ABSTRACT

Development of an Inhibition Based Biosensor for Arsenic (III)

by Sean Minderlein

A rapid, user friendly method to determine arsenic concentrations in water sources is highly desired. Biosensors are known for their simplicity and sensitivity. Arsenic is known to inhibit several enzymes, notably acetylcholinesterase. Several inhibition based sensors have been developed using this enzyme. Using these as models, a prototype of an arsenic based inhibition sensor was constructed. Investigating several electrochemically active products of the enzyme is the first step in generating the sensor. Thiocholine was determined to operate well with a screen printed electrode mediated with Cobalt- Phthalocyanine. Several different methods were considered when immobilizing the enzyme to the electrode surface. The preferred method was using glutaraldehyde with bovine serum albumin. It was then shown that the enzyme is inhibited by arsenic, and could be monitored on an electrode. In order to show decent inhibition, pretreatment of the electrode to the arsenic solution was required.
DEVELOPMENT OF AN INHIBITION BASED BIOSENSOR FOR ARSENIC (III)

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ABBREVIATIONS

ACHE  Acetylcholinesterase
EPA   Environmental Protection Agency
WHO   World Health Organization
As    Arsenic
As(III) Arsenic in the third oxidation state
As (V) Arsenic in the fifth oxidation state
CV    Cyclic Voltammetry
A     Amperage
PVA-SbQ Poly (vinyl alchohol) styrylpyridinium
GFP   green fluorescent protein
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CHAPTER I – Biosensors for Arsenic Detection
Sean Minderlein

I.1 Introduction

The ability to detect toxins in the environment is an important task to maintain the health of society. Arsenic is a known toxin that causes skin cancers, bladder cancer, as well as an array of other detrimental effects (Aposhian). Yet, the detection of heavy metals, such as arsenic, are notorious for their difficult and costly tests that can only be performed in a laboratory (Abernathy). There are several well-known areas where the arsenic concentration is high, such as Bangladesh and Argentina (Smedley). In 2000, the National Resource Defense Council did a report on 25 states drinking water, and found that over 34 million Americans in those states were at risk for cancer due to arsenic contamination in their drinking water (Ahuja). The other 25 states did not report their findings to the Environmental Protection Agency who worked with the National Resource Defense Council. There is a need for a fast, cheap, and reliable test for arsenic that can be used by a consumer with little training.

I.2 Detection of Arsenic

Arsenic is a metal that naturally exists in the earth’s crust and is the 20th most abundant element present in the earth crust (Mandal). This metal is found in two forms, organic and inorganic, where the latter is primarily found in subterranean drinking water and is toxic with long term exposure leading to cancer (Abernathy). Long term exposure to arsenic has caused other symptoms, usually dermatological in nature, such as skin lesions (Hughes). Of inorganic arsenic there are several oxidation states that the metal can exist in, -3, 0, 3, and 5 (Sigrist). The two most common found oxidation states of arsenic in ground water are 3 and 5. The ratio between the two states is dependent on the pH, redox potential, and salinity of the water. Arsenic 3 has also been found to be 25-60 times more toxic than arsenic 5 while also being several hundred times more toxic than organic arsenic (Kim, Nrigu, Haack). It is also important to note that speciation of inorganic arsenic is difficult because arsenic can change oxidation states, with arsenic 3 favorably turning into arsenic 5 under oxidizing conditions. Proper sampling and testing of arsenic in aqueous solutions is not a trivial matter (Thomas).
Table 1. Different forms of arsenic commonly found in the environment

<table>
<thead>
<tr>
<th>Arsenate</th>
<th>Arsenite</th>
<th>Methylarsenic Acid</th>
<th>Dimethylarsinic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic 5</td>
<td>Arsenic 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

According to the World Health Organization (WHO) inorganic arsenic exposure occurs typically from contaminated ground water sources, and is a serious concern in some regions. The WHO also has a published value of 10 µg of arsenic per liter of water as a guideline limit (Smedley). The Environmental Protection Agency (EPA), published a value of 50 µg of arsenic per liter as being the maximum allowable amount but recently announced that it will be lowering the limit to 10 µg of arsenic per liter (Thomas).

The actual measurement of a metal can be quite costly and difficult, and it is often easier to quantify the binding of the metal to another compound. For example, proteins containing sulfur groups, such as Pyruvate Dehydrogenase, readily bond with arsenic (Aposhian). If an enzyme, or a catalytic protein, contains the sulfur groups near an active site, then the binding of arsenic could inhibit the enzyme from functioning. Measuring the products or reactants of one or more of these enzymes would allow us to indirectly measure the levels of this metal in solution.

Currently there is no standard method to sample well water sources for the oxidation states of arsenic, however there are standard methods for the simple detection of arsenic. The simple detection of arsenic involves large and expensive equipment typically requiring transportation of samples to a laboratory. The Environmental Protection Agency recommends several testing methods to measure small arsenic concentrations in water sources that use one key method - atomic absorption (ATSDR). The method of atomic absorption atomizes the sample and shines a light through the mixture measuring the wavelengths absorbed. The wavelengths allow us to identify specific atoms, including arsenic. This process requires large equipment as well as trained technicians. Another method that can be used is inductively-coupled plasma atomic emission spectrometry (ICP), which works on the same principle as absorption but looks at the wavelengths emitted from the exited state of the analyte to identify the sample.
There are other methods that are also accepted, including electrochemical methods, however these methods often susceptible to interferences.

I.3 Biosensors for arsenic

The term biosensor is used to describe an array of devices that are able to quantify an environmental factor, using a biological transducing agent. This biological element produces a signal which is then transformed into a signal that can be quantified. Some common methods of transforming this signal are optical, piezoelectric, and electrochemical (Kaur). Typically, these sensors utilize an enzyme, or an organic catalyst to create a product that can be measured. These types of sensors are used every day and can be extremely accurate due to the inherent selective nature of this transducing agent. These detection methods are often very portable due to the ease of sample preparation and the small size of the sensor. There are several different types of biosensors, each having different advantages. Each type of biosensor is categorized by the type of biological transducing agent that is implemented.

I.3.1 Microbial Biosensors

Utilizing whole cells, microbial biosensors provide high selectivity and a physical transducer that creates a signal that is directly proportional to the concentration of the compound of interest. It is the whole cell interaction with the environment that provides the selectivity, however these sensors have several issues.

This type of sensor detects arsenic using several methods, the primary investigated method uses the ars operon, which is a string of genetic material that certain bacteria can have that is stimulated by the presence of arsenic. This gene is activated when arsenic is present which causes a signal cascade that can be used to produce a molecule which are able to be measured. Some molecules that are produced are luciferase and green fluorescent protein, these proteins can be detected using optical devices (Kaur). This type of sensor has a detection limit to arsenic III anywhere from 0.0007-10 µg/L (Kaur).

An issue with microbial biosensors is the response time, or the time it takes for the sensor to provide useful information, is long in the case of microbial sensors and can possibly take days. The variability from cell to cell also causes issues with microbial biosensors when it is desired to quantify the concentration of arsenic in solution. Another issue is that most sensors created this way need to measure florescence to determine useful information about a system, which is a more complex and costly piece of equipment (Skoog).
I.3.2 Aptamer Biosensors

Another type of biosensor is an aptamer biosensor. An aptamer is a single stranded nucleotide sequences that are artificially created. These molecules are highly selective with binding to other biomolecules as well as other compounds. Since arsenic can bind to the aptamer Ars-3 it is the most obvious choice to use for a sensor. The sensor is created by modifying this aptamer with gold nanoparticles such that when arsenic binds to the nucleotide sequence aggregation of the gold nanoparticles occurs. This type of sensor has a detection limit for arsenic III anywhere from 0.05-77,000 µg/L (Kaur). Although this method is selective, fast, and reliable it still requires a device that can measure the optical absorbance of the aptamer-sample mixture, which is usually a large and costly device.

I.3.3 Protein Biosensors

The last type of biosensor is a protein biosensor that utilizes a protein to transduce information about a system. Proteins are a type of biomolecule that can perform an array of functions. One function a protein can perform is a catalyst, this type of protein is called an enzyme. Arsenic is known to affect certain enzymes, specifically it is the substrate for arsenic oxidase and it inhibits acetylcholinesterase. Inhibiting an enzyme refers to arsenic's ability to decrease the catalytic activity of the enzyme.

Other types of sensors use proteins such as antibodies, that are not necessarily enzymes however they still interact with the analyte of interest. The interaction between the analyte and the protein is measurable, this type of detection is usually very similar to the aptamer style sensor but using a protein instead of aptamers.

While not all protein biosensors use an enzyme a great many of them do. Typically the sensor is used to measure a compound such as glucose or lactose (called a substrate) that is oxidized by the enzyme. It is desired to create a sensor that is dependent of the mass transfer of the substrate. The mass transfer depends on the bulk concentration of the analyte, therefore an excess of enzyme is used when constructing the sensor.

It is also not uncommon to have sensors that are dependent on two coupled enzymatic reactions. This is because the product of the first reaction may not be easily detected, so another reaction based off of the first reaction's product is. This type of sensor is used when the analyte reacts with the enzyme to produce products. Arsenic has this relationship with a few enzymes; however it is more common that this toxin inhibits the enzymatic reaction. Luckily there are
other sensors that can measure when an enzyme is inhibited; these are called inhibition biosensors. This is not a new paradigm in sensor technology. In fact, inhibition sensors have been used to measure various toxins from pesticides to arsenic. This type of sensor has a detection limit to arsenic III anywhere from 0.015-692 µg/L (Kaur).

**1.4 Inhibition based biosensors**

Since enzymes are organic catalysts, they facilitate a reaction. If a compound is known to disrupt the enzymatic reaction then it is possible to detect this analyte by monitoring the reaction rate. Typically, the reaction rate decreases proportionally with the concentration of the inhibitor, and monitoring of the reaction is done by either monitoring the presence of substrate or generated product. Inhibition based detection is a common method used to detect toxic molecules such as pesticides and heavy metals. The first step in creating an inhibition based sensor is to first determine what enzymes the analyte inhibits. Then proper substrates must be chosen that can be measured or react with the enzyme to produce a measurable product.

Since arsenic will inhibit acetylcholinesterase, the difference in measured current from the inhibited and uninhibited electrode will be measured and compared to see if there is a relation between inhibition and current generation. While other materials would also inhibit the acetylcholinesterase they are not typically found in well water.

There are several types of enzyme inhibition, with two main categories of models; competitive and non-competitive. Competitive inhibition is when the inhibitor ‘competes’ for the active site of the enzyme, which is where the reaction that the enzyme facilitates occurs. This type of inhibition is dependent on both the concentration of the inhibitor and the enzymatic substrate. Uncompetitive inhibitors disrupt the enzyme function from a location that is not the active site, and these inhibitors only depend on the concentration of the inhibitor.

These two types have another sub classification; reversible and irreversible. Reversible inhibition is where the inhibitor is able to unbind from the enzyme, thus allowing it to function again. Irreversible inhibition is not able to regenerate and is permanently disrupted (Arduini). It is proposed that arsenic III irreversibly inhibits acetylcholinesterase with a competitive binding site (Wilson and Silman), this type of inhibition is shown below in figure 1.
Figure 1. proposed mechanism for arsenic inhibition of acetylcholinesterase

Using the knowledge of what type of inhibitor arsenic is on various enzymes, it is possible to better design how the detector operates to increase sensitivity to this analyte (Arduini). There are several enzymes that are inhibited by arsenic, including but not limited to Acetylcholinesterase, pyruvate dehydrogenase, and acid phosphatase. These enzymes are summarized in table 2.

Table 2, summary of existing proteins that detect arsenic

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Arsenic Detection Limit</th>
<th>Sensitivity</th>
<th>Limitations</th>
<th>Reference</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>4.3 μM</td>
<td>$(1.1-4.5)*10^{-8}$ (A/μM)</td>
<td>Not selective</td>
<td>Fuku</td>
<td>N/A</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>2nM</td>
<td>$1.186$ (A/(M*cm²))</td>
<td>20min incubation time</td>
<td>Cosnier</td>
<td>Reversible</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>2.0μM</td>
<td></td>
<td>Only reacts with arsenic V</td>
<td>Román</td>
<td>Reversible</td>
</tr>
<tr>
<td>AChE</td>
<td>35.9μM</td>
<td></td>
<td>Requires a certain buffer</td>
<td>Román</td>
<td>Irreversible</td>
</tr>
<tr>
<td>Laccase</td>
<td>13μM arsenite 132 μM arsenate</td>
<td>0.9 mV/mM</td>
<td></td>
<td>Wang</td>
<td></td>
</tr>
<tr>
<td>AChE</td>
<td>$1.1*10^{-8}$ M</td>
<td></td>
<td></td>
<td>Sanllorente-Mendez</td>
<td>Irreversible</td>
</tr>
<tr>
<td>AChE</td>
<td>$2<em>10</em>10^{-4}$ mol/l</td>
<td></td>
<td></td>
<td>Stoytcheva (magnin)</td>
<td>Irreversible</td>
</tr>
<tr>
<td>ACHE</td>
<td>$2*10^{-10}$ mol/l</td>
<td></td>
<td></td>
<td>Stoytcheva (panayotova)</td>
<td>Irreversible</td>
</tr>
</tbody>
</table>
1.5 Electrochemical Biosensors

Biosensors that monitor biological binding events or catalytic activity electrochemically are referred to as electrochemical biosensors. There are two main types: potentiometric and amperometric.

Amperometric biosensors measure an electrical current moving through an electrode surface with a constant applied voltage and can typically provide a quantitative result. One of the more common amperometric biosensor is a blood glucose meter.

The enzymes that can be monitored either produce or consume an electrochemically active species. The sensing process occurs through oxidation or reduction of the species at an electrode surface. These redox reactions occur with slight variation between the two species of interest, but the simplistic view of the reaction mechanism is that when a species is reduced it gains an electron and when a species is oxidized it loses an electron. This loss of an electron from a species allows for it to be picked up through a conduit creating current that can be measured and quantified. For some redox reactions an electrical potential difference needs to be applied to facilitate the reaction, at lower voltages fewer reactions occur, while at higher voltages more reactions occur.

Electrochemistry is great for computational analysis of a system because it can easily be measured through an electrode, since not much signal processing needs to happen as the signal is already in an electrical form unlike optical or piezoelectric systems. An electrode is used to gather information about a system using three surfaces, called the working, reference, and the counter electrode. Each one of these smaller electrodes has an important role in the sensor; the reference electrode is an electrode that has a known electrode potential. The auxiliary or counter electrode is used to make sure that current does not pass through the reference electrode, providing an electron source or sink. Finally, the working electrode is the electrode that interacts with the environment of interest; it usually has an embedded medium that aids in electron transfer. This embedded medium is also required to ensure that there is uniform electron transfer throughout the electrode. Summarizing, the working and reference electrodes are held at the same voltage, which is measured from the reference electrode, and the current flow from the working electrode through the counter electrode is measured. This is shown in the below circuit diagram of the system.
Figure 2, Circuit diagram of amperometric sensor.

Figure 2 shows a box that represents the electrochemical cell. Inside the cell R stands for reference electrode, W stands for working electrode, and A stands for auxiliary electrode. Some other abbreviations that were not discussed include V, the Voltmeter which measures the potential difference of the system in Volts. A is the ammeter a measurement device for current, and SW is the sliding resistor to change the applied potential difference of the system.

An example of a typical 3 electrode configuration is shown below in figure 3.

Figure 3, The working diagram of a Dropsens electrode.
This electrode has a large amount of complexity to measure the properties about the system. Figure 4 shows a schematic of each step in the electrochemical inhibition based biosensor.

Figure 4, inhibition based biosensor schematic

For the design of an electrochemical biosensor, there are three distinct parts of the sensor that need to be investigated. The first is the electrochemically active product from the enzyme and its interaction with the electrode. The second is the enzyme and the enzyme substrate, and specifically how it interacts with the electrode. The final piece to investigating an inhibition based biosensor is how the inhibitor interacts with the enzyme. These layers of complexity are shown above in figure 4.

I.6. Hypothesis and Objectives

Conventional methods for determining arsenic in water are slow, involve expensive analytical equipment, and require taking samples from the source to a laboratory. Amperometric biosensors can be easily miniaturized and built using low cost components. It is hypothesized that an amperometric inhibition based biosensor can be constructed using the enzyme acetylcholinesterase for the detection of arsenite in water.

The objectives of this study include;

1. Create and characterize an electrochemical biosensor using the enzyme acetylcholinesterase
2. Demonstrate the utility of the acetylcholinesterase biosensor for the detection of arsenite in water
CHAPTER II Characterizing Electroactive Substrates for Acetylcholinesterase Biosensors

Sean Minderlein

II.1 Introduction

Acetylcholinesterase (AChE) facilitates several reactions and is known to be inhibited by arsenic (Page, Wilson). Choosing a substrate for this enzyme that can be electrochemically monitored is necessary to create the sensor. The primary reaction that AChE catalyzes is the hydrolysis of acylcholine to produce choline and acetic acid; however, the products and substrate of this reaction are not electrochemically active meaning that they cannot be monitored amperometrically. It is possible to couple this reaction with another enzyme such as choline oxidase, yet this would complicate the system which is not desired (Kaur).

Therefore, another substrate must be chosen to monitor the activity of this enzyme. One possible substitution would be using acylthiocholine as a substrate. This would produce thiocholine which is electrochemically active (Bucur). There are two forms of this substrate depending on the type of salt formed a chloride version and an iodide version. These two salts have different electrochemical properties that could prove to be important (Bucur). Thiocholine can also be used to monitor the enzymatic rate spectrophotometrically by reacting with Ellman’s reagent (Page, Wilson).

Acetylcholinesterase hydrolyzes acetylthiocholine, figure 7, to produce thiocholine, figure 8, and acetic acid. The thio-choline is able to be oxidized and reduced by Co-Pthalocyanine at low voltages, figure 9 (Stoytcheva).
In this case cobalt Phthalocyanine acts as a mediator transferring electrons from the electrochemically active compound to the less active product (Skladal). This compound lowers the activation energy of the oxidation of thiocholine meaning the required potential is lower. This is of interest as the higher the applied potential the more interferences will interact with the
electrode. There are other mediators that can be used (Arduini), but cobalt Phthalocyanine has been extensively studied (Skladal) (Hartley).

Another possible substrate is Phenol acetate (Liu et. Al.). This compound is hydrolyzed by ACHE to give acetic acid and electrochemically active hydroquinone. These two compounds are shown below in figures 10 and 11, respectively.

![Figure 8. Phenol acetate](image1)

![Figure 9. Hydroquinone](image2)

Hydroquinone can reversibly oxidize to form another compound called 1,4-Benzoquinone (Buleandra). This oxidation naturally occurs at low potentials and at non-modified electrodes, which is beneficial in reducing cost of electrode selectivity (Buleandra). 1,4-Benzoquinone is also colored, which introduces the possibility of monitoring the redox chemistry spectrophotometrically along with the electrode. Since Phenol acetate can be the substrate of acetylcholinesterase, it is feasible to monitor the rate of reaction of this enzyme by the redox chemistry of the product, Hydroquinone, to 1,4-Benzoquinone.

![Figure 10. 1,4-Benzoquinone](image3)

It is also important to note that it has been shown that some forms of acetylcholinesterase are inhibited by hydroquinone (Wang et. al.2007).

Since a disposable test strip is desired as the end product, screen printed electrodes are used to develop the sensor. Screen printed electrodes are of interest due to the low cost and ability to be mass produced. Typically, these electrodes are a carbon paste that is spread over a ceramic backing with a silver reference electrode (Skladal). Screen printed electrodes are typically made out of a carbon paste that is put onto a non-conductive backing that ensures that the various electrodes are insulated from each other. If another material is desired to interface
with the electrochemical cell, then that material is coated overtop the carbon. Often, a silver chloride solution is coated over the reference electrode. As mentioned previously a mediator can be used to decrease the potential required to react the compound of interest and increase the current obtained from the analyte. This typically occurs at the working electrode.

### II.2 Electrochemical theory

In order to begin the process of creating the electrode with the enzymatic reaction the first steps to investigate the electrode without the enzyme. In this case, the electrode will be investigated in the presence of the product; thiocholine. This is to learn about the various variables that are present in an electrochemical cell. There are five main classes of these variables that are dependent upon various parts of the electrolytic cell, these include: electrode variables, mass transfer variables, solution variables, electrical variables, and external variables (Bard, Faulkner).

Electrode variables arise from the small differences that occur in the product, these include the material, surface area, geometry and surface condition. Ideally, the conditions would be the same for each electrode produced.

This leads to solution variables which come directly from the solution, these include the bulk concentration of the electroactive species, concentration of other species (pH and other electrolytes), and the solvent. These parameters should be able to be controlled initially but need to be considered when the electrode is able to be field tested, as they could possibly impact the sensors ability to operate.

Mass transfer variables come from the solution being tested and its ability to get to the electrode. It combines the solution variables and the electrode variables. This type of variable includes the mode of mass transfer (diffusion or convection), the concentration of the analyte at the electrodes surface, and adsorption of the analyte into the electrode. These variables are not known and could be of value to the optimization of an electrode.

Electrical variables are considered more from the instrument prospective and include the potential, current, and the quantity of electricity. These variables are monitored through the potentiostat. The final set of variables are external variables which include various parameters such as temperature, pressure, time, and ambient electric fields.

Two tests, amperometry and cyclic voltammetry, are performed to determine several variables about the system. Amperometry is typically used to determine the concentration of a
compound given several constants, which is why it is of interest to determine concentrations of the products of an enzymatic reaction. Cyclic voltammetry is used to determine, qualitatively, several parameters such as the required potential to react the electrochemically active compound. This technique is commonly used to find general electrochemical properties of a system.

**II.2.1 Testing Methods**

All electrodes were purchased from Dropsens. The non-mediated electrode utilized was DS 150 with a carbon working electrode, platinum counter and silver, silver chloride reference electrode. The Co- Phthalocyanine electrode was DS 410 and had a carbon working electrode doped with Co- Phthalocyanine, carbon counter, and a silver reference. The DS 410 electrode was found to be optimal for monitoring thiocholine (Skladal).

There are two tests of interest that are performed, amperometry and cyclic voltammetry. Results from these allow for determination of different information about the system. In cyclic voltammetry, the applied potential is varied between two set points and the current that is produced is then measured. This can be performed at various scan rates and has the ability to show at what potentials the solution being tested is electrochemically active. Amperometry is where a constant potential is applied to a system, and the current response is recorded.

The tests were performed by putting the electrode of interest into a 10ml solution without mixing. For amperometry, the electrode is polarized then allowed to come to steady state which takes approximately 360 seconds. The steady state current is then recorded. For the cyclic voltammetry, the conditions varied for each test, and the conditions are listed with the results of interest.

An Auto Lab PG stat was the main instrument used in the measurements; however a Dropsens Microstat 400 and a Digiivy system were also used.

**II.2.2 Cyclic Voltammetry Results**

First, a cyclic voltammetry scan is done to prove that the electroactive species produced by acetylcholinesterase is active. This scan also shows the optimal potential to operate the cell amperometrically. Since a mediator can be utilized, a test between a mediated and non-mediated electrode in a thiocholine solution can show that the mediator does in fact decrease the potential
required to oxidize the thiocholine. The cyclic voltammetry results for thiocholine iodide on the cobalt phthalocyanine mediated electrode are shown below in figure 11.

![Thiocholine Oxidation](image)

**Figure 11.** Thiocholine iodide CV scan on DS 410, 0.1 M phosphate buffer pH 7.4, ~ 4 mM Thiocholine, Scan rate; 0.1V/sec, from -0.4 V to 0.8V, Anode positive, dropsens microstat 400

Analyzing the data from the preliminary scan shows a peak from thiocholine present at around 0.2V. Since an over potential is required to decrease migration and other electrochemical effects a voltage of 0.35V is recommended for measurement of thiocholine on the mediated electrode. It is important to note that the species investigated above is a salt that includes iodide as the anion which is also electrochemically active. This species is active around 0.6V which could cause interferences when attempting to make a sensor sensitive to thiocholine. Due to this inference, another salt with less interference would be desirable, such as a chloride salt.

Figure 12 compares the mediated results with the non-mediated electrode, which uses just screen printed carbon as a working electrode and a platinum counter electrode.
In the non-mediated electrode, the potential required for thiocholine oxidation is shifted higher, up to 0.5V. The salt, chloride, was used to avoid the iodide peak that would also be visible at the same potential. This shift might not be significant when considering the potential that is required to change arsenic(III) to arsenic(V), as well as other possible species that could react with the electrode at the increased potential.

Alterating the rate at which the cyclic voltammetry changes the applied potential to the cell, allows for the extraction of various information about an electrochemically active species, such as the diffusivity of the analyte. Figure 13 and 14 shows the results of this technique with thiocholine iodide and chloride, respectively. The numbers that correspond to the color of the lines is the scan rate in volts/second.
Figure 13. 1mM Thiocholine iodide CV scans with various scan rates in v/s

Figure 14. 1mM Thiocholine chloride, various scan rates in v/s

Using the above data from the CV scans the peak height can be extracted to determine information about the analyte. First, the peak height from thiocholine is obtained, then the
faradic current is also obtained. The faradic current is obtained by making a line between the two base points of the analyte peak. Then, to find the current that corresponds to the potential that has the max current from the thiocholine we extrapolate through the peak (Bard, Faulkner). By removing the faradic current from the peak current the result is the current from just the thiocholine, which forms a linear relationship to the square root of the sweep rate, this is shown below in Figure 15 (Bard, Faulkner).

![Figure 15. Thiocholine peak height-faradic current, as a function of sweep rate.](image)

The dark line above is a least squares linear fit of the data. This line relates the peak height to the square root of the sweep rate and is forced through the origin. This linear fit can then be compared to the below equation where each of the parameters can be calculated or estimated.

\[
i_p = 0.4463 \, nFAC \left(\frac{nFDv}{RT}\right)^{\frac{1}{2}}
\]

Figure 16, peak height of a CV scan as a function of sweep rate
The above equation has several variables; \( n \) is the number of electrons transferred from one molecule of analyte, \( F \) is the faradic constant, \( A \) is the area of the electrode, \( C \) is the concentration of the analyte, \( v \) is the sweep rate, \( D \) is the diffusivity of the analyte, \( R \) is the gas constant, and \( T \) is the temperature (Bard, Faulkner). Utilizing the known constants and applying the known concentration of thiocholine and temperature, allows the equation to be solved for diffusivity. It is important to note that the value of the electrochemical area is estimated. The values that are utilized are shown below in Table 3.

<table>
<thead>
<tr>
<th>( n )</th>
<th>( A )</th>
<th>( D )</th>
<th>( C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1 )</td>
<td>( 0.125663706 ) cm(^2)</td>
<td>( 1.02057 \times 10^{-7} ) cm(^2)/s</td>
<td>( 0.000005 ) mol/cm(^3)</td>
</tr>
</tbody>
</table>

The calculated diffusivity value is compared the published value of \( 1 \times 10^{-8} \) cm\(^2\)/s (Gogoi). Considering the true surface area of the electrode was unknown this is a close estimate to the value.

The next electrochemically active species investigated is hydroquinone, which does not require a mediated electrode but does require anodic pretreatment of the carbon electrode. This pretreatment of the electrode decreases the required potential for reacting hydroquinone and increases the current that is measured from the analyte. A cyclic voltammetry graph is presented in Figure 17. In this graph, various concentrations of hydroquinone were investigated to see the response of peak height. Hydroquinone is electrochemically active at a relatively low potential after anodic treatment of the electrode and running the system amperometrically at a potential of 0.3 V would be sufficient to detect the compound without any interference from migration or other effects.
II.2.3 Amperometric Results

Looking at a system amperometrically has the benefit of finding a semi-steady state where the current is dependent on one of two properties, rate of diffusion of the electroactive compound to the electrode surface or the rate of reaction of the electroactive compound on the electrode producing the electrons (Skoog). Due to the relative difficulty of ensuring each electrode is identical, diffusion controlled sensors are easier to produce than reaction controlled (Bard, Faulkner). Determining what concentrations the electrode operates at that are still defined by the diffusional control domain is important when considering creating the electrode. This can be done by operating the electrode at various concentrations of the electroactive compound to determine where the measured current is linearly proportional to the concentration of the analyte (Bard, Faulkner). Determination of this parameter needs to be done with both thiocholine and hydroquinone.

Figure 17, Cyclic voltammetry of various concentrations of hydroquinone
Figure 18, amperometric response of thiocholine on dropsens electrode 410, at 0.35V

Figure 18 shows the response of current is linear to thiocholine concentration. This implies that the system is operating in a diffusional capacity (Bard, Faulkner). This data set was obtained with three separate electrodes and several trials at each concentration. Utilizing information about this system, it is possible to again calculate the diffusivity of the electroactive compound. The equation below is utilized.

\[ i_{ss} = 4nFD_0C_0r_0 \]

Figure 19, amperometric steady state model

This equation relates the current obtained when operating at steady state in a diffusion controlled environment to the concentration of the analyte. Where \( i_{ss} \) is the current value, \( n \) is the number of electrons transferred from one molecule reacted, \( F \) is Faraday’s constant, \( D_0 \) is the diffusivity, \( C_0 \) is the concentration in the bulk solution, and \( r_0 \) is the radius of the electrode surface (Bard, Faulkner). Due to the complex nature of the electrode, this equation is not an accurate description of what is occurring at the electrode surface since it assumes only one dimension of mass flux into the electrode surface this is not true as there is flux parallel to the electrode surface as well. The equation also fails to account for surface roughness that might be present with the electrode. While this might be important to consider when creating a thiocholine
sensor, these issues should not affect an arsenic sensor because the change in thiocholine concentration should be larger than this error. The slope, or sensitivity, of this calibration curve is $4.77 \times 10^{-6}$ A/mM thiocholine. This is similar to published values of $14 \times 10^{-6}$ A/mM thiocholine (Bukur). The differences could be accounted by the difference in testing methods.

Since this compound uses a mediated electrode to transfer the electron, a non-mediated electrode was looked at to determine if there was a change in any of the parameters. The cyclic voltammetry of thiocholine chloride was utilized to pick a potential of 0.8V to operate amperometrically, and the results of this calibration curve is shown below in figure 20.

![Graph showing the calibration curve of thiocholine chloride on DS 150 at 0.8V amperometric](image)

**Figure 20, thiocholine chloride on DS 150 at 0.8V amperometric**

As expected, the slope of this curve is similar to slope that was obtained from the mediated electrode which would lead to similar diffusivity value. However, the increased potential is unacceptable for the situation that the sensor is being designed to operate in. This is because the increased potential can increase the amount of side reactions and arsenic can react at this potential (Skoog).

A point of interest is that since thiocholine has an exposed sulphur, it is possible that arsenic can interact with sulphur groups (Kaur). Testing the electrode in a known concentration of thiocholine followed by testing in a known concentration of thiocholine and arsenic is necessary to determine if this occurs. The summary of this test is presented below in Figure 21.
Figure 21, steady state current values from amperometry of thiocholine and arsenic III

Showing that arsenic does not react with the thiocholine is important because if the inhibition that was measured was not due to the arsenic inhibiting the enzyme but instead arsenic binding to the product then a biosensor based off of inhibition of the enzyme would be pointless as a sensor based off of thiocholine would be simpler and less selective. Since this substrate is non-reactive with arsenic it is viable to use in creation of an arsenic biosensor. This test also shows that there is no appreciable current increase from the added heavy metal.

II.3 Summary

Cyclic voltammetry is typically used as a preliminary form of testing and is not usually used as a detection method. The scans will often change from test to test due to various reasons. One reason is if the electrode is run anodically and cathodically can change surface properties of the electrode. Another issue in this type of measuring is the possibility of the silver chloride reference electrode interacting with the solution. When this material changes the applied potential will also change causing a drift in the measured results. There is a slew of various other issues that can cause this scanning method to be inaccurate, and why an amperometric detection method is desired to create this sensor.
It has been shown that screen printed electrode can be used to detect thiocholine and hydroquinone, however hydroquinone requires pre-treatment of the electrode. These electrodes have also been shown to behave in a diffusion controlled manner, and the diffusivity of the electrochemically active compound can be calculated. Hydroquinone was not selected for further development due to the variability of the bare electrode responses.

Other interesting points, the first is that the electrode can oxidize iodide into iodine, which can inactivate ACHE, so the applied potential needs to be chosen appropriately. Arsenic III also did not react with thiocholine, or the electrode at the conditions investigated. Acetylthiocholine chloride salt was selected for further electrode development.
III.1 Introduction to Immobilized Enzymes

Immobilization of proteins achieves two main goals. The first goal is that it creates a more stable enzyme product (Walt). This is important because the enzyme product can last longer and overall be more robust. The immobilization process makes it harder for the protein to denature when exposed to a harsh environment including high temperature, pH, and other harsh conditions. The second goal of immobilization is that it creates a layer of enzyme that prevents it from washing off the immobilized surface.

There are various ways to immobilize an enzyme onto an electrode surface. One such way is to use liquid glutaraldehyde and Bovine Serum Albumin to directly crosslink the proteins to each other. That will then creating a film that can attach to the electrode (Broun). The liquid glutaraldehyde can react with functional groups such as thiols and amines, as well as itself forming polymers (Migneault). Proteins have an abundance of amine groups, which are typically on the surface of the protein that can readily react with glutaraldehyde (Migneault). Figure 22 shows glutaraldehyde reacting with amino groups on proteins (Migneault).

![Figure 22. Reaction of glutaraldehyde with amine groups on the protein surface](image)

This covalent reaction creates a gel matrix attaching the enzyme to the electrode surface. A visualization of this is presented below in Figure 23.
There are numerous variables that affect how the chemical reaction proceeds. One such variable is the concentration of bovine serum albumin added to the immobilization mixture. The primary effect of bovine serum albumin is protecting the enzyme from the harsh crosslinking of the glutaraldehyde. Acetylcholinesterase can denature when exposed to the crosslinking agent rendering the enzyme useless. The bovine serum albumin prevents this denaturation (Migneault). Bovine serum albumin can also effect the thickness of the final gel. When more of the bovine serum albumin is present, the gel is thicker. The second variable that affects how the reaction proceeds relates to glutaraldehyde. This includes the concentration of the applied solution, the method of application of the reactive compound, and the timing of the addition of the compound (Migneault). The third variable determining the reaction and how the electrode will operate is the amount of acetylcholinesterase added to the immobilization mixture. As more enzyme is added, the faster the substrate is converted to the product.

Another form of immobilization is to crosslink a gel around the protein to entrap the enzyme. This is a gentler method of immobilization as it does not directly react with the enzyme. The compound Poly (vinyl alchohol) styrylpyridinium (PVA-SbQ) crosslinks with itself under ultraviolet light to form a polymer network that would hold the proteins in place (Bai). This reaction is shown below in figure 24.
Figure 24, reaction of PVA-SbQ with UV light

Since this type of immobilization does not directly crosslink with the protein the enzyme is able to diffuse out of the immobilizing matrix while the electrode is in use. This issue needs to be addressed and fixed by altering the immobilizing conditions to control the crosslink density to slow diffusion rate.

It is important to note that it is desired to operate the biosensor so that the rate limiting step is the enzymatic rate. In order to obtain this condition, the rate of reaction of the enzyme should be slower than the rate of reaction at the electrode, and the substrate should be in abundance as the diffusion of the substrate to the electrode surface should not be limiting.

III.2.1 Method of immobilized enzyme electrode generation

*Method 1 – Liquid Glutaraldehyde*

The first method to create an immobilized enzyme electrode was to mix all three reagents (BSA, Glutaraldehyde, and enzyme) together and immediately apply the mixture to the electrode surface (Walt). The three reagents were added to a micro centrifuge tube, mixed, and 3 µL of the mixture was applied to the working electrode. Table 4 below shows the concentration used for each of the three reagents.

Table 4. Typical concentrations of reagents applied to electrode in method 1.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Eel ACHE (2000 u/ml)</th>
<th>BSA (50mg/ml)</th>
<th>Glutaraldehyde (25%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume added</td>
<td>25 µL (2.5 units per electrode)</td>
<td>25 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

The electrode was created by mixing sodium phosphate buffer 0.1M at a pH of 7.4, bovine serum albumin, eel acetylcholinesterase, and glutaraldehyde in a separate container then to add the mixture to an electrode surface where it can react to form the gel.

The generated immobilized enzyme mixture was often not completely on the working electrode surface, as shown in Figure 26.

![AChE/BSA/Glutaraldehyde](image)

Figure 26, an electrode generated via method one, glutaraldehyde liquid immobilization

*Method 2 – Glutaraldehyde Vapor*

The second method of creating an immobilized enzyme electrode involved applying the protein to the electrode and allowing it to dry on the lab bench for 45-60 minutes. Then, electrodes were placed into a small chamber and held at 40°C along with 1 ml of liquid glutaraldehyde for 20 minutes (Li). The vapor exposure chamber used is shown below and was a closed chamber that was held at 40°C with the electrodes and 1ml of 25% glutaraldehyde (Li). The chamber was pre-saturated with the glutaraldehyde vapor before the electrodes were placed inside.
Table 5 shows the amount of each of the reagents that was used to immobilize the enzyme using this method

Table 5. Volumes of the reagents used in method 2 immobilization, glutaraldehyde vapor

<table>
<thead>
<tr>
<th>Stock solution u/ml</th>
<th>Amount BSA Stock (ml)</th>
<th>Amount ACHE solution (ml)</th>
<th>Amount of BSA in 10uL (mg)</th>
<th>Amount of ACHE in 10uL (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0.375</td>
<td>0.125 (2000u/ml)</td>
<td>0.375</td>
<td>5</td>
</tr>
<tr>
<td>300</td>
<td>0.2</td>
<td>0.3 (500u/ml)</td>
<td>0.42</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>0.33</td>
<td>0.16 (300u/ml)</td>
<td>0.47</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>0.25</td>
<td>0.25 (100u/ml)</td>
<td>0.48</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>0.45</td>
<td>0.05 (100u/ml)</td>
<td>0.49</td>
<td>0.1</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 26. glutaraldehyde vapor chamber
Method 3 – PVA-SBQ Method

The third method of creating an immobilized enzyme electrode involves use of poly (vinyl alchohol) styrylpyridinium (PVA-SbQ) to entrap the AChE. Since the polymer was viscous it had to be ‘screen printed’ onto the electrode. To create the electrode, a mask was applied, then the mixture was applied and the excess mixture was scraped away. The mask was 0.12mm thick and had a hole the size of the working electrode (4mm diameter). The reaction mixture contained 1.04 u/ml enzyme and 4% PVA-SbQ (Bai).

III.2.2 Testing immobilized enzyme electrodes

Electrodes were tested to determine the rate of enzyme reaction in several different ways including amperometrically, cyclic voltammetry, and impedance spectroscopy. The two methods used primarily were amperometric and cyclic voltammetry. The first test performed was a wash test to ensure that the electrodes were properly immobilized. During this test, the electrode was introduced to the buffer environment and a potential was applied. After steady state was obtained, the substrate was added and the system was allowed to obtain steady state again. After the test was completed, the electrode was removed, rinsed, and put into another solution. This was repeated multiple times until it was certain that the enzyme was not leaching off of the electrode.

Using the first immobilization method of electrode generation, the liquid glutaraldehyde method, it was shown that successful immobilization can be obtained. The results of this test is presented below in Figure 27, where the values are the steady state values. It was shown that the enzyme electrode was reusable and not leaching enzyme from the immobilized layer.
Figure 27. Results from a typical wash test of a method one electrode Digi-Ivy DY2000 Potentiostat, at 350 mV, Sampling time = 0.1 s, Sensitivity 1*10-4 A/V, Cathode positive

The next method of immobilization that was investigated involved applying the ACHE bovine serum albumin mixture to the working electrode. After the mixture applied to the electrode dried, this paste was put into an environment of glutaraldehyde vapor for the amount of time that allowed for cross linking of the protein slurry. Since the enzyme dried on the electrode, it is possible that some of the enzyme bound non-specifically to the non-polar surface. The figure below shows this phenomenon of non-polar deposition, as a dried electrode was not exposed to the glutaraldehyde vapor and a wash test was performed.
When glutaraldehyde is not used, the enzyme can be washed off the electrode. Therefore, the paste was introduced to a 40°C environment with the glutaraldehyde vapor. The wash test of the resulting electrode is shown below in Figure 29.

Figure 28. Wash test of no glutaraldehyde electrode

Figure 29. glutaraldehyde vapor method exposed for 10 mins
The data shows that some enzyme is able to leach off the electrode surface. If the same amount leached off everytime, there would not be an issue. Yet, the amount leached off was not reproduced. Below is data presented for a crosslinking time of 5 mins with two separate electrodes (Figures 30 and 31).

Figure 30. Glutaraldehyde vapor method 5 minute expose (1)

Figure 31. Glutaraldehyde vapor method 5 minute expose (2)

Comparing the 5-minute crosslinked data to the 10 minute exposed, shows that the longer the electrode is exposed to the glutaraldehyde vapor, the less enzyme leaches off and the smaller the initial signal. Crosslinking prevents enzyme leaching, but the film is more crosslinked so substrate diffusion is slower. Also, more crosslinking means the enzyme is modified more
increasing the chance that it is inactivated. There was a lack of reproducibly immobilizing the enzyme using the vapor method, therefore another method was investigated.

Figure 32. Picture of PVA-SbQ immobilized enzyme electrode

Since PVA-SbQ does not directly crosslink with the enzyme, wash tests were performed to prove proper immobilization. This is shown below in figure 33 where the autolab system was used. The value presented is the difference between the buffer and the substrates steady state current.
The results show that the enzyme can be properly immobilized and there is large variability in the background buffer current yet, the substrate response remains fairly consistent. The steady state values does not tell the whole story for these amperometric tests. Another issue that needs to be considered is time. These electrodes take more time to come to the steady state then the glutaraldehyde electrodes investigated. The response time of the PVA-SbQ electrodes is shown in the second electrode wash test (Figure 34). The equilibrium requires over 180 seconds to be obtained.

Figure 33. Wash test of PVA-SbQ electrode

Figure 34. PVA-SbQ typical ampermetric test
III.3.1 Results from varying immobilization conditions

Next the amount of enzyme added and the amount of glutaraldehyde was investigated to determine effect on the steady state value that would be obtained using 1mM of acetylthiocholine iodide. This is presented below in figure 35 where the electrodes tested were generated via method one.

The value of 1% glutaraldehyde and 2.5 u of enzyme was not obtained as the immobilized mixture peeled the electrode surface off. This introduced a new issue with using screen printed electrodes with this form of immobilization. As the gel thickness increased the risk of the carbon electrode paste de-laminating became greater. These results also show an interesting trend. The less glutaraldehyde added to the electrode the greater the response. This implies that this crosslinking compound is directly interacting with the enzyme. The interaction either inactivates some of this catalyst or diffusion was decreased. Also it shows that the greater amount of enzyme added to the electrode the greater the response obtained, which also shows that the system is behaving as enzyme controlled.

Figure 35. Responses of electrodes with different glutaraldehyde mixtures and enzyme.
III.3.2 Kinetic Parameters of immobilized enzymes

Determining the kinetic parameters of the electrode ensures that the limiting step of the system is the enzyme. Michaelis Menten kinetics are used to predict how the electrode would react and this equation is shown below.

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

In the Michaelis Menten equation, \( v \) refers the to reaction rate, \( V_{\text{max}} \) is the maximum rate of enzymatic reaction, \( K_m \) is the Michaelis constant, and \([S]\) refers to the concentration of the substrate. The main method of determining these constants is to vary the substrate concentration, then measure the rate of reaction of the enzyme. This can be performed by measuring the amperometric current from the electrochemical cell when it is exposed to various concentrations of the substrate, as the current is believed to be directly proportional to the rate of enzymatic reaction.

Again, performing amperometric tests as discussed previously was used. This time the concentration of the substrate was varied. The steady state currents produced from the various substrate concentrations were recorded and plotted as a function of the substrate as shown in Figure 36.
The data was then fit using a non-linear least squared approach to determine the kinetic parameters of the immobilized enzyme system. The constants from the Michaelis Menten equation were found where the max rate of enzymatic reaction is directly linked to the observed steady state current values and was determined to be -4.9 µA, and the $K_m$ value was found to be 0.69mM thiocholine. The $K_m$ value is comparable to the value that was obtained with the native enzyme.

**III.4 Conclusions**

Immobilization is an important part in electrode manufacturing as it allows for reuse of the electrode as well as increases the stability of the product. Out of the several methods of immobilization investigated, glutaraldehyde proved to be the most valuable. There were several methods of glutaraldehyde immobilization that could be performed; however, using liquid
glutaraldehyde was the most investigated in this study. The electrodes produced using this method created gels that reacted according to Mechaelis Menten kinetics PVA-SBQ immobilization requires a long amount of time to achieve steady state and is not investigated further.
Chapter IV– Arsenic Inhibition

Sean Minderlein

IV.1 Introduction to enzyme inhibition

The sensors ability to measure arsenic is dependent on how well the enzyme is inhibited by arsenic. If the enzyme is not inhibited by the desired concentration of arsenic, then a sensor based on the inhibition is not feasible. In other words, inhibition of the enzyme must be quantifiable at the concentrations of interest in order to design a sensor that would operate in the concentration ranges of interest. It is also important to determine the method of inhibition, as the different types of inhibition have different methods of reaction with the enzyme, which will impact how the sensor will be used.

Acetylcholinesterase is inhibited by arsenic, it is believed that arsenic acts as a quasi-reversible competitive inhibitor, which means that the inhibitor is actively competing with the substrate for the active site (Wilson and Silman). It is also believed that arsenite inhibits AChE through covalent bonding to a non-sulfhydryl site on the enzyme, such as tyrosine (Wilson and Silman).

Since the inhibition is competitive, and a small concentration of arsenic is desired to be detected, high concentrations of substrate present would prevent inhibition. A method to solve this issue is to incubate the electrode with the arsenic solution for some time before adding the substrate. This incubation period will depend on the reaction rate of the inhibitor with the enzyme.

Since the inhibition of this enzyme is directly dependent not only on the inhibitors ability to bind to the enzyme, but also the rate that it is released from the enzyme, these two rate terms are dependent on several factors in the reaction matrix. Since a sensitive sensor is desired, the rate of inhibition (K₁) should be larger than the rate of re-activation (+I). There are several factors that can aide in shifting the equilibrium to the inhibited state, such as the addition of ligands, such as oximes, and changing the pH (Wilson and Silman).
IV.2 Inhibition of soluble enzymes

The characterization of acetylcholinesterase from electric eel by arsenic was performed in buffer. The cholinesterase enzyme (0.5 to 1 U/ml) was incubated with 0.5 to 10 mM As(III) prepared in 25 mM Tris-HCl buffer at pH 7.0 (bubbled with nitrogen for at least 30 minutes to remove oxygen). Small aliquots of the enzyme/arsenic solution were removed and diluted 10x into the enzyme substrate/buffer to determine residual enzyme activity by Ellman’s assay. The change in enzymatic activity was monitored over time to determine the rate of enzymatic inactivation (Figure 37).
Figure 38. Inhibition of Electric Eel AChE by As(III)

The inhibition rate constant is published to be 140 M⁻¹ min⁻¹ (Page, Wilson). This rate is relatively slow in consideration for a real time sensor with the substrate and the inhibitor both present in solution. Investigating parameters to accelerate this rate of inhibition is desired to create a sensor that is sensitive to this toxin. The first parameter is the buffer, as it has been shown that the pH and buffer strongly affect the rate at which arsenite can inhibit this enzyme (Page, Dilman). More specifically, the presence of amines increases the rate at which the enzyme is inactivated (Page, Silman). This phenomenon was investigated in our studies, and similar results were obtained (data not shown).

IV.3 Inhibition of AChE Biosensor

Electrodes were created using the BSA and Glutaraldehyde method to immobilize the enzymatic protein layer directly onto the electrode surface. Then, the electrodes were tested using various testing methods. In the first method, an immobilized electrode was polarized in the buffer solution, then introduced it to a substrate environment (1.5mM ATCCl) obtaining the steady state current. Next, arsenic was added to the solution to monitor the response. This proof of concept is shown below in figure 38, where an AChE electrode was exposed to arsenic.
The addition of the substrate resulted in the predicted response of an increased current, then the addition of the inhibitor yielded a decreased response. This decreased current from the arsenic was expected, however the amount of arsenic added to the solution to see a decreased current (100 µM arsenic) was much greater than the amount desired to be detected. Since arsenic inhibition of AChE is believed to be competitive, only high concentrations of arsenic will be able to inhibit the enzyme. Adding the arsenic to the enzyme without the substrate present would lead to a higher percent inhibition.

The second method of inhibition involves incubating the electrode in the arsenic solution. After obtaining the initial steady state current from the uninhibited electrode response to the substrate, the electrode was put into an arsenic solution for 30 minutes. The electrode was then tested in the toxic solution by adding thiocholine to make the concentration of the solution 1mM.
The percent inhibition was then calculated by comparing the two values. This method allows for the inhibition to equilibrate as well as lower the chance that arsenic could pop off of the enzyme. This method was performed with various concentrations of arsenic and is shown below in figure 40.

![Inhibition Curve with As(III) Concentration Variation](image)

**Figure 40. incubation inhibition of enzyme electrode**

The inhibition of the enzyme electrode appears to be the greatest in the second method of testing, which is logical as the arsenic is not competing for the active site with thiocholine, and it does not have the ability to pop off of the enzyme. The method of incubating the electrode in the arsenic solution then adding the substrate to the incubation mixture appears to be the optimal way to inhibit the electrode.

**IV.4 Conclusions**

Determination of arsenic at a concentration at or lower than 10 µg per liter utilizing the inhibition of acetylcholinesterase and electrochemical properties is likely not feasible due to the inhibition kinetics of the inhibitor. However, other enzymes can be investigated to determine if it is likely to generate a sensor using the techniques that were developed.
It was also shown that arsenic behaves as a quasi-reversible competitive inhibitor of acetylcholinesterase when it is immobilized on an electrode. This enzyme was also successfully immobilized onto an electrodes surface and different methods were used to ensure the successful crosslinking of the proteins.
Resources


