacitance

while keeping the scan rate constant. There were multiple electrodes on each chip, but of slightly different dimensions. The dimensions of the electrodes on Chip #1 were: A = 0.20 cm x 0.40 cm, B = 0.20 cm x 0.20 cm, C = 0.20 cm x 0.20 cm, and D = 0.20 cm x 0.40 cm. The electrode dimensions for Chip #2 were: A = 0.20 cm x 0.30 cm, B = 0.20 cm x 0.20 cm, C = 0.20 cm x 0.20 cm, and D = 0.20 cm x 0.30 cm.

Chip #1, shown in Figure 1, was placed into a vial containing 20 mL of 0.1 M KCl. The chip was characterized using a Ag/AgCl (saturated KCl) reference electrode and a platinum flag counter electrode, with the bare gold serving as the working electrode. A study of capacitance was performed using CV with the following parameters: initial potential: 0.0 V; switching potential: 0.5 V, and sensitivity setting of  $1 \times 10^{-5}$  A. Each electrode was characterized using those parameters and with different scan rates, and the resulting CV responses were used to determine the capacitance before polymerization. The scan rates studied were 0.01 V/s, 0.05 V/s, 0.1 V/s, 0.5 V/s, and 1.0 V/s. The capacitance was also measured and monitored on another gold chip similar to the one in Figure 1, Chip #2, however the scan rate was kept constant at 0.1 V/s. The current density was calculated for a specific scan rate by assuming the current was similar to a charging current and normalizing the electrode for area. The scan rate was kept constant because there was not enough time to complete a full scan rate study.

### *Polymerization*

Polymerization of the monomer to create a conducting polymer carried out using a potentiostat and a three-electrode cell. To determine the best polymerization conditions, four solutions were prepared and used to deposit polymer on four different electrodes on the same chip. The first solution contained 0.01 M phosphate buffered saline (PBS), 0.01

M HM-EDOT, and 0.001 M  $\beta$ -Cyclodextrin sulfated sodium ( $\beta$ -CDSS) , which serves as the solbulizer; the second solution contained 0.01 M PBS, 0.01 M HM-EDOT, and 0.01 M  $\beta$ -CDSS; the third solution contained 0.01 M HM-EDOT, 0.01 M  $\beta$ -CDSS, and water; and the fourth solution contained 0.01 M HM-EDOT, 0.001 M  $\beta$ -CDSS, and water. Each solution was sonicated for 1 h prior to polymerization. Electropolymerization was performed through cyclic voltammetry, where each cycle consisted of the following conditions: initial potential: 0.0 V, switching potential: 1.2 V, final potential: 0.0 V scan rate: 0.005 V/s and sensitivity:  $1 \times 10^{-4}$  A. Each electrode was continuously cycled three times for the deposition.

Using the polymerization solution that was found to give the largest change in capacitance while using the simplest approach, the polymerization of HM-EDOT was repeated several times to ensure the results were reproducible using the same polymerization parameters that were used to deposit the polymer on the gold electrode surface. The solution providing optimal results was the fourth solution because it resulted in films with the greatest change in capacitance, it also allowed for less of the  $\beta$ -cyclodextrin to be consumed during each polymerization since the concentration was lower.

#### *Characterization of Polymer Films*

Following polymerization, the Chip #2 used to determine optimal conditions was placed in 20 mL of 0.1 M KCl and the change in capacitance was calculated by keeping the scan rate steady at 0.1 V/s. The scan rate study was repeated on the electrodes with the modified surface to determine the change in capacitance due to polymerization on Chip #1. Another study to determine the change in capacitance was performed at only

one scan rate, was repeated on Chip #2 after polymerization of the HM-EDOT in 0.1 M KCl.

## Results

### *Characterization of Chips Before Polymerization*

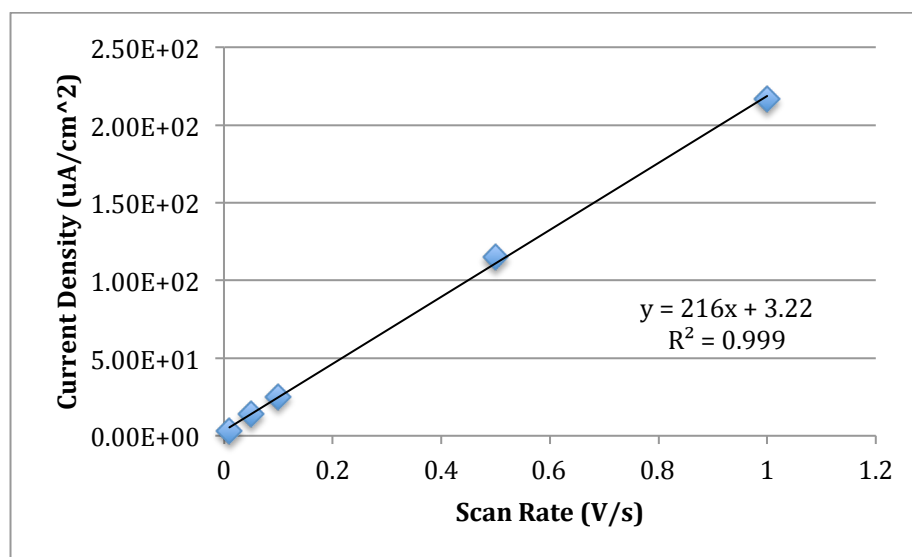
The results of the scan rate study were graphed as current density versus scan rate, which gives the capacitance as the slope of the line, according to Equation 1. The values are reported in current density so that the results are normalized for area because the area of the electrodes varies and the resulting current is dependent on area. The results must be normalized for area so they can be directly compared. Figure 3 is an example of a graph produced from the scan rate study before polymerization, and it shows the capacitance to be  $216 \mu\text{F}/\text{cm}^2$ .

In order to determine the effect of the polymer on the electrode the equation

$$i_c = vC_dA \quad (1)$$

is used to determine the current density and capacitance before and after polymerization, which suggests the polymer properties, such as the accessibility of the polymer for electron transfer events and the speed with which the polymer can transmit electrons (conductivity), respectively. This equation is used to find the charging current of an electrode, and in order to use this equation, it must be assumed that the current before and after polymerization results from charging of the double layer at the electrode/solution interface. The current can be treated as a charging current because it can be assumed that the uncompensated resistance is negligible and the faradic resistance is infinite. Also, although there are likely to be underlying faradaic processes occurring within the polymer, the shape of the CV response and the dependence of current density on scan rate

are characteristic of double layer charging. Thus, this is the model that is used throughout this thesis.



**Figure 3.** The current density versus scan rate for electrode C on Chip #1 before electropolymerization of HM-EDOT in 0.1 M KCl, giving the capacitance of the bare gold electrode as 216  $\mu\text{F}/\text{cm}^2$  and an  $R^2$  value of 0.999.

The capacitance of each electrode before polymerization was determined by graphing the data according to Equation 1, and the data are arranged into Table 1 to show that there was no polymer on the surface of the electrode prior to polymerization because the capacitance for a bare electrode is typically around 100  $\mu\text{F}/\text{cm}^2$ .

**Table 1.** The area and capacitance for characterization of each electrode on Chip #1 following the scan rate study in 0.1 M KCl

Electrode	Area ( $\text{cm}^2$ )	Capacitance ( $\mu\text{F}/\text{cm}^2$ )
A	0.080	115
B	0.040	43.5
C	0.040	216
D	0.080	103

The results of the capacitance study, using CV with a single scan rate, were used to calculate the current density and capacitance for each electrode before polymerization using Equation 1. The values that were calculated for current density and capacitance at a scan rate of 0.1 V/s and are arranged into Table 2.

**Table 2.** The values for area, current density, and capacitance ( $\pm 1$  standard deviation,  $N = 5$  characterizations per electrode) for characterization of Chip #2 in 0.1 M KCl at a scan rate of 0.1 V/s before polymerization of HM-EDOT

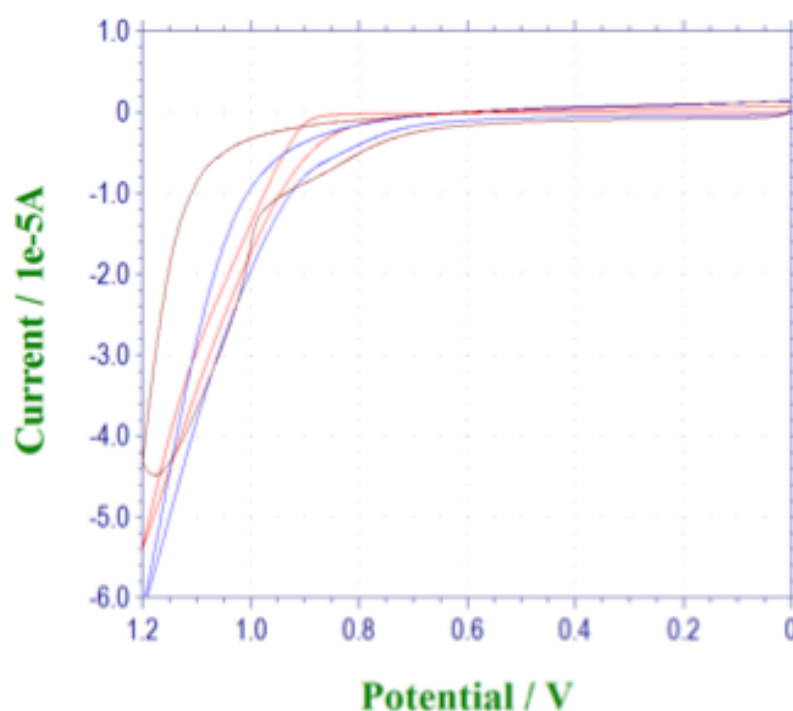
Electrode	Area (cm <sup>2</sup> )	Current Density ( $\mu\text{A}/\text{cm}^2$ )	Capacitance ( $\mu\text{F}/\text{cm}^2$ )
A	0.060	$11.0 \pm 1.6$	$110 \pm 16$
B	0.040	$9.9 \pm 2.3$	$99 \pm 23$
C	0.040	$9.8 \pm 3.3$	$99 \pm 33$
D	0.060	$11.1 \pm 4.3$	$111 \pm 43$

The data use the values of current at the midpoint of the characterization (0.25 V vs. Ag/AgCl) because that is the point where the current is the most consistently flat, while also being a value that is kept constant throughout all the experiments.

#### *Polymerization of HM-EDOT*

The experiment using four different solutions to test optimal polymerization conditions indicated that using water over PBS solution was more advantageous, and resulted in more stable polymers, that did not fall off the electrode. The capacitance and current densities for the polymers deposited in water were higher, illustrating that they have the ability to conduct more current. Table 3 shows the results from the optimization experiment.

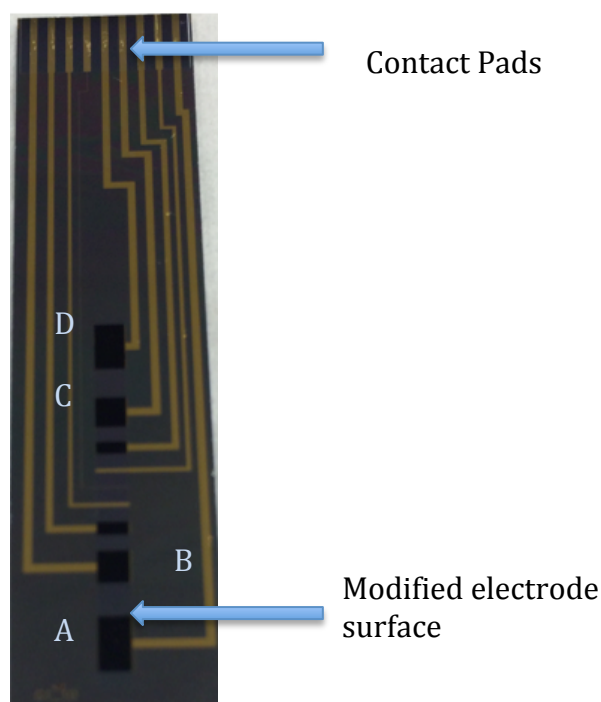
Polymerization occurs when the monomer oxidizes and reacts with other monomers, linking together and allowing it to deposit on the gold electrode surface. There is some speculation that chemisorption of the sulfur in the thiophene moiety to the gold surface helps in the adhesion of the polymer film that grows with every cycle. Figure 4 shows the CV of a typical polymerization scan, showing all three cycles, with growing charging current in the region 0.8 V to 0 V, with every cycle, indicative of a growing thickness of the polymer.



**Figure 4.** A representative cyclic voltammogram of the polymerization of HM-EDOT on the gold surface of an electrode, with the three cycles overlaid on the scan. The first cycle is represented by the brown scan, the second cycle is the red scan, and the third cycle is the blue scan. It illustrates the oxidation occurring at the electrode surface when the polymer is deposited. Conditions: area = 0.080 cm<sup>2</sup>, scan rate = 0.005 V/s, solution: 0.001 M  $\beta$ -cyclodextrin sulfated sodium, 0.01 M HM-EDOT in water. The first scan shows a lower amount of current generated, as well as, a more positive potential before oxidation begins.



Because the third and fourth solutions yielded similar polymerization results, the fourth one was chosen for use in the remaining experiments because it allowed for the conservation of resources. The results of the subsequent polymerizations yielded visible films on the electrode surface, such as those shown in Figure 5. The conducting polymer of HM-PEDOT is shown on the surface of the gold electrodes as a dark blue film covering the entire electrode surface.



**Figure 5.** The chip after the polymerization of HM-EDOT has occurred. The dark blue polymer film is present at the modified electrode surface. (The gold-colored features are the electrode leads that are insulated by BCB, and therefore cannot be polymerized.)

The figure shows an electropolymerized film completely covering each of the modified electrode surfaces. This indicates that the film is stable with a good adherence to the gold surface. The film is considered stable because it withstood multiple experiments of

cycling in pure electrolyte and multiple rinsings with water, and the electrochemical activity did not deviate from day to day.

### *Characterization of Polymer Films formed from HM-EDOT*

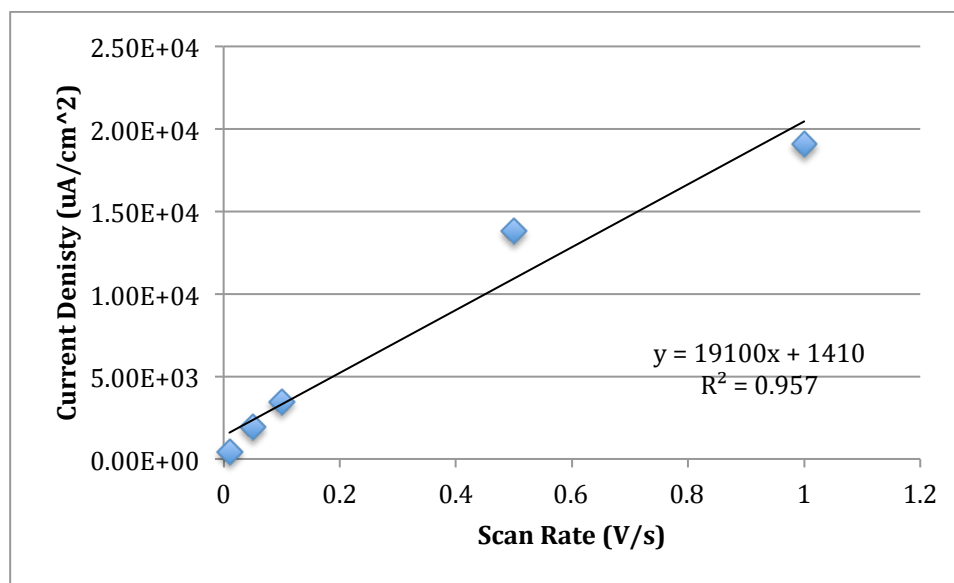
The chip containing the polymer films deposited with varying polymerization solutions was characterized, and Equation 1 was used to determine the current density and capacitance to indicate the best conditions for polymer deposition. The best conditions were determined to be the one that produced the greatest increase in current density and capacitance from before polymerization.

**Table 3.** Area, current density, and capacitance before and after polymerization for the experiment in which four polymerization solutions were used to determine optimal conditions

Electrode	Solution Composition (with 0.1 M HM-EDOT in water)	Area (cm <sup>2</sup> )	Current Density Before (μA/cm <sup>2</sup> )	Current Density After (μA/cm <sup>2</sup> )	Capacitance Before (μF/cm <sup>2</sup> )	Capacitance After (μF/cm <sup>2</sup> )
A	0.01 M PBS 0.001 M β-CD	0.080	12.5	75.0	125	750
B	0.01 M PBS 0.01 M β-CD	0.040	7.50	18.8	75.0	188
C	No PBS 0.01 M β-CD,	0.020	16.6	368	166	3680
D	No PBS 0.001 M β-CD	0.080	24.9	413	249	4130

It is evident that the solutions without PBS as electrolyte produced films with higher capacitances and current densities, therefore, those films can carry more current per unit area. The presence of the polymer indicates that HM-EDOT is capable of being electropolymerized. The conducting properties of the resulting polymer were further

explored using the same characterization procedure from before polymer deposition. The results of the scan rate study of the polymer films were graphed, as before, as current density versus scan rate. Figure 6 shows the graph for the same electrode shown in Figure 3 with capacitance shown as the slope of the graph.



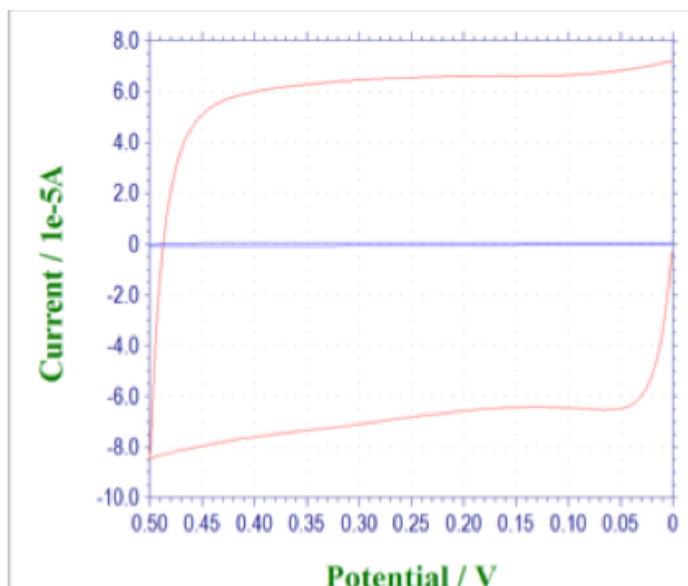
**Figure 6.** The graph of current density versus scan rate for Electrode C of Chip#1 after polymerization of HM-EDOT, in 0.1 M KCl resulting in a capacitance of 19.1 mF/cm<sup>2</sup>. The R<sup>2</sup> value was 0.957.

Comparing the two graphs and their slopes, it is evident that the capacitances before and after polymerization greatly differ. The original capacitance of the bare gold was 216 μF/cm<sup>2</sup>, and the capacitance after polymerization is 191 μF/cm<sup>2</sup>. The results for all the electrodes on Chip #1 are given in Table 4. Also, it appears that the data do not fit the model of a line as well with the polymer, suggesting that other processes, such as faradaic ones that involve mass transfer limitations for charge compensation, are also playing a role.

**Table 4.** The area and capacitance for characterization of each electrode on Chip #1 following the scan rate study in 0.1 M KCl after polymerization of HM-EDOT

Electrode	Area (cm <sup>2</sup> )	Capacitance (μF/cm <sup>2</sup> )
A	0.080	7440
B	0.040	12900
C	0.040	19100
D	0.080	9660

The results indicate that the capacitance of Chip #1 greatly increased due to the electropolymerization of the HM-EDOT. The results from this experiment are reproducible because the large change in capacitance was seen following each polymerization. The large variation seen between electrode sizes could have been due to mass transport. The smaller electrodes have a limited amount of space for the electrons to flow through the interface at the electrode surface. The modified electrode surface is capable of carrying more current than the bare gold electrode. Another way to show the increase in current due to the electropolymerization is to overlay the cycle voltammograms of the characterization of the electrodes in 0.1 M KCl before and after polymerization.



**Figure 7.** A representative cyclic voltammogram of an overlay of the charging current of an electrode in 0.1 M KCl before and after polymerization. The polymerization of HM-EDOT greatly increases the charging current of the bare gold electrode. The blue scan is the scan of the charging current before the electropolymerization, and the red scan is the charging current after the electropolymerization. Conditions: area = 0.080 cm<sup>2</sup>, scan rate = 0.1 V/s, solution = 0.1 M KCl.

As seen in Figure 7, the blue scan around zero Amperes throughout the whole scan is the charging current of the electrode before the electropolymerization, and the red scan is after the polymerization. The large increase in charging current shows the modified electrode surface is capable of carrying much larger amounts of current, therefore, better suited to transfer electrons between the gold surface and the polymer. The capacitance study, that was repeated using a steady scan rate, gave similar results, which are shown in Table 5.

**Table 5.** The values for area, current density, and capacitance for characterization of Chip #2 in 0.1 M KCl at a scan rate of 0.1 V/s after polymerization of HM-EDOT

Electrode	Area (cm <sup>2</sup> )	Current Density (μA/cm <sup>2</sup> )	Capacitance (μF/cm <sup>2</sup> )
A	0.060	1100 ± 26	10960 ± 260
B	0.040	1080 ± 78	10790 ± 780
C	0.040	1120 ± 25	10940 ± 250
D	0.060	1090 ± 63	10880 ± 630

The values for current density and capacitance before and after electropolymerization can be directly compared between electrodes because the electrodes have been normalized for area. The values seen in the table are reproducible following the same polymerization conditions.

## Conclusion

The data suggest that the electropolymerization of HM-EDOT produce a robust and stable film on the electrode surface because the polymers produced are not easily removed from the electrode surface. The large increase in current density and capacitance is due to the deposition of the polymer film. The addition of the polymer increases the size of the electrode, as well as its conducting properties allowing for more electrons and, therefore, current to flow through to the electrode surface.

The ability of the HM-EDOT to be electropolymerized is important for the future because the functional alcohol group allows for the conjugation and further derivatization of the monomer. The HM-EDOT can also be deposited and electrochemically stable in aqueous solutions, which is vital for the possible production of biofuel cells. Also, it is

hopeful that HM-EDOT can be used for biological purposes since much of the human body functions in an aqueous environment.

The success of the polymerization of HM-EDOT allows for the continuation of the project to conjugate the enzyme horseradish peroxidase (HRP) to the conducting polymer and working toward the future development of enzymatic biofuel cells.

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- <sup>2</sup> Kros A, Sommerdijk NAJM, Nolte RJM. Poly(pyrrole) versus poly(3,4-ethylenedioxythiophene): Implications for biosensor applications. *Sensors Actuators B: Chem* 2005 /4/29/;106(1):289-95.



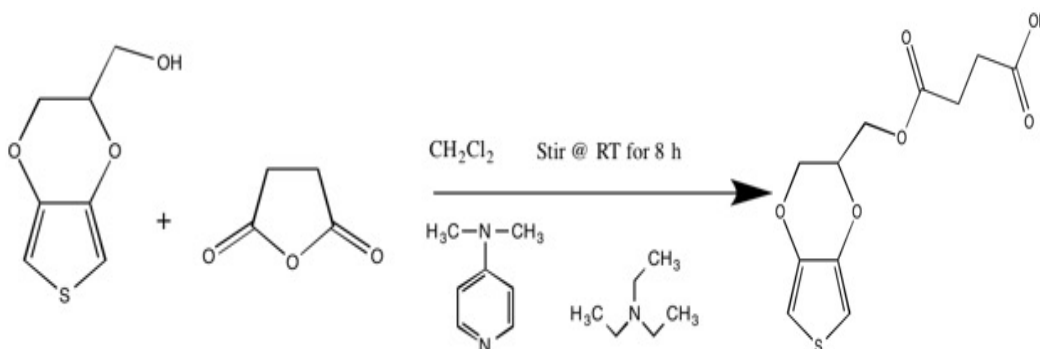
### Introduction

The characterization of the polymer of hydroxymethyl-3,4-ethylenedioxythiophene (HM-EDOT) showed that the derivative of EDOT greatly increased the current and capacitance of the modified electrode. Adding an enzyme to a conducting polymer, such as PEDOT, with the addition of a mediator, can help catalyze the oxidations and reductions in a biofuel cell.<sup>1</sup> The enzyme being used in these experiments is horseradish Peroxidase (HRP), which contains a heme group as the catalytic site. HRP is activated by hydrogen peroxide,  $H_2O_2$ , as the  $H_2O_2$  removes electrons, oxidizing the HRP. This allows the HRP to then oxidize other substrates by removing electrons. The activity of HRP can be easily detected when combined in solution with  $H_2O_2$  and 3,3',5,5'-tetramethylbenzidine (TMB) because after the activation of the HRP, the HRP then oxidizes the TMB, and the oxidized form of TMB has a visual color change and absorbs light at 450 nm. The structure of HRP is shown in Figure 1, and it can be seen that HRP contains a heme group surrounded by a porphyrin ring and  $\alpha$ -helices around the catalytic center.<sup>2</sup> The lysine groups of the HRP are highlighted in blue because they are integral in the conjugation of the enzyme to other molecules through amide bonds.



**Figure 1.** An image of the enzyme HRP. The  $\alpha$ -helices are seen surrounding the heme group at the center within the porphyrin ring. The lysine residues are highlighted in blue and are important in enzyme conjugation.<sup>3</sup>

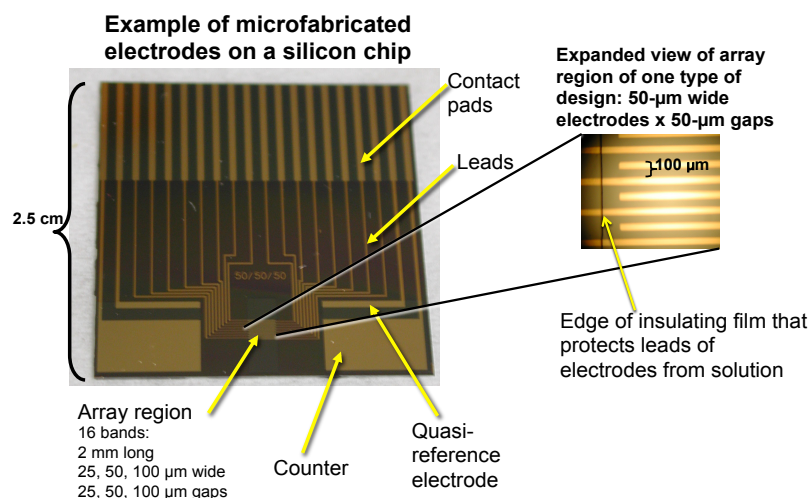
In order for the enzyme to be conjugated to the conducting polymer, a monomer with an organic tether and more accessible functional group had to be synthesized. A carboxylic acid form of 3,4-ethylenedioxythiophene (COOH-EDOT) was synthesized from the HM-EDOT using succinic anhydride.<sup>4</sup> The succinic anhydride and HM-EDOT are mixed with dimethyl-aminopyridine (DMAP) and triethylamine ( $\text{Et}_3\text{N}$ ) in dichloromethane. Figure 2 shows a reaction schematic.



**Figure 2.** The synthesis of COOH-EDOT from HM-EDOT, succinic anhydride, DMAP,  $\text{Et}_3\text{N}$ , and dichloromethane.

The synthesis of COOH-EDOT allows for conjugation of the enzyme to the polymer backbone because it has the ability to become an activated ester that can easily bind to primary amine groups, like those in lysine. The COOH-EDOT is integral in the development of possible biofuel cells because it can electropolymerized and used for the immobilization of the enzyme HRP.

The polymerization of the COOH-EDOT was performed using a chip with a microelectrode array because in the future it will allow for two electrodes to be used as a possible biofuel cell. Figure 3 shows an example of a chip with the microelectrode array, which contains sixteen microelectrodes that are 50  $\mu\text{m}$  wide with 50  $\mu\text{m}$  gaps between electrodes. Each electrode has an area of 0.10  $\text{mm}^2$ .



**Figure 3.** Example of the chip containing the microelectrode array that has electrodes that are 50  $\mu\text{m}$  wide with 50  $\mu\text{m}$  gaps, and 2 mm long electrodes.

## Experimental

### *Materials*

The materials used in these experiments were HM-EDOT, succinic anhydride, DMAP, Et<sub>3</sub>N, and acetonitrile. All materials were received from Sigma US. The potentiostat models used were CHI760B and CHI650A from CH Instruments (Austin, Texas).

The electropolymerization of COOH-EDOT, as well as all characterizations, were done using a platinum flag counter electrode and a Ag/AgCl (saturated KCl) reference electrode.

The chips containing the microelectrode array were microfabricated by others within the research group, and then plasma cleaned using a Harrick PDC-32G model for fifteen minutes at a pressure of 60 mTorr, which uses oxygen gas to generate an oxygen plasma that cleans the chips. They were then stored in 18.2 MΩ water before use.

### *Synthesis of COOH-EDOT*

The synthesis of COOH-EDOT was performed by combining the HM-EDOT and dry dichloromethane (using aluminum oxide) in a three-neck round bottom flask that had been thoroughly evacuated with nitrogen gas (N<sub>2</sub>). The solution is stirred on a magnetic stir plate and kept under N<sub>2</sub> atmosphere throughout the reaction. In another vial, the succinic anhydride, DMAP, Et<sub>3</sub>N, and dry dichloromethane are combined and mixed. Using a syringe the solution is injected drop-wise through a rubber septum into the round bottom flask. The mixture is stirred and kept under N<sub>2</sub> at room temperature overnight.

The product of the synthesis was then transferred to a conical vial, and washed with 10% hydrochloric acid (HCl) by mixing the phases five times, and extracting the

aqueous phase. It was then washed three times with saturated NaCl solution, extracting the aqueous phase after each wash. The organic phase was dried with magnesium sulfate ( $\text{MgSO}_4$ ) by adding  $\text{MgSO}_4$  to the vial until there was no clumping and waiting five minutes. The product was filtered, using glass pipets and glass wool, into a new conical vial and dried again with  $\text{MgSO}_4$  for ten minutes. The final product is transferred into a clean vial after filtering the  $\text{MgSO}_4$ , and the solvent was evaporated under  $\text{N}_2$  gas until a yellow honey-like substance remained. To determine successful synthesis, a small amount of product was combined with deuterated chloroform and a nuclear magnetic resonance (NMR) was taken. In addition to NMR, infrared (IR) spectroscopy, and mass spectrometry are used to determine a successful synthesis.

#### *Characterization of Microelectrode Array Before Polymerization*

The chip containing the microelectrode array, Chip #3, was characterized in 6 mL of a solution containing 50% water and 50% acetonitrile with 0.1 M lithium perchlorate ( $\text{LiClO}_4$ ) as the electrolyte prior to polymerization using a scan rate study. The characterization conditions were: initial potential: 0.0 V, switching potential: 0.5 V, and sensitivity:  $1 \times 10^{-7}$  A. The scan rates used were: 0.01 V/s, 0.05 V/s, 0.1 V/s, 0.5 V/s, and 1.0 V/s.

#### *Polymerization of COOH-EDOT*

The polymerization of COOH-EDOT was performed in aqueous conditions using a mixture of acetonitrile and water (1:1) with 0.1 M  $\text{LiClO}_4$  as electrolyte<sup>5</sup>. A small amount of the COOH-EDOT was added to the vial. The microelectrode array was immersed in solution and the conditions for polymerization were: initial potential: 0.0

V,high potential: 1.2 V, scan rate: 0.005 V/s and sensitivity:  $1 \times 10^{-7}$  A. Each electrode was cycled six times for the deposition.

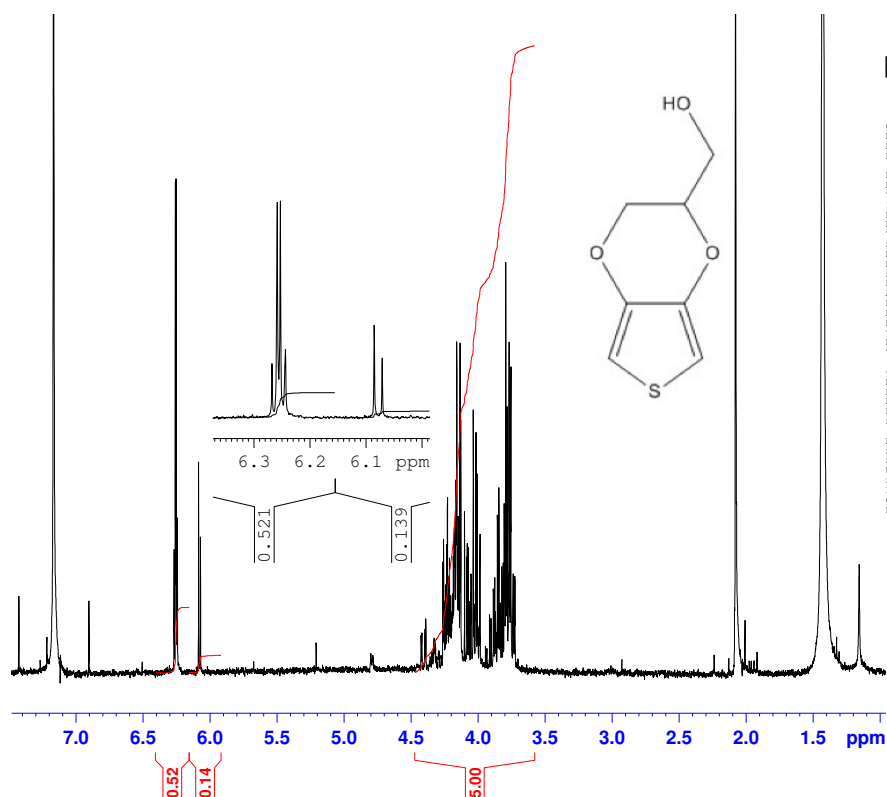
#### *Characterization of Microelectrode Array After Polymerization*

The chip containing the microelectrode array was characterized in 50% water and 50% acetonitrile with 0.1 M LiClO<sub>4</sub> as electrolyte after polymerization, using a scan rate study. The characterization conditions were: initial potential: 0.0 V, high potential: 0.5 V, and sensitivity:  $1 \times 10^{-7}$  A. The scan rates used were: 0.01 V/s, 0.05 V/s, 0.1 V/s, 0.5 V/s, and 1.0 V/s.

## **Results**

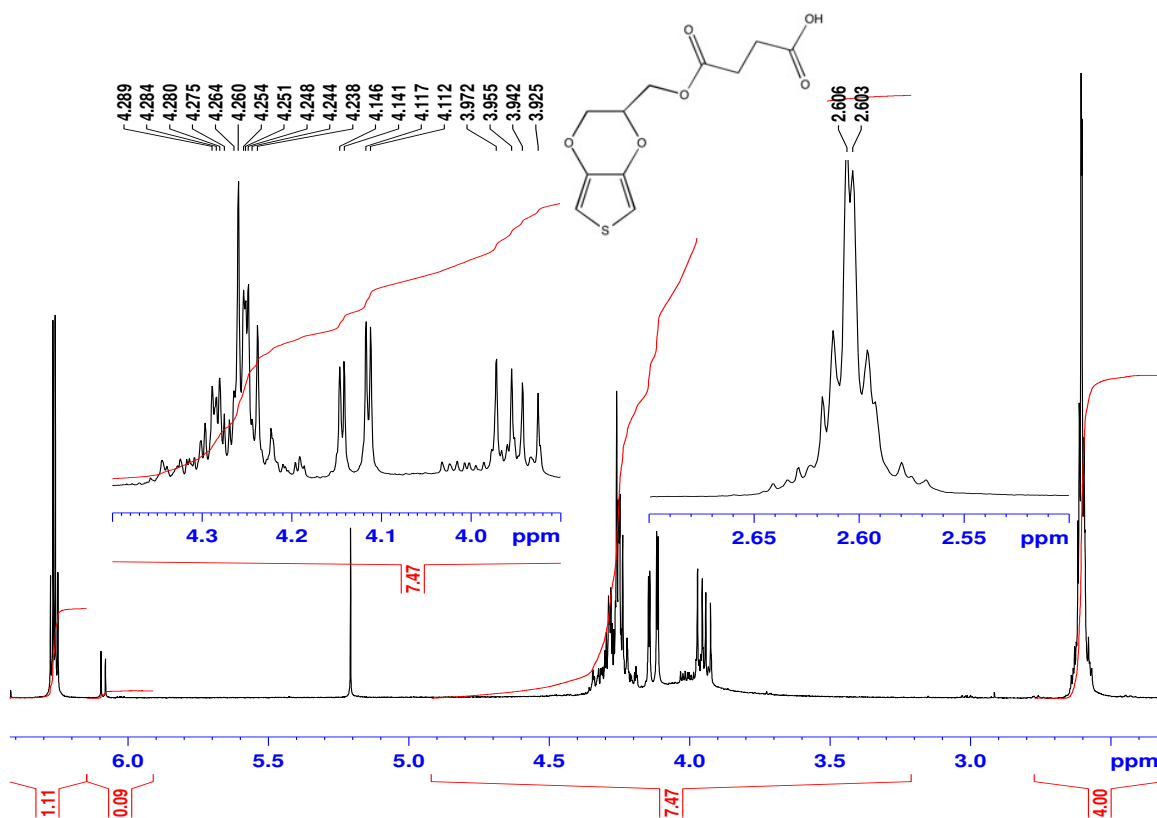
#### *Synthesis of COOH-EDOT*

After the completion of the COOH-EDOT synthesis reaction, a sample of the product was used in order to obtain an NMR scan to determine if the desired product was synthesized. The proton NMR for HM-EDOT was also obtained in order to compare the peaks to that of the COOH-EDOT. The HM-EDOT <sup>1</sup>H NMR is shown in Figure 4, which shows a complex set of peaks around 4.0 ppm that may be attributed to the protons in the ethylene groups and the hydrogens in the methyl functional group. The peaks around 6.1 ppm and 6.2 ppm are representative of the hydrogen atoms within the thiophene ring near the sulfur. The solvent chloroform-d is seen in the large peak at 7.2 ppm, and acetone is the large peak near 2.0 ppm.



**Figure 4.** The proton NMR for HM-EDOT where the peaks at 4.0 ppm are the protons in the ethylene groups and the methyl group, the peaks 6.1 ppm and 6.2 ppm represent the hydrogens within the thiophene ring, and the peak at 7.0 ppm is from the chloroform-d solvent. The large peak at 2.0 ppm is due to acetone that was used to rinse the NMR tube.

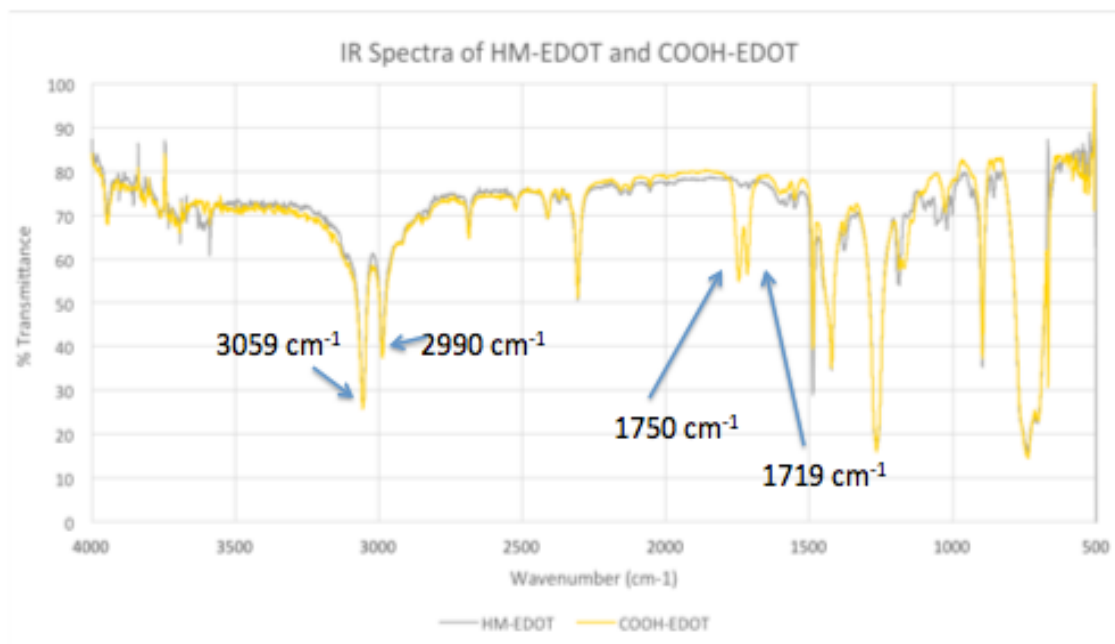
The  $^1\text{H}$  NMR for the product showed a unique peak at 2.6 ppm that represents the protons between the carbonyl groups in the organic tether. The addition of the organic tether also caused the proton signal for the thiophene ring to shift to 4.1 ppm. The peak near 5.3 ppm is due to excess dichloromethane in the product. Figure 5 shows the NMR for the COOH-EDOT synthesis product.



**Figure 5.** The  $\text{H}^+$  NMR spectrum for COOH-EDOT, where the peak at 2.6 ppm is representative of the hydrogens between the carbonyl groups in the tether. The peaks at 4.0, 4.1, and 4.3 ppm are due to the protons within the thiophene ring and the ethylene groups. The peak at 5.3 ppm is due to excess dichloromethane, and the peak at 6.3 ppm is due to the NMR solvent, chloroform-d.

The peak at 2.6 ppm has an integration of 4.00, which coordinates with the hydrogens between the carbonyls suggesting that the synthesis was a success. The product of the synthesis was also determined using infrared resonance spectroscopy (IR). The spectra for HM-EDOT and COOH-EDOT were overlaid and are shown in Figure 6. It shows that both molecules contain similar bands and stretches within the IR, except the COOH-EDOT has an extra band near  $1750\text{ cm}^{-1}$ , which is indicative of the stretch of a carbonyl.

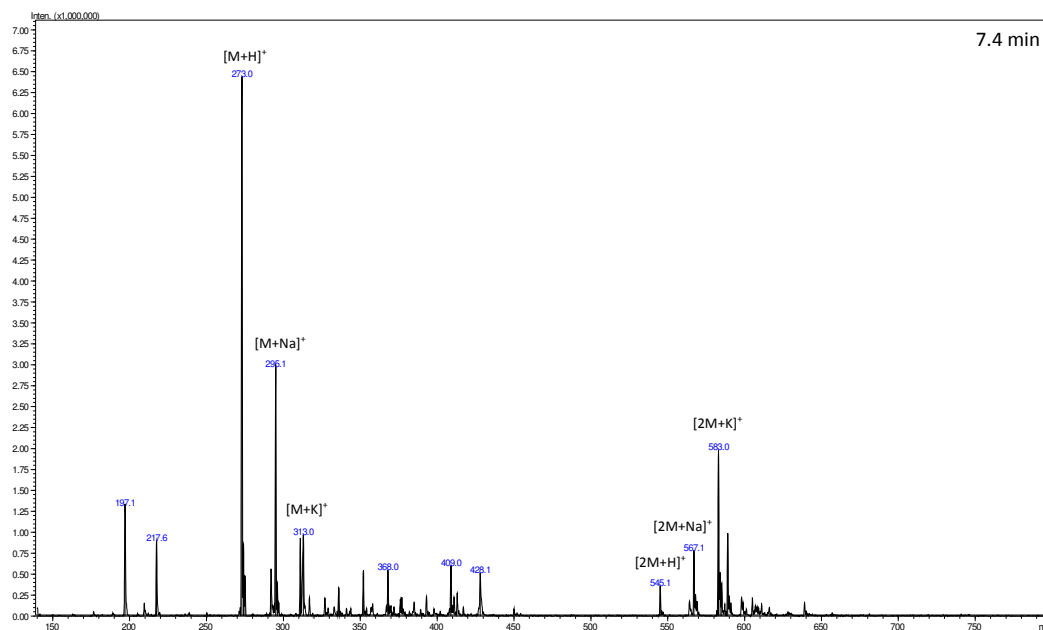




Functional Group	Wavenumber Peak
Carboxylic acid (COOH)	1719 cm <sup>-1</sup>
Ester (OCO)	1750 cm <sup>-1</sup>
Alkene (=C-H)	3059 cm <sup>-1</sup>
Alkane (C-H)	2950 cm <sup>-1</sup>

**Figure 6.** The IR spectra of HM-EDOT overlaid with COOH-EDOT that shows they have similar molecular elements except for the stretch around 1750 cm<sup>-1</sup>. The table indicates the peaks of the major functional groups within the molecules.

The presence of the unique band near 1750 cm<sup>-1</sup> represents a carbon that is double bonded to an oxygen, like those in carboxylic acids. This shows that the synthesis product is the expected product, COOH-EDOT. One last way to determine the purity of the synthesis product was for an electrospray ionization mass spectrometry scan (EIS) to be performed. This allows the product to be ionized, and the spectrum shows the molecular mass of the injected molecule when protons have been added. As seen in Figure 7, the molecular mass of the product when protonated is 273 g/mol, which is what is expected when calculated.



**Figure 7.** The ESI spectrum for COOH-EDOT, which shows that the molecular mass of product when protonated is 273 g/mol, which is the expected mass of COOH-EDOT.

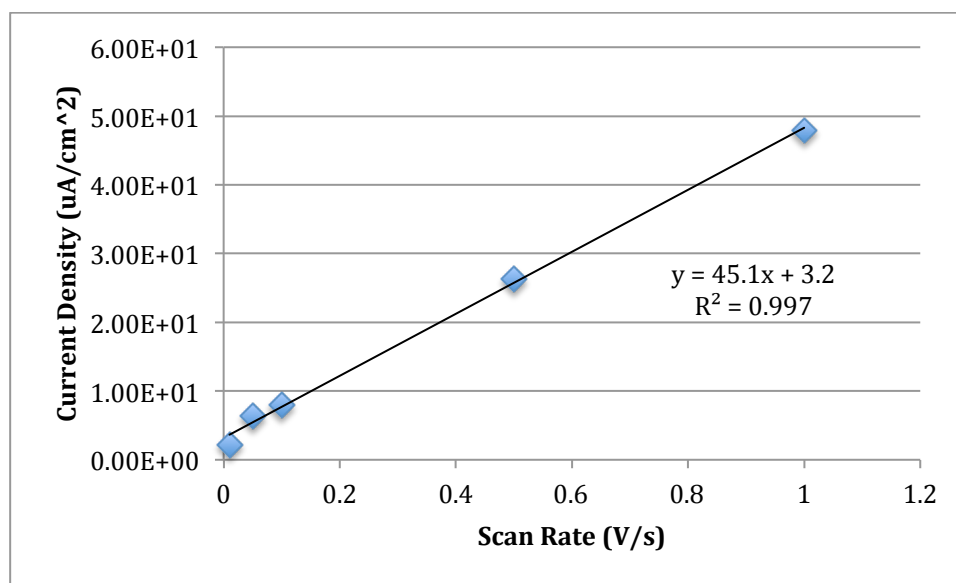
#### *Characterization of the Microelectrode Array before Polymerization*

The scan rate study results for the electrode used in polymerization were graphed by current density versus scan rate. The slope of the graph gives the capacitance for the electrode, indicating the amount of electrochemical energy that can be stored. The results are graphed using the equation

$$i_c = \nu C_d A \quad (1)$$

which can be used to determine the double layer capacitance. This equation can be used because it is assumed that the current produced during cyclic voltammetry (CV) is similar to that of a charging current, that the uncompensated resistance is negligible, and that the faradic resistance is infinite. The graph of an electrode before polymerization is shown in Figure 8, and the capacitance calculated is 45.1  $\mu\text{F}/\text{cm}^2$ . The capacitance would be equal

to  $45.1 \mu\text{F}/\text{cm}^2$ , which indicates that the electrode has no prior modification before polymerization.

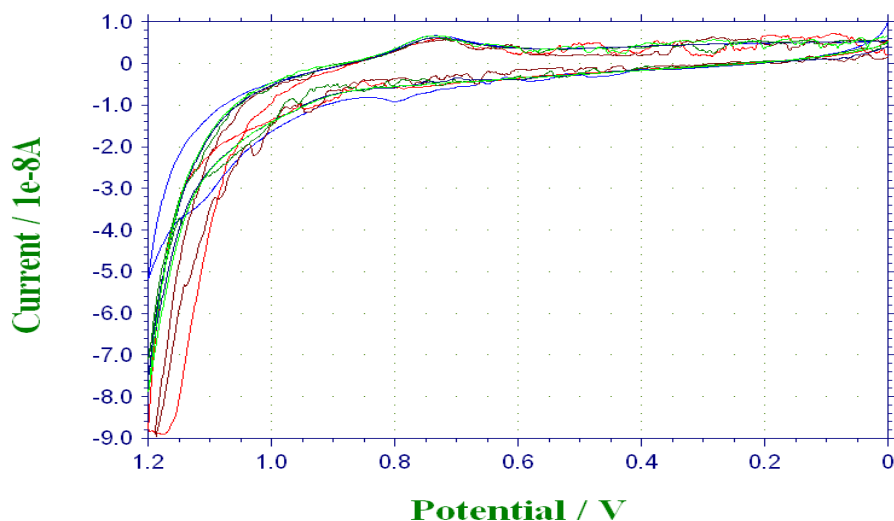


**Figure 8.** The graph of current density versus scan rate before polymerization, showing that the capacitance of the electrode is  $45.1 \mu\text{F}/\text{cm}^2$ . The characterization was done in a 50% acetonitrile, 50% water solution containing 0.1 M  $\text{LiClO}_4$ . The  $R^2$  value was 0.997.

The characterization of the electrode in the microelectrode array also ensured that there was no modification to the electrode surface prior to polymerization.

#### *Polymerization of COOH-EDOT*

The electropolymerization of COOH-EDOT was unsuccessful in the acetonitrile/ $\text{H}_2\text{O}$  (1:1) mixture. Organic solvents were used for the electropolymerization because polymerization was unsuccessful in aqueous conditions. The use of organic solvents was decided because after the synthesis, the product is dissolved in the organic phase. The CV of the electropolymerization is shown in Figure 9, which indicates that there was an oxidation occurring at the electrode surface.

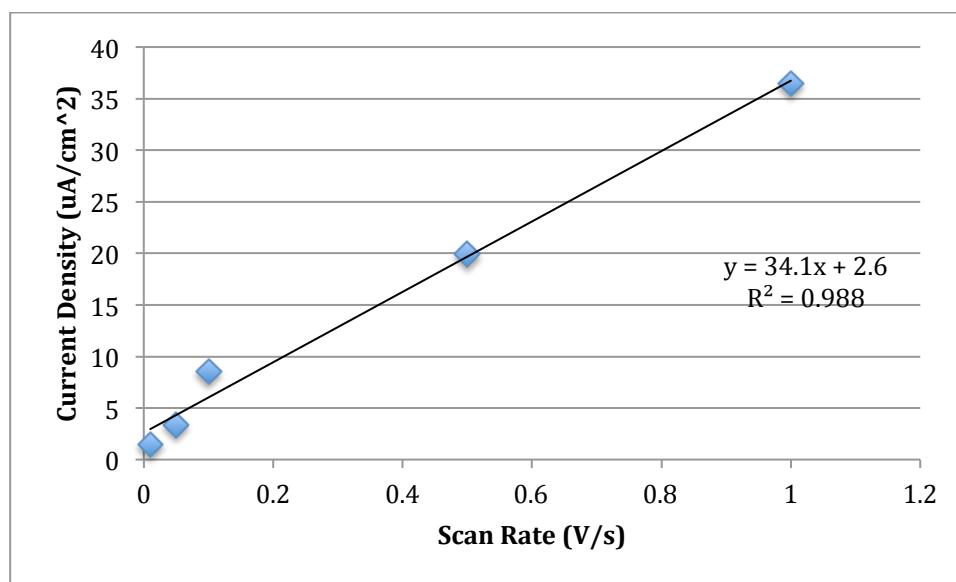


**Figure 9.** The overlay of the six polymerization cycles for deposition of COOH-EDOT. There is evidence of an oxidation occurring due to the peak near 1.0 V. The blue scan represents the first cycle, red is the second cycle, brown is the third cycle, teal is the fourth cycle, navy is the fifth cycle, and green is the sixth cycle. Conditions: area = 0.10 mm<sup>2</sup>, scan rate = 0.005 V/s, solution = acetonitrile/water (1:1), 0.1 M LiClO<sub>4</sub>, and 0.01 M COOH-EDOT.

While there was an oxidation occurring during the deposition, there was no visible appearance of a polymer on the electrode surface after electropolymerization. A thin film may have developed on the surface, but it was not visible. In order to determine if any polymer deposited on the surface, characterization of the electrode was repeated.

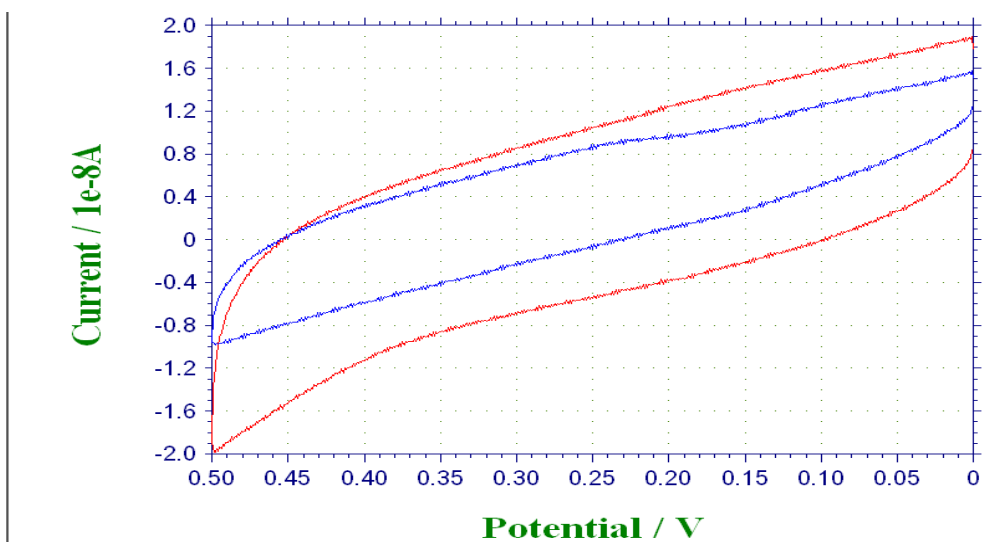
#### *Characterization of the Microelectrode Array after Polymerization*

The scan rate study was repeated on the electrode used for electropolymerization, and the results were graphed as before. Figure 10 shows the graph of current density versus scan rate of the electrode with the capacitance as the slope. The capacitance was found to be 34.1  $\mu\text{F}/\text{cm}^2$ .



**Figure 10.** The graph of current density versus scan rate after polymerization, showing that the capacitance of the electrode is  $34.1 \mu\text{F}/\text{cm}^2$ . The characterization was done in a 50% acetonitrile, 50% water solution containing 0.1 M  $\text{LiClO}_4$ . The  $R^2$  was 0.988.

The graph indicates that there was a decrease in the capacitance after the attempted polymerization. This means the amount of energy that can be held within the system decreased after the attempted electropolymerization. The flow of electrons through the system also decreased, shown by the overall decrease in current density. This can also be seen when the charging current of the electrode in electrolyte before polymerization is overlaid with charging current after polymerization, as shown in Figure 11. The decrease in capacitance could have been caused by insulation of the carboxylic acid groups, which would not allow a charge to build up at the double layer. A thin polymer may have developed at the electrode surface, however there was no visible sign of the polymer. The lack of polymer does not mean it is not there, it just indicates further tests and experimentation would need to be performed in order to confirm if there is a polymer at the electrode surface.



**Figure 11.** The overlay of the current before and after polymerization in a acetonitrile/H<sub>2</sub>O (1:1) solution with 0.1 M LiClO<sub>4</sub> as electrolyte. The blue scan is the current after polymerization, and the red scan is before polymerization. Conditions: area = 0.10 mm<sup>2</sup>, scan rate = 0.1 V/s, solution = acetonitrile/water (1:1), 0.1 M LiClO<sub>4</sub>.

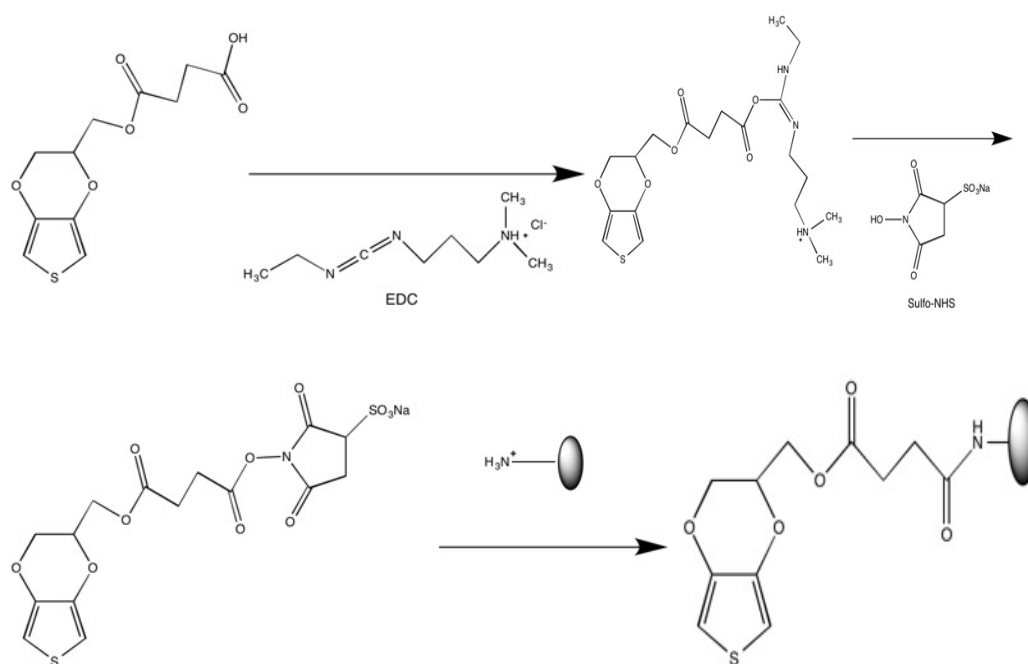
The blue scan is the current after polymerization further emphasizing that the current density and capacitance decreased after the attempted electropolymerization of COOH-EDOT.

The main factor in determining that minimal deposition occurred was the lack of a visible polymer. A thin polymer may have developed from the electropolymerization since there is an oxidation occurring at the electrode surface, which would account for the decrease in capacitance. If no polymer was present, then the capacitance should have stayed the same, but the decrease indicates some insulation due to the carboxylic acid groups. Since there is no visible evidence of the polymer, the project cannot be continued until it can be confirmed.

## Conclusion

The synthesis of COOH-EDOT was a success as shown by the NMR, IR, and EIS, and it is an important step because it allows a product that is commercially available to be used toward the development biofuel cells. The COOH-EDOT allows for the covalent attachment of an enzyme to the conducting polymer through the reactive carboxylic acid group, and the organic tether allows the enzyme to be close enough to the polymer to retain its catalytic activity without compromising its shape. The polymerization of COOH-EDOT, while unsuccessful thus far, is an integral step because it must be done before the conjugation of HRP to the polymer backbone. Further studies and conditions for the electropolymerization of COOH-EDOT will need to be conducted to determine the proper conditions to achieve a stable polymer.

The future work of this project is to conjugate the HRP to the conducting polymer. The conjugation of the enzyme to the conducting polymer will be done by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to create an active ester complex. Then, *N*-hydroxysulfosuccinimide (sulfo-NHS) will be added to reaction, which protects the active ester and is also a good leaving group. The lysine residues in the HRP contain primary amine groups that will react with the active ester, forming an amide bond, and the sulfo-NHS will leave. Figure 12 illustrates the reaction between the EDC/NHS and the COOH-EDOT to form an amide bond with the HRP.



**Figure 12** The conjugation reaction of the COOH-EDOT polymer to HRP using EDC/NHS, which will form an amide linkage between the HRP and EDOT.

After the conjugation of the HRP to the conducting polymer, an enzyme assay will be performed to determine if the enzyme has retained activity after the conjugation. Once catalytic activity is established, it can be used toward the production of an enzymatic biofuel cell because the HRP will catalyze reactions at either the anode or cathode of a cell.



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## Chapter IV: Conclusion

The electropolymerization of HM-EDOT into stable, robust polymers, illustrated by the large increase in current density and capacitance, is important because it is a building block for future research. The HM-EDOT is a stepping-stone for the production of other monomers, such as COOH-EDOT, that can be used for conjugation to molecules important for biofuel cells, like enzymes or mediators. The successful synthesis of COOH-EDOT from HM-EDOT illustrates the impact of HM-EDOT and its function group, because it allows for a new monomer with a more reactive functional group and an organic tether to be produced.

While unsuccessful, the attempted polymerization of COOH-EDOT indicates that the monomer does not form a polymer under aqueous conditions, which are integral in the production of biofuel cells. The next step in this research is to determine the optimal electropolymerization conditions for the COOH-EDOT that produces a stable polymer. Once a stable polymer can be generated, the conjugation of the HRP to the polymer backbone can be completed using EDC/NHS, and then the enzyme can be assessed for activity using an enzyme assay. If the catalytic activity of the HRP is relatively unchanged when conjugated to the polymer, then half of a biofuel cell has been produced.