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Electrochemical Detection of Antioxidants

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Electrochemical Detection of Antioxidants

Senior Honors Thesis

Garrett Thompson
Spring 2016

Advisor: Jeffrey Halpern PhD

1 Introduction

Beta-carotene is a common carotenoid found in plant pigments. Beta-carotene's unique structure allows it to act as a potent antioxidant; it is highly reactive with singlet oxygen and free radical oxygen species (Yue *et. al* 2012). Beta-carotene's mechanism of action is theorized to provide protection against oxidative species within the body. Initial studies have shown that beta-carotene may lower risk for a multitude of disease states including cancer, cardiovascular disease, and macular degeneration (Mayne 1996). Beta-carotene's cardio protective properties are of acute interest as heart disease is the leading cause of death among Americans (National Center for Health Statistics 2014). Acute myocardial infarctions, heart attacks, are the most lethal of all cardiac related disease states. In the United States, acute myocardial infarctions (AMI) are endemic. On average, an AMI occurs every 43 seconds resulting in over 734,000 AMIs annually; 14% of AMIs are fatal (CDC 2011).

Beta-carotene is a mechanistically unique antioxidant. Like most antioxidants, it has free radical trapping behaviors. Beta-carotene is special because it only operates under oxygen levels of less than 150 torr. Higher oxygen partial pressures have been shown to cause prooxidant behavior, but because of observed low oxygen partial pressures in human tissue, this behavior is not typically seen.

In a study completed in 1990, researchers found that there was an increased risk of an AMI in subjects with low serum levels of beta-carotene and lutein (Street *et al.* 1994). When compared to the control groups (380 nM beta-carotene and 245 nM lutein) subjects who suffered an AMI had on average 16.6% ($p < .02$) lower levels of beta-carotene (317 nM) and 7.9% ($p < .09$) lower levels of lutein (228 nM). Multiple other studies confirmed the trend which showed

increased risk for an AMI with decreasing levels of antioxidant (Tiina *et al.* 2001, Koh *et al.* 2011, and Rejon *et al.* 2002). With the importance of antioxidants demonstrated, following their levels in serum would provide a critical tool for tracking risk factors of AMIs.

Serum antioxidant levels are presently being detected through high performance liquid chromatography (HPLC), HPLC is operationally prohibitive to most hospitals and clinics due to its inability to handle high throughput volumes and high labor demands. HPLC is currently used as a clinical detection method, but is limited to a small number of laboratories due to its requirement of a highly technically skilled operator (Vogesser and Seger 2008). Due to these constraints HPLC is far from widespread clinical application. Other non-invasive methods such as RAMAN spectroscopy have been developed to detect antioxidants. The RAMAN technique involves a laser based spectroscopic technique using a holographic rejection filter. It has been shown to be highly reproducible (Hata *et. al.* 2000). Reflection spectrometry based detection of carotenoid levels in skin has been shown to correlate with serum levels of carotenoids (Stahl *et. al.* 1998). The RAMAN technique has also been used to detect macular antioxidants. Lutein and zeaxanthin were detected due to double bond vibrations at a signal intensity of 1525^{-1} cm (Bernstein *et. al.* 2001). Lutein and zeaxanthin levels were found to inversely correlate with age and risk of age-related macular degeneration (Bernstein *et. al.* 2001). Currently the RAMAN method is limited to skin based detection of antioxidants.

Due to the increasing need for tracking predictive AMI indicators, a novel and fast method for antioxidant detection is desired. A quick and clinically feasible indicator would allow doctors to guide their patients to healthier lifestyles. It would also allow researchers to better understand what factors effect serum antioxidant levels.

Measuring electrochemical interactions is proposed to detect antioxidants in human serum. This method would provide many advantages over the current detection method: HPLC. An electrochemical antioxidant test would have a much higher throughput and would take only a fraction of the time. This would allow the creation of a point of care detection method.

2 Unmodified Electrode

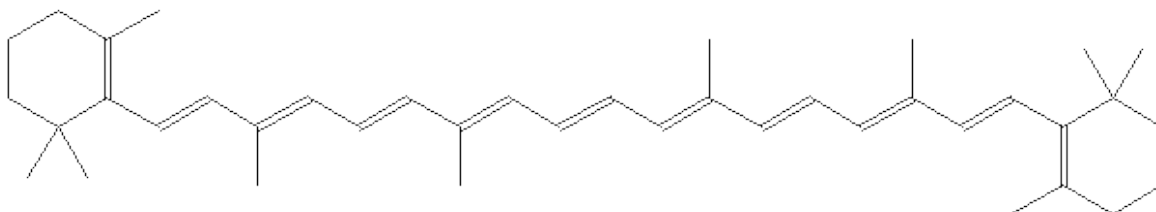
2.1 Introduction:

Previously published work has shown that beta-carotene and lutein are electrochemically active. Furthermore good calibration ($R^2 = .999$) is shown at high concentrations (0.5-76 μM) of lutein with a limit of detection of about 0.1 μM (Yue et. al 2012) in a tetrahydrofuran and absolute ethanol solvent. The experimental setup utilized a bare glassy carbon working electrode, a platinum counter electrode, and an Ag/AgNO₃ working electrode (Yue et. al 2012).

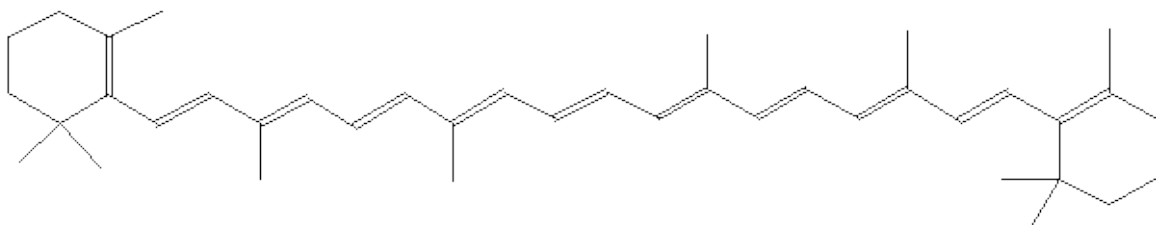
With the knowledge that carotenoids are electrochemically active, the goal of the unmodified experiments was to create replicative and sensitive (nM scale) calibration curves for beta-carotene and lutein.

It was expected that lutein and beta-carotene would display similar electrochemical properties as they are extremely similar in structure (Scheme 1). The molecules differ only by the direction of one double bond at a terminus.

a)



b)



Scheme 1:

a) lutein molecule and b) beta-carotene molecule

To achieve the first objective, serial dilutions of lutein and beta-carotene were run under similar conditions to the Yue experiment (Yue et. al 2012).

2.2 Methods:

2.2.1 Reagents

Beta-carotene (97%) and lutein (90%) were purchased from Acros organics. Other reagents are of analytical grade. The stock solution was 0.1M tetrabutylammonium hexafluorophosphate in dimethyl sulfoxide.

2.2.2 Apparatus and measurement methods

All electrochemical tests were run on a Gamry Instruments reference 600. The electrode setup included a glassy carbon macroelectrode, a platinum counter electrode and an Ag/AgNO₃ reference electrode. The macroelectrode setup was used for all electrochemical experiments.

2.2.3 Sample Preparation

Solutions of both beta-carotene and lutein were prepared in 0.1M tetrabutylammonium hexa-fluorophosphate in dimethyl sulfoxide.

2.2.4 Electrochemical Tests

All tests run in BASi C3 cell stand static cell. Nitrogen was bubbled through the solution to create an anoxic environment. Cyclic voltammograms were run from -0.6 to +0.6 V for 6 cycles. A scan rate of 100 mV/s and max current of 0.001 mA were used. Cyclic voltammograms were created by plotting voltage vs. current.

2.3 Results:

Using the macroelectrode setup (glassy carbon macroelectrode), a serial dilution including a background (0M), high concentrations (250uM, 150uM, 80uM, 40uM, 19uM, and 1uM), and low concentrations (900nM, 500nM, 100nM, 75nM, 25nM) of beta-carotene yielded a cyclic voltammogram with two clear oxidation peaks. The oxidation peaks were seen at 256mV and 420mV (Figure 1). The serial dilution for lutein yielded a cyclic voltammogram with two clear oxidation peaks at 338mV and 412mV (Figure 2).

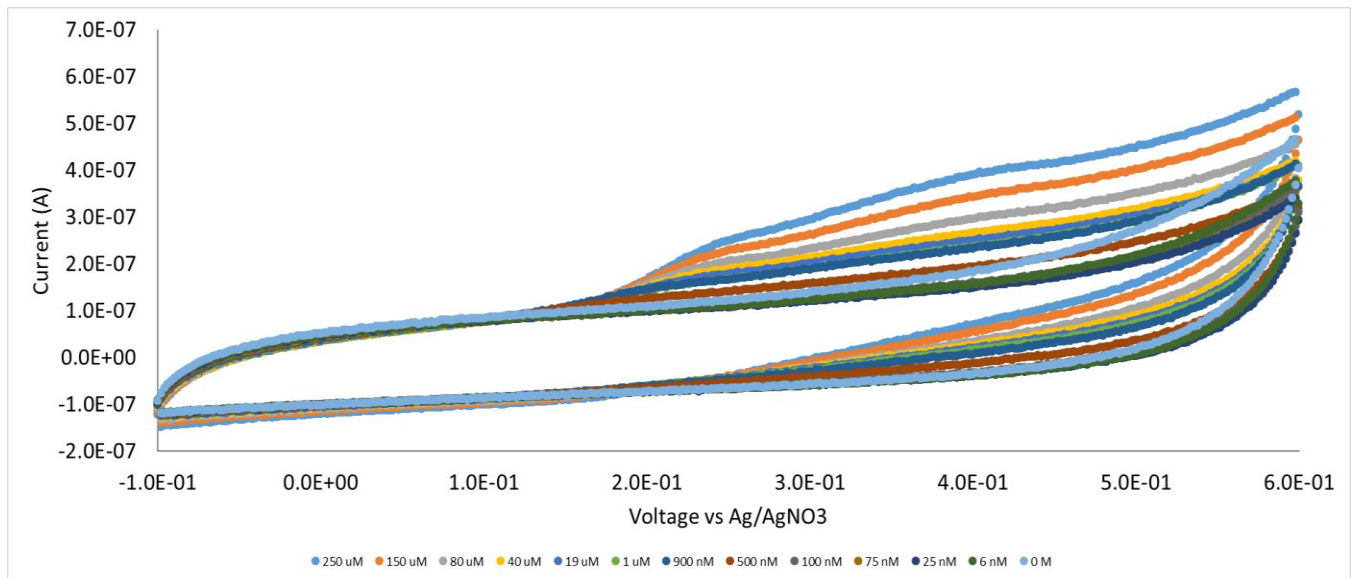


Figure 1. Cyclic voltammogram of beta-carotene serial dilution (see appendix pg 38) using an unmodified glassy carbon macro electrode serial dilution which shows oxidation peaks at +256 mV and +420 mV.

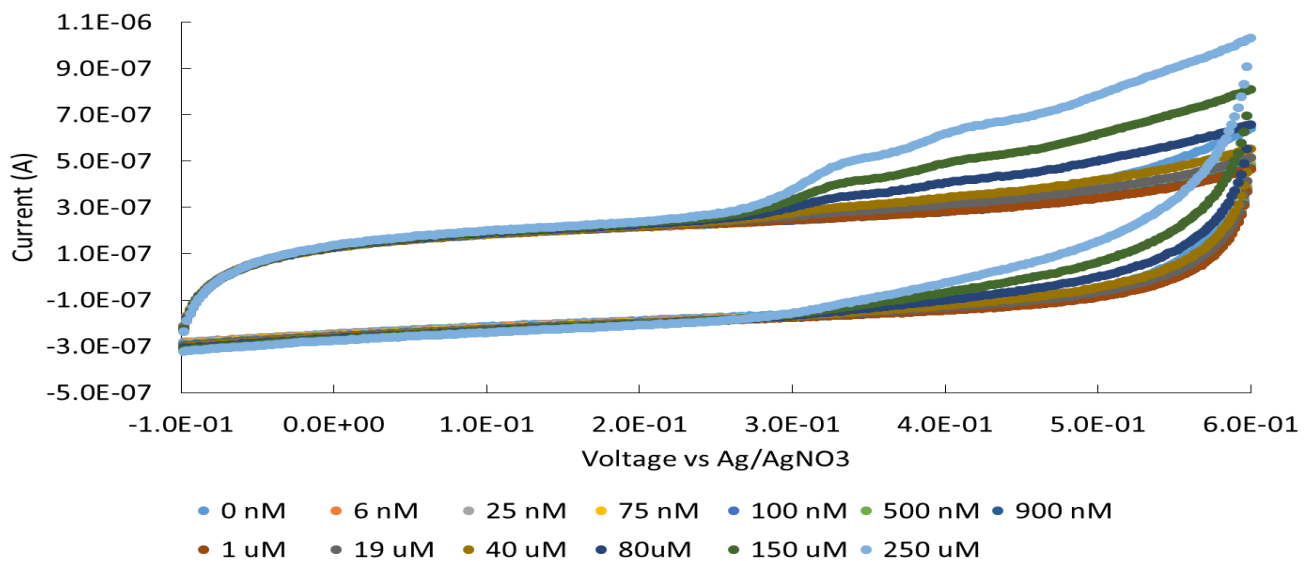


Figure 2. Cyclic voltammogram of lutein serial dilution using a bare glassy carbon macro electrode which shows oxidation peaks at +338 mV and +417 mV.

2.4 Discussion:

Using an unmodified glassy carbon electrode, beta-carotene and lutein were shown to be electrochemically active. Both antioxidants showed very similar electrochemical activity, each with two peaks which were in relatively the same area. The cyclic voltammograms revealed good and consistent calibration ($R^2 > 0.9$) for both antioxidants at high concentrations (923 nM – 231 μ M) (Figure 4 and 6). Poor calibration, for both lutein and beta-carotene, was observed for concentrations less than 923 nM (Figure 5 and 7).

Serum levels of beta-carotene and lutein are on the nanomolar scale. The antioxidant concentration for diseased individuals was 16.6% and 7.9% lower respectively for beta-carotene and lutein when compared to the control (Street et. al 1994). The sensitivity produced by the bare electrode method was not within range to accurately detect low levels of antioxidants in serum on the nanomolar scale (Figure 5 and 7).

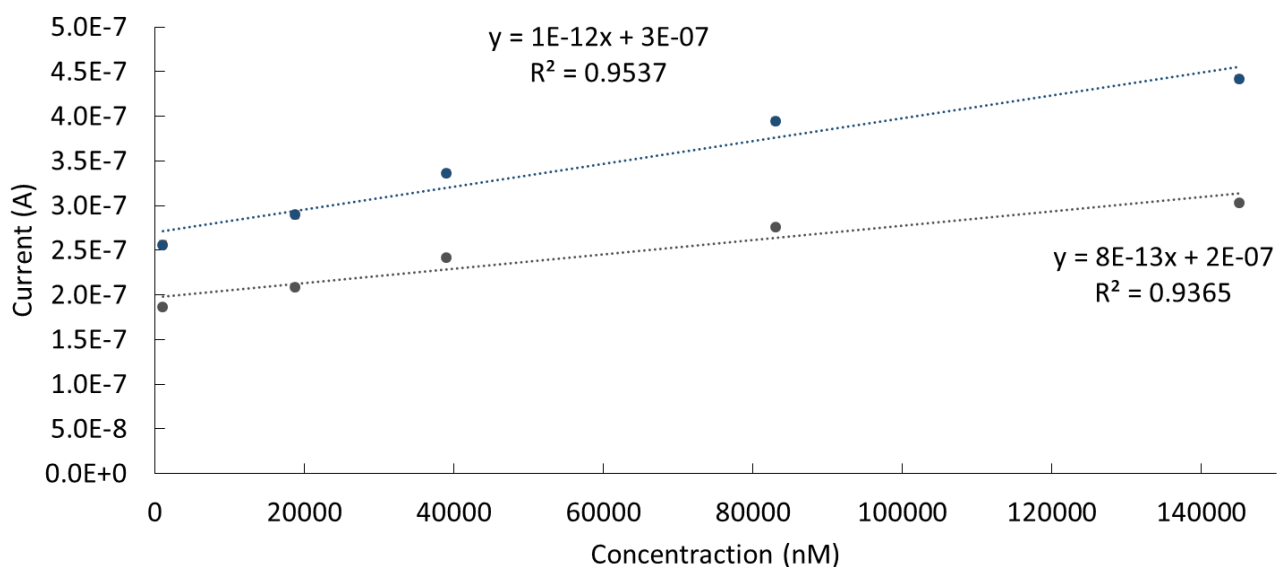


Figure 4

A beta-carotene serial dilution(see appendix pg 38) CV calibration curve at nM concentrations of 5.7, 25.7, 76.4, 103, 513, 923, and μ M concentrations of 1.3, 18.8, 39, 80.3, 145, and 231. Peak voltages were +452mV and +250mV respectively.

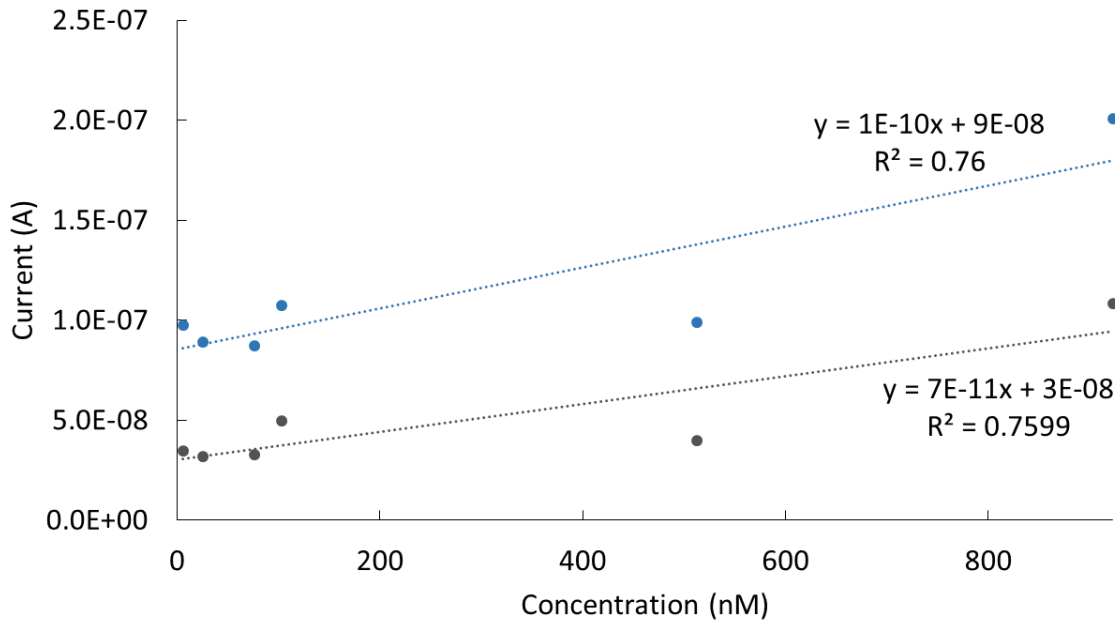


Figure 5.
A beta-carotene serial dilution CV calibration curve at nM concentrations of 5.7, 25.7, 76.4, 103, 513, and 923. Peak voltages were +0.452 V and +0.25 V respectively.

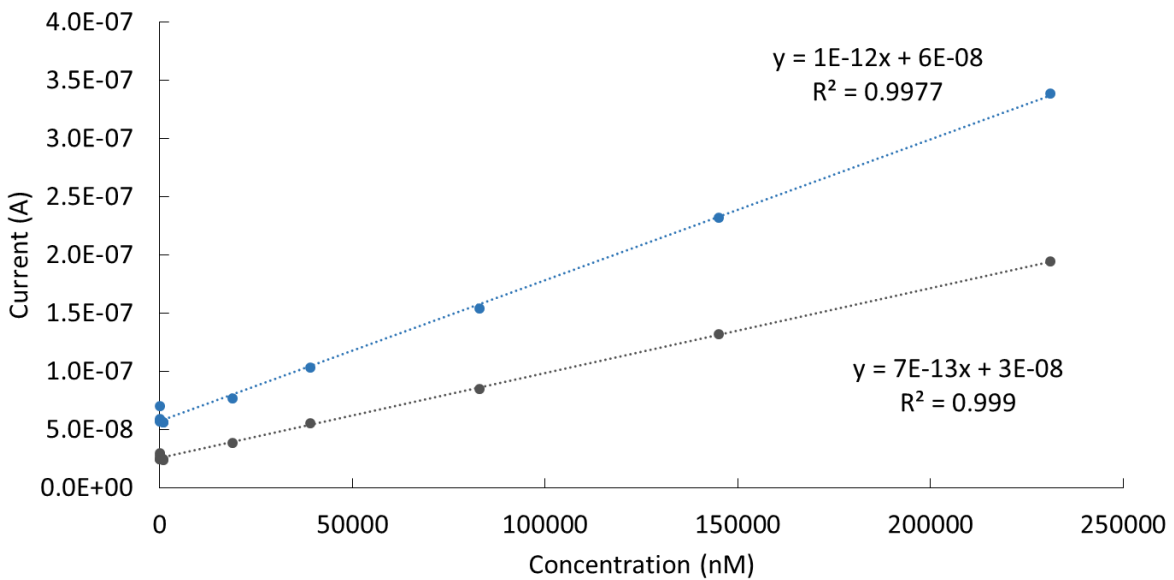


Figure 6.
A lutein serial dilution CV calibration curve at nM concentrations of 5.7, 25.7, 76.4, 103, 513, 923, and μ M concentrations of 1.3, 18.8, 39, 80.3, 145, and 231. Peak voltages were +417mV and 339mV respectively.

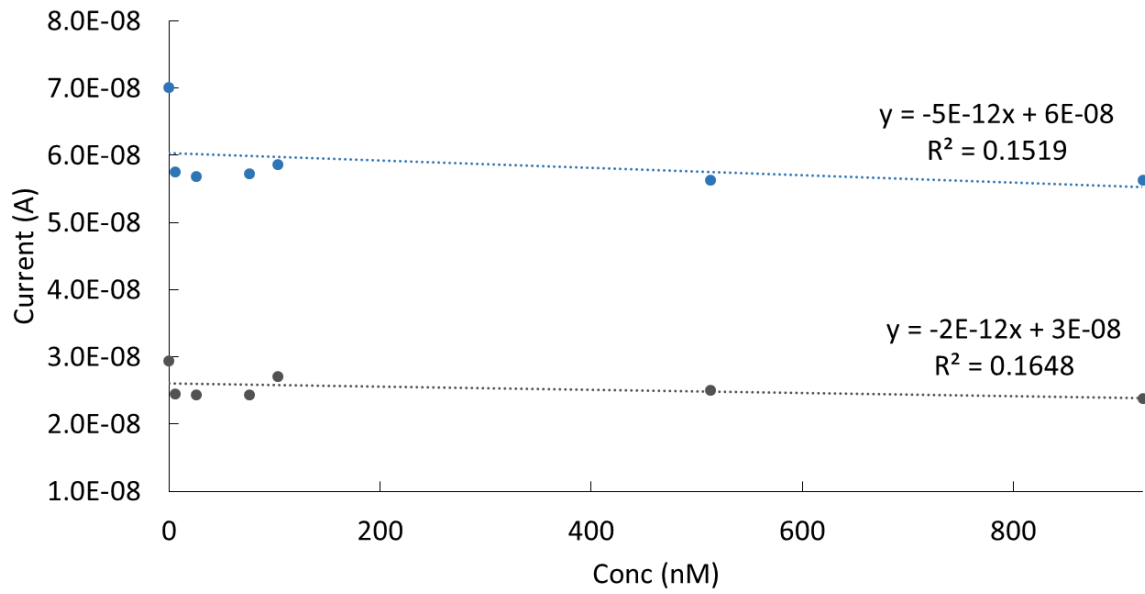


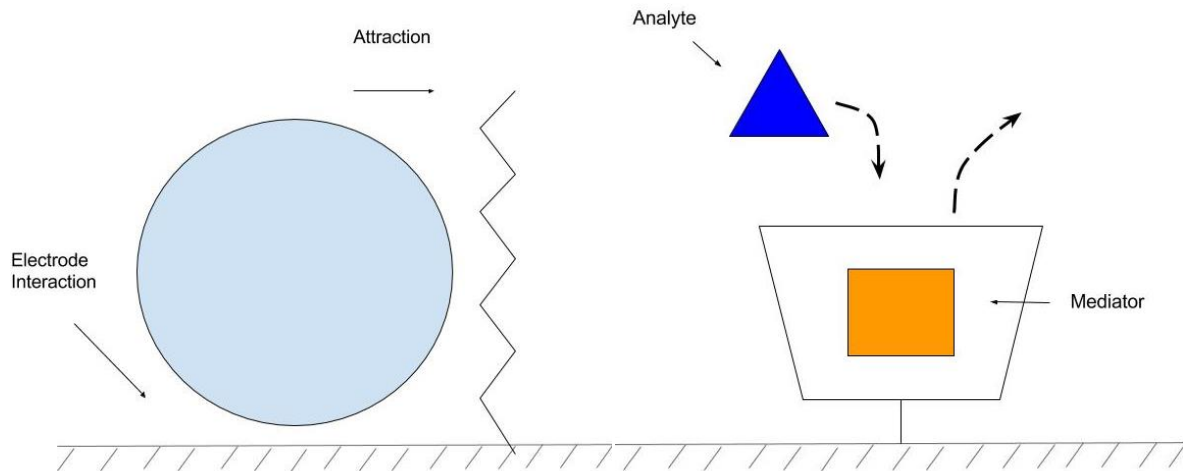
Figure 7

A lutein serial dilution CV calibration curve at nM concentrations of 5.7, 25.7, 76.4, 103, 513, and 923. Peak voltages were 417mV and 339mV respectively.

3 Cyclodextrin Modified Electrodes:

Tests using a bare carbon electrode are limited in their sensitivity. Although both beta-carotene and lutein were detected at unmodified electrodes, the sensitivity was not high enough for serum detection. Electrode surface modifications were performed, with the hopes of increasing sensitivity.

Two surface modification strategies were used within the scope of this thesis. First, surface modifications can serve to attract molecules to the electrode surface (Scheme 2). The attraction allows greater contact between the analyte and the electrode surface which increases sensitivity. Second, surface modification with mediator molecules allows the detection of electrochemically inactive (or low activity) molecules. The use of a mediator facilitates the detection of an electrochemically inactive molecule by serving as a direct or indirect molecule that indicates concentration of the desired analyte (electrochemically inactive molecule) (Scheme 2). The mediator strategy allows surface modification to increase detection possibilities for analytes that are normally undetectable via traditional methods. In one study done by Zang and Gorski it was shown that the use of an azure dye mediator for the detection of NADH amplified current 35x (Zang and Gorski 2005).



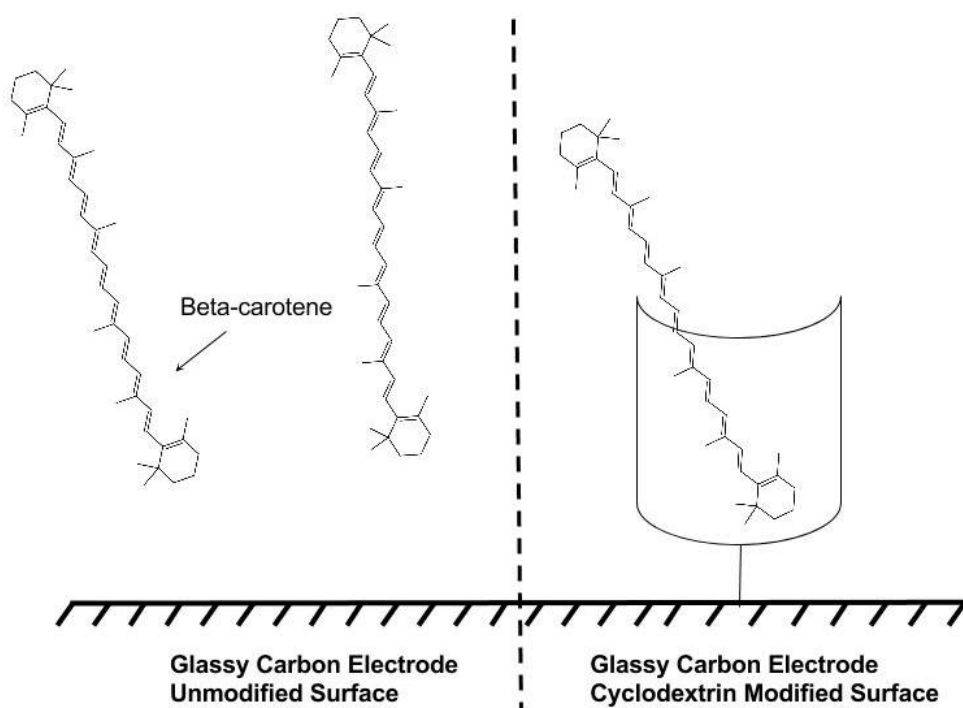
Scheme 2:

A modified electrode surface showing: Left) Surface modifications attract analytes to the electrode surface. Right) Redox active mediators serve to indirectly detect electrochemically inactive molecules. The mediator ,the orange molecule, is being displaced by the blue analyte.

3.1 Surface Modification: Without Mediator

3.1.1 Introduction

In an attempt to increase sensitivity, a glassy carbon electrode was modified with beta-cyclodextrin (Scheme 3). This modification is similar in design to the electrode modification shown on the left of scheme 2. It was theorized that beta-cyclodextrin's large hydrophobic core would attract beta-carotene (which is also large and hydrophobic). Beta-carotene's interaction with the cyclodextrin was thought to increase its interaction with the electrode surface, and therefore, result in improved sensitivity. Molecular modeling studies indicated that beta-cyclodextrin was the most promising of the cyclodextrin species to form complexes with beta-carotene (Bikadi et. al 2006).



Scheme 3.

Left) unmodified glassy carbon electrode interacting with beta-carotene.

Right) a beta-cyclodextrin modified glassy carbon electrode interacting with beta-carotene.

3.1.2 Methods:

3.1.2.1 Reagents

Beta-carotene (97%), and lutein (90%) were purchased from Acros organics.

Beta-cyclodextrin was purchased from Sigma Aldrich. All other reagents are of analytical grade.

The stock solution was dimethyl sulfoxide with 0.1M tetrabutylammonium hexa-

fluorophosphate.

3.1.2.2 Apparatus and measurement methods

All electrochemical tests were run on a Gamry Instruments Reference 600. A

three electrode setup was utilized including a glassy carbon macroelectrode, a platinum counter electrode and an Ag/AgNO₃ reference electrode. Nitrogen was bubbled through the solution to create an anoxic environment.

3.1.2.3 Sample Preparation

Solutions of beta-carotene and lutein were prepared in 0.1M tetrabutylammonium hexa-fluorophosphate in dimethyl sulfoxide.

3.1.2.4 Electrochemical Tests

Refer to section 2.2.4

3.1.2.5 Electrode Preparation

The surface modification procedure was adapted from a paper published by Hernandez et. Al in 2014. A bare glassy carbon macroelectrode was cleaned and polished with alumina powder on a felt pad for over two minutes (see appendix pg 39). The electrode was then washed and placed in a 28.5mM beta-cyclodextrin, 1mM ferrocene, and 0.6M tetrabutylammonium methoxide tetra(n-butyl)ammonium hexafluorophosphate solution and an applied electric field of -0.5V - +0.8V vs Ag/AgNO₃.

Based on the paper, cyclodextrin was covalently bound to the electrode surface through radical chemistry in a manner to create a permanent attachment that could be used multiple times (Hernandez et. al 2014). A standard operating procedure (SOP) was drafted by a Nicholas Cook (a previous Halpern Lab group member, see appendix pg 40), and this SOP was followed for all cyclodextrin attachments unless specified.

3.1.3 Results:

Glassy Carbon electrode modification with beta-cyclodextrin yielded a cyclic voltammogram (Figure 8). Changes in current (see from +0.3 to +0.5 V vs AgNO₃) decreased as the cycles progressed. The difference in current signifies level of surface attachment.

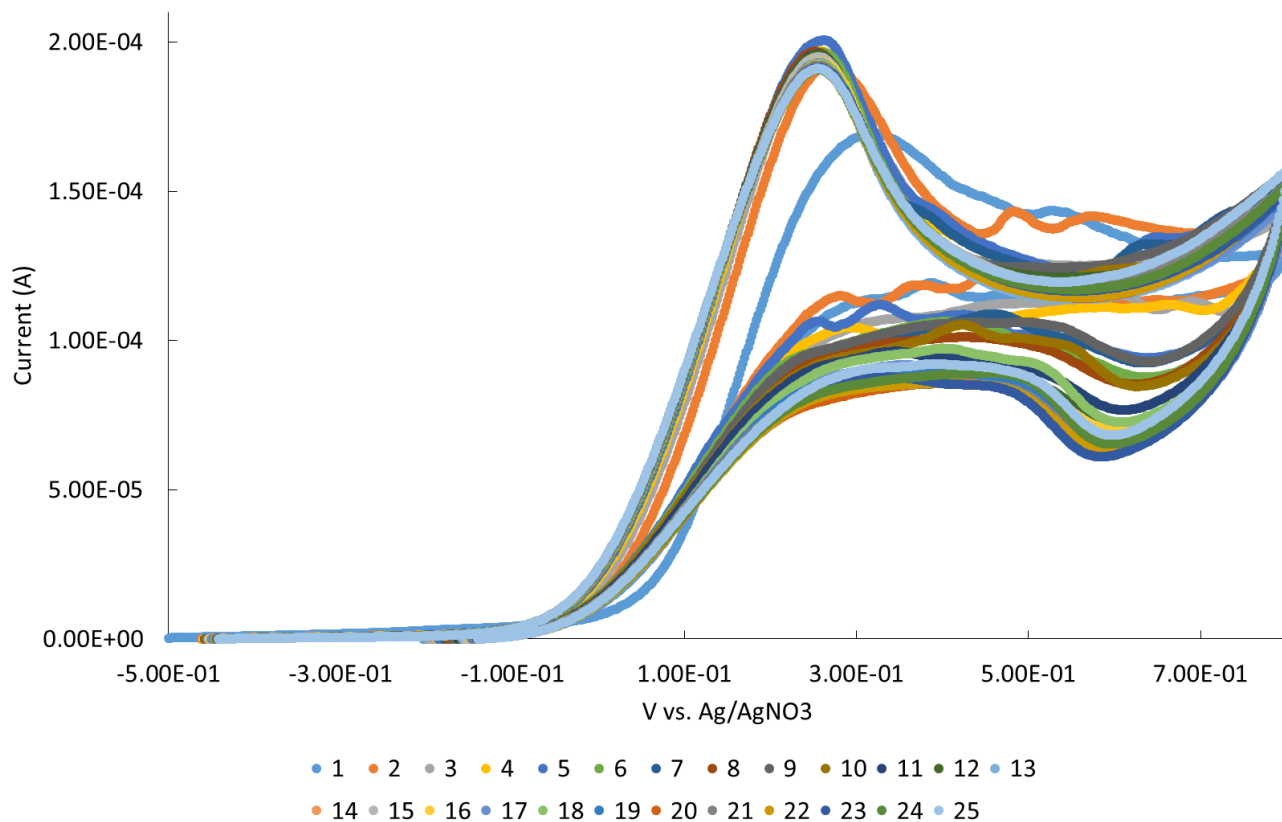


Figure 8

Cyclic voltammogram of beta-cyclodextrin attachment to a bare glassy carbon electrode shows a change in current at differing cycles in the +0.3-+0.7 V vs Ag/AgNO₃.

A serial dilution including both high (uM) and low (nM) concentrations of beta-carotene yielded a cyclic voltammogram with two clear oxidation peaks. The oxidation peaks were seen at +250mV and +920mV (Figure 9). The serial dilution for lutein yielded a cyclic voltammogram with one clear oxidation peak at +412mV (Figure 10).

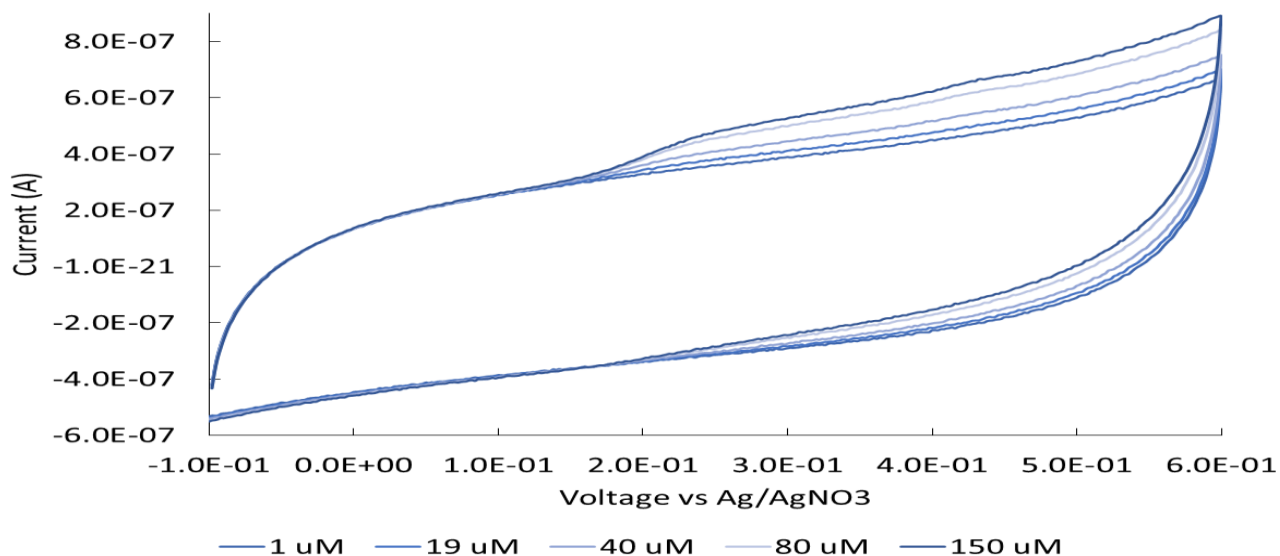


Figure 9
Cyclic voltammogram of beta-carotene serial dilution using beta-cyclodextrin modified glassy carbon electrode.

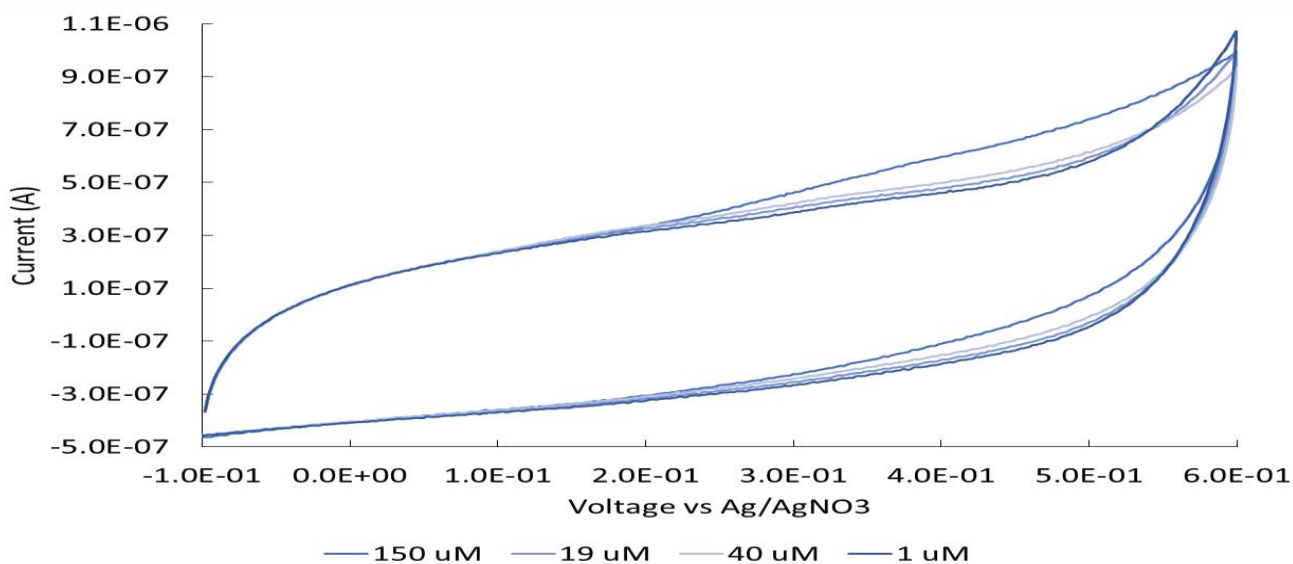


Figure 10
Cyclic voltammogram of lutein serial dilution using a cyclodextrin modified glassy carbon electrode.

3.1.4 Discussion

When compared to the unmodified electrodes, the cyclodextrin modified electrodes showed no improvement in sensitivity (Figure 10). The lutein dilution showed only one peak

(+412mV), unlike the two peaks noted on the lutein serial dilution with the unmodified glassy carbon electrode (Figure 10). Furthermore the second beta-carotene peak (920mV) was significantly shifted to the right ($\Delta 500$ mV) (Figure 9).

The cyclic voltammograms revealed good and consistent calibration ($R^2 > 0.93$) for both antioxidants at high concentrations (923nM - 231 μ M) (Figure 11 and 14). Poor calibration was shown for concentrations less than 923 nM (Figure 12). Furthermore no significant difference was noted between the cyclodextrin modified and unmodified glassy carbon electrode calibration curves for both antioxidants (Figure 13 and 15).

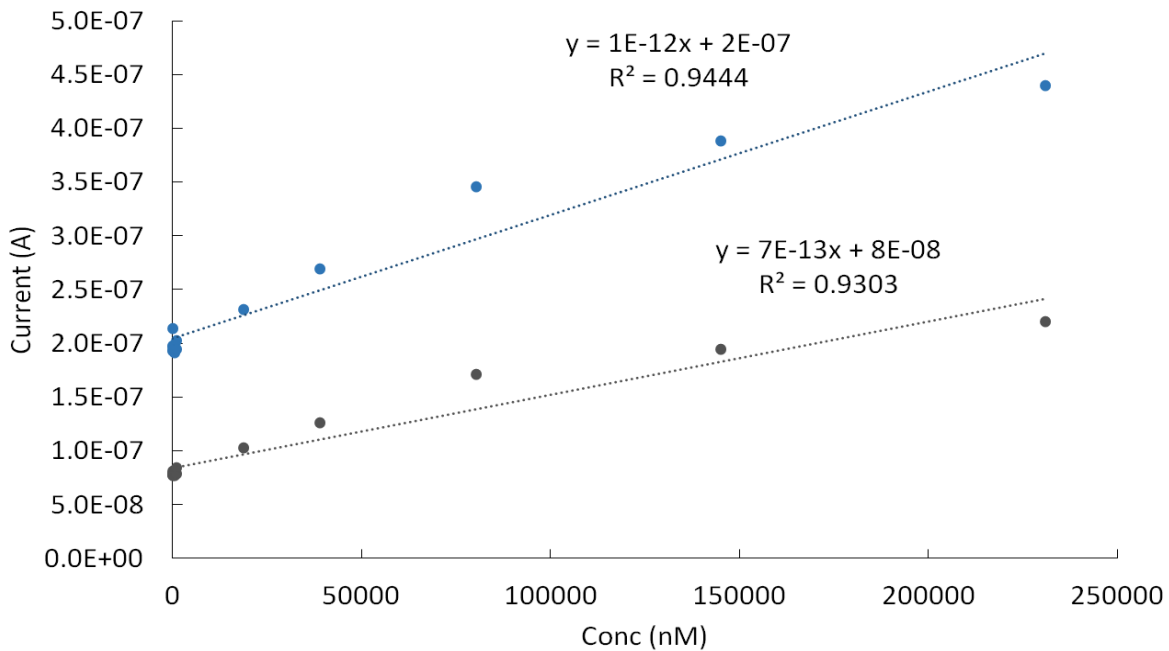


Figure 11. A beta-carotene serial dilution with a beta-cyclodextrin modified glassy carbon electrode calibration curve at nM concentrations of 5.7, 25.7, 76.4, 103, 513, 923, 1,030, 18,800, 39,000, 83,000, 145,000, and 231,000. Peak voltages were +0.942V and +0.25V respectively.

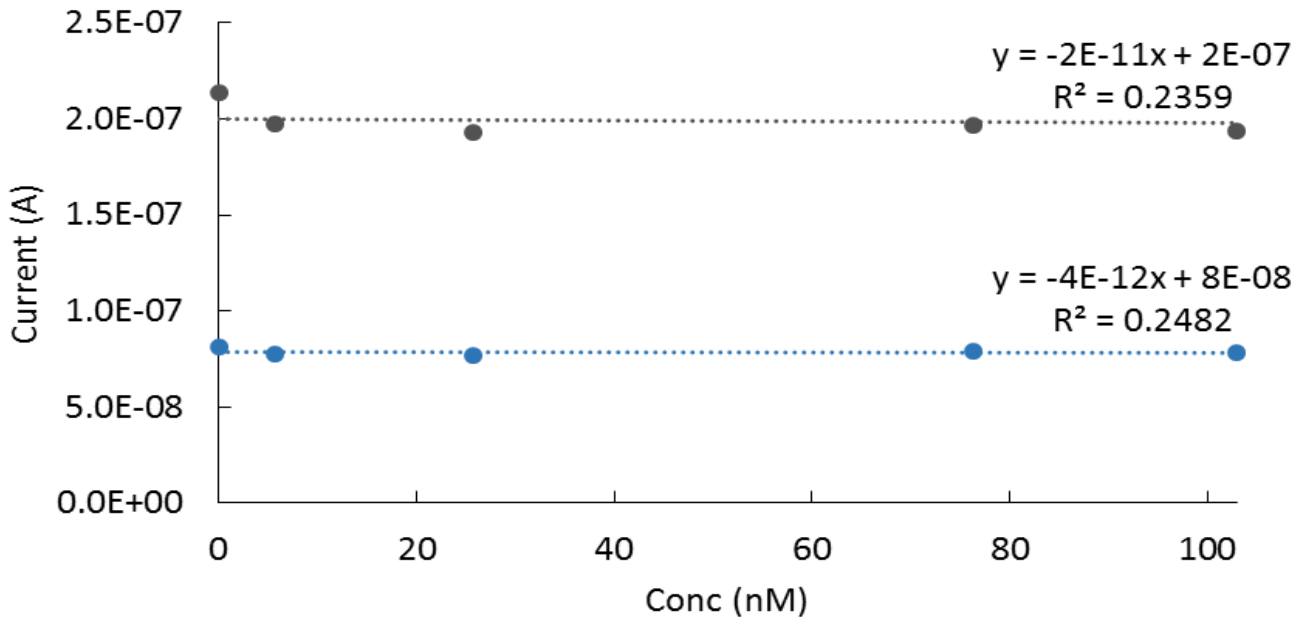


Figure 12. A beta-carotene serial dilution with a beta-cyclodextrin modified glassy carbon electrode calibration curve at nM concentrations of 5.7, 25.7, 76.4, and 103. Peak voltages were +0.942V and +0.25V respectively.

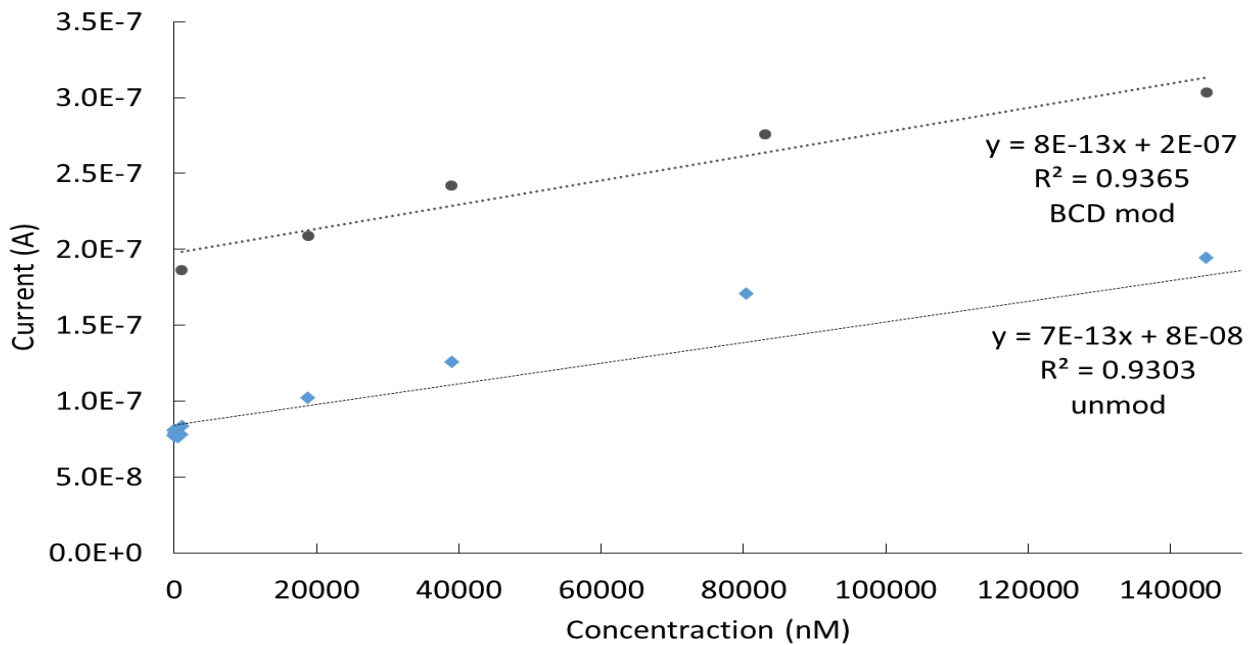


Figure 13. Comparison beta-carotene serial dilution calibration curve for a beta-cyclodextrin modified glassy carbon electrode and an unmodified glassy carbon electrode at nM concentrations of 5.7, 25.7, 76.4, 103, 513, 923, 1,030, 18,800, 39,000, 83,000, and 145,000. Peak voltage was +0.25V.

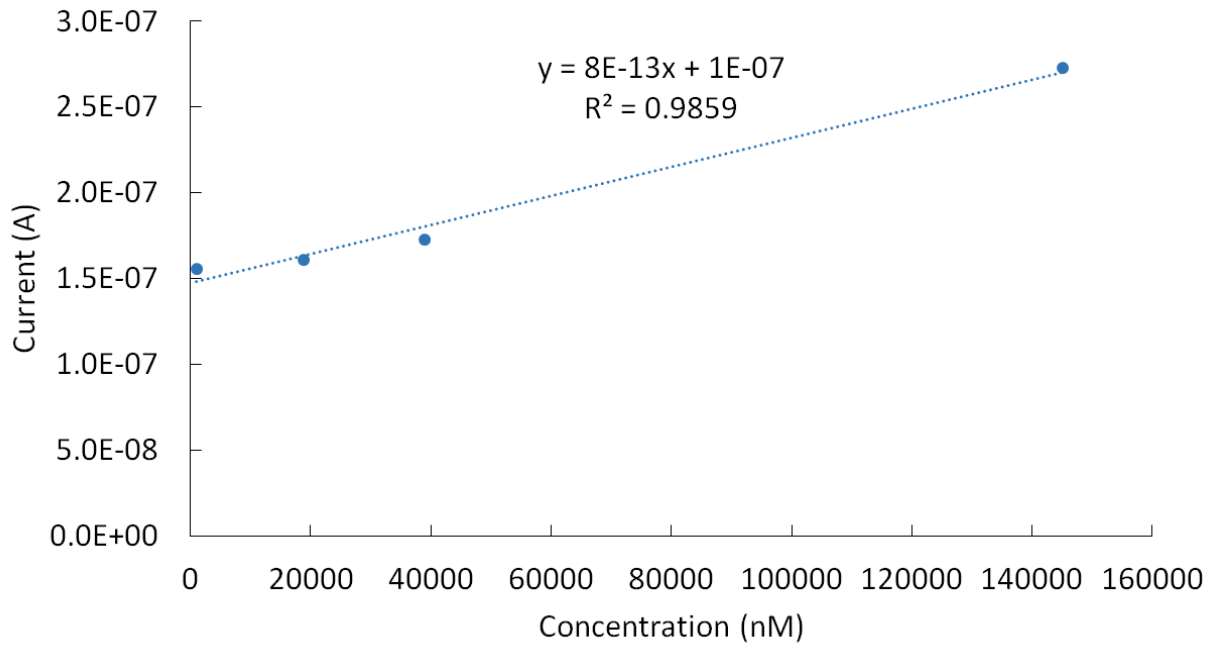


Figure 14. A lutein serial dilution with a beta-cyclodextrin modified glassy carbon electrode calibration curve at nM concentrations of 1,030, 18,800, 39,000, and 145,000. Peak voltage was +0.412V.

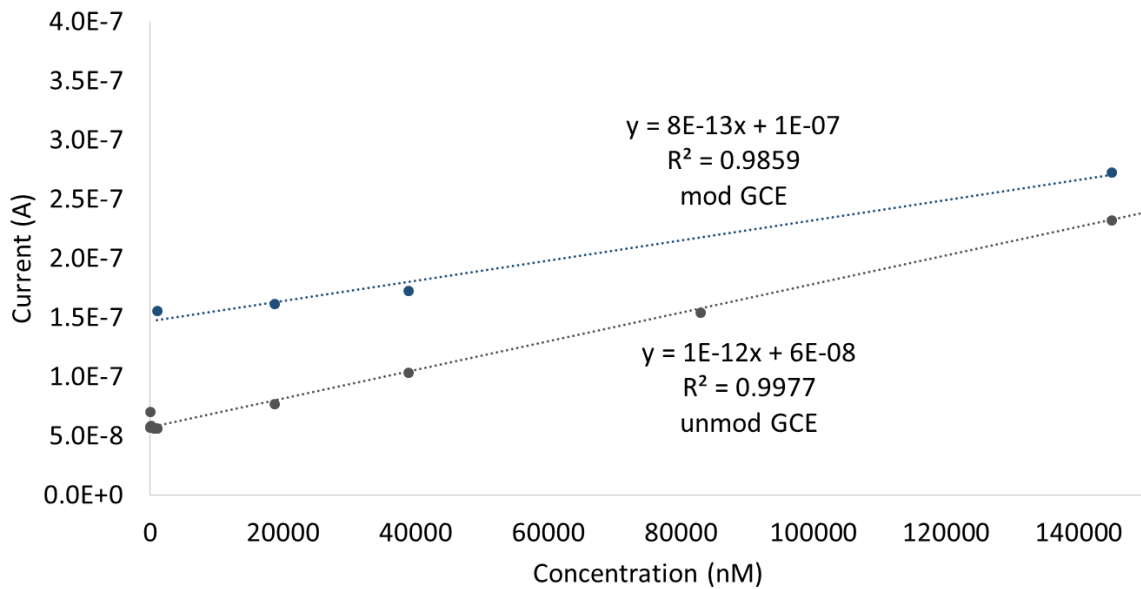
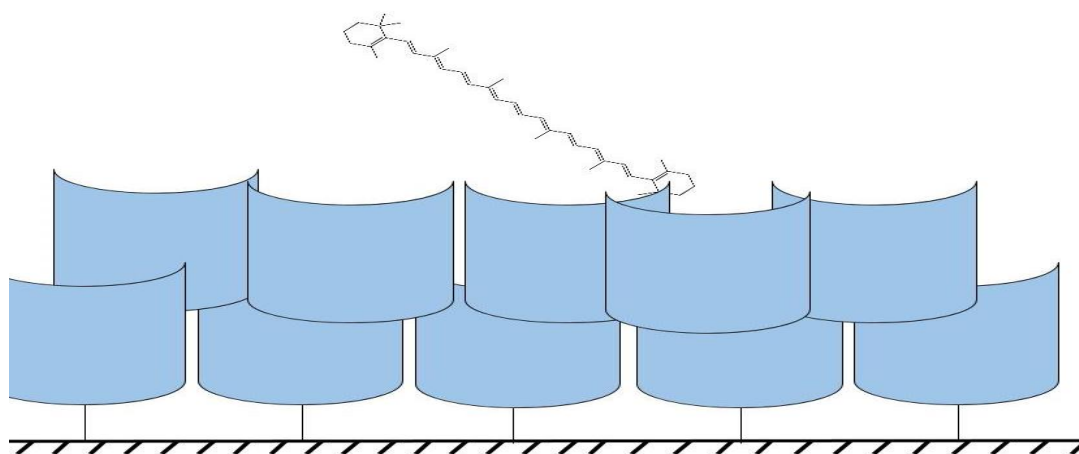


Figure 15 Comparison lutein serial dilution calibration curve for a beta-cyclodextrin modified glassy carbon electrode and an unmodified glassy carbon electrode. Peak voltage was +0.412V.

We theorize that the lack of sensitivity increase was due to beta-carotene's decreased interaction with the electrode surface caused by cyclodextrin pocket interactions. Furthermore Bikadi et. al showed that cyclodextrin can self-aggregate. A layering of cyclodextrin molecules could have occurred (Scheme 4). This would have further exacerbated the decreased interaction between antioxidant and electrode surface therefore reducing sensitivity.



Scheme 4.
Cyclodextrin laying on the electrode surface.

3.2 Surface Modification: With a Mediator

3.2.1 Introduction

In addition to plain surface modification, there are further ways to increase electrode sensitivity. The use of a mediator facilitates the detection of an electrochemically inactive molecule by serving as a direct or indirect molecule that indicates concentration of the desired analyte (electrochemically inactive molecule). Mediators are often highly electrochemically active molecules that can be easily detected at very low concentrations. The use of a redox active mediator as a means of detecting a particular molecule is extremely promising as many analytes are not highly electrochemically active (Scheme 2). Furthermore this method has the potential to

increase selectivity as well due to the fact that specific reactions such as custom formatted immunoglobulin reactions can be designed.

3.2.1.1 Detection of Cholesterol

Previously published work has shown that methylene blue can act as a redox active mediator for the detection of cholesterol on a cyclodextrin modified graphene sheet (Agnihotri et. al 2015). Agnihotri et. al proposed that graphene, modified by cyclodextrin, will house methylene blue molecules, within the cyclodextrin core, and the methylene blue elutes from the cyclodextrin core as it is replaced by cholesterol. Methylene blue, a redox active analyte, is then detected in solution. The amount of methylene blue that is detected is thought to be in direct proportion to the amount of cholesterol added. This allows the creation of a calibration curve to determine a predictive relationship between concentration and current. Figure 15 shows an attempt to replicate the Agnihotri et. al experiment with a beta-cyclodextrin modified platinum electrode. The replication attempt revealed good sensitivity, showed a reduction peak as opposed to an oxidation peak found in the published data. It is thought that the scan range published in Aginhotri et al is actually positive to negative as opposed to negative to positive as published.

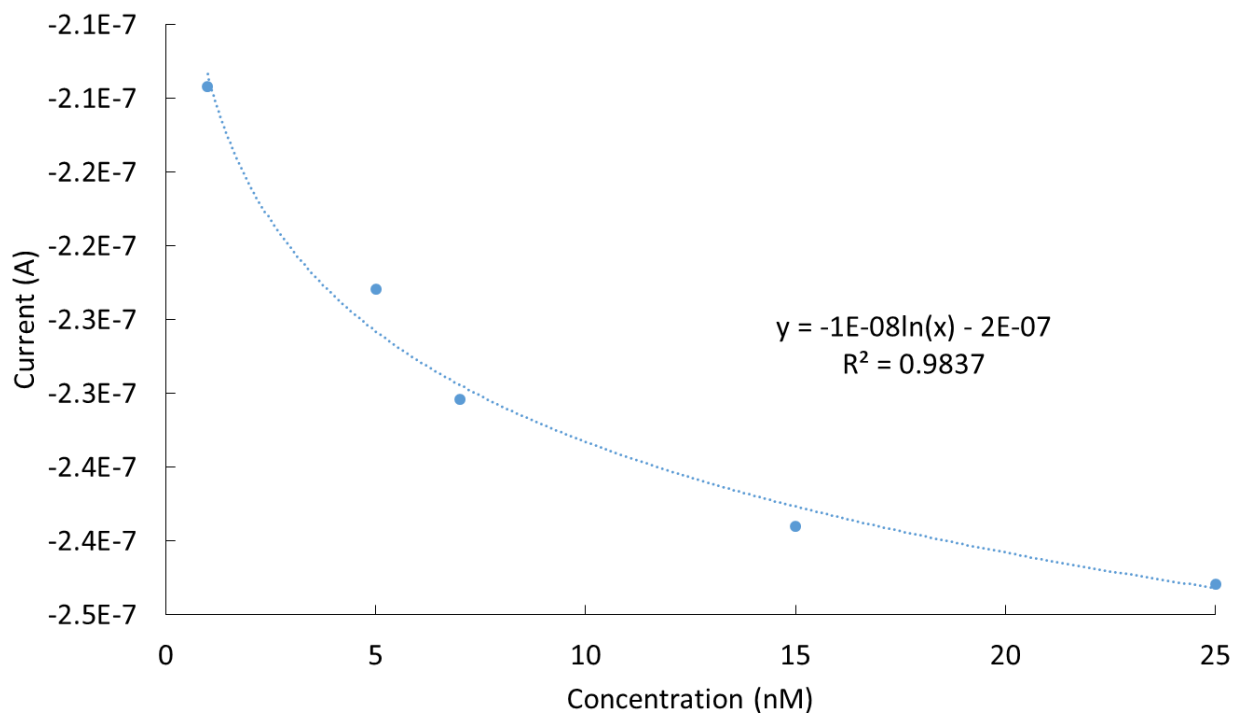


Figure 16. Serial dilution DPV attempt to mimic Agnihotri et. al. Nanomolar concentrations at 1, 5, 7, 15, and 25. Peak voltage was 210mV vs. Ag/AgCl.

Cholesterol serial dilutions measured with a MB-CD sensor showed that the cholesterol calibration curve was dependent of the ethanol solvent used to solubilize cholesterol (Figure 17). Ethanol, a potent solvent, caused methylene blue to elute from the cyclodextrin independent of cholesterol. Because the analyte sensitivity is dependent on the solvent, MB-CD electrodes might be an inaccurate sensor approach when ethanol is used to solubilize the analyte.

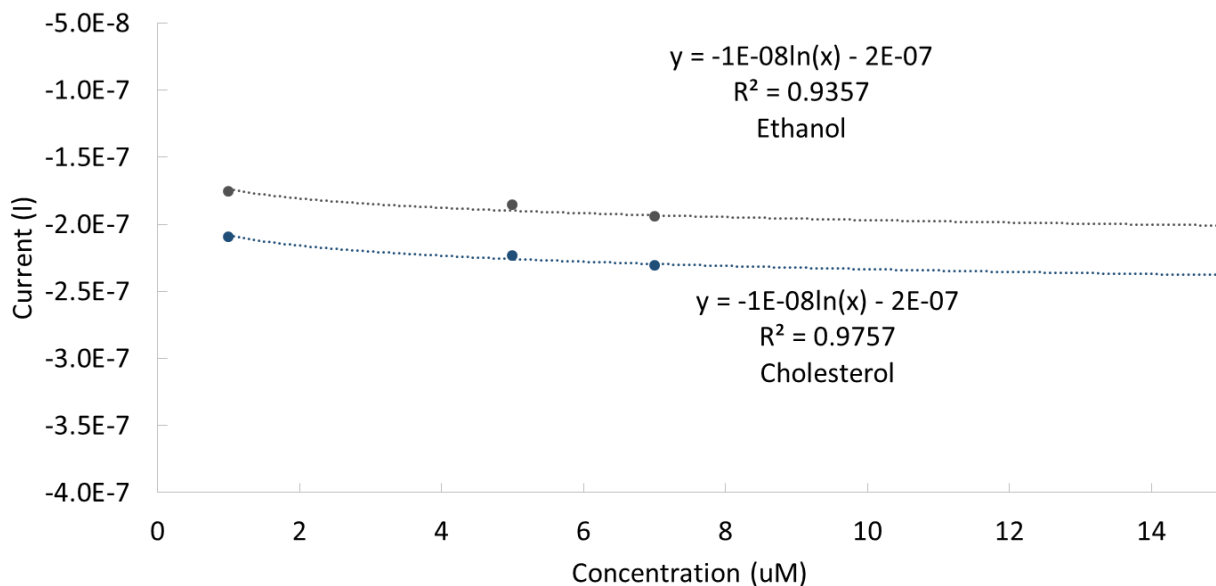


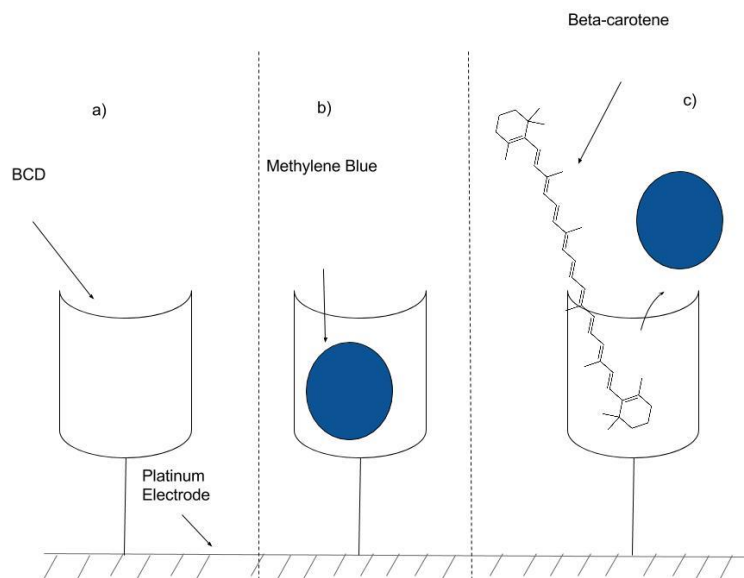
Figure 17.

Overlaid calibration curves of cholesterol and ethanol serial dilutions using the same ethanol volumes. No Significant difference is seen between the two. This was done by adding μL ethanol volumes (0, 9.52, 38.2, 19.2, and 78) which corresponds to μM cholesterol concentrations (1, 5, 7, and 15). Peak voltage was 210mV.

3.2.1.2 Detection of Beta-Carotene with MB-CD sensors

In an attempt to modify the work performed by the Agnihotri group, methylene blue was used as a redox mediator for the detection of beta-carotene on a beta-cyclodextrin modified platinum electrode. In accordance with the principles of the Agnihotri experiment, it was believed the large hydrophobic core of cyclodextrin would provide housing for methylene blue molecules after the electrode was soaked in a solution of methylene blue. Once the electrode was placed in a PBS solution and exposed to beta-carotene, it was theorized that the methylene blue would elute from the core as it was replaced by beta-carotene (due its higher attraction and tendency to complex with beta-cyclodextrin) (Scheme 5). The methylene blue that eluted would be in direct proportion to the beta-carotene added (Scheme 5). The methylene blue would be

measured electrochemically, thus creating a viable means of detecting nM levels of beta-carotene (via a proportional relationship).



Scheme 5

A) a beta-cyclodextrin modified electrode, B) a beta-cyclodextrin modified electrode housing methylene blue in its core, and C) a beta-cyclodextrin modified electrode with methylene blue exposed to beta-carotene.

3.2.2 Methods:

3.2.2.1 Reagents

See 3.1.2.1 Reagents

3.2.2.2 Apparatus and measurement methods

All electrochemical tests were run on a Gamry Instruments reference 600. A three electrode setup was utilized including a beta-cyclodextrin modified platinum electrode, a platinum counter electrode and an Ag/AgCl reference electrode. Stirring occurred in between electrochemical tests.

3.2.2.3 Sample Preparation

A 10 mM Methylene blue solution was prepared to soak electrodes. Solutions of beta-carotene and lutein were prepared in a 1% tween 20/PBS solution (tween acted as a solvent for the antioxidants). Phosphate buffer saline was used as the stock solution for the serial dilution.

3.2.2.4 Electrochemical Tests

All tests run in BASi C3 cell stand static cell. Cyclic voltammograms were run from +0.55 to -0.4 V. A max current of .003 mV, frequency of 10 Hz, and pulse size of 25mV were used. The solution was stirred between dilutions. Square wave voltammograms (SWV) were created by plotting voltage vs. current.

3.2.2.5 Electrode Preparation

A bare platinum macroelectrode was cleaned and polished with alumina powder (.05 μm) on a felt pad for over two minutes (see appendix pg 39). After the electrode was polished its surface was modified with beta-cyclodextrin. To combat self-aggregation the number of attachment cycles was reduced to one and the n-Bu₄NPF₆, ferrocene, and beta-cyclodextrin solutions were reduced by a factor of ten (refer to appendix pg 40).

3.2.3 Results

A cyclic voltammogram was produced during the attachment of beta-cyclodextrin to a platinum electrode surface (Figure 18).

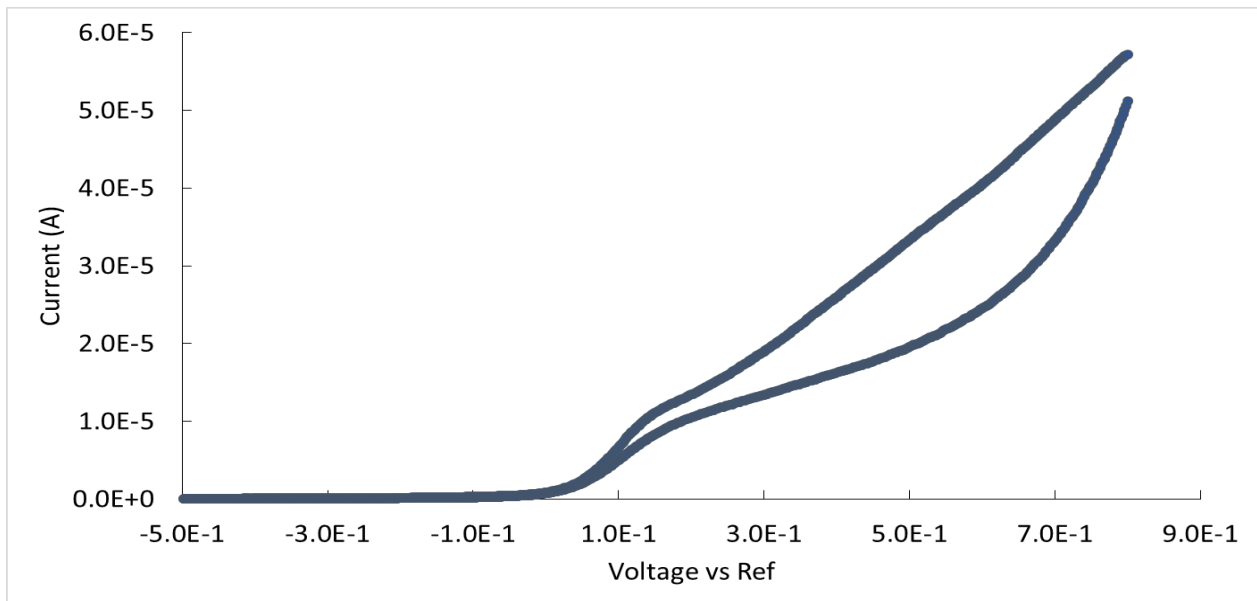


Figure 18.
Cyclic voltammogram of the attachment of beta-cyclodextrin to a platinum electrode

A serial dilution with beta-carotene using the beta-cyclodextrin/methylene blue modified electrode yielded a square wave voltammogram with peaks at -33 mV and +79 mV vs. Ag/AgCl (Figure 19). Peaks were clear at all concentrations (including the background).

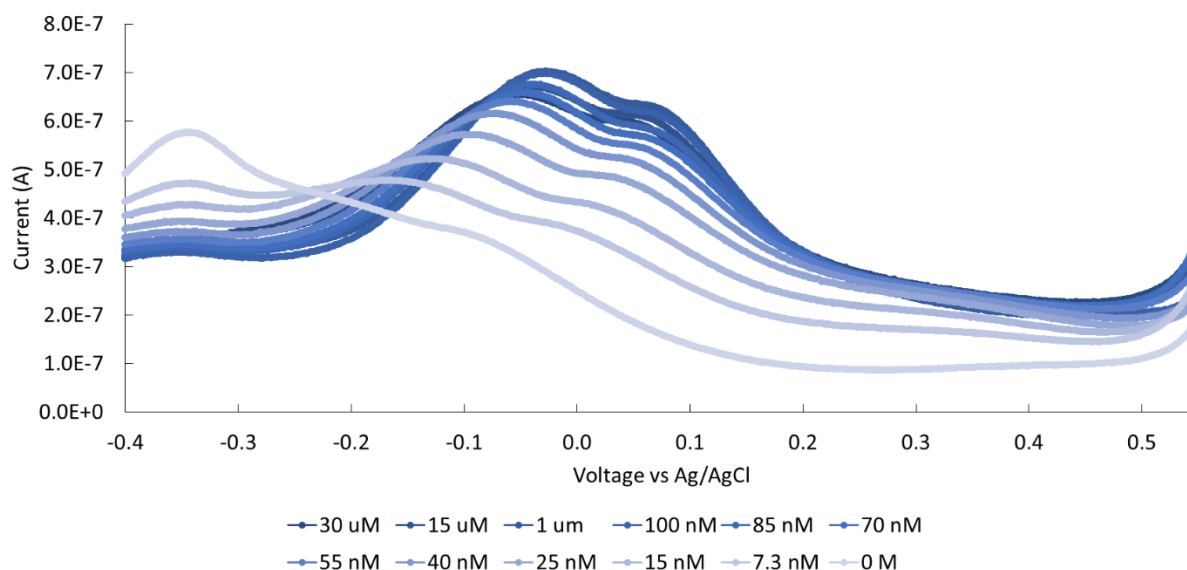


Figure 19. SWV of beta-carotene serial dilution with a beta-cyclodextrin methylene blue modified platinum electrode. Peaks are observed at -33mV and +72mV vs. Ag/AgCl.

3.2.4 Discussion

For the beta-cyclodextrin methylene blue modified electrode setup, square wave (SWV) was shown to have better calibration than cyclic voltammetry. Due to the fact that SWV was a significantly quicker and more sensitive test, it was used for all further electrochemical tests.

Both glassy carbon and platinum electrodes were tested. Initial results showed platinum electrodes to be more promising. All further testing occurred with platinum electrodes.

The initial serial dilutions of beta-carotene with a beta-cyclodextrin/methylene blue modified platinum electrode showed more sensitive and repeatable results when compared to the

unmodified electrode. The calibration was logarithmic, but was able to consistently detectable levels of beta-carotene on the low (<20) nanomolar scale (Figure 20).

The peaks (-33mV and +72 mV) detected were significantly shifted from beta-carotene peaks (+256mV and +420mV) detected via the unmodified glassy carbon electrode set-up. This was not of concern because surface modification change the oxidative properties of the electrode surface.

Further study of MB-CD serial dilutions, showed that the beta-carotene calibration curve was independent of beta-carotene. A 1% Tween 20/PBS serial dilution revealed nearly identical data compared to the beta-carotene dilution (Figure 20). Tween 20 is such a potent solvent that it could cause methylene blue to elute from the cyclodextrin independent of beta-carotene.

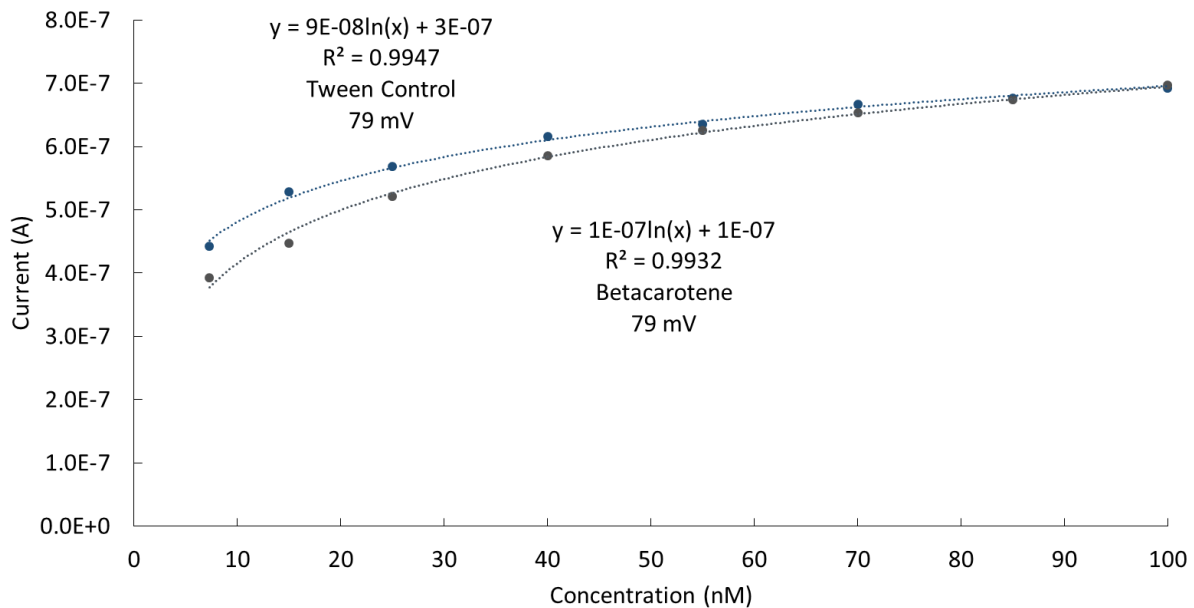
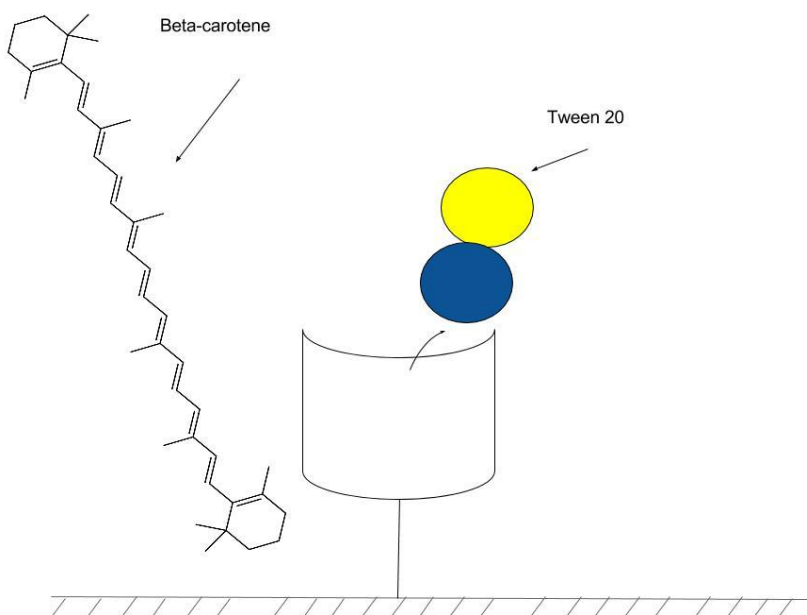


Figure 20
Comparison of the beta-carotene and tween calibration curves. Nanomolar concentrations at 7.3, 15, 25, 40, 55, 70, 85, and 100. Peak voltage was 3mV.



Scheme 6

A beta-cyclodextrin modified platinum electrode with methylene blue house in its core being exposed to a tween/beta-carotene solution. It is shown that tween is pulling methylene blue out of the cyclodextrin not beta-carotene.

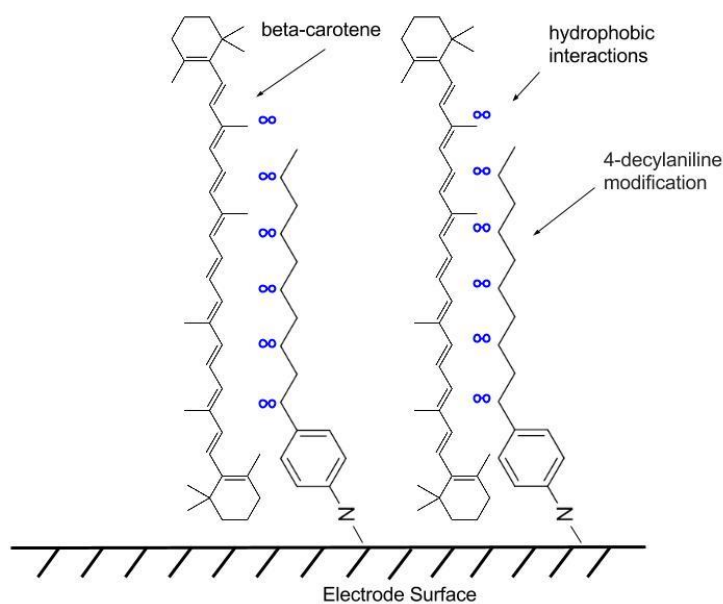
The lack of specificity for this test is not shocking as the attempts to replicate the Aginhotri et. al experiments yielded similar findings. This mediator method of electrode modification would only work in when the desired analyte is soluble in phosphate buffer saline and has a higher association constant with cyclodextrin than methylene blue. Experiments using other solvents such as dimethyl sulfoxide (DMSO) and ethanol failed. Unfortunately antioxidants require strong solvents such as tween, DMSO, and ethanol.

4 Decylaniline Modified Electrode

4.1 Introduction

In a final attempt to increase sensitivity, another modification was attempted. Both a glassy carbon and platinum electrode were modified with a decylaniline compound. The modification was intended to increase surface area interaction and therefore increase sensitivity.

The theory was that decylaniline, which is structurally similar to beta-carotene, would attract beta-carotene to the electrode surface (Scheme 7). The increased interaction at the electrode surface was theorized to increase sensitivity.



Scheme 7

An electrode surface is covalently modified with decylaniline. The decylaniline will recruit beta-carotene to the electrode surface and therefore increasing interaction.

Beyond increasing sensitivity, Roy et. al showed that a decylaniline modification can produce a more specific response to analytes that are in an acidic solution (Roy et. al 2003). It was shown that dopamine and ascorbic acid peaks were separated by over 300 mV with the decylaniline modified electrode (Roy et. al 2003). Under unmodified electrode conditions, the

two peaks were interfering with each other (Roy et. al 2003). This is applicable to beta-carotene as it is found in serum with many other compounds that could potentially interfere with its electrochemical signal.

4.2 Methods:

4.2.1 Reagents

Beta-carotene (97%) was purchased from Acros organics. 4-Decylaniline (97%) was purchased from Alfa Aesar. Acetate buffer was produced in house to a pH of 3 (See appendix pg 42). All other reagents are of analytical grade.

4.2.2 Apparatus and measurement methods

All electrochemical tests were run on a Gamry Instruments reference 600. A three electrode setup was utilized including a 4-decylaniline modified glassy carbon electrode, a platinum counter electrode and an Ag/AgCl reference electrode. Stirring occurred in between electrochemical tests.

4.2.3 Sample Preparation

Solutions of beta-carotene were prepared in a 1% tween/Acetate buffer solution (tween acted as a solvent for the antioxidants). Acetate buffer was used as the stock solution for the serial dilution (refer to appendix pg 42).

4.2.4 Electrochemical Tests

All tests run in BASi C3 cell stand static cell. Cyclic voltammograms were run from -0.2 to +0.6 V vs Ag/AgCl. A max current of .003 mA, frequency of 10 Hz, and pulse size of 25mV were used. Solution was stirred between dilutions. Square wave voltammograms (SWV) were created by plotting voltage vs. current.

4.2.5 Electrode Preparation

A bare glassy carbon macroelectrode was cleaned and polished with alumina powder on a felt pad for over two minutes. After the electrode was washed its surface was

modified with 4-decyylaniline. A standard operating procedure was drafted by Elnaz Mohammadi and was followed for this attachment (see appendixpg 43)

4.3 Results

Decylaniline attachment to a glassy carbon electrode yielded a three cycle cyclic voltammogram (Figure 21).

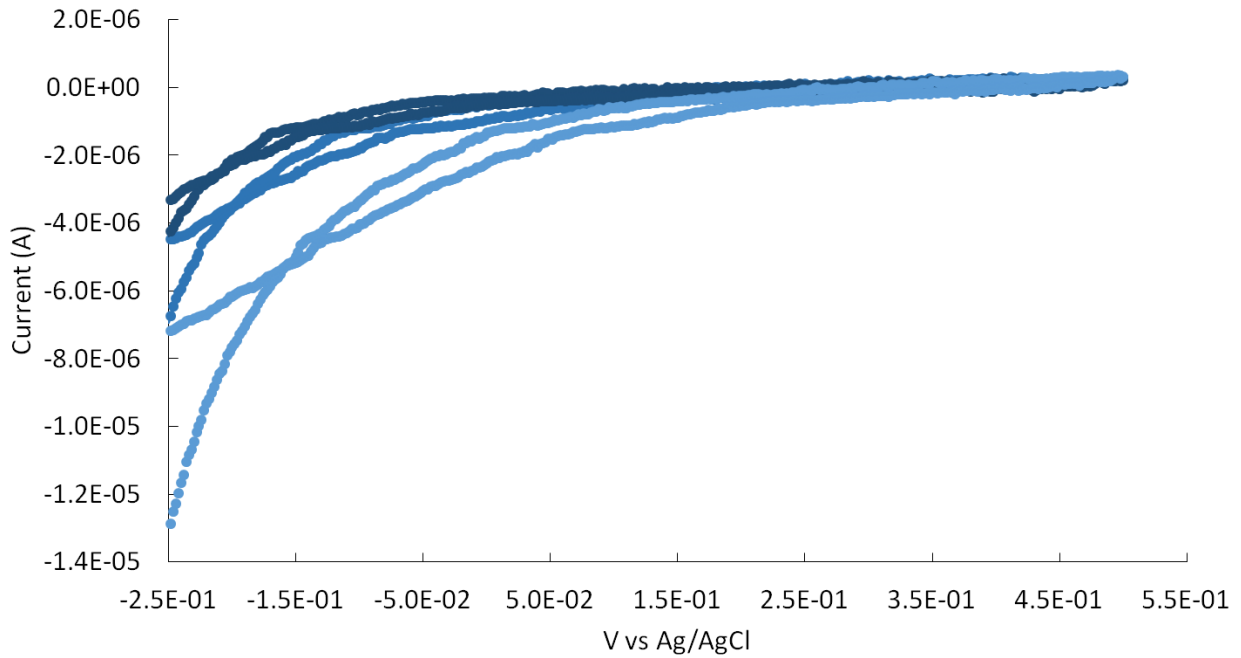


Figure 21
Cyclic voltammogram of the attachment of 4-decyylaniline to a glassy carbon electrode.

A serial dilution including both high (1uM, 3uM, and 5uM) and low (25nM, 75nM, 100nM, 250nM, 500nM) concentrations of beta-carotene yielded a square wave voltammogram with no clear oxidation peaks (Figure 22).

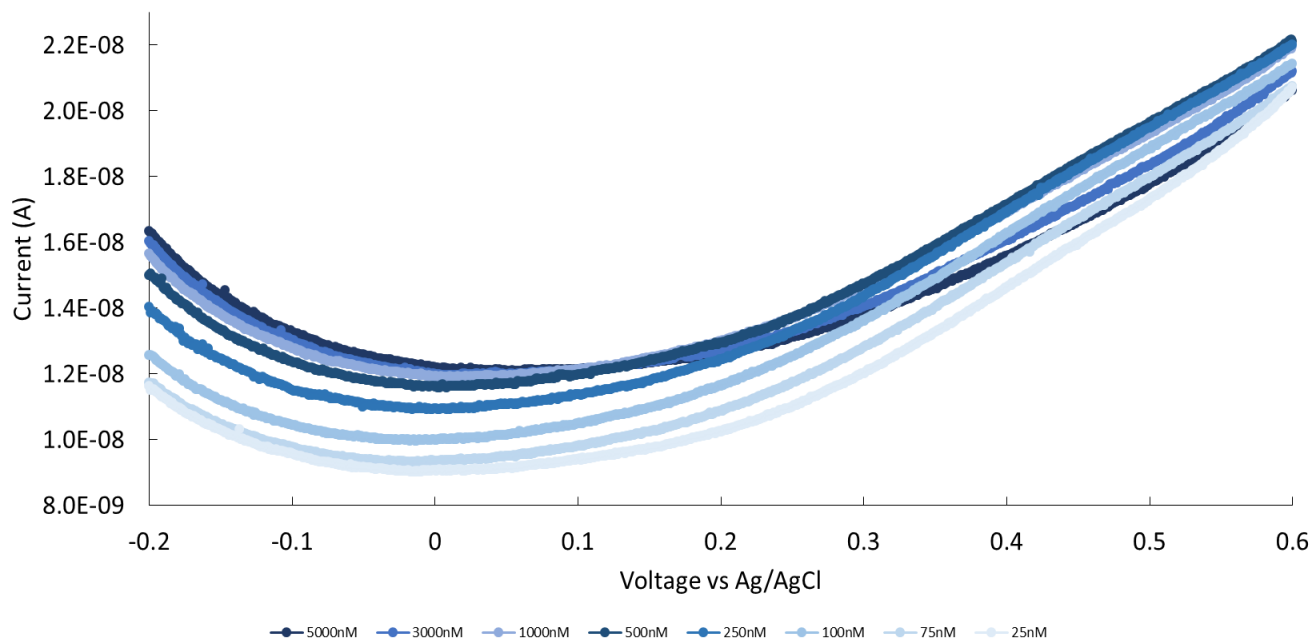


Figure 22
SWV of a decylaniline modified glassy carbon macroelectrode beta-carotene serial dilution with no obvious peaks.

4.4 Discussion

Testing with an acetate buffer stock solution (to minimize pH affects) revealed no detectable peaks. Without clear electrochemical activity no correlation could be made between concentration and current. A calibration curve was created using the +420 mV site due to the fact beta-carotene showed a peak there with the unmodified electrode. Small correlation between current and concentration existed, but the results were nearly identical to that of a tween/acetate control dilution (Figure 23).

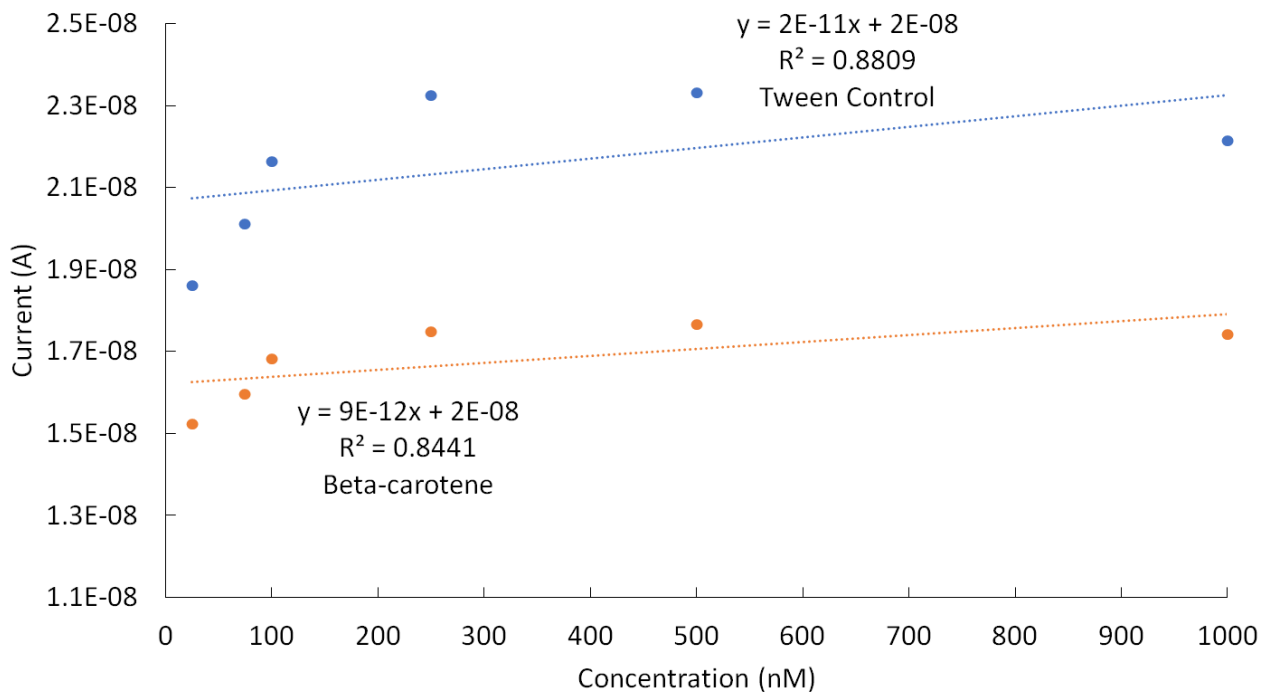


Figure 23

A calibration curve for a beta-carotene serial dilution with a decylaniline modified glassy carbon electrode. Nanomolar concentrations at 35, 75, 100, 250, 500, and 1000. Peak voltage was +420mV.

5 Future Work

The above mentioned body of work has demonstrated that carotenoids are electrochemically active. Their presence can be detected at high (μM) concentrations. The work failed to detect antioxidants at low concentrations similar to serum levels. Of the techniques attempted, the only successful method proved to be the unmodified glassy carbon electrode. No surface modification techniques were successful. To continue pursuing the creation of a point of care electrochemical sensor for antioxidants future work must be completed.

Fast scan cyclic voltammetry (FSCV) is thought to provide a more sensitive test. Microelectrode's can operate under higher scan rates. Higher scan rates are shown to increase the current signal (Yue et. al 2012). With higher outputted currents, a higher sensitivity was thought

to be achieved. A macroelectrode system outputs lower currents which produces similar results that thought to be less sensitive.

Initial attempts were made at this method, but due to excessive noise and inconsistent data FSCV was abandoned in favor of cyclic voltammetry (CV) using a macro glassy carbon electrodes. It is thought that the noise issues could be due to inconsistencies in the in-house manufacturing process of the microelectrodes or due to the fact that stirring occurred constantly during the electrochemical tests (see appendix pg 46). Later experiments showed that constant stirring led to excessive noise under certain electrode conditions. Future tests will be run in attempt to utilize FSCV as a viable method for detecting antioxidants.

My role in this project has ended. All future work will be performed by current and future members of the Halpern Lab group. Future work will continue to explore detecting antioxidants using electrodes both unmodified and surface modified..

The need for a point of care serum sensor of carotenoids is great and the benefits are evident. The initial ground work had been laid for future Halpern Lab members to further towards the creation of a serum sensor.

6. Citations:

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Images:

Scheme 1.

a) <http://www.caslab.com/Chemical-Search/Chemical-Structure/7235-40-7.gif>

b) <http://img.guidechem.com/pic/image/6811-73-0.gif>

7. Appendix:

Beta-Carotene Serial Dilution		MW	weight needed (mg)	Stock Volume (ml)			
Solution		536	N/A	10			
Stock Solution 1 (uM): 51.4		536	8.82	8.2			
Stock Solution 1 (uM): 2000							
	Added Volume (uL)	Added Volume (L)	Concentration of Added Volume (M)	Volume in cell	mol	Concentration in Cell	Conc. in Cell uM
0	1000	1.00E-02	0	0.01000	0	0.00E+00	0
1	1.1	1.10E-06	5.14E-05	0.01000	5.65E-11	5.65E-09	0.0057
2	3.9	3.90E-06	5.14E-05	0.01001	2.57E-10	2.57E-08	0.0257
3	9.9	9.90E-06	5.14E-05	0.01001	7.65E-10	7.64E-08	0.0764
4	5.1	5.10E-06	5.14E-05	0.01002	1.03E-09	1.03E-07	0.103
5	80.8	8.08E-05	5.14E-05	0.01010	5.18E-09	5.13E-07	0.513
6	82.2	8.22E-05	5.14E-05	0.01018	9.40E-09	9.23E-07	0.923
7	20.8	2.08E-05	5.14E-05	0.01020	1.05E-08	1.03E-06	1.03
8	91.4	9.14E-05	0.002	0.01030	1.94E-07	1.88E-05	18.8
9	106	1.06E-04	0.002	0.01040	4.06E-07	3.90E-05	39.0
10	223	2.23E-04	0.002	0.01062	8.53E-07	8.03E-05	80.3
11	372	3.72E-04	0.002	0.01100	1.60E-06	1.45E-04	145
12	532	5.32E-04	0.002	0.01153	2.66E-06	2.31E-04	231

Electrode Polishing Instructions

Garrett Thompson

How to Polish a Glassy Carbon Electrode

1. remove the micro-cloth disk (should be pink in color)
2. rinse the electrode with DI water then methanol, wipe dry
3. wet the micro-cloth disk with DI water
4. add two drops of the alumina suspension onto the center of the cloth
 - a. should be a milky white color
5. add a drop of alumina to the GC surface
6. place the electrode face down onto the pad
 - o apply gentle pressure
 - o go in a figure 8 motion
 - change direction frequently
7. once done polishing clean with distilled water
8. if there is residual debris on the tip sonicate the electrode
9. rinse with methanol and dry

Cyclodextrin Attachment Standard Operating Procedure:

Standard Operating Procedure
Electrochemical Grafting of β -CD to Carbon Fiber surface
Nicholas M. Sweeney Cook
October 22, 2014

Objective:

β -cyclodextrin (β -CD) functionalized Carbon Fibers (CFs) are prepared for use as electrodes in a chemical analyte sensor. The functionalization is achieved by the mediated oxidation of β -CD monoanions by ferrocenium in the presence of a carbon surface. The functionalized CFs will be electrochemically tested.

Safety Considerations:

Equipment:

Chemicals:

β -cyclodextrin (β -CD) $\geq 99\%$ $C_{42}H_{70}O_{35}$

MSDS: <http://www.sigmaaldrich.com/catalog/product/sigma/c4767?lang=en®ion=US>

CAS#: 7585-39-9

Tetrabutylammonium methoxide 20%*** in methanol

MSDS: <http://www.sigmaaldrich.com/catalog/product/aldrich/86906?lang=en®ion=US>

CAS#: 34851-41-7

Tetrabutylammonium hexafluorophosphate (TBHFP) $\geq 99\%$

MSDS: <http://www.sigmaaldrich.com/catalog/product/fluka/86879?lang=en®ion=US>

CAS#: 3109-63-5

Nitrogen gas (5.0 grade) - N_2

MSDS: <https://www.airgas.com/msds/001004.pdf>

CAS#: 7440-37-1

Ferrocene (Fc) 98%

MSDS: <http://www.sigmaaldrich.com/catalog/product/aldrich/f408?lang=en®ion=US>

CAS#: 102:54-5

Dimethyl sulfoxide (DMSO) $\geq 99\%$

MSDS: <http://www.sigmaaldrich.com/catalog/product/sigma/d8418?lang=en®ion=US>

CAS#: 67-68-5

PPE:

- Eye goggles
- Nitrile Gloves
- Fume hood
- Lab coat

Other

- Lab Computer
- Cyclic voltammetry software
- Universal potentiostat
- Carbon fiber electrodes
- Platinum electrode
- Ag/AgNO₃ electrode
- 250-mL beaker
- Round bottom electrochemical flask
- 5.0 N₂ gas cylinder
- Squeeze wash bottle

Procedure

Preparation of solutions

- **Make a 10mL 250mM BCD 100mM n-Bu₄NPF₆ solution in DMSO.**
 - o Place 0.3874 g. n-Bu₄NPF₆ in a 10 mL volumetric Flask
 - o Fill flask with approximately 5 mL DMSO.
 - o Mix until dissolved
 - o Add 2.8375 g BCD to make a 1 M solution
 - 2.4321g if ACD
 - 3.2428g if GCD
 - o Fill to about 7 mL total
 - o Mix until dissolved
 - o Fill up to 10 mL
- **Prepare a concentrated ferrocene solution in DMSO (75 mM)**
 - o Add 0.1395 g ferrocene to a 10 mL volumetric flask
 - o Fill 5 mL of DMSO
 - o Mix until dissolved
 - o Fill to 10 mL
- **Dilute the previous two solutions to achieve 28.5 mM CD and 1 mM ferrocene in 10 mL DMSO in a electrochemical cell**
 - o Add .206 mL concentrated BCD solution
 - o Add 0.026 mL concentrated Fc mixture
 - o Add 9.76 mL 0.1 M n-Bu₄NPF₆ in DMSO

Electrochemical Grafting

- Add 0.5 mL 0.6 M tetrabutylammonium methoxide in methanol
- Set up c3 cell stand with CFE WE, Pt CE, and a Ag/AgNO₃ RE
- Purge with 5.0 N₂ for 10 min
- Scan at 0.1 V/s 1 (fill in accordingly) cycles from -0.5 V to +0.8 V while stirring at 250 RPM in the c3 cell stand. Multiple CF electrodes can be run with the same setup. All variables should be recorded in the notebook. Variables can include the CD concentration, type of CD, scan rate, scan window, and number of scans.

Analysis

- Review the electrochemical signal and observe a decrease in the ferrocene reaction.
- Perform FSCV on functionalized electrode with a hormone known to bind to CD

Acetate Buffer Standard Operating Procedure
Garrett Thompson
April 12, 2016

Goal: Creation of an acetate buffer solution pH of 3.

Equipment:

NO Micropipeters are to be used

- Gloves
- Goggle
- Lab coat
- 500 ml flask
- 100 ml flask
- 10 ml flask
- Large graduated cylinder
- Small graduated cylinder

Glass pippeters

Chemicals:

Acetic Acid, 99.7+%, A.C.S. Reagent

CAS 64197

SDS: <https://cems.unh.edu/unh/CEMS/index.html#4>

Sodium Acetate, 99+%, A.C.S. Reagent

CAS 127093

SDS: <https://cems.unh.edu/unh/CEMS/index.html#7>

Preparation of 100 ml .1M acetic acid

- Measure 100 ml of HP water in 100 ml flask
- Slowly add .58 ml of acetic acid to the PBS

Preparation of 10 ml .1M sodium acetate

- Weigh 82 mg of sodium acetate
 - Place in 10 ml flask
- Add 10 ml of HP water to the flask

Preparation of pH 3 acetate buffer

- Measure 98.23 ml of .1M acetic acid
 - Place in 100 ml flask
- Measure 1.77 ml .1M sodium acetate
 - Place in 100 ml flask

Experimental Design: DA attachment

Elnaz Mohammadi

July 1st, 2015

Garrett Thompson

March 25, 2016

Hypothesis:

Confirm that direct modification of a carbon electrode can occur with an aminophenyl group via *in situ* fabrication of the diazonium salt in an acidic aqueous media.

Approach:

According to Lyskawa and Belanger 2006, an aniline can be attached to a gold surface via *in situ* diazonium salt fabrication and electrochemical grafting. 4-bromoaniline will be used because of the distinct bromine peak in XPS. The experiment in Lyskawa and Belanger will be retested and tested with a carbon surface. As a proper control, an electrode will be inserted into the solution with no applied potential. Therefore 4 surfaces will be tested

<u>Surface</u>	<u>Expected XPS Result</u>
Gold (control)	No bromine present.
Gold (test)	Bromine present
Carbon (control)	No bromine present
Carbon (test)	Bromine present.

Equipment:

- Potentiostat
- Computer
- Cyclic voltammetry software
- One-compartment electrochemical cell with three electrode configuration
- Reference electrode Ag/AgCl (saturated KCl)
- Platinum counter electrode
- Two Gold wires
- Two carbon foil sheets
- Fume hood
- 25-mL shot glass
- 10 mL volumetric flask
- Stir bar for electrochemical experiments

- Goggles
- Lab Coat
- Gloves

Chemicals:

4-n 4-n Decylaniline, 98+% (C₁₆H₂₇N)

CAS#: 37529-30-9

MSDS: file:///C:/Users/Biosensor%20lab/Downloads/PrintMSDSAction.pdf

Gold wire, 0.05 mm (0.002in) diameter, Premion 99.999% (metals basis)

CAS#: 7440-57-5

Carbon foil,

Ethyl alcohol (CH₃CH₂OH)

CAS#: 64-17-5

MSDS:

<http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=16368&brand=SIAL&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsial%2F16368%3Flang%3Den>

Sodium nitrite (NaNO₂)

CAS #:7632-00-0

<http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=237213&brand=SIAL&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fsearch%3Finterface%3DAI%26term%3Dsodium%2Bnitrite%26N%3D0%26focus%3Dproduct%26lang%3Den%26region%3DUS>
[Trifluoroacetic acid anhydride, TFAA \(Aldrich\)](#)

Hydrochloric acid (HCl)

CAS#: 7647-01-0

MSDS: <http://www.sigmaaldrich.com/catalog/product/sial/320331?lang=en®ion=US>

Acetone

Electrode Preparation

- Polish (see SOP above)

Solution preparation for in situ reduction of diazonium:

1. Clean all glassware that will be used and rinse 5 times with ultra-high pure water. Place in the oven to dry. Do not use unless completely dried.
2. Add 8.84 mg Sodium nitrite (NaNO_2) to have equal equivalents of 4-Bromoaniline and NaNO_2
3. Weigh 29.17 mg (32 μ L) of 4-n Decylaniline (Molecular weight: 233.39) and place in a 25 mL volumetric flask (5mM)
4. Place 25 ml of 0.5M HCl in the volumetric flask. Mix until dissolved.
5. Transfer approximately 15-20 mL immediately into a shot glass and place in the BASi Faraday cage under N_2 purge.
6. Purge for 5 min of N_2 while the rest of the cell is hooked up:
 - Working electrode: Pretreated platinum or carbon electrode
 - Reference electrode: Ag/AgCl
 - Counter electrode: Platinum electrode
7. Using cyclic voltammetry technique, use the following settings:
 - 100 mV/s scan rate
 - Initial voltage +0.5 V
 - Peak voltage -0.25 V
 - 3-cycles
 - PStat off
 - Max Current = .6 mA
8. After modification, ultrasonic the electrode in ultra-high pure water for 5 min.
9. 5 min of ultrasonic cleaning in acetone
10. Repeat steps 5-9 for carbon. Repeat steps 5 and 8-9 for both gold and carbon as a control. The control should not be connected to potentiostat, but should be placed in the solution for 2 min.
11. Rinse with excessive water, UHP and Isopropanol

Standard Operating Procedure
Manufacturing of Carbon Fiber Electrodes
Garrett Thompson
May 18, 2015

Objective:

The objective of this SOP is to provide a standard manufacturing process for carbon fiber electrodes used in electrochemical analysis of analytes which pass over the carbon fiber surface.

Chemicals:

m-Phenylenediamine

MSDS: <https://cems.unh.edu/unh/CEMS/index.html#5>

CAS# 108-45-2

Epon Resin 828

MSDS: <https://cems.unh.edu/unh/CEMS/index.html#7>

CAS# 25068-38-6

Chemical Safety Considerations

- m-Phenylenediamine
 - eye irritation
 - toxic if swallowed
- Epon Resin 828
 - Eye, skin, and respiratory irritation
 - Non-toxic if ingested
- Analyte
 - Varied depending on analyte and solution
 - Refer to specific analyte and solution MSDS and take the appropriate cautions

Safety Considerations:

- small glass capillaries pose a hazard when they are broken
 - handle with caution while wearing gloves and safety glasses
- dispose of broken glass into a marked and approved sharps container
- pipette puller contains extremely hot coil when active use caution to avoid the coil at all times
- use standard caution when cutting electrodes
- if cut with broken glass: remove the glass from the tissue and sanitize wound with appropriate agent and cover the wound

PPE

Throughout this procedure gloves, safety goggles, and a lab coat must be worn at all times

- powder free nitrile gloves
- safety goggles
- lab coat
- first aid kit

Equipment/Supplies:

- 10 cm O.D 1.0 mm I.D 0.50 borosilicate fire polished glass capillaries
 - Sutter instrument
- Spooled carbon fiber 7 micrometer diameter at least 12 cm long
- Tape
- Blank white paper
- High intensity illuminator
- Glass microscope slides
- Repositionable adhesive
- Spooled Carbon fiber Scalpel

- Pipette puller
 - Sutter Instrument Co. Model P-30
- Scissors
- Microscope – x200 magnification
- Sink with perpendicular hose nozzle
- Sink attachment suction hose
- Repositionable/moldable adhesive
- Water bath
- BV 10 Micropipette beveling
 - Sutter instrument company
- Oil
- UHP/DI water

Procedure

Preparation of Carbon Fiber

1. Pull non-epoxied carbon fiber from spool
2. Cut portions of carbon fiber 1.25 x 10cm= 12.5 cm long
3. Use caution to not tangle the threads
4. Place in labeled letter envelope

Threading Capillaries

1. Spread multiple threads of carbon fiber across a blank white sheet of paper
 - a. Set up an adjacent sheet of white paper, leave that piece void of any fibers
 - b. Set up high intensity illuminator to light up the work area
2. Select an individual fiber and drag it across to the blank sheet of paper
 - a. Make sure that it is longer than the glass capillary by at least 2 cm
 - b. Make sure it is a singular fiber and not multiple
3. Tape the end of the fiber to the paper
4. Attach the suction hose to the nozzle on the sink
5. Attach a glass capillary to the end of the nozzle
6. Turn the water on
 - a. Test for suction

7. Use the suction to pull the singular carbon fiber through the glass capillary
 - a. Stop when there is at one cm of fiber outside of the capillary
 - b. shut off the water
 - c. cut the carbon fiber
8. Remove the capillary from the hose
9. Visualize the carbon fiber in the capillary either by microscope
 - a. Ensure its placement
10. Place the threaded capillaries in a designated container

Pulling Electrodes

1. Place threaded glass capillary into the grooved top clamp of the pipette puller
 - a. Loosen the clamp so that the capillary can easily be pushed down, but does not fall under the force of gravity
2. Push the threaded capillary down through the circular heating coil so that its center aligns with the coil
3. Move the bottom clamp to its highest vertical position
4. Tighten the bottom and top clamp by turning the fastening screw to the right
 - a. Ensure that the electrode is securely fastened
5. Set the puller in accordance to the below specifications
 - a. Heat- 985
 - b. Pull- 465
6. Press the pull button then shut the transparent door
7. Allow the glass capillary to be pulled in half
8. Open the door after the heating coil is no longer red hot
9. Cut the thin thread connecting the two electrodes
10. Remove each electrode with caution avoiding any contact with heating coil and place them into a marked container
11. Shut the machine off and replace the cover

Cutting the Electrodes

1. Place a penny size piece of repositionable adhesive on the short end of a glass microscope slide
2. Place the uncut end of the pulled electrodes onto the adhesive
3. View the electrode under the microscope
 - a. Ensure that the carbon fiber travels the distance of the capillary
 - b. Ensure the capillary contains only one continuous carbon fiber
 - c. Discard any with discontinuous, multiple, absent, or short carbon fibers
4. View the pulled end of the electrode
5. Cut the electrode with the scalpel at the point where the width of the void space is the same width as the carbon fiber see figure 1 for a visual representation

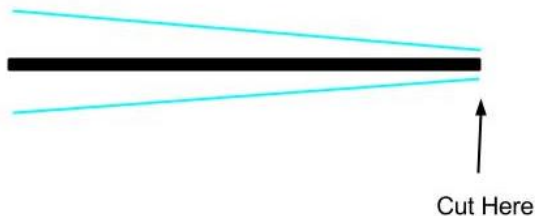


Figure 1

6. Remove cut electrode and place in a designated area

Epoxying the Electrodes

1. Zero a scale with a 50 ml vial
2. Pour Epon Resin 828 into a 50 ml vial until it fills 4/5th of the vial
3. Weigh the vial to get weight of Epon Resin 828 added
4. Heat the vial in a water bath until the Epon is no longer highly viscous
5. Add m-Phenylenediamine (1.0 : 0.14 Epon Resin : m-Phenylenediamine) to the vial
6. Mix a 1 : 0.14 ratio of Epon Resin 828 : in a water bath at approximately 77 degrees Celsius
7. Once fully mixed, dip the electrodes into the solution for approximately 15 sec
 - a. Make sure the electrodes tip is fully covered in epoxy
8. Harden the epoxy in the oven at 85 degrees Celsius for approximately 2 hours
 - a. Place the unused epoxy in a vial in the oven, when that epoxy is fully hardened the electrodes will be done

Beveling the electrodes

1. Assembling the beveller
 - a. Assemble the pedestal
 - i. Separate the lower and upper retaining ring by unscrewing the screws found on the upper retaining ring
 - ii. Place the grinding plate with the abrasive side up in-between the retaining rings
 - iii. Secure the rings together via tightening the screws
 - iv. Place two drops of oil on the quartz optical flat
 - v. Lower the retaining rings with grinding plate on the optical flat, stop when you feel magnetic resistance
2. Operating the beveller
 - a. Turn on
 - i. Ensure that the pedestal is rotating evenly
 1. If not, stop immediately to prevent any damage
 - a. The apparatus may need cleaning or is not set up properly
 - b. Wet the wick and place it on the grinding plate
 - i. Wet the grinding plate
 - c. Adjust the angle plate, on the manipulator, so that it is at a 30 degree angle
 - d. Place electrode in V shaped clamp on the manipulator
 - e. Move the manipulator towards the grinding plate with the electrode angled away from the operator
 - f. Place the manipulator so that the electrode tip is directly above the grinding plate
 - g. Lower the electrode with the course adjustment until the tip is at the surface of the water
 - h. Visualize the electrode with the scope
 - i. While visualizing lower into the water with the course adjustment

- ii. Lower the electrode with the fine adjustment until it is grinding (shadow will be at the tip)
 - iii. Continuously adjust the fine adjustment to achieve the desired cut
- 3. Disassembling
 - a. Removing the electrode
 - i. When finished beveling raise the electrode with the fine adjustment
 - ii. After using the fine adjustment use the coarse adjustment to completely raise
 - iii. Move the manipulator away from the grinding plate and remove the electrode from the clamp
 - b. Disassembling the grinding plate
 - i. Turn off, allow plate to come to a complete stop
 - ii. Remove the wick from the plate cautiously
 - iii. Unscrew and remove the upper retaining ring
 - iv. Dry the stop of the lower retaining ring as well as the top of the grinding plate with a Kim wipe
 - v. Remove the grinding plate, only touching the sides, while pulling straight and slightly up with gentle force
 - vi. Clean the bottom of the grinding plate with a NEW Kim wipe
 - 1. It is crucial to not get water on this side and to not get oil on the grinding surface
 - vii. Place the grinding plate in cheese cloth and then in its protective case
 - viii. Clean the quartz optical flat with a NEW Kim wipe
 - ix. Place a new Kim wipe over the optical flat
 - x. Cover the scope
- 4. Check the pipettes
 - a. Visualize the pipettes under 200x mag
 - i. Ensure they contain carbon fiber
 - ii. Ensure they are epoxied from the top to $\frac{1}{2}$ - $\frac{3}{4}$ of the slide view
 - iii. Set aside any faulty electrodes