I, Robert Wilson, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Chemistry.

It is entitled: Spectroelectrochemical Sensing and Detection of Zinc in Serum by Anodic Stripping Voltammetry on a Bismuth Film Electrode

Student's name: Robert Wilson

This work and its defense approved by:

Committee chair: William Heineman, PhD
Committee member: Michael Baldwin, PhD
Committee member: Apryll Stalcup, PhD
Spectroelectrochemical Sensing and Detection of Zinc in Serum by Anodic Stripping Voltammetry on a Bismuth Film Electrode

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by

Robert A. Wilson
M.S., University of Cincinnati, 2010
B.S., Northern Kentucky University, 2007
B.A., Northern Kentucky University, 2006

Committee:
William R. Heineman (Chair)
Michael J. Baldwin
Apryll M. Stalcup
ABSTRACT OF DISSERTATION

The work described in this dissertation was done as a part of a project to further develop spectroelectrochemical sensors for detection of biomarkers of human exposure to polycyclic aromatic hydrocarbons, pyrene metabolites 1-hydroxypyrene (1-PyOH) and 1-hydroxypyrene glucuronide (1-PyO-glu) and to develop a down-well spectroelectrochemical sensor. The second part of this work is focused on the development of a lab-on-a-chip sensor for zinc detection in serum by anodic stripping voltammetry.

The spectroelectrochemical sensor developed in our research group offers high analyte selectivity. Most of the sensors developed by other members of our group include a glass waveguide coated by an optically transparent indium tin oxide (tin-doped indium oxide or ITO) electrode. An optically transparent thin layer electrochemical (OTTLE) cell and holder that can be used with a conventional spectrophotometer for both absorbance and luminescence measurements was optimized and characterized as a part of this project. This OTTLE cell consists of an ITO electrode and a quartz cover slide. The holder is designed to hold the OTTLE at 45° to the incident light and has dimensions of 1 x 1 x 4 cm which allows the holder to fit most conventional spectrophotometers. The reference and auxiliary electrodes are fixed in space in the opposite corners of the holder. The cell was characterized by thin-layer cyclic voltammetry and coulometry of ferricyanide/ferrocyanide. Spectroelectrochemistry of tris-(2, 2'-bipyridine) ruthenium (II) chloride (Ru(bpy)₃Cl₂) and 1-hydroxypyrene (1-pyOH) was done with commercially available bench-top absorbance and fluorescence spectrophotometers. The good correlation between the results obtained and the known properties of each compound demonstrate that the OTTLE cell and holder provide an effective means for making...
spectroelectrochemical measurements in bench-top absorbance and fluorescence spectrophotometers.

Spectroelectrochemical sensing in an optically transparent thin layer electrode cell (OTTLE) was used for detecting the polycyclic aromatic hydrocarbon (PAH) biomarkers 1-hydroxypyrene (1-pyOH) and 1-hydroxypyrene-glucuronide (1-pyOglu) in phosphate buffer and artificial urine. This approach uses selective electrochemical modulation of a fluorescence signal by sequentially oxidizing the analytes in an optically transparent thin layer electrode (OTTLE) to distinguish between their overlapping fluorescence spectra. This technique allows for complete oxidation and signal modulation in approximately 15 min for each analyte; a mixture of 1-pyOH and its glucuronic acid conjugate can be analyzed in 30 min. Calibration curves consisting of the fluorescence change vs. analyte concentration for 1-pyOH and 1-pyOglu yielded linear ranges from 10 nM to 1 µM and from 1 nM to 1 µM, respectively.

To further the spectroelectrochemical technique a down-well spectroelectrochemical cell was developed. The down-well spectroelectrochemical sensor was shown to provide sufficient electrochemical and preconcentration of Ru(bpy)$_3^{2+}$. The uptake of Ru(bpy)$_3^{2+}$ into a Nafion thin film provides the possibility for signal enhancement and demonstrates the cell’s ability to uptake an analyte and regenerate a film. A fluorescence change associated with the oxidation of Ru(bpy)$_3^{2+}$ to Ru(bpy)$_3^{3+}$ at 1100 mV shows the probe’s ability to spectroelectrochemically detect the analyte if the optical bundle is positioned properly.

The laser noise present in the optical signal prevents the cell from being calibrated in its current state. However, modifications to the cell could result in the elimination of the laser noise. This would allow for successful spectroelectrochemical detection of the model analyte Ru(bpy)$_3^{2+}$. A down-well probe for fluorescence spectroelectrochemical detection can be
beneficial for a number of analytes and the design presented here can be easily scaled up or down in size to make the probe even more versatile.

The second part of this work focuses on the detection of zinc (Zn) by anodic stripping voltammetry (ASV) on electrodeposited bismuth electrodes. The electrochemical sensor is a three-electrode system consisting of the electrodeposited bismuth film working electrode, the electrodeposited Ag/AgCl reference electrode, and a Au counter electrode. The working potential window of the bismuth film electrode was investigated by cyclic voltammetry, while square wave ASV was used for measurement of Zn in acetate buffer and blood serum. Conditions critical to sensing, such as preconcentration potential, preconcentration time, and buffer pH were optimized for Zn detection. The sensor was successfully calibrated with pH 6 acetate buffer in the physiologically-relevant range of 5 μM to 35 μM. Detection of Zn in unspiked and spiked serum samples was done by two digestion methods. The initial 0.2 M HCl digestion method allowed for Zn detection in serum but did not exhibit adequate reproducibility. The second digestion method resulted in the expected Zn stripping peaks for post digestion spikes but peaks could not be obtained for Zn spikes prior to digestion.
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## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASV</td>
<td>Anodic stripping voltammetry</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflection</td>
</tr>
<tr>
<td>BiFE</td>
<td>Bismuth film electrode</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HMDE</td>
<td>Hanging mercury drop electrode</td>
</tr>
<tr>
<td>HPLC</td>
<td>High precision liquid chromatography</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium-tin oxide</td>
</tr>
<tr>
<td>MFES</td>
<td>Mercury film electrodes</td>
</tr>
<tr>
<td>MIR</td>
<td>Multiple internal reflections</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>OTEs</td>
<td>Optically transparent electrodes</td>
</tr>
<tr>
<td>OTTLE</td>
<td>Optically transparent thin layer electrode</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
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Chapter 1. Introduction

1.2 Spectroelectrochemical Sensing

1.1.1 Spectroelectrochemical Technique

Spectroelectrochemistry has been used since the early 1960s to simultaneously obtain electrochemical and optical data.[1-4] Initially, this approach utilized only two modes of selectivity: potential and absorbance. In this work optically transparent electrodes (OTEs) such as transparent thin films of Au, Pt, indium-tin oxide (ITO) or metal minigrid electrodes (generally 100-200 wires/inch) were used. The optical signal was monitored by either transmission of an optical beam through the transparent electrode or attenuated total reflection (ATR).[5,6] ATR couples a beam of light at an appropriate angle to allow for total internal reflection within the OTE. As the light travels through materials with different refractive indices, the beam will be refracted and reflected, Figure 1.1. If the light is passing from a medium of higher refractive index to a medium of lower refractive index and the angle of incidence is equal to or greater than the critical angle the beam will be totally reflected at the point of incidence, Figure 1.1. This optical phenomenon cannot occur when light passes from a medium of lower refractive index to a medium of higher refractive index. The critical angle is determined by Equation 1, where \( n_2 \) is the refractive index of the medium at the boundary with the lower refractive index and \( n_1 \) is the refractive index of the medium with the higher refractive index.

Equation 1. \[ \theta_c = \arcsin \left( \frac{n_2}{n_1} \right) \]
While the entire wave is reflected back to the medium with the higher refractive index, at the reflection points an evanescent wave penetrates approximately one wavelength into the medium of lower refractive index and decays exponentially with the distance from the surface, Figure 1.2. Optical monitoring occurs when species present at or near the electrode surface interact with light from the evanescent field. The main advantage of ATR is the increased optical pathlength due to the numerous reflections within the planar OTE, which allows for lower detection limits.

![Figure 1.1](http://en.wikipedia.org/wiki/File:R%C3%A9flexion_total.svg)

Figure 1.1  A) Angle of incidence less than the critical angle. B) Partially reflected light from A. C) Partially refracted light from A. D) Angle of incidence is equal to or greater than the critical angle and all light is reflected. Public domain image is adapted from http://en.wikipedia.org/wiki/File:R%C3%A9flexion_total.svg.
1.1.2 Spectroelectrochemical Sensor

More recently, a third mode of selectivity was added by incorporating a thin ion-exchange film.[7] The thin ion-exchange film selectively preconcentrates ions of a specific charge and prevents ions of the opposite charge from partitioning into the film. This improves the detection limit by increasing the concentration of analyte at the electrodes surface and reduces the possible interferences. This sensing method allows for three modes of selectivity which require an analyte to: 1) preconcentrate into an ion-exchange thin film, 2) be electrolyzed at the selected potential applied to the working electrode, 3) absorb light at the selected wavelength in its oxidized or reduced state. The sensor concept is illustrated by the schematic diagram shown in Figure 1.3.

In this illustration an anion exchange thin-film is shown. This film provides the first mode of selectivity by preventing transport of cations into the film as shown in Figure 1.3, uptaking/preconcentrating anions as shown, and having no effect on neutral species (not shown). The second mode of selectivity involves the oxidation or reduction of the analyte or possible interferences at the selected potential applied to the working electrode. Anions that previously
preconcentrated into the film but do not undergo an electrochemical reaction at the selected potential will not be measured as shown in Figure 1.3. Anions transported into the film that undergo an electrochemical reaction must also exhibit an optical change at the selected wavelength associated with electrolysis to be monitored. Quantification of the analyte is achieved because this optical change is proportional to the analyte concentration at the electrode surface.

Figure 1.3 Illustration of a prototypical spectroelectrochemical sensor. The major components of the spectroelectrochemical sensor (the optical waveguide, transparent electrode, and chemically-selective film) are shown in a cross-section view of the sensor. Vertical arrows denote transport into the film. Half-circle arrows denote electrolysis at the applied potential. Large X indicates an analyte’s inability to meet one of the three modes of selective and therefore cannot be measured.
1.1.3 Proof of Concept

The capabilities of the spectroelectrochemical sensing method featuring three modes of selectivity was first demonstrated on the model analyte ferrocyanide \([\text{Fe(CN)}_6]^{4-}\). Ferrocyanide is an ideal model analyte because it has well-defined reversible electrochemistry which results in an optical change at 420 nm. This sensor consisted of a sol-gel processed poly(dimethyldiallyl ammonium chloride (PDMDAAC-SiO2) anion-exchange film on an ITO electrode for preconcentration \([\text{Fe(CN)}_6]^{3-/4-}\). This work clearly demonstrated the ability to quantify the concentration of \([\text{Fe(CN)}_6]^{4+}\) based on the optical change at 420 nm, associated with the electrolysis of \([\text{Fe(CN)}_6]^{4+}\) to \([\text{Fe(CN)}_6]^{3-}\). In the sensor’s unoptimized condition a detection limit of \(8 \times 10^{-6}\) was achieved. The concept was also demonstrated in the presence of direct interferences to the \([\text{Fe(CN)}_6]^{3-/4-}\) couple. This work demonstrated that the sensor was capable of detecting an analyte in the presents of charged, optical, and electrochemical interferences. The versatility of the spectroelectrochemical detection method was shown by expanding the detection method to the hexacyanoruthenate \(\text{Ru(CN)}_6^{4-/3-}\) couple by changing the optical wavelength monitored and electrochemical potentials used.

The spectroelectrochemical sensor was later used for detection of \(\text{Fe(CN)}_6^{4+}\) in a complex waste tank simulant and radioactive waste samples from a Hanford storage tank. The waste simulant sludge introduced a number of issues including high concentrations of possible interfering ions and high pH. The high concentration of ions in the solution increase the competition for uptake of the analyte and can also lead to film degradation. However, the results presented in this work showed the 900 nm PDMDAAC-SiO2 coated on an ITO was sufficiently rugged under these adverse conditions to give reproducible response for ferrocyanide for 40 min. While the signal was reduced due to ion competition an absorbance change
corresponding to 8.0 mM Fe(CN)₆⁴⁻ was obtained. This compares favorably to the 8.2 mM obtained by FTIR. These results were promising enough to develop a field portable spectroelectrochemical sensor for ferrocyanide detection in real Hanford waste storage samples. For this work a standard addition method was developed which resulted in a linear response due to the change in absorbance from 5 x 10⁻⁵ to 5 x 10⁻³ M.

In an effort to improve detection limits a fluorescence based spectroelectrochemical sensor was developed. This sensor used an attenuated total reflection (ATR) method which measured fluorescence at 90° to the OTE surface. This allows for the emitted light from the analyte preconcentrated in the film to be monitored. This unoptimized fluorescence-based sensor for the Ru(bpy)₃²⁺ ion showed a linear response between 1 × 10⁻¹¹ and 1 × 10⁻⁷ M with a calculated 2 × 10⁻¹³ M detection limit.

1.1.4 Optically transparent thin layer electrode cells

One development which has proven to be extremely useful is monitoring an analyte solution confined in a thin layer next to a transparent electrode. The first use of an optically transparent thin layer electrode (OTTLE) for optical monitoring of an electrochemically generated species was reported in 1967. This electrode was designed to monitor the absorbance of an analyte during electrolysis on a gold minigrid sandwiched between two microscope slides. The transparency of this electrode is due to the physical holes in the grid and is dependent on the quality of micromesh selected (generally 100-200 wires/inch). Consequently, the optical range of this OTTLE is determined only by the choice of material for the cover slide. Alternatively, OTEs consisting of a thin layer (100 to 5000 Å) of optically transparent and conductive material such as gold, platinum, carbon, or tin-doped indium oxide (ITO) can be used. The transparent properties of these OTEs depend on the thinness of the conductive layer and the
material selection. Thinner conducting layers increase OTE transparency, which is beneficial for the spectroscopy, but have an adverse effect on resistance, which is detrimental to electrolysis. The major disadvantage of using ITO OTEs is the large resistance resulting from the thin layer of tin oxide. The resistance of the electrode becomes a larger issue when used in the construction of an OTTLE because thin layer cells already have a pronounced solution resistance due to the relatively thin solution layer. The resistance problem is further exacerbated by the large current resulting from the large electrode area typically used.

The main advantage of an OTTLE is the fast and complete electrolysis with simultaneous optical monitoring due to restricted diffusion in the thin layer of solution. Depending on the area of the electrode exposed to solution and the optical path length, a relatively small volume of solution is electrolyzed (30-50 µL), which usually requires only a few minutes or less for complete electrolysis.

Previous studies have incorporated an OTTLE into a standard quartz cuvette. One such design places the OTTLE cell at a 45° angle within a standard cuvette. By placing the cell at a 45° angle to the excitation and emission slits, fluorescence measurements can be obtained, but the cell suffers from high variability between experiments due to inconsistencies in cell positioning. Another approach to incorporating an OTTLE into a standard cuvette involves cutting away the majority of three sides of the cuvette, leaving only the base intact to hold the OTTLE. While this modification is easy to make, the resulting cell remains unsuitable for making fluorescence measurements in spectrophotometers that measure at a 90° angle. Another spectroelectrochemical cell is the Thin Layer Quartz Glass Spectroelectrochemical cell made by Bioanalytical Systems, Inc. This cell is similar in size to a conventional cuvette and is designed for use with the UV-absorbance spectrophotometers commonly available in research
facilities. However, this design is not compatible with standard bench-top fluorescence spectrophotometers that measure emission at an angle of 90°.

1.2 Polycyclic aromatic hydrocarbons (PAHs)

1.2.1 PAH sources and health hazards

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds formed from the incomplete combustion of organic material. As the name implies, PAHs contain two or more fused benzene rings. The smallest PAH is naphthalene C_{10}H_{8} and contains two fused rings (Figure 1.4). Some of the major sources of PAH contamination are combustion of fossil fuels from power plants, waste incineration, cigarette smoke, automobile emission, and various industrial processes.\textsuperscript{[27]} PAH contamination is a widespread problem and PAHs have been found in over 600 out of 1,408 hazardous waste sites in the EPA National Priority list.\textsuperscript{[28]} PAHs are absorbed via oral, inhalation, and dermal exposure.\textsuperscript{[29-31]} Though absorption through the gastrointestinal tract has been shown to be relatively poor and results in a low percentage of excretion compared to exposure and high deviation.

![Naphthalene](image1.png)

![Anthracene](image2.png)

![Pyrene](image3.png)

Figure 1.4 Examples of commonly found PAHs.
Many PAHs have been shown to have carcinogenic effect to humans.\textsuperscript{[32-35]} Several studies of PAHs have led to an understanding of their mechanism of tumor initiation.\textsuperscript{[36, 37]} PAHs are enzymatically activated and form stable bonds with DNA by two main pathways: one-electron oxidation to produce reactive intermediate radical cations and monooxygenation to bay-region diol epoxides.\textsuperscript{[36, 37]} The reactive intermediates formed by these two mechanisms, radical cations and diol epoxides, can bind to DNA to produce adducts that initiate the process of tumor formation unless removed during DNA repair.\textsuperscript{[37, 38]}

1.2.2. Pyrene metabolism and detection

The pyrene metabolites 1-pyOH and 1-pyOglu have been extensively used as biomarkers for indirect assessment of total polycyclic aromatic hydrocarbon (PAH) exposure.\textsuperscript{[40-45]} The structure of 1-pyOH and 1-pyOglu can be seen in Figure 1.5. These metabolites have been found to be effective biomarkers for PAH exposure assessment because pyrene is a major constituent in virtually all PAH mixtures\textsuperscript{[46, 47]} and its metabolites are readily excreted in urine.\textsuperscript{[48, 49]} The metabolism of pyrene involves the initial formation of the phase I metabolite 1-pyOH by enzymatic conversion by cytochrome P450.\textsuperscript{[50]} 1-pyOH is converted to the more water soluble phase II metabolite 1-pyOglu by the addition of glucuronic acid in an enzymatic process catalyzed by UDP-glucuronosyltransferases (UGT) UGT1A6, UGT1A7, and UGT1A9.\textsuperscript{[50]} These metabolites are then excreted in urine. While the relative concentration of 1-pyOH and its conjugates in urine largely depend on the person’s genetically determined enzymatic pattern for metabolic PAH conversion assessment of human urine has shown 1-pyOglu accounts for approximately 80\% of the pyrene metabolites excreted.\textsuperscript{[50]}
Figure 1.5  Structure of the pyrene metabolites 1-hydroxypyrene (1-pyOH) and 1-hydroxypyrene-glucuronide (1-pyOglu).

Due to the high concentration of conjugated metabolites, the standard approach for human assessment involves an initial enzymatic or acid hydrolysis of pyrene conjugates to 1-pyOH. The resulting sample of 1-pyOH can then be analyzed by HPLC with fluorescence detection.\textsuperscript{[40]} Though this technique is highly reproducible and offers detection limits in the low µg/L range, it requires multiple experimental steps and has an extended analysis time (overnight). A variety of other detection methods have been developed to expedite this process, including: HPLC-FL for direct measurement of 1-pyOglu; immunoaffinity chromatography with synchronous fluorescence spectroscopy (SFS); GC-MS; and LC-MS/MS.\textsuperscript{[51-59]} All of these techniques have been shown to offer detection limits within a suitable range for pyrene metabolite detection (1 nM).\textsuperscript{[60]}

1.2.3 Sensors for PAH detection

Optical and electrochemical sensors have previously been used for the detection of multiple PAHs. A phosphorescence-based flow-through sensor was developed for the detection of Benzo[a]pyrene in a sample with multiple PAH interferences.\textsuperscript{[61]} A group of PAHs (pyrene, benzo[c]phenanthrene, triphenylene, and coronene) have been detected on dithiocarbamate calix[4]arene functionalized Ag nanoparticles by using surface-enhanced Raman scattering
The voltammetric detection of 1-pyOH has been shown on molecularly imprinted polymer-modified screen-printed carbon electrodes and on carbon paste electrodes modified with cyclodextrin derivatives and double stranded deoxyribonucleic acid (dsDNA).

1.3 Anodic stripping voltammetry (ASV) and Zinc detection

1.3.1 Anodic stripping voltammetry

Anodic stripping voltammetry (ASV) is a commonly used electrochemical technique for quantitative analysis of electroactive species such as metals ions. ASV uses a three electrode cell consisting of working, auxiliary, and reference electrodes. The reference electrode establishes the electrical potential against which other potentials may be measured. To ensure accurate potential measurements the stability of the reference electrode is controlled by using a saturated buffer solution with a well known redox potential. The auxiliary electrode is used to balance the current produced at the working electrode and is commonly referred to as the counter electrode because of this. The major purpose of the auxiliary electrode is to prevent current from passing through the reference electrode and compromising its potential. The working electrode is where the electrochemical reaction of interest takes place.

ASV consists of two major steps: an electrochemical deposition step and a stripping step (Figure 1.6). During the deposition step the analyte is reduced and electroplated on the working electrode surface. This allows for analyte pre-concentration and provides the ability to obtain very low limits of detection. In most cases only a small fraction of the total analyte in the sample is deposited on the working electrode during the deposition step. Complete deposition of all the analyte would be time-consuming and is usually unnecessary because over short deposition times adequate stripping peaks are obtained from the amount of analyte deposited. Since the deposition is not exhaustive, it is important to control the amount of analyte deposited. To do
this the electrode surface area, deposition time, and stirring must be carefully controlled throughout the experiment. Deposition times can vary widely depending on the analyte concentration, the type of electrode, and the stripping technique used. The less concentrated solutions require longer deposition times to give adequate stripping peaks.

Figure 1.6  Schematic of the pre-concentration and stripping steps for ASV.

Following the deposition step the electroplated metal is oxidized during the stripping step and the current is measured. The stripping step can use a number of waveforms. Some of the most widely used are linear, staircase, pulse, and squarewave. When a linear waveform is used the potential is swept linearly with time following deposition. Alternatively a staircase waveform can be imposed on the linear sweep which produces a series of steps. The current can be measured at any point in the step, though most of the time the current is measured at the end of the step just before potential change. This waveform will result in a minimized charging current. In normal pulse voltammetry each potential step begins at the same potential and the amplitude of each step increases in small increments over time. This results in the capacitive current decaying exponentially while the faradic current decays as the square root of time and therefore the analyte signal can be better discriminated from the background. When using a squarewave (Figure 1.7) form the differential current is measured. A measurement is taken at a single point
for each half-wave and the differential current is then plotted as a function of potential. This allows for the subtraction of the capacitive current and provides a fast and reproducible method with very low detection limits.

Figure 1.7 Illustration of the squarewave form. Public domain image adapted from http://en.wikipedia.org/wiki/File:Squarewave_Potential_Sweep.JPG.

1.3.2 Mercury electrodes

Historically, hanging mercury drop electrodes (HMDEs) and mercury film electrodes (MFEs) were widely used for ASV. HMDE is a drop of mercury suspended in the sample, usually on the end of a capillary. The main advantages of the HMDE are the very low residual current, the excellent negative potential range that pure mercury exhibits, and the fresh electrode surface formed for each measurement. The major advantage of using mercury as the working electrode for ASV is that many metals dissolve in mercury to form amalgams. The MFE is prepared by the electrochemical reduction of $\text{Hg}^{2+}$ to $\text{Hg}^0$ on a conductive substrate such as glassy carbon.
An advantage of the MFE results from the greater electrode surface area/volume ratio of a film as compared with the spherical HMDE. The larger surface area enables more analyte to be concentrated into a given amount of mercury during deposition. This results in greater sensitivity for the MFE compared with the HMDE. The MFE is also compatible with stirring rates that would dislodge a HMDE. The ability to use greater stirring rates or to rapidly rotate the electrode improves mass transport during the concentration step, which reduces the time required to deposit sufficient analyte to give adequate stripping peaks. For both the MFE and HMDE the major advantage for stripping voltammetry is the solubility of the deposited metals in mercury. This allows for each metal atom to diffuse to the electrode independently without interference from other co-deposited metals.

1.3.3 Bismuth electrodes

Recently, bismuth film electrodes (BiFE) have become a popular alternative to the MFE and HMDE due to the rising concern of mercury toxicity and the advent of microfabricated cells which mercury is not well suited for. BiFEs, like MFEs are prepared by reduction of the ion Bi$^{3+}$ to Bi$^{0}$ (s) on a conductive electrode. Bismuth films can be electroplated on a number of conductive electrodes.\textsuperscript{[65]} This means BiFEs will be compatible with the majority of microfabricated cells. Also, BiFEs exhibit a high overvoltage for hydrogen evolution resulting in a negative potential window similar to that of MFE.\textsuperscript{[66, 67]} This negative working window has been utilized to detect Pb\textsuperscript{[66-69]}, Cd\textsuperscript{[66-69]}, Zn\textsuperscript{[66-69]}, Ti\textsuperscript{[66, 67, 70]}, In\textsuperscript{[67]}, Cu\textsuperscript{[67]}, and Sn\textsuperscript{[71]} by ASV. Another advantage of BiFEs is their low toxicity compared to MFEs. The lower toxicity of the BiFE allows for the development of disposable devices, which can be utilized for a variety of point of care and field use studies.
While BiFEs and MFEs have similar electrochemical properties, there are some important differences that must be taken into consideration. For instance, mercury deposits on the electrode as liquid mercury droplets while bismuth deposits as a solid film at room temperature. Solid films exhibit a number of disadvantages compared to the liquid MFE. In the MFE metal atoms are soluble and can diffuse independently of each other resulting in little to no distortion of stripping peaks. On solid surfaces metals are deposited on top of each other leading to multiple and distorted stripping peaks. Solid surfaces can also have issues with fouling due to metals adsorbing on the surface. Another disadvantage of the BiFE compared to MFE is the decreased potential window. While the negative potential windows are similar the working window of BiFE is decreased due to the negative potential required for oxidation of Bi\textsuperscript{0} to Bi\textsuperscript{3+}. This leads to an upper limit for the BiFE around -200mV, compared to 200 mV for MFE.

A number of solid electrodes such as glassy carbon, Au, and Pt have also been used for stripping analysis. Some of these electrodes like Au and carbon have been used extensively in microfabricated devices. However, the major disadvantage of these solid electrodes is the limited negative working region they offer compared to MFE and BiFE. This results in the inability to detect many important analytes like Zn. Another disadvantage is the issue of peak shifting and distortion due to metal interactions at the electrode surface similar to the BiFE.

1.3.4 Zinc in serum

Zinc is an essential element required for a number of enzymes and transcription factors.\textsuperscript{[72]} The flexibility of Zn coordination geometry allows for rapid protein conformation changes required for many biological reactions.\textsuperscript{[73]} Due to this Zn provides an essential catalytic function for a number of enzymatic reactions. Pediatric and adult studies have consistently demonstrated abnormally low zinc levels in critically ill patients and inflammation and infection are associated
with reduced serum levels of zinc.\textsuperscript{[74-79]} Zn supplementation may be a beneficial therapeutic strategy in critically ill patients.\textsuperscript{[80-83]} However, for this strategy to work safely, zinc levels must be monitored constantly because in blood plasma, zinc is bound to and transported by albumin and transferrin.\textsuperscript{[84]} Since transferrin also transports iron, excessive zinc reduces iron absorption, and vice-versa. A similar reaction occurs with copper.\textsuperscript{[85]}

Conventional methods for Zn analysis requires blood samples to be collected and shipped to a laboratory for analysis by atomic absorption spectrophotometry (AAS) or inductively coupled plasma mass spectroscopy (ICP-MS).\textsuperscript{[86,87]} These methods are accurate but costly and can be time consuming due to the need for shipping the collected samples. In order to determine the possible benefits of Zn supplementation for critically ill patients an on-site method for Zn quantification in serum is required.

ASV has been used for Zn detection on multiple electrodes. Initially, HMDEs and MFEs were used for the detection of Zn in a mixture of Pb and Cd.\textsuperscript{[88]} More recently a perfluorinated anion-exchange polymer modified MFE has been used for Zn detection.\textsuperscript{[89]} Mercury electrodes have also been used for the detection of Zn in sugar cane spirit\textsuperscript{[90]} and natural water.\textsuperscript{[91]} As mercury decreased in popularity due to the disposal issues the use of the BiFE has greatly increased for Zn detection by ASV. ASV of both in situ and ex situ deposited bismuth on Nafion-coated glassy carbon electrodes has been shown to offer detection limits of 0.4 µg/L.\textsuperscript{[92]} The detection of Zn has also been shown on BiFE plated on commercially available screen-printed strips\textsuperscript{[93]} and even pencil-lead.\textsuperscript{[94]} The performance of BiFE has been studied on different substrates such as glassy carbon electrode, carbon paste and impregnated graphite, graphite-epoxy composite electrodes (Bi-GECE), carbon nanotubes (Bi-CNT), and bismuth/poly(p-aminobenzene sulfonic acid) film electrode have also been reported.\textsuperscript{[95-97]}
1.4 Project objectives

This work focuses on three main project; 1) exploring the possibility of developing a spectroelectrochemical sensor for PAH biomarkers, 2) exploring the possibilities of developing a down-well spectroelectrochemical cell, and 3) investigating the possibility of developing a lab-on-a-chip sensor for detection of Zn by anodic stripping voltammetry. The goal of the first spectroelectrochemical sensing project was to explore the possibilities of developing a sensor for the biomarkers of human PAH exposure, 1-hydroxypyrene and 1-hydroxypyrene-glucuronide. First, the electrochemistry of the analytes was investigated on an optically transparent indium tin oxide electrode in order to determine the redox potentials to be used for oxidation of each analyte. Next, the change in fluorescence was monitored at specified wavelengths during the electrochemical redox reactions. Calibration curves were obtained for each analyte based on the change in fluorescence signal due to oxidation. A mixture of the analytes was monitored in buffer and urine simulant solutions.

The second spectroelectrochemical project explores the possibility of expanding the spectroelectrochemical technique to a down-well probe using fluorescence detection. First, the electrochemical performance of the cell was investigated by cyclic voltammetry of the model compound Ru(bpy)$_3^{2+}$. The uptake of Ru(bpy)$_3^{2+}$ into a Nafton thin film and subsequent removal was investigated by cyclic voltammetry. Finally, the optical performance of the cell was investigated.

The third project explores the possibilities of developing a lab-on-a-chip sensor with an electrodeposited bismuth film working electrode for monitoring Zn in serum by anodic stripping voltammetry. First, the electrochemical properties of the BiF and the buffer solution were
optimized for ASV. Next, the electrochemical parameters for Zn stripping were optimized (deposition time and deposition potential). These optimized conditions were used to calibrate the sensor for Zn detection in an acetate buffer solution. Next two digestion methods were used to digest human and bovine serum. The effectiveness of these digestion methods were investigated by ASV and ICP-MS.
1.4 References


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Chapter 2. Rapid prototyped optically transparent thin-layer electrode holder for spectroelectrochemical measurements

2.1 Introduction

Spectroelectrochemistry is a proven technique for monitoring electrochemically generated species by absorption\cite{1-3} and fluorescence\cite{4} spectroscopy. This technique couples the ability to change the oxidation state of an analyte by applying an electrical potential with the detection capabilities of spectroscopy. Quantification of the analyte is based on the magnitude of change in the optical signal due to the applied potential, which is proportional to the concentration of analyte in a sample.

One development which has proven to be extremely useful is monitoring an analyte solution confined in a thin layer next to a transparent electrode. The first use of an optically transparent thin layer electrode (OTTLE) for optical monitoring of an electrochemically generated species was reported in 1967\cite{5}. This electrode was designed to monitor the absorbance of an analyte during electrolysis on a gold minigrid sandwiched between two microscope slides (Figure 2.1). The transparency of this electrode is due to the physical holes in the grid and is dependent on the quality of micromesh selected (generally 100-200 wires/inch). Consequently, the optical range of this OTTLE is determined only by the choice of material for the cover slide. Alternatively, optically transparent electrodes (OTEs) consisting of a thin layer (100 to 5000 Å) of optically transparent and conductive material such as gold, platinum, carbon, or tin-doped indium oxide (ITO) can be used.\cite{6-13} The transparent properties of these OTEs depend on the thinness of the conductive layer and the material selection. Thinner conducting layers increase OTE transparency, which is beneficial for the spectroscopy, but have an adverse effect on resistance, which is detrimental to electrolysis.
Figure 2.1  Optically transparent thin-layer electrode (gold minigrid generally 100-200 wires/inch).  Left assembly of cell front and bottom view.  Right side view of components before assembly.  A) Glass microscope slide (1 x 3 in).  B) Teflon spacers.  C) Transparent Au micromesh.  D) Glass microscope slide.  Path length of cell is dependent on the thickness of the spacers and Au micromesh.  All parts held together by epoxy.

The main advantage of an OTTLE is the fast and complete electrolysis with simultaneous optical monitoring due to restricted diffusion in the thin layer of solution.  Depending on the area of the electrode exposed to solution and the optical path length, a relatively small volume of solution is electrolyzed (30-50 µL), which usually requires only a few minutes or less for complete electrolysis.
Since the initial OTTLE cell, several spectroelectrochemical cells have been designed for use with standard spectrophotometers. The majority of this work involves absorbance based measurements in a long optical path thin-layer cell (LOPTLC). In a LOPTLC the optical beam is oriented parallel to the electrode, and offers improved optical sensitivity because of the increased optical path length. LOPTLC are capable of rapid and complete electrolysis due to the large electrode area and the small solution volume required for thin-layer cells. Non-thin-layer, long optical path cells have also been designed for use with standard spectrophotometers, including one designed for both absorbance and fluorescence measurements. This cell consists of narrow optical channels drilled at 90° angles through a reticulated vitreous carbon (RVC) electrode. However, a 25-30 min equilibration time is required for each measurement due to the 2 mm diameter of the channel.

Previous studies have incorporated an OTTLE into a standard quartz cuvette. One such design places the OTTLE cell at a 45° angle within a standard cuvette. By placing the cell at a 45° angle to the excitation and emission slits, fluorescence measurements can be obtained, but the cell suffers from high variability between experiments due to inconsistencies in cell positioning. Another approach to incorporating an OTTLE into a standard cuvette involves cutting away the majority of three sides of the cuvette, leaving only the base intact to hold the OTTLE. While this modification is easy to make, the resulting cell remains unsuitable for making fluorescence measurements in spectrophotometers that measure at a 90° angle. Another spectroelectrochemical cell is the Thin Layer Quartz Glass Spectroelectrochemical cell made by Bioanalytical Systems, Inc. This cell is similar in size to a conventional cuvette (Figure 2.2) and is designed for use with the UV-absorbance spectrophotometers commonly available in
research facilities. However, this design is not compatible with standard bench-top fluorescence spectrophotometers that measure emission at an angle of 90°.

Figure 2.2 Thin Layer Quartz Glass Spectroelectrochemical cell (Bioanalytical Systems, Inc.)

Here we optimize and characterize a thin layer spectroelectrochemical cell to be used with conventional spectrophotometers for both absorbance and fluorescence measurements. The cell consists of two parts: an optically transparent thin layer cell and a unique cell holder. The cell
holder is designed to house the thin layer cell, a reference electrode, and an auxiliary electrode. The cell holder allows the OTTLE cell to be used for both absorbance and fluorescence measurements and standardizes the distance between electrodes. The OTTLE cell and holder presented here provide an inexpensive and reproducible way to perform spectroelectrochemical experiments in any instrument compatible with a standard 1 cm cuvette.

The unique OTTLE cell holder (Figure 2.3B) was designed and fabricated using rapid prototyping technology. This technique was first introduced in the late 1980s and is now used extensively to produce small models and parts. Rapid prototyping is based on taking a computer-designed model and automatically transforming it into a physical object by the sequential delivery of material to specified points in space. This process creates a physical model identical to the virtual design. The use of computer-aided design (CAD) software makes creating virtual models of specified dimension quick and easy. CAD software and rapid prototyping technology allows for the creation of complex structures in an inexpensive and timely manner.
Figure 2.3 Optically transparent thin-layer electrode cell and holder. A) Assembly of cell front and side view. B) Cell holder, 3D and top down view.

2.2 Experimental Section

Reagents and Materials

The following chemicals were used without further purification: potassium ferrocyanide (Aldrich); potassium nitrate, sodium chloride, sodium bicarbonate, sodium hydroxide (all from Fisher Scientific); tris-(2,2’-bipyridine) ruthenium (II) chloride hexahydrate ([Ru(bpy)3Cl2 6H2O], GFS Chemicals); 1-hydroxypyrene ([1-pyOH] Toronto Research Chemicals Inc.); and...
200 proof ethyl alcohol (Pharmco-AAPER). Fe(CN)$_6^{3-}$ and Ru(bpy)$_3^{2+}$ solutions were prepared by dissolving appropriate amounts in 1.0 M KNO$_3$ (prepared with deionized water from a Barnstead purification system). 1-pyOH was prepared by dissolving appropriate amounts in 1.0 M NaCl/0.2 M pH 10.5 carbonate buffer/20% EtOH.

The OTTLE cell is constructed from a quartz glass slide (ESCO products) cut to (1.90 x 1.00 cm), 0.018 cm-thick silicone spacers (Specialty Manufacturing Inc., Pineville, NC), and indium tin oxide (ITO)-coated glass slides (Corning 1737F and 7059, 11-50 Ω/square, 130-nm-thick film on 1.1-mm glass, Thin Film Devices, Anaheim, CA) with dimensions of 4.00 x 1.00 cm. Silicone spacers cut to approximately 1.90 x 0.20 cm are placed onto the edges of the ITO glass slide and sandwiched between a quartz slide and the ITO (Figure 2.3A). Two-part quick-set epoxy (Loctite) is applied along the edges of the spacers and allowed to cure for 2 h to hold the components together. OTTLE cells made in this manner were capable of approximately 8 hr of continuous use. The exposed ITO above the quartz slide is used for electrical contact.

**Instrumentation**

For all experiments the electrochemical cell consisted of a Pt wire auxiliary electrode, a miniature Ag/AgCl reference electrode (3 M KCl, Cypress Systems), and an OTTLE. Thin layer cyclic voltammetry and coulometry of ferricyanide/ferrocyanide in 1.0 M KNO$_3$ was performed on a BAS 100 Electrochemical Analyzer (Bioanalytical Systems).

Nernst plots of absorbance and emission were constructed by controlled potential electrolysis of Ru(bpy)$_3^{2+}$ and 1-pyOH with an Epsilon Electrochemical Workstation (Bioanalytical Systems). Absorption spectra of 1.00 mM Ru(bpy)$_3^{2+}$ in 1.0 M KNO$_3$ were obtained with a Varian Cary 50 Bio UV-Visible Spectrophotometer. Emission spectra of 0.10 mM 1-pyOH (ex.
280 nm) were obtained using a Varian Cary Eclipse Fluorescence Spectrophotometer with an excitation slit width of 5 nm, an emission slit width of 1.5 nm, and a PMT voltage of 670 V.

Emission spectra of 10.0 µM Ru(bpy)$_3^{2+}$ in 1.0 M KNO$_3$ were acquired using a custom-made system. This system consists of a laser (441.6 nm HeCd model 1K4153R-C, Kimmon Electric Co.), light control modules (shutter, attenuator, and focusing optics), a monochromator (0.3 m focal length, triple grating turret), a photon-counting phototube (Acton Research Corp.), and a computer and control electronics (NCL and Spectra-Sense software, Acton). Light from the laser was focused onto the polished end of a 6-around-1 fiber optic bundle (RoMack Inc.). Laser power was attenuated to 0.5 mW and the sample was exposed to the laser light only during data acquisition to minimize photodegradation.

2.3 Results and Discussion

OTTLE cells have previously been shown to be useful for optical characterization of electrochemical reactions. The majority of these cells have focused on absorbance based spectroelectrochemical detection. By incorporating this unique cell holder (Figure 2.3B.), created using Solid Works software and rapid prototyping technology, the OTTLE cell can be easily used for both absorbance and fluorescence based measurements. The outer dimensions of the holder are identical to the standard cuvette commonly used in spectrophotometers (1 x 1 x 4 cm). However, unlike a standard cuvette, all four side walls have windows for light passage. Two of the inside diagonal corners have slots for the OTTLE cell. This design positions the thin layer cell at a 45° angle with respect to each of the holder’s walls. The remaining two corners are designed to hold a commercially available Ag/AgCl miniature reference electrode and a Pt wire auxiliary electrode. The bottom of the holder is designed to accommodate 1 mL of analyte solution. The material cost for the OTTLE and cell holder presented here are relatively low due
to the low-cost of materials used for the holder (< $10) and the commercial availability of inexpensive ITO thin film electrodes.

While numerous OTEs can be used, ITO has become a popular choice due to its low cost, good optical transparency over the visible range, and durability. The major disadvantage of using ITO OTEs is the large resistance resulting from the thin layer of tin oxide. The resistance of the electrode becomes a larger issue when used in the construction of an OTTLE because thin layer cells already have a pronounced solution resistance due to the relatively thin solution layer. The resistance problem is further exacerbated by the large current resulting from the large electrode area typically used. In order to diminish this effect the scan rate was reduced to 2 mV/s and a high concentration of supporting electrolyte was used (1.0 M). Several modifications to the OTTLE were investigated to further reduce this effect.

In an attempt to reduce the resistance resulting from the thin layer of tin oxide the exposed portion of the ITO was coated with a layer of Au. Initially, the Au film was sputter coated directly on the exposed ITO and used for cyclic voltammetry experiments. However, the ΔEp obtained from the cyclic voltammogram of 20.0 mM Fe(CN)₆ increased from ~320 mV for the unmodified ITO electrode to 480 mV for the Au modified electrode (Figure 2.4). The cyclic voltammogram obtained from Au modification no longer exhibits the characteristic thin-layer behavior of complete analyte oxidation and reduction (Figure 2.4). The conductivity of the Au film was increased by annealing the Au film to the ITO electrode. Annealing the Au ensures a uniform Au surface is formed. As Figure 2.5 shows annealing the Au resulted in a large decrease in the peak to peak separation and an increase in peak current for the oxidation and reduction of Fe(CN)₆ when compared to the unannealed Au film. Annealing the Au film resulted in a cyclic voltammogram that exhibits the characteristic thin-layer properties (Figure 2.5).
However, annealing the Au did not result in a significant decrease in resistance compared to the unmodified ITO ($\Delta E_p \sim 315$ mV and $\sim 320$ mV, respectively). The unmodified ITO electrodes were selected for use due to the lack of improvement from use of sputter coated Au films.

In another attempt to reduce the pronounced resistance of the OTTLE the quartz cover slide was reduced from 3.0 to 1.9 cm. Figure 2.6 shows the cyclic voltammogram of 20.0 mM Fe(CN)$_6$ with the 1.9 cm quartz slide positioned 1.0 from the bottom of the ITO electrode. In order to prevent electrolysis from occurring on the exposed ITO a nonconductive paint was applied to the 1.0 cm area. This resulted in a $\Delta E_p$ of 770 mV and does not exhibit the characteristic thin-layer behavior. Positioning the 1.9 cm quartz slide at the bottom of the ITO and not using the insulating paint resulted in a $\Delta E_p$ of 280 mV (Figure 2.7). This resulted in a 40 mV decrease in $\Delta E_p$ compared to the 3.0 cm quartz cover slide (Figure 2.8). The 1.9 and 3.0 cm cover slides result in complete oxidation and reduction of the analyte as seen in Figure 2.8.

![Figure 2.4 Cyclic voltammogram of 20.0 mM Fe(CN)$_6$ in 1.0 M KNO$_3$, scan rate of 2 mV /s versus Ag/AgCl reference electrode with an Au film (unknown thickness) sputter coated on an ITO working electrode.](image)
Figure 2.5  Cyclic voltammogram of 20.0 mM Fe(CN)$_6^{3-}$ in 1.0 M KNO$_3$, scan rate of 2 mV/s versus Ag/AgCl reference electrode with an annealed Au film (unknown thickness) sputter coated on an ITO working electrode.

Figure 2.6  Cyclic voltammogram of 20.0 mM Fe(CN)$_6^{3-}$ in 1.0 M KNO$_3$, scan rate of 2 mV/s versus Ag/AgCl reference electrode and a 2 cm quartz cover slide moved up from the bottom of the ITO. Exposed ITO at bottom of cell is coated with insulating paint.
Figure 2.7 Cyclic voltammogram of 20.0 mM Fe(CN)$_6^{3-}$ in 1.0 M KNO$_3$, scan rate of 2 mV/s versus Ag/AgCl reference electrode with a 2 cm quartz cover slide.

![Cyclic voltammogram](image)

$\Delta E_p = 280$ mV

Figure 2.8 Thin-layer cyclic voltammogram of 2.00 mM Fe(CN)$_6^{4-}$, 1.0 M KNO$_3$, 2 mV/s versus Ag/AgCl in a 1.9 and 3.0 cm OTTLE.

![Thin-layer cyclic voltammogram](image)

Typical cyclic voltammograms for the model ferri/ferrocyanide reversible couple in the 1.90 cm and 3.0 cm OTTLE cell can be seen in Figure 2.8. Voltammograms recorded at a 2 mV/s scan
rate resulted in a formal potential for the ferri/ferrocyanide redox reaction of 283 mV in the 1.90 cm OTTLE. This result is slightly more positive than the previously reported literature value.\[26\]

The volume of the cell was determined by coulometry of a 4.00 mM \( \text{K}_3\text{Fe(CN)}_6 \) in 1.0 M \( \text{KNO}_3 \) solution. The total charge (\( Q_T \)) was measured by complete electrolysis of ferricyanide to ferrocyanide by a potential step from 600 mV to 0 mV (Figure 2.9). As ferricyanide is reduced the charge (\( Q \)) begins to level off and a linear equation is fitted to this portion of the line. The linear equation was extrapolated to \( x = 0 \) and the resulting y value at the intercept equals \( Q_T \) as shown in Figure 2.9. Similarly, the background charge (\( Q_B \)) was measured in the supporting electrolyte solution (Figure 2.9 inset) and subtracted from \( Q_T \) to give the Faradic charge (\( Q_F \)). Substituting \( Q_F \) and other known values into Faraday’s law allows for the determination of the volume of the thin-layer cell.

\[
Q_F = Q_T - Q_B = nFVC
\]

Experimentally, the \( Q_F \) was found to be \( 1.07 \times 10^{-2} \) C, resulting in a thin-layer solution volume of 27.7 \( \mu \)L. Using this volume and the measured area of the cell, the thickness of the cell was determined to be \( 1.46 \times 10^{-2} \) cm. To verify this result a simple calculation of a newly made cell’s thickness was done using absorbance measurements and Beer’s Law, giving a path length of \( 1.60 \times 10^{-2} \) cm. This path length results in a calculated cell volume of 30.3 \( \mu \)L. The discrepancy in these results is most likely due the incomplete reduction of ferricyanide to ferrocyanide during the single step, coulometry experiment as seen in Figure 2.9.
Figure 2.9 Thin-layer coulometry of a 4.00 mM K$_2$Fe(CN)$_6$ in 1.0 M KNO$_3$ and coulometry of 1.0 M KNO$_3$. Potential step from 600 mV to 0 mV vs. Ag/AgCl.

The cell was evaluated spectroelectrochemically by recording spectra of the model analyte Ru(bpy)$_3$$^{2+}$. Figure 2.10 shows the absorbance spectra of Ru(bpy)$_3$$^{2+}$ in an OTTLE for a series of applied potentials. The spectrum with the highest absorbance value was recorded after application of +900 mV for 4 min, which caused complete reduction of Ru(bpy)$_3$$^{3+}$ ([O]/[R] < 0.001). The spectrum with the lowest absorbance value was recorded after application of +1200 mV for 4 min, causing complete oxidation of Ru(bpy)$_3$$^{2+}$ ([O]/[R] > 1000). Intermediate spectra correspond to the intermediate values of $E_{\text{applied}}$. For each value of applied potential, the equilibrium value of the ratio [Ox]/[Red] in the thin solution layer was calculated from the
Appropriate spectra using the previously reported method [27]. A Nernst plot of $E_{\text{applied}}$ vs. log ([Ox]/[Red]) at 454 nm gave an $E^\circ$ value of $1.07 \pm <0.01$ V vs. Ag/AgCl reference electrode, which is in close agreement with the previously reported values.\textsuperscript{[28, 29]} The slope of the plot is $-59.3 \pm 0.3$ mV ($R^2 = 1.00$), which corresponds to an $n$ value of 0.995. The slope of the plot is only slightly higher than the theoretical value of 59.2 mV and gave an $n$ value of $\sim 1$, which corresponds nicely to the actual value.

Figure 2.10 Thin-layer absorbance spectra and a plot of $E_{\text{applied}}$ vs. log ([O]/[R]) at 454 nm of 1.00 mM Ru(bpy)$_3^{2+}$, 1.0 M KNO$_3$ for different values of $E_{\text{applied}}$. Cell thickness 1.60 x $10^{-2}$ cm. From top to bottom 0.900, 1.020, 1.050, 1.062, 1.074, 1.086, 1.098, 1.110, 1.122, 1.134, and 1.200 V versus Ag/AgCl.
Similar results were obtained for the emission spectra of 10 µM Ru(bpy)$_3^{2+}$ using the custom optical system previously described. Utilizing the potentials from Figure 2.11, a Nernst plot was obtained for data resulting in a log ([Ox]/[Red]) between ~1 and -1. This plot of $E_{\text{applied}}$ vs. log ([Ox]/[Red]) for the emission data results in a slope of $-58.0 \pm 4$ mV ($R^2 = 0.968$), which corresponds to an n value of 1.02. The $E^0'$ value determined was $1.07 \pm < 0.01$ V vs. Ag/AgCl. These values are in good agreement with the values obtained from the absorbance based measurements and from the literature.[28, 29]

The main advantage of this cell and holder design is its ability to be used with any optical setup which measures 1.00 cm above the base of the cuvette. Here we have shown the use of this design with a model analyte on a standard absorbance spectrophotometer and a custom fluorescence spectrophotometer. In order to show the OTTLE cell and holder’s versatility, the more complex irreversible electrochemical oxidation of 1-pyOH was monitored using a bench-top Cary Eclipse fluorescence spectrophotometer. 1-pyOH undergoes a one-electron electrochemical oxidation at a pH-dependent potential, ultimately resulting in an ECE mechanism.[30] This analyte is a good representative of analytes which often do not exhibit the ideal reversible electrochemistry shown by model compounds.

At pH 10.5, 1-pyOH is in the deprotonated 1-pyO$^-$ form, which results in a broad emission spectrum from ~400 to 500 nm (Figure 2.12). 1-pyO$^-$ was incrementally converted from its reduced to its oxidized form. Spectra for the fully reduced, fully oxidized, and intermediate forms of 1-pyO$^-$ are shown (Figure 2.12). A Nernst plot corresponding to this data gave an $E^0'$ of $162 \pm 1$ mV vs. a Ag/AgCl reference electrode. The slope of the plot was $-58.1 \pm 2.4$ mV ($R^2 = 0.992$), which results in an n value of 1.02. Though in a different solution matrix, this data is consistent with the previously published results.[30]
Figure 2.11 Thin-layer emission spectra (A) and a plot of $E_{\text{applied}}$ vs. $\log ([O]/[R])$ excitation at 441.6 nm. (B) at 610 nm of 1.00 mM Ru(bpy)$_3^{2+}$, 1.0 M KNO$_3$ for different values of $E_{\text{applied}}$. Cell thickness $1.60 \times 10^{-2}$ cm. From top to bottom 1.020, 1.050, 1.062, 1.074, 1.086, 1.098, 1.110, 1.122, 1.134, and 1.200 V versus Ag/AgCl.
Figure 2.12 Thin-layer emission spectra (ex. 280 nm.) and a plot of $E_{\text{applied}}$ versus log ([O]/[R]) at 452 nm of 1.00 x $10^{-4}$ M 1-pyOH for different values of $E_{\text{applied}}$. From top to bottom 0, 0.145, 0.150, 0.160, 0.170, 0.180, 0.190, 0.200, 0.210, and 0.400 V versus Ag/AgCl.

2.4 Conclusion

The design of a new rapid prototyped OTTLE holder for spectroelectrochemical measurements in standard bench-top absorbance or fluorescence spectrophotometers has been described. The performance of this cell holder has been evaluated by spectroelectrochemical detection of multiple analytes in a variety of optical setups. The ability to couple this cell with fluorescence detection offers the possibility of lower detection limits than similar cells that are only capable of absorbance detection. The rapid and complete electrolysis of the analyte in the OTTLE and the versatility provided by the rapid prototyped holder make this design a useful method for spectroelectrochemical detection in any laboratory.
2.5 References


Chapter 3. Spectroelectrochemical Sensing of Pyrene Metabolites 1-Hydroxypyrene and 1-Hydroxypyrene-glucuronide

3.1 Introduction

Here we demonstrate a technique based on spectroelectrochemical sensing in an optically transparent thin layer electrode (OTTLE) cell to detect the PAH biomarkers 1-hydroxypyrene (1-pyOH) and 1-hydroxypyrene-glucuronide (1-pyOglu). Thin-layer spectroelectrochemistry in an OTTLE couples the ability to change the oxidation state of a compound confined in a thin layer of solution next to an optically transparent electrode (OTE), with the detection capabilities of spectroscopy. The advantages of this technique are: rapid and complete analyte electrolysis; available optical window; and the selectivity and sensitivity of the spectroelectrochemical approach. Depending on the thickness of the OTTLE, complete electrolysis can occur within a few minutes. Increasing the thickness of the cell increases the optical path length which provides an increased optical signal. However, this also increases the time required for complete electrolysis of the analytes. OTEs consisting of a thin layer (100 to 5000 Å) of optically transparent and conductive materials such as Au, Pt, carbon, or tin-doped indium oxide (ITO) have been used for OTTLE. The optical range of the OTTLE is normally determined by the selection and thickness of the conductive thin film. However, for front-side fluorescence measurements like those made here the optical window depends only on the choice of OTTLE cover slide. In this case a quartz window allows for measurements over the visible and UV spectral range.

The pyrene metabolites 1-pyOH and 1-pyOglu have been extensively used as biomarkers for indirect assessment of total polycyclic aromatic hydrocarbon (PAH) exposure. The structure of 1-pyOH and 1-pyOglu can be seen in Figure 3.1. These metabolites have been found to be
effective biomarkers for PAH exposure assessment because pyrene is a major constituent in virtually all PAH mixtures\textsuperscript{[17,18]} and its metabolites are readily excreted in urine.\textsuperscript{[19,20]} The metabolism of pyrene involves formation of the phase I metabolite 1-pyOH, which undergoes phase II metabolism with conjugation to glucuronic acid.\textsuperscript{[21]} Assessment of human urine has shown 1-pyOglu accounts for approximately 80\% of the pyrene metabolites excreted.\textsuperscript{[21]}

![Figure 3.1 Structure of the pyrene metabolites 1-hydroxypyrene (1-pyOH) and 1-hydroxypyrene-glucuronide (1-pyOglu).](image)

Due to the high concentration of conjugated metabolites, the standard approach for human assessment involves an initial enzymatic or acid hydrolysis of pyrene conjugates to 1-pyOH. The resulting sample of 1-pyOH can then be analyzed by HPLC with fluorescence detection.\textsuperscript{[11]} Though this technique is highly reproducible and offers detection limits in the low µg/L range, it requires multiple experimental steps and has an extended analysis time (overnight). A variety of other detection methods have been developed to expedite this process, including: HPLC-FL for direct measurement of 1-pyOglu; immunoaffinity chromatography with synchronous fluorescence spectroscopy (SFS); GC-MS; and LC-MS/MS.\textsuperscript{[22-30]} All of these techniques have been shown to offer detection limits within a suitable range for pyrene metabolite detection (1
However, they are all still multi-step processes involving an initial separation step for selectivity, followed by detection. Ideally, quantification of these metabolites in a solution mixture could be done in a single analysis that required no dilution or separation step.

Quantification of 1-pyOH and 1-pyOglu without separation is difficult and requires high levels of selectivity and sensitivity due to the metabolites similar optical properties and the low concentrations commonly found in urine. The spectroelectrochemical technique used here offers a high level of selectivity because it requires an analyte to be electrochemically active at the potential selected and either the analyte or the electrolysis product must absorb or emit light at the selected wavelength. In this case, 1-pyOH and 1-pyOglu are detected by the change in optical spectra associated with their oxidation. Excellent sensitivity is achieved by using fluorescence detection.

3.2 Experimental Section

3.2.1 Reagents and Materials

The following chemicals were used as received: ammonium chloride, calcium chloride, citric acid, magnesium sulfate, potassium phosphate dibasic, potassium phosphate monobasic, sodium bicarbonate, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, sodium sulfate, uric acid (all from Fisher Scientific); 1-hydroxypyrene (1-pyOH), 1-hydroxypyrene-glucuronide (1-pyOglu) (both from Toronto Research Chemicals, Inc.); bovine albumin, lactic acid (both from Sigma-Aldrich); urea (J.T. Baker Chemical Co.); and 200 proof ethyl alcohol (Pharmco-AAPER). 1-pyOH and 1-pyglu solutions were prepared by dissolving appropriate amounts in 0.1 M NaCl/0.05 M pH 7.2 phosphate buffer/30% EtOH (prepared with deionized water from a Barnstead Nanopure system).
The OTTLE cell, which has been previously described,\cite{10} is constructed from a silica slide (1.90 x 1.00 cm), 0.018 cm-thick silicone spacers (Specialty Manufacturing Inc., Pineville, NC), and indium tin oxide (ITO)-coated glass slides (Corning 1737F and 7059, 11-50 Ω/square, 130-nm-thick film on 1.1-mm glass, Thin Film Devices, Anaheim, CA) with dimensions of 4.00 x 1.00 cm. Silicone spacers cut to approximately 1.90 x 0.20 cm are placed onto the edges of the ITO glass slide and sandwiched between a silica slide and the ITO. Two-part quick setting epoxy (Loctite) is used along the edges of the spacers and allowed to cure for 2 h to hold the components together. The optical path length of the cell is determined by the thickness of the spacers used in cell construction. The exposed ITO above the silica slide is used for electrical contact. For this work three OTTLE cells were constructed and used, each having an approximate lifetime of 5 h. After 5 h of continuous use the epoxy would fail from exposure to the ethanol in the solvent and the quartz slide would begin to separate from the ITO electrode.

An artificial urine simulant solution containing 2.0 mM citric acid, 1.1 mM lactic acid, 0.4 mM uric acid, 0.5 mM albumin, 25 mM sodium bicarbonate, 170 mM urea, 2.5 mM calcium chloride, 90 mM sodium chloride, 2.0 mM magnesium sulfate, 10 mM sodium sulfate, 7 mM dipotassium hydrogen phosphate, 7 mM potassium dihydrogen phosphate, and 25 mM ammonium chloride was prepared by dissolving appropriate amounts in deionized water.\cite{32} The artificial urine solution was diluted by 30% with EtOH spiked with 5.0 x 10^{-7} M 1-pyOH and 1.0 x 10^{-7} M 1-pyOglu.

Urine samples were acquired on the day of sampling. These samples were spiked with appropriate amounts of 1-pyOH and 1-pyOglu to make 1.0 x 10^{-8} through 5.0 x 10^{-7} M samples. Separation of 1.0 x 10^{-8} spiked urine samples was done on a disposable Varian C-18 Bond Elut
extr
action column with 30% EtOH and methanol. The column was conditioned with methanol before the sample was passed through the cartridge.

3.2.2 Instrumentation

For all experiments the electrochemical cell consisted of a Pt wire auxiliary electrode, a miniature Ag/AgCl reference electrode (Cypress Systems), and an OTTLE. Thin layer cyclic voltammetry was performed on a BAS 100 Electrochemical Analyzer (Bioanalytical Systems).

Absorbance and emission spectra of 5.0 x 10^{-5} M 1-pyOH and 1-pyOglu in a 1 cm quartz cuvette were acquired using a Varian Cary 50 Bio UV-Visible Spectrophotometer and a Varian Cary Eclipse Fluorescence Spectrophotometer, respectively. Spectroelectrochemical detection of 1-pyOH and 1-pyOglu was performed using the instrumentation arrangement shown in Figure 3.2, consisting of: a laser (325 nm HeCd model 1K3202R-D Kimmon Electric Co.); light control modules (shutter, attenuator, and focusing optics); a monochromator (0.3 m focal length, triple grating turret); a photon-counting phototube (Acton Research Corp.), and a computer and control electronics (NCL and Spectra-Sense software, Acton). Light from the laser was focused onto the polished end of a 6-around-1 silica fiber optic bundle (RoMack, Inc.) with a microscope objective (10x, NA=0.25, Newport). Laser power was attenuated to 0.5 mW at the OTTLE with a variable neutral density filter (Newport). The sample was exposed to the laser light only during data acquisition to minimize photodegradation. A Bioanalytical Systems Epsilon potentiostat was used for controlled potential electrolysis of 1-pyOH and 1-pyOglu at 750 and 1200 mV, respectively, for 15 min.
3.3 Results and Discussion

3.3.1 Optical properties

The novel sensing method presented here allows for detection of the pyrene metabolites 1-pyOH and 1-pyOglu in a single 30 min. analysis. This detection is achieved by using a spectroelectrochemical approach which allows for selective electrochemical modulation of what would otherwise be overlapping absorption and emission spectra of the metabolites in a mixture. At pH 7.2, the absorption and fluorescence spectra largely coincide (Figure 3.3 and 3.4). The absorption spectra of 1-pyOH and 1-pyOglu at this pH are distinguished only by a 5 nm bathochromic shift for 1-pyOH and by the difference between their molar absorptivities. The fluorescence spectra obtained by excitation at the wavelength of maximum absorbance for 1-
pyOH and 1-pyOglu (345 and 340 nm, respectively) exhibit characteristic pyrene emission peaks (Figure 3.4). These peaks (vibronic structure) are again separated by only a 5 nm red shift for 1-pyOH along with systematic differences in intensity.

An additional complication is the strong absorbance of 1-pyOH and 1-pyOglu in the UV region (Figure 3.3). Detection in the UV can be problematic for spectroelectrochemistry because excitation in the UV limits the materials for prisms and waveguides to silica. The previously described OTTLE cell and holder used for this work alleviates this problem by allowing for front side measurements through a silica slide, which is suitable for excitation in the near UV range.\textsuperscript{[10]}

Figure 3.3  Normalized absorption spectra of 5.0 x 10\textsuperscript{-5} M 1-pyOH and 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer and 30\% EtOH. Fluorescence excitation at absorption maxima for 1-pyOH and 1-pyOglu (345 and 340 nm, respectively).
Figure 3.4  Normalized fluorescence spectra of $5.0 \times 10^{-5}$ M 1-pyOH and 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer and 30% EtOH. Fluorescence excitation at absorption maxima for 1-pyOH and 1-pyOglu (345 and 340 nm, respectively).

### 3.3.2 Cyclic voltammetry of 1-pyOH and 1-pyOglu

The electrochemical oxidation of 1-pyOH at pH 7.2 has been previously studied and shown to involve a complex electrochemical-chemical-electrochemical (ECE) and an electrochemical-electrochemical-chemical-electrochemical (EECE) mechanism.\[^{33}\] Figure 3.5 shows similar results for the CV of 1-pyOH at an ITO electrode. Using an initial potential of -0.2 V and switching potentials of +1.2 and -0.2 V, two irreversible oxidation peaks at +0.396 and +0.742 V
on the initial positive sweep were observed. Following the initial oxidation a new, quasi-reversible couple is seen with Epc = -0.054 and Epa = +0.074 V.

Figure 3.5 also shows the CV of 1-pyOglu in the same solution matrix as 1-pyOH at an ITO electrode, which has not been previously investigated. As expected given their structural similarities, the oxidation of 1-pyOglu results in a CV similar to that obtained by the oxidation of 1-pyOH. The voltammagram reveals an initial, irreversible oxidation at +1.050 V leading to a new quasi-reversible couple at Epc = -0.112 and Epa = +0.060 V. The major difference in the CVs is the potential required for the initial oxidation of the metabolites. 1-pyOH is much easier to oxidize than its glucuronic acid conjugate at this pH. As shown in Figure 3.5 1-pyOglu requires a potential of approximately +1.050 V vs. the +0.742 V required for oxidation of 1-pyOH. While the metabolites are easily distinguishable by electrochemical techniques on the basis of their oxidation potentials, the detection limits for these methods cannot reach the low nM range required for detection of 1-pyOH or 1-pyOglu in practical applications. In order to reach the detection limits required for these metabolites and have the selectivity to distinguish between each in a solution mixture, the potential differences can be used in combination with the sensitivity of fluorescence detection.
Figure 3.5 Thin layer cyclic voltammograms of $1.0 \times 10^{-4}$ M 1-pyOH and 1-pyOglu were obtained from -200 to 1200 mV at a scan rate of 5 mV/s in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at an ITO electrode. Enlargement from 300 to -200 mV of the redox products from the oxidation of the parent PAH metabolite.

3.3.3 Spectroelectrochemistry

The spectroelectrochemical technique used here involves measuring a change in the optical signal associated with electrochemical oxidation or reduction of the analyte. Figure 3.6A shows how the oxidation potential determined by CV can be used to change the fluorescence signal for $1.0 \times 10^{-6}$ 1-pyOglu over 15 min. in an OTTLE cell. The oxidation of 1-pyOglu results in a decrease in fluorescence intensity across the emission spectrum of 1-pyOglu. The greatest change in signal occurs at the fluorescence maximum (382 nm). Using this emission
wavelength, Figure 3.6B depicts the change in fluorescence during controlled potential oxidation. At time zero no potential is applied, this point corresponds to the maximum intensity seen in Figure 3.6A. The fluorescence decreases over the first 5 min for this concentration then begins to level off as the electrolysis approaches completion. The point at 15 min in Figure 3.6B corresponds to the fluorescence counts at 382 nm for the spectrum of lower intensity in Figure 3.6A.

Figure 3.6  (A) Fluorescence of 1.0 x 10\(^{-6}\) M 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH before and after the application of 1.2 V for 15 min. (B) Fluorescence of 1.0 x 10\(^{-6}\) M 1-pyOglu at 382 nm during oxidation at 1.2 V for 15 min. All potentials vs. Ag/AgCl and excitation at 325 nm.

A similar response was obtained for the oxidation of 1-pyOH at 750 mV. This oxidation resulted in the largest change in fluorescence signal at the emission maximum for 1-pyOH of 387 nm. Monitoring this wavelength over the 15 min of potential application resulted in an initial linear portion followed by leveling off of the signal similar to what is shown in Figure 3.6B.
Due to the irreversible nature of the oxidation of 1-pyOglu and 1-pyOH, multiple measurements on a single sample cannot be obtained. This irreversibility results in a single signal modulation for each sample instead of an average of multiple modulations which would decrease the error associated with each measurement by signal averaging.\[^{34}\]

The dynamic linear range was determined by generating a calibration curve for each analyte in separate solutions. Solutions of 1-pyOH and 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH were excited at 325 nm in an OTTLE cell as previously described. The optical response was monitored at 1 min. intervals for 16 min. For the final 15 min. of monitoring a constant potential sufficient for oxidation of the analyte being monitored was applied (750 mV for 1-pyOH and 1200 mV for 1-pyOglu). After 15 min of electrolysis, the final spectrum was subtracted from the initial spectrum (at which no potential was applied). The difference in photon counts at the wavelength of maximum fluorescence for each analyte was used for generation of the calibration curves (382 nm for 1-pyOglu and 387 nm for 1-pyOH).

Following each trial the OTTLE was washed twice with 100% EtOH. The small error associated with each randomly introduced concentration in Figure 3.7A and B confirms that this is an appropriate cleaning method that results in little or no carry over from trial to trial.

The calibration curve generated for 1-pyOH is shown in Figure 3.7A. A linear relationship between the log of the difference in photon counts, Δ, and the log of [1-pyOH] in sample solutions ranging from 10 nM to 1 µM was observed. The equation for the linear range was \( y = (1.09 \pm 0.03)x + (11.59 \pm 0.19) \) (\( R^2 = 0.997 \)). The detection limit for 1-pyOH was calculated to be \( 1 \times 10^{-8} \) M using the previously described method of three times the experimentally determined standard deviation of the blank (261 counts) and the slope of the linear equation obtained from the calibration curve.\[^{35}\]
Figure 3.7B shows the calibration curve generated for 1-pyOglu. Again, a linear relationship between the log of Δ and the log of [1-pyOglu] in sample solutions ranging from 1 nM to 1 µM was observed. The equation for the linear range was \( y = (0.62 \pm 0.02)x + (9.28 \pm 0.15) \) \((R^2 = 0.993)\). The detection limit for 1-pyOglu was calculated to be \( 9 \times 10^{-11} \) M. The lower limit of detection for 1-pyOglu compared to 1-pyOH was expected due to the approximately 3-5x larger quantum yield of 1-pyOglu.[14] A contributing factor was the decreased standard deviation of the blank at 382 nm (238 counts). These two factors allow for the calculated detection limit of 1-pyOglu to be lower than the expected physiological range for individuals exposed to PAHs.

![Figure 3.7 Calibration curves for the change in fluorescence signal of (A) 1-pyOH in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at 387 nm with 750 mV over 15 min and (B) 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH at 382 nm with 1200 mV over 15 min. Each point is the average of a minimum of 3 trials. Excitation at 325 nm and potentials vs. Ag/AgCl.

The difference in redox potentials enables the fluorescence change associated with each analyte in a mixture to be measured. The large separation between the oxidation potential of 1-
pyOH and 1-pyOglu allows 1-pyOH to be oxidized without oxidizing the 1-pyOglu in solution. The application of 750 mV to a solution of 1-pyOglu results in little to no optical change over 12 mins (Figure 3.8). The maximum change in counts was only 2% of the total signal and occurred half way through the 12 min experiment. Plotting the photon counts at 385 nm for each spectrum acquired over the 12 min shows no trend due to the potential application (Figure 3.8B). This confirms a sequential oxidation method can be used because there is sufficient separation between the analytes oxidation potentials.

Figure 3.8  (A) Fluorescence of 1-pyOglu in 0.1 M NaCl, 0.05 M pH 7.2 phosphate buffer, and 30% EtOH with the application of 0.75 V for 12 min.  (B) Fluorescence of 1-pyOglu at 382 nm during oxidation vs. time.  All potentials vs. Ag/AgCl and excitation at 325 nm.

Figure 3.9 shows the sequential fluorescence modulation of 5.0 x 10^{-7} M 1-pyOH (A) and 1.0 x 10^{-7} M 1-pyOglu (B) in a single analysis. The spectrum of highest intensity in Figure 3.9 is attributed to the unoxidized 1-pyOH and 1-pyOglu in solution. It is clear from this spectrum that quantifying the concentration of analytes by fluorescence alone is difficult. However, by sequentially changing the potential from 750 mV to 1200 mV the fluorescence signal associated
with each analyte can be selectively modulated. A potential of 750 mV was first applied to the mixture for 15 min, resulting in the oxidation of 1-pyOH (Figure 3.9A). As with the calibration curve, the change in fluorescence was determined by subtracting the final spectrum after 15 min of electrolysis from the initial spectrum at which no potential was applied. This calculation resulted in a modulation amplitude of 48,000 counts, which correlates nicely with the 52,000 ± 5,000 counts obtained from the calibration curve obtained with the single analyte. Following the 15 min. oxidation at 750 mV the potential was stepped to 1200 mV, resulting in the oxidation of 1-pyOglu (Figure 3.9B). The change in fluorescence signal associated with this was 46,000 counts, which again falls well within a standard deviation of the mean (± 8,000 of 44,000 counts) obtained from the calibration curve.

It is important to note that complete oxidation of 1-pyOH is required in the initial potential step because any unoxidized 1-pyOH will oxidize during the sequential oxidation step. Oxidation of 1-pyOH during the 2nd sequential step will result in a fluorescence modulation which would be incorrectly associated with 1-pyOglu concentration due to the overlap in fluorescence spectra. The complete oxidation of 1-pyOH can clearly be seen in Figure 3.9 by the leveling off and ultimately coinciding spectra at 387 nm, near the end of the initial oxidation.
Figure 3.9 Fluorescence modulation of a mixture of $5.0 \times 10^{-7}$ M 1-pyOH (A. modulation at 750 mV over 15 min) and $1.0 \times 10^{-7}$ M 1-pyOglu (B. modulation at 1200 mV for 15 min). The change in signal for each analyte is measured at their respective fluorescent $\lambda_{\text{max}}$ following the application of potential sufficient for oxidation. Each scan is 1 min. Excitation at 325 nm and all potentials vs. Ag/AgCl.

Similar plots were obtained for $5.0 \times 10^{-7}$ M 1-pyOH and $1.0 \times 10^{-7}$ M 1-pyOglu in an artificial urine solution. Electrochemical modulation using the same procedure described for the buffered solution resulted in a signal modulation of $54,000 \pm 1,000$ for 1-pyOH and $40,000 \pm 2,000$ for 1-pyOglu (Table 3.1). Both of these values fall within the expected range determined by the single analyte calibration solutions ($52,000 \pm 5,000$ for 1-pyOH and $44,000 \pm 8,000$ for 1-pyOglu).
<table>
<thead>
<tr>
<th>1-pyOH ( \Delta ) Counts</th>
<th>Average</th>
<th>Std Dev</th>
<th>1-pyOglu ( \Delta ) Counts</th>
<th>Average</th>
<th>Std Dev</th>
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<tr>
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<td>600</td>
<td>37,900</td>
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<td>54,500</td>
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<td>40,600</td>
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</table>

Table 3.1  Fluorescence modulation of a mixture of \( 5.0 \times 10^{-7} \) M 1-pyOH (at 750 mV over 15 min) and \( 1.0 \times 10^{-7} \) M 1-pyOglu (at 1200 mV for 15 min). The change in signal for each analyte is measured at their respective fluorescence \( \lambda_{\text{max}} \) following the application of a potential sufficient for oxidation. Excitation at 325 nm and all potentials vs. Ag/AgCl.

3.3.4 Spectroelectrochemistry in urine

Figure 3.10 shows the cyclic voltammogram of human urine in the OTTLE cell. Using an initial potential of -1.5 V and switching potentials of +2.0 and -1.5 V, two irreversible oxidation peaks at -0.5 and a broad oxidation peak +1.5 which begin at +0.5 V were observed on the initial positive sweep. In the reverse sweep a large reduction current is seen below -1.0 V. This reduction current is most likely due to the limited negative working window of the ITO OTE used as the working electrode. In the second forward sweep the initial irreversible oxidation peak at -0.5 V is no longer present however, the large current due to oxidation from 0.5 to 2.0 V is still present.
Figure 3.10  Thin layer cyclic voltammograms of urine from -1.5 to +2.0 V vs. Ag/AgCl at a scan rate of 5 mV/s at an ITO electrode.

Oxidation at the optimum potentials for 1-pyOH (750 mV) and 1-pyOglu (1200 mV) occurs at similar potentials as the oxidation seen in urine. Oxidation of urine at the potentials applied to oxidize the analytes of interest can be problematic if this oxidation results in an emission signal modulation at the selected optical wavelengths. To assess this potential problem, the optical modulation of urine was monitored at 0.4, 0.75, and 1.2 V over 1 min intervals (Figure 3.11). Figure 3.11 shows oxidation of urine at 0.4 V results in a decrease in emission intensity from 350 to 450 nm. After the first few minutes of potential application the decrease in counts begins to level off as the potential is applied. Increasing the potential from 0.4 to 0.75 V results in another decrease in photon counts over the wavelengths monitored. Following the application of 0.75 V the potential was stepped to 1.2 V. Oxidation at 1.2 V results in a decrease in counts from 350 to 450 nm similar to the application of 0.75 and 0.4 V. In order to accurately determine the concentration of 1-pyOH and 1-pyOglu in solution the modulation due to the urine has to be
taken into account. If this is not accounted for an elevated quantification of the analytes would be obtained.

Figure 3.11, also shows the background counts increase from 350 to 425 nm before leveling off, similar to the backgrounds seen in buffer and simulant solutions. However, the urine sample results in an increased background compared to the buffer and simulant solutions over the wavelengths of interest. The increased background counts can have an adverse effect on the detection limit for 1-pyOH and 1-pyOglu in urine.

![Figure 3.11](image)

**Figure 3.11**  Fluorescence modulation of urine at 400, 750 mV, and 1200 mV. Each scan is 1 min. Excitation at 325 nm and all potentials vs. Ag/AgCl.

Figure 3.12 shows the fluorescence spectrum obtained for a mixture of $5.0 \times 10^{-7}$ M 1-pyOH and $1.0 \times 10^{-7}$ M 1-pyOglu in urine in the OTTLE cell. Unlike the fluorescence spectra obtained for a mixture of these analytes in buffer and urine simulant the characteristic pyrene spectrum is
not seen. The spectra acquired for the mixture in urine is similar to the unspiked urine background spectra obtained over the same wavelengths. Increasing the concentrations of 1-pyOH and 1-pyOglu to $1.0 \times 10^{-6}$ M in urine resulted in a similar spectrum. The reason for the loss of fluorescence signal is unknown. Figure 3.13 shows the fluorescence spectrum of $1.0 \times 10^{-7}$ M 1-pyOglu in urine in a 1 cm quartz cuvette. The spectrum obtained in the 1 cm cuvette is similar to the spectrum obtained for the mixture of 1-pyOH and 1-pyOglu in the OTTLE.

Figure 3.13  Fluorescence spectrum of $5.0 \times 10^{-7}$ M 1-pyOH and $1.0 \times 10^{-7}$ 1-pyOglu in urine. Fluorescence excitation at 325 nm.
Figure 3.13  Fluorescence spectrum of $1.0 \times 10^{-7}$ M 1-pyOglu in urine. Fluorescence excitation at 325 nm.

Urine samples spiked with $1.0 \times 10^{-8}$ M 1-pyOglu were eluted on a C-18 separation column with 30% EtOH/H$_2$O and methanol. The initial urine fraction was collected after passing through the column and the fluorescence spectrum of the resulting solution was obtained in a 1 cm quartz cuvette (Figure 3.14). Like the previous urine spectra an increase in counts is seen from 350 to 425 nm before leveling off and beginning to decrease. Figure 3.14 also shows the spectrum obtained from the faction collected following elution with 30% EtOH. The eluted samples resulted in spectra with similar characteristics as the initial fraction and the spiked urine prior to separation (Figure 3.13). The eluted samples offer a decreased background but the characteristic pyrene spectrum is not seen. This indicates the separation method used here is not adequate to separate the pyrene conguate from the interferences preventing the fluorescence spectrum of 1-pyOglu from being monitored.
3.4 Conclusions

The spectroelectrochemical technique was shown to be capable of quantitatively distinguishing between two analytes with overlapping fluorescence spectra without the need for an initial separation step. The analytes selected (1-pyOH and 1-pyOglu) are a good model system for this detection method because of their near identical absorbance and fluorescent spectra and the large difference in their initial oxidation potentials. This approach could be used for other analytes and more complex mixtures as long as the analytes redox potentials are sufficiently separated.

In this work spectroelectrochemical sensing was used for detection of a mixture of 1-pyOH and 1-pyOglu in a single 30 min analysis in a phosphate buffer and an artificial urine solution. This approach provided a calculated limit of detection for 1-pyOglu within the required range for physiological measurements in urine to evaluate exposure to PAHs. However, the 10 nM detection limit obtained for 1-pyOH is adequate for some physiological measurements, but not down to the lowest exposure levels of interest. A lower limit of detection can be obtained by
increasing the thickness of the OTTLE by simply increasing the thickness of the spacers used for cell construction. The increased optical path length would require longer electrolysis times, but would result in larger changes in fluorescence for each analyte concentration.

Urine spiked with 1-pyOH and 1-pyOglu did not result in the characteristic pyrene spectra seen in the buffer and urine simulant solutions. This was seen in the OTTLE cell and standard cuvette. Finally, spiked urine was eluted on a C-18 separation column with multiple solutions. Fluorescence spectra of the collected fractions gave similar results and did not provide adequate separation of the pyrene conjugate from the interferences preventing the fluorescence spectrum from being monitored.
3.5 References


Chapter 4. Down-well Spectroelectrochemical Sensor for Fluorescence Detection

4.1 Introduction

For the past several decades spectroelectrochemical sensing based on the combination of electrochemistry, optical spectroscopy, and analyte partitioning into a chemically selective thin-film has allowed for optical monitoring of electrochemical products.[1-11] Initially, the spectroelectrochemical sensor focused on absorbance based detection of the analyte. In an effort to improve detection limits a fluorescence based spectroelectrochemical sensor was developed.[12] This sensor used an attenuated total reflection (ATR) method which uses an evanescent wave to excite an analyte within one wavelength of the electrode’s surface. By collecting light at 90° to the optically transparent electrodes (OTE) surface the emitted light from the analyte preconcentrated in the film can be monitored.

Since the development of the first spectroelectrochemical approach[13] a variety of cells have been designed to allow for simultaneous optical and electrochemical monitoring. The majority of these have been optically transparent thin layer electrodes (OTTLEs)[14-22] or flow spectroelectrochemical cells.[12] While easier to construct and use due to the ability to couple the cell with standard absorbance and fluorescence instruments, OTTLE cells can be problematic when trying to incorporate an ion selective film. Flow cells allow for the incorporation of a selective film however can be difficult to setup and make. However, when incorporated with a selective thin film these techniques have shown high levels of selectivity and sensitivity in response to a variety of analytes and possible interferences.[1-12]

A portable absorbance based spectroelectrochemical sensor using a flow cell design has been successfully used to determine the concentration of Fe(CN)$_6^{3/-4}$ in a Hanford Site sample of nuclear waste.[23] For this work a standard addition method was developed which resulted in a
linear response due to the change in absorbance from $5 \times 10^{-5}$ to $5 \times 10^{-3}$ M. While the sensor was effective in determining the concentration of Fe(CN)$_6^{3/-4}$ in a Hanford waste site sample the sample collection method and laboratory requirements results in expensive waste monitoring. Due to the inherent cost of working with radioactive material in a laboratory on site monitoring is the only way to reduce the cost. Ideally, a cell could be lowered into a waste tank and left in the tank following the measurement. This would drastically reduce the cost of sampling by avoiding the expensive radioactive monitoring and disposal of contaminated material.

To improve our ability to utilize the spectroelectrochemical technique in the field we have developed a down-well spectroelectrochemical sensor. This design incorporates the essential components needed for spectroelectrochemical detection in a single device. This device can be lowered into a sample and a spectroelectrochemical measurement can be made. To increase sensitivity the sensor uses fluorescence based detection.

A down-well probe for fluorescence spectroelectrochemical detection can be beneficial for a number of analytes. One analyte of particular interest is technetium Tc. All technetium isotopes are radioactive and the prevalent isotope takes $10^4$ to $10^6$ years to decay. Tc forms anionic pertechnetate which is not removed during the radioactive waste treatment which is designed to remove cationic species. The current method for disposal is burial. This presents a problem because the anionic pertechnetate does not adsorb onto the surface of rocks and can be washed away. This can result in environmental contamination due to leaching into the water supply. Consequently, developing a way to monitor Tc is important. However, working with radioactive material is expensive and hazardous. To reduce the cost of method development the ruthenium Ru ion was selected. This analyte forms similar complexes as Tc (Figure 4.1) but is not radioactive and therefore much less expensive to work with.
The work presented here evaluates the down-well spectroelectrochemical cells performance with the model analyte tris-(2,2’”-bipyridine) ruthenium (II) (Ru(bpy)$_3^{2+}$). This analyte was selected because it has been shown to partition into a Nafion film, undergo an electrochemical reaction that leads to an optical change[12], and provides a good model system for the detection of Tc. One key aspect of this design is it can easily be scaled up or down in size to make the probe even more versatile.

4.2 Experimental

4.2.1 Materials

The following chemicals were used: tris tris-(2,2’”-bipyridine) ruthenium (II) chlorine hexahydrate ([Ru(bpy)$_3$Cl$_2$6H$_2$O], GFS Chemicals), potassium nitrate (Fisher Scientific), and
Nafion (5% solution, Aldrich). All reagents were used without further purification. Reagents were prepared by dissolving appropriate amounts in 0.1M KNO₃ (prepared with deionized water from a Barnstead purification system). Indium tin oxide (ITO) coated glass slides had dimensions of 1”x 1”.

4.2.2 Preparation of Nafion film

Nafion films were prepared by using a spin coater. Film thickness was determined using a variable-angle spectroscopic ellipsometer (VASE) and software (Woodlam Co.). The 5% stock solution was diluted to the desired concentration (1%) with a 4:1 isopropanol-water mixture. A 75 uL aliquot of diluted solution was pipette onto an ITO slide and spun at 600 rpm for 30 seconds. The resulting films were approximately 24 nm thick.

4.2.3 Spectroelectrochemical Sensor

Figure 4.2 is an illustration of the down-well spectroelectrochemical sensor. This sensor consist of three components: the laser bundle, the probe body, and the probe head. A 441.6 HeCd laser (model 1K4153R-c Kimmon Electric) and a six around one laser bundle are used. As the name implies this bundle consists of six optical fibers used for excitation centered around one fiber used for collection of the fluorescence signal (Figure 4.3). The advantage of this is the bundle can be quickly and easily installed and positioned. The probe body is made from black delrin (5.0” long and diameter of 1.75”). The body has a hollow center (0.5” diameter) and four tapped holes for the fastening screws. The final and most complex component is the probe head. Like the body it is made of black delrin and has a hollow center and holes for the fastening screws (all of the same dimensions). This portion of the probe contains all of the electrochemical components. The working electrode is connected by two spring loaded pins that are compressed when the optically transparent and conductive electrode is in place. An O’ring
between the electrode and the probe head completes the seal and prevents solution from reaching
the pins themselves. Two through holes allow for the reference (Ag/AgCl wire) and auxiliary (Pt
wire) electrodes to be placed inside the cell. After assembling the cell these holes are sealed and
only the covered wire is exposed to solution.

4.2.4 Instrumentation

For all experiments the electrochemical cell consisted of a Pt wire auxiliary electrode, an on
board Ag/AgCl reference electrode, and an ITO working electrode. Cyclic voltammetry was
performed on a BAS 100 Electrochemical Analyzer (Bioanalytical Systems).

Emission spectra of Ru(bpy)$_3^{2+}$ were obtained using the instrumentation arrangement shown
in Figure 4.4, consisting of a laser (441.6 nm HeCd); light control modules (shutter, attenuator,
and focusing optics); a monochromator (0.3 m focal length, triple grating turret); a photon-
counting phototube (Acton Research Corp.); and a computer and control electronics (NCL and
Spectra-Sense software, Acton). Light from the laser was focused onto the polished end of a 6-
around-1 silica fiber optic bundle (RoMack, Inc.) with a microscope objective (10x, NA=0.25,
Newport). Laser power was attenuated to 0.5 mW at the OTTLE with a variable neutral density
filter (Newport). The sample was exposed to the laser light only during data acquisition to
minimize photodegradation. A Bioanalytical Systems Epsilon potentiostat was used for
controlled potential electrolysis of Ru(bpy)$_3^{2+}$. 
Figure 4.2 Illustration of down-well spectroelectrochemical sensor for fluorescence based measurements
Figure 4.3  Illustration of down-well spectroelectrochemical sensor and six around one laser bundle for fluorescence based measurements
4.3 Results and Discussion

To evaluate the sensors performance the model analyte Ru(bpy)$_3^{2+}$ was selected because it has been well characterized and shown to meet all requirements for spectroelectrochemical sensing. This includes undergoing an electrochemical reaction which leads to an optical modulation and pre-concentrating into a thin film. In order for the down-well sensor to be used for spectroelectrochemical sensing it must have the ability to control the electrochemical reaction, monitor the optical change, and be able to use film modified electrodes. Figure 4.5 shows the cyclic voltammogram for the reversible reaction of Ru(bpy)$_3^{2+}$ in the down-well spectroelectrochemical cell. Oxidation of Ru(bpy)$_3^{2+}$ to Ru(bpy)$_3^{3+}$ occurs at $E_{pa} = 900$ mV and reduction of Ru(bpy)$_3^{3+}$ back to Ru(bpy)$_3^{2+}$ occurs at $E_{pc} = 815$ mV vs. the internal Ag/AgCl wire. This is similar to the previously reported results for the Ru(bpy)$_3^{2+/3+}$ reaction.
shows the down-well sensor is providing sufficient electrochemical response and provides the potentials needed for reduction and oxidation of the analyte. Similar results were seen for the oxidation and reduction of Ru(bpy)$_3^{2+/3+}$ over several months and multiple trials indicating the internal Ag/AgCl reference electrode provides good stability and ruggedness. While not seen in this work if the Ag/AgCl reference electrode failed to provide a stable potential it can be replated within the cell by placing the cell in a plating solution and applying the correct potential for deposition of Ag/AgCl.

![Cyclic voltammetry of 1.0x10$^{-4}$ Ru(bpy)$_3^{2+}$ in 0.1 M KNO$_3$ solution vs. Ag/AgCl wire. Scan rate 75 mV/s.](image)

Figure 4.5  Cyclic voltammetry of 1.0x10$^{-4}$ Ru(bpy)$_3^{2+}$ in 0.1 M KNO$_3$ solution vs. Ag/AgCl wire. Scan rate 75 mV/s.

The down-well probe provides adequate electrochemical control of Ru(bpy)$_3^{2+}$. In order to be used for the spectroelectrochemical method previously described the cell must also allow for analyte preconcentration in a chemically selective film. Film selection is a very important aspect
of the sensor. Using a film that is selective for the analyte of interest will allow for better selectivity and preconcentration that improves the limit of detection. A Nafion film was selected for this work because it has been shown to be quite useful at preconcentrating $\text{Ru(bpy)}_3^{2+}$.[12] It is important to note that the film and electrode can be changed by simply removing the film coated electrode and placing a new film coated electrode in its place. The electrode or the film can be any number of combinations as long as the electrode is sized properly.

Figure 4.6 shows the uptake of $\text{Ru(bpy)}_3^{2+}$ into a 24 nm thick Nafion film. The cyclic voltammogram of $\text{Ru(bpy)}_3^{2+}$ in a Nafion film results in similar redox potentials as Figure 4.5. Both the oxidation and reduction peaks exhibit increasing current values as $\text{Ru(bpy)}_3^{2+}$ partitions into the film. This uptake is complete within the first 25 min of exposure to the analyte. This is seen in Figure 4.6 by the overlapping cyclic voltammograms following 25 min. At this point the film is equilibrated and further uptake does not occur. Previous work with films of this thickness in flow cells has shown complete film saturation of similar concentrations within 12 minutes.[12] The longer time interval reported here is to be expected when comparing preconcentration in a flow to preconcentration in the cell used here.

Film regeneration is another important factor to consider. The ability to reuse the film can allow for multiple uses from a single device. However, film regeneration like film preconcentration with this setup is inherently more difficult when compared to the use of a flow cell. Previous work using a flow cell setup has shown that $\text{Ru(bpy)}_3^{2+}$ can be removed from a film by flowing 95% EtOH through the film.[12] This process was shown to require only a few minutes of flow before the film was completely regenerated. The probe reported here was lowered into a stirred 95% EtOH solution. After every five minutes the probe was removed and placed in a supporting electrolyte solution and cyclic voltammograms were obtained. Figure 4.7
shows the cyclic voltammogram after twenty minutes of EtOH exposure. This shows Ru(bpy)$_3^{2+}$ is being removed from the Nafion film by placing the probe in the stirred solution. While uptake and removal of Ru(bpy)$_3^{2+}$ in a Nafion film is less efficient in a down-well design, this task can still be accomplished in a reasonable amount of time.

Figure 4.6  Uptake over 90 minutes, of 1.0x10$^{-5}$ M Ru(bpy)$_3^{2+}$ in a 24 nm thick Nafion film on ITO. Potential vs. Ag/AgCl wire.
Figure 4.7  Cyclic voltammogram showing the removal of Ru(bpy)$_3^{2+}$ from Nafion film after 20 minutes in stirred 95% EtOH solution. Potential vs. Ag/AgCl wire.

The results indicate the down-well cell is capable of making electrochemical measurements and preconcentrating Ru(bpy)$_3^{2+}$ into a Nafion film, two of the three required components of making spectroelectrochemical measurements. The final component requires the down-well cell to be able to monitor the optical change associated with oxidation of the analyte. Initial attempts at measuring the fluorescence spectrum of Ru(bpy)$_3^{2+}$ from 550 to 700 resulted in noisy and unquantifiable spectra. Figure 4.8 shows a normal spectrum obtained from these intial trials of Ru(bpy)$_3^{2+}$ in the down-well probe. The spectrum shows large fluorescence spikes at 555 nm and 580 nm and smaller spikes at 608, 640, and 680 nm. The expected broad Ru(bpy)$_3^{2+}$ spectrum from 550 to 700 nm can be seen but the intense spikes make quantification difficult. It
is expected these spikes are attributed to laser noise by their narrow bandwidths and their intensity.

![Graph of Phosphorescence](image)

Figure 4.8 Phosphorescence of $1.0 \times 10^{-5}$ Ru(bpy)$_3^{2+}$ in 0.1 M KNO$_3$ solution in down-well probe using a 441.6 HeCd laser for excitation.

In order to obtain the expected spectrum without the intense interference the fiber optic bundle was modified and repositioned inside the cell. By modifying the fiber optic to fit all the way inside the cell it could be placed near the back of the ITO slide at an unknown angle. This method does not allow for reproducible positioning of the optical bundle because you can no longer use the set screw in the probe body (Figure 4.3) which was design to control the distance between the ITO working electrode and the optical bundle. This would prevent reproducible results from being obtained and therefore make calibrating the cell in its current configuration impossible. However, using the arrangement shown in Figure 4.4 with the fiber optic bundle pushed inside the cell near the back of the ITO fluorescence measurements in the down-well probe yields the expected broad Ru(bpy)$_3^{2+}$ spectrum from 550 to 700 nm (Figure 4.9).
Figure 4.9  Phosphorescence of \(1.0 \times 10^{-5}\) \(\text{Ru}(\text{bpy})_3^{2+}\) in 0.1 M KNO\(_3\) solution in down-well probe using a 441.6 HeCd laser for excitation.

Figure 4.10 shows the reduction of \(\text{Ru}(\text{bpy})_3^{2+}\) phosphorescence signal by oxidation at 1100 mV vs. Ag/AgCl wire to \(\text{Ru}(\text{bpy})_3^{3+}\) over 10 min. Each point is the phosphorescence signal following another 1 min of oxidation. The initial signal decreases rapidly for the first minute due to the oxidation of \(\text{Ru}(\text{bpy})_3^{2+}\) at the working electrodes surface. This is followed by a leveling off of the optical signal from 3 to 10 min. After 10 min of oxidation a large optical signal is still present due to the \(\text{Ru}(\text{bpy})_3^{2+}\) in solution that has not been oxidized. Following 10 min of oxidation the change in counts at 600 nm is 235,000 counts. This change is proportional to the concentration of \(\text{Ru}(\text{bpy})_3^{2+}\) in solution.
Figure 4.10 Reduction of Ru(bpy)$_3^{2+}$ phosphorescence by oxidation at 1100 mV vs. Ag/AgCl wire to Ru(bpy)$_3^{3+}$ over 10 min. Each point is 1 min, 441.6 HeCd laser used for excitation.

To further investigate the noise obtained in the initial fluorescence spectra the optical bundle was removed from the down-well cell and used to measure Ru(bpy)$_3^{2+}$ in a cuvette. As Figure 4.11 shows positioning the bundle at 90° to a solution of Ru(bpy)$_3^{2+}$ in a standard 1 cm cuvette results in similar noise in the spectra. This shows the noise is due to the laser itself and not caused by the down-well probe or any external light source. Positioning the laser at 45° to the cuvette results in the expected Ru(bpy)$_3^{2+}$ spectrum and does not show any peaks from laser noise. This is because it is not being reflected back to the optical fiber collecting the emitted light. As Figure 4.13 shows, positioning the optical bundle at a slight angle (~15° to the electrode surface) results in the expected spectrum.

The down-well probe presented here prevents the optical bundle from being placed at an angle other than 90° and therefore results in the collection of the laser noise. This makes calibrating the down-well probe using the current arrangement (Figure 4.4) difficult. In order to
complete the evaluation of the down-well probe a redesign which allows for the optical bundle to be placed at an angle other than 90° to the electrode surface is required. This would allow for optical monitoring of Ru(bpy)$_3^{2+}$ because the noise would not be present. This modification would require a redesign of the probe body to allow for the optical bundle to be held at a controlled angle. As Figure 4.13 shows even a small angle of ~15° to the electrodes surface allows for the elimination of the laser noise.

![Laser at 90° to solution](image)

Figure 4.11 Phosphorescence of 1.0x10$^{-5}$ Ru(bpy)$_3^{2+}$ in 0.1 M KNO$_3$ solution in a 1 cm cuvette using a 441.6 HeCd laser for excitation. Collection and excitation at 90° to the solution.
Figure 4.12  Figure 4.11 Phosphorescence of $1.0 \times 10^{-5} \text{Ru(bpy)}_3^{2+}$ in 0.1 M KNO$_3$ solution in a 1 cm cuvette using a 441.6 HeCd laser for excitation. Collection and excitation at $45^\circ$ to the solution.

Figure 4.13  Figure 4.11 Phosphorescence of $1.0 \times 10^{-5} \text{Ru(bpy)}_3^{2+}$ in 0.1 M KNO$_3$ solution in a 1 cm cuvette using a 441.6 HeCd laser for excitation. Collection and excitation at $\sim 15^\circ$ to the solution.
4.4 Conclusions

The down-well spectroelectrochemical sensor presented here has been shown to provide sufficient electrochemical and preconcentration of Ru(bpy)$_3^{2+}$. The uptake of Ru(bpy)$_3^{2+}$ into a Nafion thin film provides the possibility for signal enhancement and demonstrates the cell’s ability to uptake an analyte and regenerate a film. A fluorescence change associated with the oxidation of Ru(bpy)$_3^{2+}$ to Ru(bpy)$_3^{3+}$ at 1100 mV shows the probe’s ability to spectroelectrochemically detect the analyte if the optical bundle is positioned properly.

The laser noise present in the optical signal prevents the cell from being calibrated in its current state. However, modifications to the cell could result in the elimination of the laser noise. This would allow for successful spectroelectrochemical detection of the model analyte Ru(bpy)$_3^{2+}$. A down-well probe for fluorescence spectroelectrochemical detection can be beneficial for a number of analytes and the design presented here can be easily scaled up or down in size to make the probe even more versatile.
4.5 References


Chapter 5. Anodic Stripping Voltammetry

5.1 Introduction

Zinc is an essential element required for a number of enzymes and transcription factors.\textsuperscript{[1]} The flexibility of Zn coordination geometry allows for rapid protein conformation changes required for many biological reactions.\textsuperscript{[2]} Due to this Zn provides an essential catalytic function for a number of enzymatic reactions. Pediatric and adult studies have consistently demonstrated abnormally low zinc levels in critically ill patients and inflammation and infection are associated with reduced serum levels of zinc.\textsuperscript{[3-8]} Zn supplementation may be a beneficial therapeutic strategy in critically ill patients.\textsuperscript{[9-12]} However, for this strategy to work safely, zinc levels must be monitored constantly because in blood plasma, zinc is bound to and transported by albumin and transferrin.\textsuperscript{[13]} Since transferrin also transports iron, excessive zinc reduces iron absorption, and vice-versa. A similar reaction occurs with copper.\textsuperscript{[14]}

Conventional methods for analysis of serum for Zn requires blood samples to be collected and shipped to a laboratory for analysis by atomic absorption spectrophotometry (AAS) or inductively coupled plasma mass spectroscopy (ICP-MS).\textsuperscript{[15,16]} These methods are accurate but costly and can be time consuming due to the need for shipping the collected samples. In order to determine the possible benefits of Zn supplementation for critically ill patients an on-site method for Zn quantification in serum is required.

Anodic stripping voltammetry (ASV) is a commonly used electrochemical technique for quantitative analysis of electroactive species such as metals ions. This technique has been shown to offer low limits of detection due to the pre-concentration step that enormously increases analyte concentration at the electrode surface. The pre-concentrated metal is then analyzed in a separate stripping step that gives a signal proportional to the concentration of the analyte (Figure
5.1. In addition to a very low limit of detection, stripping voltammetry requires relatively simple and inexpensive instrumentation, with exceptional suitability for miniaturization. These features, especially the latter, have stimulated interest in developing microfabricated electrochemical sensors for point-of-care use that could allow for on-site Zn quantification.

**Figure 5.1** Schematic of the deposition and stripping steps for ASV.

Historically, hanging mercury drop electrodes (HMDEs) and mercury film electrodes (MFEs) were widely used for ASV. Recently, bismuth film electrodes (BiFEs) have become a popular alternative to the MFE and HMDE. BiFEs, like MFEs are prepared by reduction of the ion Bi$^{3+}$ to Bi$^0$s on a conductive electrode. Bismuth films can be electroplated on a number of conductive electrodes.$^{[17]}$ This means BiFEs will be compatible with the majority of microfabricated cells. Also, BiFEs exhibit a high overvoltage for hydrogen evolution resulting in a negative potential window similar to that of the MFE.$^{[18,19]}$ This negative working window has been utilized to detect Pb$^{(18-21)}$, Cd$^{(18-21)}$, Zn$^{(18-21)}$, Ti$^{(18,19,22)}$, In$^{(19)}$, Cu$^{(19)}$, and Sn$^{(23)}$ by ASV. Another advantage of BiFEs is their low toxicity compared to MFEs. The lower toxicity of the
BiFE allows for the development of disposable devices, which can be used for a variety of point of care and field use studies.

While BiFEs and MFEs have similar electrochemical properties, there are some important differences that must be taken into consideration. For instance, mercury deposits on the electrode as liquid mercury droplets while bismuth deposit as a solid film at room temperature. Solid films exhibit a number of disadvantages compared to the liquid MFE. In the MFE metal atoms are soluble and can diffuse independently of each other resulting in little to no distortion of stripping peaks. On solids surfaces metals are deposited on top of each other leading to multiple and distorted stripping peaks. Solid surfaces can also have issues with fouling due to metals adsorbing on the surface. Another disadvantage of the BiFE compared to the MFE is the decreased potential window. While the negative potential windows are similar, the working window of BiFE is decreased due to the negative potential required for oxidation of $\text{Bi}^0 (s)$ to $\text{Bi}^{3+}$. This leads to an upper limit for the BiFE around -200 mV, compared to 200 mV for the MFE.

A number of solid electrodes such as glassy carbon, Au, and Pt have also been used for stripping analysis. Some of these electrodes like Au and carbon have been used extensively in microfabricated devices. However, the major disadvantage of these solid electrodes is the limited negative working region they offer compared to MFE and BiFE. This results in the inability to detect many important analytes like Zn. Another disadvantage is the issue of peak shifting and distortion due to metal interactions at the electrode surface similar to the BiFE.

In our work, a miniaturized sensor was developed using *ex situ* electrodeposited Bi electrodes and was successfully applied to the determination of Zn by ASV. The sensor integrated a silver/silver chloride (Ag/AgCl) reference electrode and a gold auxiliary electrode.
5.2 Experimental

5.2.1 Materials and reagents

All reagents were used as purchased. Acetate buffer was prepared from sodium acetate salt (Fisher Scientific) and de-ionized water. Zinc stock solution was prepared from zinc acetate salt in acetate buffer. AAS standard solution of Bi (III) ion in 2% HNO₃ (Fisher Scientific) was used to make the bismuth plating solution and a concentration of 500 mg/L was made in acetate buffer. Commercially available silver-plating solution from Technic, Inc. was used for the fabrication of the Ag/AgCl reference electrode. Wet etchants for the gold etching and the Ti etching were made from salts and acids: the Au etchant was made from 20 g I₂ :5g KI: 200 ml of DI water (w/w/v) and the Ti etchant was made from HNO₃, HF and water in the ratio 1:2:7 (v/v/v). Hyclone fetal bovine serum (Thermo Scientific).

5.2.2 Instrumentation

The sensor designed and fabricated by the Papautsky group (University of Cincinnati BioMicroSystem Lab, School of Electronic and Computing Systems) consists of a three-electrode system inside a polydimethylsiloxane (PDMS) well (Figure 5.2). The electrodes were fabricated by electrodeposition on a 200 nm thick gold layer evaporated on a glass substrate. Photolithography was used for patterning the sensor electrodes during device fabrication. The Ag/AgCl reference electrode was formed by electrodeposition of ~2 µm Ag (5 mA /cm² for 15 s), followed by chloridization in 1 M KCl solution to form an AgCl layer. Unmodified gold surface was used as the auxiliary electrode. The working electrode consisted of a bismuth film, ~1 µm thick. The bismuth working electrode was fabricated by electrodepositing Bi by reduction of Bi³⁺ from a solution of 0.1 M acetate buffer and 500 mg/L bismuth (III) at - 800 mV for 240 s. For Bi film studies the concentration of Bi in the plating solution was varied from 50
mg/L to 500 mg/L and was electrodeposited on gold slides of 1 cm² area. The Zn chip was completed by the fabrication of polydimethylsiloxane (PDMS) Sylgard 184 (Dow Corning) polymer well using the standard soft lithography methods as discussed in previous work. The Reference 600 (Gamry) and BAS100W (BioAnalytical Systems) potentiostats were used in both device fabrication and electrochemical measurements. ICP-MS was done on an Agilent ICP-MS 7500. Digested samples were filtered at 9000 rpm for 10 min in Microcon ultracel ym-10 regenerated cellulose 10,000 MWCO and Microcon ym-m regenerated cellulose 3,000 MWCO (Milipore).

Figure 5.2 Lab-on-a-chip sensor designed and fabricated by the Papautsky group (University of Cincinnati BioMicroSystem Lab, School of Electronic and Computing Systems). Left: complete chip showing electrode design and overall size. Right: close up of the three electrode design, showing the Bi working, Ag/AgCl reference, and Au auxiliary electrodes.

5.2.3 Serum sample preparation

Blood was drawn from a healthy adult female into metal free, no additives, sterile, Royal Blue Top Tubes (BD Vacutainer). These samples were centrifuged (Eppendorf 5810R) at 4000 rpm for 8 min at 35°C. Serum was then taken off via pipette into tube transport containers (ARUP Labs PK/100 Trace Metal Free Transport Tubes) which were stored at room temperature.
Human serum was digested in 0.2 M HCl for 24 h. Following acid digestion, the pH of the sample was adjusted to 5.7 with 0.5 M sodium acetate. The resulting serum dilution was 1:1/2. A 250 µL of the acid digested and pH adjusted serum sample was used for ASV as previously described. Bovine serum was thawed by heating at approximately 40°C. Thawed serum was spiked with Zn to the desired concentration. Spiked and unspiked serum was digested in concentrated HNO₃ for 30 min and heated to dryness. The digested serum was diluted in 0.1 M pH 6 acetate buffer up to 1 mL. For bovine serum experiments the electrochemical cell consisted of a BiF working electrode, Pt wire auxiliary electrode, and a micro Ag/AgCl reference electrode (Cypress Systems).

5.2.4 Preparation of Nafion film

Nafion films were prepared from 5% stock solutions diluted to the desired concentration (1%) with a 4:1 isopropanol-water mixture. A 75 uL aliquot of diluted solution was pipetted onto the electrodes and allowed to air dry. This resulted in films of unknown thickness.

5.3 Results and Discussion

5.3.1 BiFE optimization

Selecting the proper working electrode is a vital part of any electrochemical experiment. This is especially important when attempting to detect highly electronegative species like Zn²⁺ (stripping peak potential –1.4 V), because the working electrodes surface properties will ultimately determine the working potential window for the device. Figure 5.3 shows cyclic voltammograms of pH 8, 0.1 M sodium acetate on Pt, Au, GC, electrodeposited BiFE, and MFE. The lower limit of the working region of all these electrodes is determined by the potential where the solution breaks down and produces a large current. This drastic current increase at and below the hydrolysis potential prevents any analyte signal from being monitored. Due to this the
The upper limit of the working region on the Pt, Au, and GC solid state working electrodes is determined by the potential where $2H_2O(l)\rightarrow O_2(g) + 4H^+(aq) + 4e^-$. Like the reduction reactions that occur at negative potentials large currents are produced due to the breakdown of solution. The upper limits for the BiFE and MFE are determined by the potential at which $Bi(s)\rightarrow Bi(aq)^{3+}$ and $Hg(l)\rightarrow Hg(aq)^{2+}$. The oxidation of the Bi and Hg at x and yV respectively, results in a reduced oxidative potential window compared to that of the Pt, Au, and GC. However, the limited positive region is of little concern when attempting to detect electronegative metals by ASV.

Figure 5.3 shows the most negative potential window is achieved with the MFE working electrode, with potentials going to approximately -1.8V. The BiFE and GC electrode approach this limit with lower working windows reaching -1.7 and -1.6 V respectively. Pt and Au gave lower limits of approximately -1.0 and -1.2 V which are insufficient for $Zn^{2+}$ detection. Based on these results only the MFE, BiFE and GC electrodes are suitable for detection of $Zn^{2+}$ by ASV. However, due to the health and environmental concerns involved with the use of Hg, the BiFE was selected as the working electrode for this device.
Figure 5.3  Working windows of various electrodes in 0.1 M sodium acetate at pH 8, solutions purged with N₂ for ten minutes prior to trial. Scan rate of 100 mV/s all potentials vs. Ag/AgCl reference electrode.

A variety of methods can be used for Bi film deposition. We have investigated two of the most common methods for film deposition, constant current and constant potential deposition. Figure 5.4 shows the SEM and AFM images of the Bi films deposited on the working electrode by constant current and constant potential deposition for 4 min and the profilometry results for each deposition method after 2, 4, 6, 8, and 10 min depositions. Figures 5.4 a and d show the SEM and AFM images of the Au surface prior to deposition of the Bi film. As expected the evaporated gold surface provides a flat and uniform surface for Bi film deposition. A uniform initial surface is important because any defects in the initial Au seed layer can affect the quality of the deposited film.

Figures 5.4 b and c show the SEM images resulting from a 4 min Bi deposition by constant
current (b) and constant potential (c). The SEM image of the constant current deposition of Bi after 4 min of deposition shows an underlining smooth Bi surface plated on the Au layer with clusters of Bi seen throughout the electrode. The SEM image of the constant potential deposition method over the same time frame shows a similar underlining smooth Bi surface, but results in an increased number of Bi clusters on the surface. The increased number of clusters results in a Bi film of increased density compared to the film resulting from constant current deposition. Figures 5.4 e and f show the AFM images of the BiFE resulting from constant current (e) and constant potential (f) deposition. The surface roughness calculated from the AFM images confirms the increased density of the Bi clusters resulting from constant potential deposition (Erms = 230±49 nm) compared to constant current deposition (Erms = 610±78 nm).

Profilometery of the Bi films after deposition at various time intervals (Figure 5.4 g and h) shows a linear increase in Bi film thickness with increased deposition times for both methods. Slightly higher standard deviations (n=3) were recorded for the constant potential deposition method, but a better linear correlation was reported for this method. Figures 5.4 h shows an overall increased film thickness over the same time intervals for BiFE resulting from constant potential deposition. The Bi film thickness is important because stripping voltammograms of 50 µM Zn on BiFEs of various thicknesses shows increasing the film thickness results in larger peak currents (Figure 5.5). Films deposited by constant potential deposition for 300 s gave 2 times the peak current obtained from constant potential deposition for 100 s. Figure 5.5 shows the peak current increases linearly with deposition times from 120 s to 360 s. The upper limit of time for films deposited by this method is ~400 s because the Bi film becomes thick and unstable leading to flaking into the plating solution. Short BiF deposition times (<120 s) cause the peak currents to level off.
Figure 5.4 Comparison of Bi films electrodeposited by controlled-current (5 mA/cm²) and controlled-potential (-0.8 V), from [ref]. SEM images of the (a) Au seed layer, (b) controlled current Bi film, and (c) controlled potential Bi film illustrate surface quality. AFM scans of the (d) Au seed layer, (e) controlled current Bi film, and (f) controlled potential Bi film illustrate surface roughness. Controlled current electrodeposition method exhibited a lower film deposition rate (g) as compared with the controlled potential condition (h). Film thickness was measured over a 1 cm² area using profilometry. AFM and profilometry measurements were done by University of Cincinnati Department of Engineering.
While peak current and peak shape are some of the most important factors to consider in selecting the best deposition method, the potential window resulting from the various deposition methods needs to be considered. To determine the best BiF deposition method, cyclic voltammograms of acetate buffer solutions were obtained at BiFEs created by both methods (Figure 5.6) from -0.6 V to -2.2 V vs. Ag/AgCl. As expected the positive limit of the BiFE from each deposition method is the same due to the potential of Bi stripping. However, films deposited by constant potential deposition have larger negative potential window, which is extended to ~1.9 V compared to constant current at ~1.7 V. The constant potential deposition also results in a reduced current background at the potential of Zn stripping (~1.4 V). Due to the increased negative working window, lower background currents, and larger peak currents obtained with films deposited by the constant potential method it was selected as the preferred method for BiF deposition.
Figure 5.6  Cyclic voltammetry comparison of the working potential windows of the Bi working electrodes prepared by controlled-current (5 mA/cm²) and controlled-potential (-0.8 V). Dotted lines indicate extension of the working potential window for the Bi film prepared by controlled-potential deposition. Both results are in 0.1 M sodium acetate pH 5.75 buffer, with a scan rate of 75 mV/s; all potentials vs. Ag/AgCl reference electrode.

Similar to the working electrode the buffer composition and buffer pH can be a very important factors in determining the working window and background currents obtained in stripping voltammetry. Cyclic voltammograms of 0.1 M phosphate buffers (PBS) at pH 2, 7, and 11 show a drastic decrease in the negative working window as the pH is reduced to 2 (Figure 5.7). At pH 11 the working window approaches -1.7 V compared to the -0.6 V at which hydrolysis in the acidic PBS. At pH 11 and 7 similar negative working windows were obtained. As expected due to the stripping potential of Bi all the PBS tested resulted in similar positive working windows. Cyclic voltammograms of pH 7 phosphate, sodium acetate, and sodium chloride solutions give similar working windows with only a slight decrease in the positive working window for sodium chloride (Figure 5.7).
Figure 5.7 Effect of different supporting electrolytes and pH on Au electrode working window. Solutions were purged with N₂ for ten minutes prior to trial. Scan rate of 100 mV/s all potentials vs. Ag/AgCl reference electrode.

5.3.2 ASV of Zn²⁺ in acetate buffer

Following optimization of the BiFE and buffer solution, stripping voltammograms were obtained for Zn on BiFE used over multiple trials and on a BiFE plated prior to each experiment. Removing and plating the BiFE prior to each trial results in larger peak currents for Zn stripping (4.6 x 10⁻⁵ vs. 4.0 x 10⁻⁵) and a reduced standard deviation (8.3 x 10⁻⁶ vs. 1.3 x 10⁻⁵) compared to the BiFE used over multiple trials. Due to the increased current and better standard deviation the BiF was stripped and replated prior to each experiment in subsequent experiments.
Figure 5.8 shows the stripping voltammograms for Zn in acetate buffer using multiple deposition potentials. The peak currents for the Zn stripping peak increase as the deposition potential is reduced from -1.5 V to -1.7 V. However, the peak current for Zn decreases when more negative potentials are applied (-1.8 and -1.9 V). This is due to the increased hydrolysis at the working electrode leading to vigorous gas evolution which prevents Zn from depositing on the BiFE. To prevent excessive hydrolysis and allow for sufficient Zn deposition a preconcentration potential of -1.6 V was selected.

![Stripping voltammograms showing optimization of the preconcentration potential in 10 μM Zn in pH 6 acetate buffer.](image)

Next the preconcentration time was optimized by measuring the Zn stripping peak current following deposition at -1.6 V for varying times ranging from 30 to 900 s. Figure 5.9 shows that Zn stripping peaks can be seen using deposition times as low as 30 s. Increasing the deposition time results in a linear increase in the Zn stripping peak current from 30 to 400 s as seen in
Figure 5.10. The peak current begins to level off at 600 s. Due to the leveling off of the Zn stripping current after 600 s, this deposition time was selected for subsequent ASV experiments.

Figure 5.9  ASV of 10 µM Zn for preconcentration times from 30 to 900 s in pH 6 acetate buffer. Deposition potential -1.6 V vs. Ag/AgCl.
Figure 5.10 10 µM Zn stripping peak current at ~-1.4 V for preconcentration times from 30 to 900 s in pH 6 acetate buffer. Deposition potential -1.6 V vs. Ag/AgCl.

The optimized sensor was successfully calibrated with Zn solutions in pH 6 acetate buffer. Figure 5.11 shows the peak currents obtained from ASV of 5 to 35 µM Zn in 200 µL of solution. As Figure 5.11 shows the peak current increases linearly over this range ($y = 7 \times 10^{-8} x + -2 \times 10^{-6}$ $R^2 = 0.994$). This is sufficient for detection of the physiological range of Zn in blood serum, which is 9 to 18 µM.

Figure 5.11 ASV of increasing concentrations of Zn from 5 µM to 35 µM. Zn stripping peak currents measured at ~-1.36 V vs. Ag/AgCl in pH 6 acetate buffer.
5.3.3 Detection of Zn in serum

Detection of Zn in serum samples requires an additional serum digestion step in order to free Zn\(^{2+}\) bound by proteins. Multiple methods have been developed for protein digestion and subsequent metal ion release. For simplicity the initial digestion method consisted of a 24 h acid digestion in 0.2 M HCl. Following digestion the sample was buffered to pH 6 with sodium acetate. Figure 5.6 shows the stripping voltammogram of the digested and buffered serum sample obtained using the previously optimized stripping parameters. The Zn stripping peak in this sample can be seen at -1.28 V vs. Ag/AgCl. This peak is shifted approximately 0.1 V from the -1.36 V Zn stripping potential obtained in acetate buffer solution. This slightly positive potential shift was shown to be due to the increased Cl\(^-\) concentration resulting from the acid digestion step.

The digested and buffered serum sample was spiked in the cell with 50 and 74 µM Zn and stripping voltammograms were obtained. Similar to the unspiked serum sample, the Zn stripping potential for the spiked samples occurs at approximately -1.28 V vs. Ag/AgCl (Figure 5.12). Using the baseline correction method shown in Figure 5.13 the peak heights were corrected for as shown in the inset in Figure 5.12. The corrected peak heights for the unspiked and spiked samples were used to estimate the serum concentration of Zn by standard addition. As Figure 5.14 shows the calculated unspiked concentration of Zn in the sample was determined to 12.44 µM. This is within the expected range for a healthy adult.

It is important to note that the stripping voltammograms for all the serum samples gave lower than expected peak currents when compared to the peak currents obtained for similar Zn concentrations in buffer only. This difference could be due to several factors, including: biofouling of the bismuth film electrode, not cleaning and re-depositing Bi between each trial, or
possible interactions between the Zn\textsuperscript{2+} and the digested proteins. These factors could also explain the decreasing background current seen between the first voltammogram and the final voltammogram (Figure 5.12).

Figure 5.12  ASV of unspiked serum and 75 µM spiked serum. Inset is baseline corrected Zn peaks for spiked and unspiked serum samples.
Figure 5.13 Image of baseline correction method from Originpro 8 software. Serum sample following 24 hr HCl digestion. Potential vs. Ag/AgCl.

Figure 5.14 Standard addition method used for calculation of Zn in serum. Linear fit of unspiked serum and serum spiked with 54 and 75 µM Zn.

Several attempts to quantify Zn in serum by this digestion method revealed a lack of reproducibility in the digestion procedure leading to multiple trials where no spiked or unspiked
Zn stripping peaks could be found in serum (Figure 5.15). It is expected the factors that lead to the reduced current in the serum measurements are also contributing to the inability to replicate results. These factors include: biofouling of the bismuth film electrode, not cleaning and re-depositing Bi between each trial, or possible interactions between the Zn$^{2+}$ and the digested/undigested proteins.

To prevent biofouling the electrodes were covered by a Nafion film.[25-27] This prevents large components of the sample matrix (like proteins) from reaching the electrodes due to the small pore size of the film and can also be used to preconcentrate Zn$^{2+}$. However, no improvement was found from coating the electrodes with Nafion film (Figure 5.16).

In an attempt to reduce the interactions between the digested/undigested proteins and Zn$^{2+}$, Cr$^{6+}$ was added to the digestion solution. Figure 5.17 shows the stripping voltammograms of unspiked (Figure 5.17A) and Zn spiked serum (Figure 5.17B) following the addition of Cr$^{6+}$ to the serum sample prior to digestion. The addition of Cr$^{6+}$ resulted in increased Zn stripping peak currents compared to the previous results obtained in serum. However, sequential trials showed little improvement in reproducibility.
Figure 5.15  ASV of unspiked serum and 15, 30, and 45 µM spiked serum.

Figure 5.16  ASV of unspiked serum and 10, 20, and 30 µM spiked serum on Nafion film coated BiFE.
Figure 5.17 ASV of unspiked serum and 15 µM spiked serum following the addition of ~ 1 mM Cr\textsuperscript{6+} prior to digestion  A) Unspiked serum. B) 15 µM spiked serum. C) Baseline subtracted ASV.

To better understand the issues involved in serum digestion, bovine serum and a conventional electrochemical cell were used. Switching to bovine serum reduces the hazards involved in working with human samples and allows for increased sample volumes. To improve the reproducibility of Zn detection in serum a new digestion method was used. Concentrated HNO\textsubscript{3} and serum spiked with 20 µM Zn was heated to dryness. Caution was taken to ensure the sample did not burn. The resulting sample was dissolved in 0.1 M acetate buffer. Following digestion
ASV was done using a conventional electrochemical cell and the previously optimized parameters.

Figure 5.18 shows the ASV of a bovine serum sample spiked prior to digestion with 20 µM Zn and additional 14 and 25 µM spikes in the cell post digestion. The ASV of the pre-digestion spike results in large background peaks at ~ -1.4 and -0.2 V and no peak at the expected Zn stripping potential. Spiking the solution with 14 µM Zn following the first trial results in similar background peaks but also gives a Zn stripping peak at -1.0 V. An additional 10 µM Zn spike (25 µM total post-digestion Zn spike) results in a larger Zn stripping peak at -1.0 V. The peak increase at -1.0 V with the addition of higher concentrations of Zn confirms this peak is due to Zn stripping. The inability to detect the pre-digestion Zn spike suggests Zn could be lost during the digestion process, bound to proteins which would prevent Zn deposition, or interacting with Cu to form intermetallic complexes which interfere with Zn stripping.[19]
To test for possible Zn loss during the digestion process a serum sample was spiked with Pb, Cd, and Zn prior to digestion. Figure 5.19 shows the ASV of 20 µM Pb, 10 µM Cd, and 20 µM Zn serum spiked pre-digestion and increasing Pb, Cd, and Zn spikes following digestion. Similar to the previous results the initial Zn spike is not seen while the additional post digestion Zn spikes result in the expected stripping peaks at -1.0 V. The initial Pb and Cd spikes can be recovered following digestion as seen in Figure 5.19 at -0.45 and -0.70 V, respectively. As with Zn the additional post digestion Pb and Cd spikes result in increasing stripping peak currents with increasing analyte concentration.

Figure 5.19  ASV of 20 µM Pb, 10 µM Cd, and 20 µM Zn serum spiked pre-digestion and increasing Pb, Cd, and Zn spikes following digestion on a BiFE.
Pb and Cd recoveries following digestion suggest Zn is not lost during the digestion process. To confirm this ICP-MS was done on the digested serum sample. Table 5.1 shows the expected Zn recovery from the initial 20 µM (~1.3 ppm) Zn spike. This table also shows the solution has very little Cu present (~40 ppb) relative to the Zn concentration (~1.3 ppm). Since Cu and Zn are known to form an intermetallic complex in a 1:1 ratio \cite{19} this is most likely not the issue for the lack of Zn recovery.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Cu (ppb)</th>
<th>%RSD</th>
<th>Zn (ppb)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43.32</td>
<td>2.36</td>
<td>1273.29</td>
<td>0.82</td>
</tr>
<tr>
<td>B</td>
<td>39.45</td>
<td>0.83</td>
<td>1265.58</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Table 5.1  Digested serum spiked with ~1.3 ppm Zn.

The possibility of partially digested proteins binding Zn and preventing deposition was investigated by filtering the serum post digestion and monitoring the resulting Zn concentration by ICP-MS. The selected filter sizes (10,000 and 3,000 MWCO) should exclude a large portion of partially digested proteins and therefore result in a decrease in Zn signal if Zn is bound to the excluded protein fragment. Table 2 shows the Zn concentration does not change due to filtering so Zn is not bound to anything larger than 3,000 MWCO. The filters used for this experiment have Zn in them and result in an increased Zn background. However, after subtracting out the background increase due to Zn contamination in the filters no loss of Zn due to filtering can be seen. This indicates the digestion method used here is adequate for serum digestion.
5.4 Conclusions

In this work, a microfabricated electrochemical sensor was developed for the detection of the Zn. The sensor consists of an electrodeposited bismuth film as the working electrode along with an integrated Ag/AgCl reference and Au counter electrodes. Multiple plating and solution conditions were optimized for the detection of Zn. The stripping voltammetry parameters such as the preconcentration potential and preconcentration time for the detection of Zn were also optimized to be -1.6 V and 600 s. The sensor was successfully calibrated for acetate buffer of pH 6 for a range of 5 μM to 35 μM. The sensor demonstrated excellent reproducibility and reliability in buffer solution. Detection of Zn in unspiked and spiked serum samples was done by two digestion methods. The initial 0.2 M HCl digestion method allowed for Zn detection in serum but did not exhibit adequate reproducibility. The second digestion method resulted in the expected Zn stripping peaks for post digestion spikes but peaks could not be obtained for Zn spikes prior to digestion. Further optimization of the sensing and digestion method allows us to
envision the development of a point-of-care sensor that will reduce the detection and analysis
time in critically ill patients for bedside monitoring.
5.5 References


Chapter 6. Conclusions

6.1 Spectroelectrochemical sensing conclusions

The spectroelectrochemical technique was shown to be capable of quantitatively distinguishing between two analytes with overlapping fluorescence spectra without the need for an initial separation step. The analytes selected (1-pyOH and 1-pyOglu) are a good model system for this detection method because of their near identical absorbance and fluorescent spectra and the large difference in their initial oxidation potentials. This approach could be used for other analytes and more complex mixtures as long as the analytes redox potentials are sufficiently separated.

Urine spiked with 1-pyOH and 1-pyOglu did not result in the characteristic pyrene spectra seen in the buffer and urine simulant solutions. This was seen in the OTTLE cell and standard cuvette. Finally, spiked urine was eluted on a C-18 separation column with multiple solutions. Fluorescence spectra of the collected fractions gave similar results and did not provide adequate separation of the pyrene conjugate from the interferences preventing the fluorescence spectrum from being monitored.

The down-well spectroelectrochemical sensor presented here has been shown to provide sufficient electrochemical and preconcentration of Ru(bpy)$_3^{2+}$. The laser noise present in the optical signal prevents the cell from being calibrated in its current state. However, modifications to the cell could result in the elimination of the laser noise. The probe body would need to be modified to allow the optical bundle to be placed at a controllable angle to the electrode surface. This would allow for successful spectroelectrochemical detection of the model analyte Ru(bpy)$_3^{2+}$. A down-well probe for fluorescence spectroelectrochemical detection can be
beneficial for a number of analytes and the design presented here can be easily scaled up or
down in size to make the probe even more versatile.

6.2 Detection of zinc in serum conclusions

A microfabricated electrochemical sensor was developed for the detection of the Zn. The
optimized stripping voltammetry parameters proved capable of detecting Zn in buffer solutions
within the range required for detection in human serum. Detection of Zn in unspiked and spiked
serum samples was done by two digestion methods. The initial 0.2 M HCl digestion method
allowed for Zn detection in serum but did not exhibit adequate reproducibility. The second
digestion method resulted in the expected Zn stripping peaks for post digestion spikes but peaks
could not be obtained for Zn spikes prior to digestion. These methods did not provide
reproducible results for Zn stripping and would require optimization of the sensing and digestion
method to allow for detection of Zn in critically ill patients for bedside monitoring. The sensing
method can be optimized by improving the negative working window of the BiFE electrode.
This work will focus on producing a more uniform BiF by better controlling the deposition of Bi.
New serum digestion and Zn extraction methods will be investigated to allow for Zn detection in
serum.