MONITORING OF PSEUDOMONAS AERUGINOSA TOXINS VIA MINIATURIZED ELECTROCHEMICAL ASSEMBLIES

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Thaddaeus Andrew Webster

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ABSTRACT

The production of pyocyanin from the opportunistic pathogen *Pseudomonas aeruginosa* was probed using a variety of miniaturized electrochemical systems. **Goal 1** used disposable screen printed carbon electrodes to measure pyocyanin in medically relevant samples showing for the first time that pyocyanin can be detected at medically relevant concentrations (1-100 µM) without sample processing. **Goal 2** coupled these same electrodes with Polydimethylsiloxane growth chambers to expose *P. aeruginosa* biofilms to varying concentrations of colistin sulphate. A reduction in electrochemical signal from pyocyanin, of approximately 80% when exposed to 100 mg/L colistin sulphate, highlights this molecules relation to biofilm health. **Goal 3** addresses the question of detecting *P. aeruginosa* in patient samples if the concentration of pyocyanin is initially low/non-existent in patient samples. Disposable screen printed carbon electrodes were embedded within King’s A agar to determine whether electrochemical detection of pyocyanin could decrease the positive time to detection of *P. aeruginosa*. Measurements using bacterial loads of PA14 from $10^2$-$10^8$ cells showed a load dependence on the electrochemical time to detection. Importantly the positive identification of *P. aeruginosa* was reduced approximately by 14-18 hours. **Goal 4** looks at the miniaturization of the reference electrode using palladium as a novel pseudo reference material. The reference electrode was fabricated in a nanofluidic chamber housing a gold working electrode. The palladium electrode showed promise as it maintained a stable reference during testing and was sensitive to changes in pH only. The miniaturized two electrode system was capable of discerning pyocyanin production from the wild type and three mutants of *P. aeruginosa*. **Goal 5** combines all of the necessary components for a three electrode
electrochemical cell, within a nanofluidic channel. This was done to monitor the production of pyocyanin from small concentrations of *P. aeruginosa* confined in microfluidic channels to address whether pyocyanin production was indeed controlled by quorum sensing. Results suggest that the production and measurement of pyocyanin in this system is indeed dependent on quorum sensing. However the utilized system requires a 100 fold increase in the number of cells present to approach similar bacterial concentrations of other reported single cell quorum sensing platforms the results are inconclusive. Future research should be aimed at simplifying the fabrication and characterization processes of the three electrode nanofluidic sensors along with the miniaturization of the sensors. Decreasing sensor and growth chamber size will help to address the question of whether or not *P. aeruginosa* can successfully detect its own quorum sensing molecules leading to the production of pyocyanin.
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1.0 Introduction

The world that humans inhabit is filled with bacteria. This has been common knowledge since Antonie van Leeuwenhoek first glimpsed at this unseen world under the microscope. They fill a wide variety of niches living within hosts, the plaque found in our teeth, and even as free swimming cells in the environment. While some bacteria are beneficial to humans other bacteria can have negative effects on humans ranging from flu like symptoms to death (i.e. tuberculosis, pneumonia) leading to the need to understand how these bacteria initially interact with their human hosts [1].

Of interest is the gram negative bacteria *Pseudomonas aeruginosa* (PA) which is found everywhere in nature. This bacterium is normally benign to healthy humans but has been linked with a high mortality rate in people with compromised immune systems such as burn/wound victims or people suffering from autoimmune diseases [2]. While the bacterium’s effect on its infected host is well documented, little is known about why a normally mild bacterium becomes pathogenic when presented with certain environmental stimuli. Elucidating what these conditions are could yield insight into how to prevent the infection from occurring.

One of the modes by which bacteria communicate and infect tissues is through the use of quorum sensing molecules (QSMs). These molecules in the right concentrations allow bacteria to communicate with one another, leading to the regulation of genes that are beneficial to the community as a whole. Common QSMs for PA are acyl homoserine lactones (AHSLs), of which the two most prominent are N-butyryl-L-Homoserine lactone and N-3-oxo-dodecanoyl-L-Homoserine lactone. Another molecule pyocyanin is a redox active phenazine which has several negative implications in the host’s response to infections, as well as activity as a QSM. QSMs have been detected in bulk bacterial
biofilms as well as in bacteria confined to small volumes such as microfluidic chambers [3, 4]. Monitoring the conditions at which these QSMs are produced could help to better understand how PA responds to environmental conditions and perhaps shed light on its pathogenic properties. The fact that pyocyanin is a redox active molecule opens the possibility of using electrochemical methods to monitor the growth of single PA cells in microfluidic channels as well as their response to environmental stresses.

Recent advances in microfabrication techniques have led to the fast and reproducible development of small scale electrode assemblies that offer greater sensitivity compared to their large scale counterparts. Their small size allows for the fabrication of high density electrode assemblies that find uses in concentration gradient monitoring and parallel electrochemical measurements. One of the major drawbacks of current microscale electrode assemblies is their reliance on large scale reference electrodes. The large scale reference electrodes limit the devices uses in situations where size is an issue (inside a ventilator tube), cause IR drop, and require larger sample volumes than would normally be required in microfluidic structures. Miniaturization of reference electrodes has been shown with some success and further efforts towards miniaturization should lead to the production of complete electrochemical cells on a chip. Some progress on this front has been made through the use of commercial screen printed electrodes, containing all of the electrodes required for a three electrochemical cell.

This dissertation proposes the use of micro and nanofluidic electrochemical assemblies containing all of the electrodes necessary for electrochemical testing within the microfluidic channel. The assemblies will be used to monitor the production of Pyocyanin (a QSM) from PA biofilms and single cell cultures under various
environmental stressors and in response to QSMs (Pyocyanin) in the hopes that they can be used to elucidate what conditions lead to increased pathogenesis in PA. This dissertation contains a literature review of the pertinent literature, methodology, the results of this dissertation work, and conclusions for future work in this exciting research area.
2.0 Literature Review

2.1 Electrode Size

Advances in technology have driven the desire for smaller and smaller components. The invention of solid state transistors and resistors revolutionized the way electrical circuits were made and led to the developments of newer technologies, i.e. laptop computers that would not have been realized if the ability to shrink specific components had not been developed. In the same way microscale electrodes have gained interest in recent years as advances in microfabrication have increased.

2.1.1 Micrometer Electrodes

The simplest microfluidic electrodes consist of a working and counter electrode microfabricated onto an insulated silicon wafer with a macroscale reference electrode used to complete the circuit. Microfluidic electrodes are ideal because they offer several advantages over their commercially available counterparts such as high electrode density [5, 6], detection of molecules that would be found in small sample volumes [7-9], placement inside the microfluidic channel to obtain better results [10], and a higher sensitivity [11, 12].

Hwang et al. (2009) created a 32 x 32 array of 7 µm square microelectrodes with a center to center spacing of 37 µm as well as an 8 x 8 array of 6 µm circular microelectrodes. They showed that potassium ferrocyanide could be detected by these individual electrodes. Furthermore they scaled down the potentiostat used for controlling the electrodes so that the entire device would be on a chip compared to common lab scale
apparatus' that require large bulky potentiostats. By loading multiple sensors onto a single device the cost decreases, while the utility of the detection device increases.

Of great interest is the small size scale of microfabricated electrodes. The small size scale not only means that the electrodes can be placed within microfluidic channels, but that a smaller sample volume is required. This is important when the molecule of interest is found in a small sample volume that is uneasy to obtain, such as cerebral spinal fluid, cellular excretions, or other bodily fluids. Pihel et al. (1995) were able to detect histamine and 5-hydroxytryptamine from rat peritoneal mast cells using cylindrical carbon fiber microelectrodes [7]. The microelectrode was initially used in a chromatography column to detect the aforementioned molecules. It was then placed in close proximity to peritoneal mast cells by bringing the electrode down onto the cell and then vertically moving the electrode 1 µm away from the cell. Measurements in the chromatography column as well as the single cell measurements showed the detection of small concentrations of both histamine and 5-hydroxytryptamine showing the utility of this microelectrode set up. Kwakye et al. (2006) and Oda et al. (2008) both showed the detection of DNA with microfluidic electrode arrays [8, 9]. Due to the small sample volumes associated with these analytes (Kwakye et al. (2006) showed detection at volumes ranging from 50-200 nL in a microfluidic channel) these microelectrodes offered advantages that are not available at the large scale.

Another benefit of microelectrodes is their increased sensitivity compared over conventional electrodes. Gunasekara et al. (2011) created an internalized microfluidic electrode coupled with capillary electrophoresis [10]. Capillary electrophoresis is a concentration technique that utilizes a voltage difference to drive electrochemical species.
Under an electric field the molecules with faster mobilities will separate out from those that are slower. For detection most microfluidic electrophoresis systems utilize an electrode located at the end of the channel to minimize the electric interference that would be experienced within the channel. By incorporating an isolated potentiostat the authors were able to obtain a 2 fold higher sensitivity and 2 fold lower limit of detection compared to the end channel detection method. Being able to incorporate the electrochemical system within the microfluidic environment could lead to the development of small scale separation and detection systems.

2.1.2 Interdigitated Electrode Arrays
In the pursuit of more sensitive microfluidic electrode devices numerous researchers have looked at creating interdigitated microfluidic electrodes which consist of an array of metal fingers that are spaced at specific distances from one another. Interdigitated electrode arrays work on the principal of redox cycling whereby one pair of interdigitated fingers is held at a potential that will reduce all molecules that come in contact with it, while the other pair is held at a potential that will oxidize all molecules in contact with it. Much research and emphasis has been placed on optimizing the output of these devices along with comparing them to conventional laboratory scale electrode systems as well as their contemporary microfluidic counter parts [13].

Morf et al. (1997) showed that a low packing density of microelectrodes acted as many individually addressable electrodes increasing the output response, whereas a high packing density of microelectrodes led to the system acting as 1 large macro electrode [14]. To this end several researchers looked at optimizing the response of interdigitated electrodes. Bard et al. (1986) ran simulations of microelectrode arrays and their outputs [15]. The authors looked at the effect of spacing between electrode fingers and electrode
width on the signal response of the system. Their simulations determined that the gap spacing between the interdigitated fingers has a larger effect on the signal output than the width of the electrode. This was due to larger electrode fingers reducing the number of interdigitated fingers that could be found in a given surface area which reduced the redox cycling efficiency of these devices. Along these lines Niwa et al. (1990) looked at different geometries and found that the collection efficiency of the electrodes was dependent on the diffusion length [16]. The diffusion length in turn depended on the gap and electrode spacing. Under experimental conditions the authors fabricated interdigitated electrodes that were capable of achieving a large dynamic range (10 nM to 1 mM) as well as a steady state response under 100 milliseconds and a limit of detection of 10 nM. Strutwolf et al. (2005) ran simulations of time dependent diffusion for interdigitated electrodes that were coplanar versus elevated [17]. The authors found that the elevated electrodes were more efficient than coplanar electrodes. This was determined to be due to the increased surface area that the elevated electrodes inherently had, as well as decreased communication time due to the higher flux at these electrodes [17].

Increased sensitivities have been found when microfluidic interdigitated electrodes have been used instead of normal electrode systems. Of note Aoki et al. (1990) showed that the microfabricated interdigitated electrode system outperformed a single potential microarray electrode as well as a dual series rectangular electrode under flowing systems [13]. The interdigitated systems showed current responses of 2.8-3.9 times greater compared to the other electrode systems being tested. Furthermore interdigitated electrodes have shown much promise in the areas of diagnostic and biochemical study due to the fact that several important biochemicals have been detected using
interdigitated platforms such as catechol [18], adrenaline [19], dopamine 20, and DNA to name a few [5].

In addition to being more sensitive, interdigitated arrays have even been used to selectivity detect biologically relevant biochemicals when measured in solutions containing other electrochemically active species. Dam et al. (2007) detected dopamine in the presence of potassium ferrocyanide [20]. This was accomplished by oxidizing both molecules at 700 mV and then selectively reducing dopamine at 400 mV with a platinum interdigitated electrode array. Since potassium ferrocyanide reduces at lower potentials than dopamine the authors showed selective detection of dopamine in the presence of potassium ferrocyanide.

While interdigitated electrodes are very interesting for their ability to induce redox cycling they are not unique in this regard. Redox cycling with a microfluidic electrode system that was not an interdigitated electrode array has been shown. Straver et al. (2012) showed that by placing two working electrode on top of each other with a vertical spacing of only a few microns, it was possible to perform redox cycling leading to an increased sensitivity of the constructed devices [21]. The devices were constructed via well-known microfabrication techniques and relied on a SU-8 layer to create a 5 µm tall channel though other methods for microfluidic and nanofluidic cavity creation exist [21, 22]. The idea that two working electrodes spaced only a small distance vertically apart increased device sensitivity led to the creation of a field known as nanofluidic electrodes [23-29].

2.1.3 Nanofluidic Electrodes

Straver et al. (2012) showed that redox cycling was achievable by decreasing the spacing between two working electrodes (henceforth referred to as top and bottom
electrodes) [21]. From this premise several authors have looked at creating nanofluidic electrode assemblies (NEAs) [23-29]. These assemblies consist of a top and bottom electrode that is spaced less than a few hundred nanometers apart. The confined spacing of these devices has led to several interesting discoveries such as measuring electron kinetics, better sensitivity, selectivity, and resistance to the effect of flow over the electrodes.

The NEAs work via redox cycling that occurs within the nanofluidic chamber due to the decreased diffusion of oxidized and reduced molecules between the top and bottom electrodes. While the diffusion time in the nanofluidic channels is fast the diffusion of just a few molecules into the nanocavity can take a lot longer due to problems arising from the absorption of the redox molecule onto the electrode surface. To this end Zevenbergen et al. (2009) outlined the mathematical theory behind the diffusion of molecules into nanofluidic channels [28]. The authors looked at the experimental response of the devices and compared with theoretical models and achieved good agreement between the two for a 70 nm tall nanochannel.

Of great interest is the increased sensitivity that the NEAs were able to achieve compared to interdigitated microfluidic electrode counterparts also utilizing redox cycling. Zevenbergen et al. (2007) were able to detect as few as 70 molecules with a redox cycling efficiency of 400 [27]. With an optimized system (better insulating material, optimized gap spacing) Zevenbergen et al. (2011) were able to detect one molecule of ferrocene dimethanol in acetonitrile solution, the first time single molecule detection was reported via electrochemical detection [26].
Increased selectivity has also been achieved in these nanofluidic systems as seen in the works of Goluch et al. (2009) and Wolfrum et al. (2008) [23, 25]. The selectivity derived from these systems was accomplished due to the fact that electrochemical molecules that increased the noise of the system were not stable in either their oxidized or reduced forms. Due to the increased cycling efficiency seen in these systems the unstable molecules eventually all degraded making it possible to detect the molecules of interest with an increased signal to noise ratio.

Finally the last improvement that nanofluidic electrodes have yielded is their ability to resist changes in their current output due to fluid flow over the circuit. Rassaei et al. (2012) created nanofluidic electrodes and studied the effect convective mass transport had on the current response [24]. The authors theorized that this was due to the hydraulic resistance within the nanochannel and the diffusion based transport that occurs when the gap size was less than 70 nm. The authors concluded that little to no fluid velocity corrections were needed to be taken into account for this system showing the utility of the nanofluidic channel design compared to other microfluidic electrode systems.

2.2 Miniaturizing Reference Electrodes

The majority of microfluidic and nanofluidic electrode systems all require the use of an external macroscale reference electrode to obtain reliable and reproducible measurements. While these reference electrodes are of use for performing laboratory experiments there are several disadvantages of these systems. First the large scale of the reference electrode requires a larger sample volume to perform measurements than what would be required if the reference could be included with the working and counter electrode. Secondly if the reference electrode is not placed in close proximity to the rest
of the electrochemical cell drift known as IR drop could occur. IR drop would make it difficult to get reproducible measurements from the device. Thirdly the large scale of the reference makes it impractical for use in many real world examples such as monitoring molecule production in wounds or ventilator tubes. Some examples in the literature of complete electrochemical cells that fit into microfluidic channels have been found.

Swensen et al. (2009), microfabricated an all gold electrochemical cell that could be used in a microfluidic channel [30]. While the construction of this device could be produced in a single fabrication step there is a major disadvantage of using metals as the reference electrode. Most metals tend to drift when held at a potential for too long which in turn leads to poor measurements. Silva et al. (2009) tried to address this by creating a microfluidic electrochemical cell that utilized a gold electrode as the working electrode, a carbon composite as the counter electrode, and a silver composite pseudoreference electrode [31]. The use of silver composite as a reference was most likely chosen because of the popularity of the silver/silver chloride reference electrode that has seen wide use in electrochemistry experiments. However this system was also prone to drift which means that if researchers wish to microfabricate the entire electrochemical cell a different reference electrode material is needed.

2.2.1 Ag/AgCl Reference Electrodes

The silver/silver chloride reference electrode is one of the most popular reference electrodes used to date in electrochemical systems. It is easier to fabricate compared to the normal/standard hydrogen electrode and is not toxic, a major advantage over the saturated calomel electrode which relies on mercury. The system consists of a silver wire that has been coated with silver chloride. To maintain the silver chloride layer at the
surface of the silver wire the entire electrode sits in a solution containing chloride ions (usually KCl or NaCl). The Ag/AgCl reference electrode works via the following redox reaction:

\[
AgCl(s) + e^- \leftrightarrow Ag(s) + Cl^-(aq)
\]  

A frit that prevents the flow of chlorine ions into the test solution is commonly used to help prevent contamination of the test solution. While commercial Ag/AgCl references are available, efforts to microfabricate these electrodes have been harder to achieve. The largest problem with the scale down of these electrodes has been due to the dissolution of the electrolyte solution, which surrounds the reference electrode, into the sample. While some dissolution occurs with commercial Ag/AgCl electrodes, due to the volume of electrolyte solution the dissolution that occurs usually has little to no effect on the reference potential. However due to the small nature of microfluidic systems, even a small amount of electrolyte dissolution from the reference area could lead to an unstable reference potential.

Suzuki et al. (1999) microfabricated an Ag/AgCl reference electrode that was covered with a polyimide protecting layer [32]. The silver electrode had silver chloride deposited onto a 50 µm slit. The device was able to maintain its potential for 30 hrs in saturated KCl. By implementing a polyvinylpyrrolidone (PVP) matrix around the reference electrode it was possible to increase the stability of the electrode to 100 hrs. The authors found no effect of external solution pH and KCl concentration and that the constructed device could be used for one-chip potentiometric sensors. The complexity of the PVP matrix and the polyimide construction layer however make it harder to mass produce on chip. Fast forward to 2005 and Simonis et al. (2005) created an Ag/AgCl
A reference electrode that did not have a KCl containing membrane [33]. A protective layer that reduced the leaching of KCl from the electrode increased its usefulness from 3 hrs to 50 days. While the stability of the reference is impressive, the difficult fabrication of this system might make it impractical for scale up.

Safari-Mohsenabad (2010) attempted to address solution dissolution in microfluidic channels by creating a silver/silver chloride reference electrode over which saturated KCl was flowed [34]. The reference electrode consisted of a silver chloride coated silver wire that was inserted into a microfluidic chamber. Nanopores were created at the intersection of the test solution so that KCl could flow over the reference electrode continuously. This system had great stability but potentially suffers from two drawbacks. The first is that while the Ag/AgCl wire is easy to fabricate, inserting the wire into the microfluidic chamber can be hard to achieve, and could lead to device leakage. The need for continual KCl flow over the reference electrode, to maintain a stable reference, feeds directly into the test solution which may contaminate the test solution.

Finally Dydek et al. (2012) created a silver/silver chloride microfluidic reference electrode that did not rely on a physical barrier or salt bridge [35]. Their system relied on the slower diffusion times in microchannels to maintain a stable reference potential during measurements. The authors created a y-shaped microfluidic channel which contained the working and counter electrodes on one side of the channels and the reference electrode on the opposite side. The test solution and reference solution were flowed through the system at different rates. By maintaining a 2:1 flow rate of analyte to reference solution, the system was shown to successfully detect the redox couple of Ir^{IV}/Ir^{III} couple at 0.699 V. This shows that it is possible to create a salt bridge free
reference electrode integrated within a microfluidic channel. However the need for a reference solution along with the analyte solution could lead to issues with leaking as the more inlets and outlets involved inevitably lead to higher potentials for leaking.

Finally another improvement to the field of miniaturizing Ag/AgCl reference electrodes has come with the advent of screen printing. In the screen printing process a mesh with the desired pattern is placed over the substrate that will be used for electrode fabrication. A conductive ink is then spread over the surface of the mesh. The ink is cured, adhering the metal paste to the substrate. Several companies offer screen printed electrochemical cells containing Ag/AgCl paste reference electrodes that have been shown to offer similar response to conventional Ag/AgCl reference electrodes. While cheaper and easier to mass produce these system inevitably suffer from drift as the reference electrode is placed in direct contact with the sampling solution.

While it can't be overstated how important the silver/silver chloride reference electrode is, and that the above authors achieved impressive stability in their respective systems ease of manufacturing is a must if microfluidic electrochemical cells are to gain widespread use. To this end another reference material should at least be considered.

2.2.2 Palladium Hydride Reference Electrodes

Another potential reference electrode material is the palladium hydride system. Palladium is a noble metal that is capable of holding 900 times its volume of hydrogen within its crystal lattice [36]. The hydrogen can be deposited either electrochemically from solution, or from exposure to hydrogen gas [37, 38]. Palladium hydride reference electrodes have been shown to have a steady standard potential of 0.05 volts versus the standard hydrogen electrode [36-42]. Hydrogen stored in palladium metal undergoes
three phase changes, the alpha, alpha-beta, and beta phase, upon exposure to cathodic (negative) electrode potentials in acidic solutions [42]. Palladium metal will begin absorbing hydrogen at approximately 240 mV versus a normal hydrogen electrode (NHE), approaching the alpha hydride phase around 140 mV versus a NHE, and finally reaching the beta-hydride phase at potentials less than 40 mV versus NHE [42]. Schuldiner et al. (1958) proposed the following redox reaction for the palladium hydride system:

\[ H^+(aq) + e^- + Pd(s) \leftrightarrow (Pd - H)_\alpha \] (2)

Metal deposition is a common microfabrication technique that has been utilized for a variety of metals. All that is required to construct a palladium hydride reference electrode is for a layer of palladium metal to be deposited and then charged with hydrogen. As such it would be relatively easy to microfabricate such an electrode for use in a miniaturized electrochemical cell.

The thermodynamics and electrochemistry for palladium hydride systems have been well studied [37, 38, 40, 43-46]. Hoare et al. (1958) looked at the thermodynamics of hydrogen absorption electrolytically [38], from exposure to hydrogen gas [37], as well as elucidating what the value for the reversible palladium hydride electrode should be [41]. The authors found that the palladium hydride electrode would spontaneously approach the alpha-beta phase when exposed to acidic solutions [38, 41], and noted that the electrochemically absorbed beta hydride phase was thermodynamically different from the beta-hydride phase obtained from hydrogen gas absorption [38]. Czerwinski and his colleagues looked at the effects of pH and salt concentration on the potential for palladium hydride electrodes [43-45].
Due to palladium’s ability to absorb hydrogen from its environment the use of palladium hydride systems as pH sensors have seen use and are used as a means of characterizing the electrodes response [39, 40, 42]. Goffe et al. (1978) compared the response of palladium hydride wires that had been continuously charged with hydrogen to conventional palladium hydride electrodes. They found that their system performed as expected and had a stability of over 330 hours (13.75 days).

Several industrial examples of palladium hydride electrodes have been shown. Cormet produces a palladium hydride reference electrode that can be used in high temperature and pressure water monitoring systems [47]. Thermo Fisher Scientific created a palladium hydride system for use in chromatography column [39]. The system utilized a counter electrode that continuously produced hydrogen which could then be absorbed by the palladium hydride reference.

Microfabricated palladium hydride reference electrodes potentially offer several advantages over current microfabricated Ag/AgCl reference electrodes. Firstly the creation of the palladium hydride reference electrode involves only a metal deposition step which with current microfabrication is easily achieved. Secondly since the palladium hydride electrode can absorb hydrogen from the system there is no need to worry about continuously flowing solute over the miniaturized electrode [34, 35]. Additionally incorporating the reference electrode within the nanocavity of a nanofluidic electrode assembly should reduce the influences of flow over the surface [24].

2.2.3 Other Miniaturized Reference Electrodes
Several other electrode materials have been microfabricated. Mercury based microfabricated reference electrodes have been created. The mercury or calomel electrode works via the following half reaction.

\[
Hg_2Cl_2(s) + 2e^- \leftrightarrow 2Hg(l) + 2Cl^- (aq)
\]

To maintain a steady potential at the surface of the electrode a saturated KCl solution is used. Lill et al. (2010) created a microfabricated reference electrode by depositing mercury electrochemically on a gold wire to form an amalgam. This reference was then surrounded by an agar containing the appropriate reference electrolyte [48]. The microfabricated reference electrodes response was found to be good. However the use of even small amounts of mercury can lead to the possibility of mercury poisoning.

Iridium Oxide is another metal system that has been shown to have a good response as a reference electrode. Iridium metal is coated with an oxidizing agent which then results in a thin layer of iridium oxide forming on the surface of the electrode [36, 49]. This system works based on the following two redox reactions:

\[
\text{IrO}_2(s) + H^+(aq) + e^- \leftrightarrow \text{IrOOH}(s)
\]
\[
\text{IrO(OH)}_2(s) + H^+(aq) + e^- \leftrightarrow \text{Ir(OH)}_3(s)
\]

Franklin et al. (2005) created an on chip IrOx reference electrode for use in the detection of neurochemicals in a MEMS microelectrode array. Their devices showed good agreement with results obtained versus a commercial Ag/AgCl reference electrode [50].

2.3 Microfluidic Systems
2.3.1 Materials of Construction

The field of microfluidics has seen an explosion in the past ten years. Many researchers have shown the benefits of performing experiments in diagnostics on the micron scale whether it be in polymerase chain reaction (PCR) for DNA copying [51,52],
single cell assays [53], or for molecular diffusion studies [54]. The following is a list of different polymer materials that can be used in the construction of microfluidic channels. This is by no means an exhaustive list but is instead a review of several different materials of construction that could be used for the microfluidic studies in this dissertation.

2.3.1.1 Polydimethylsiloxane (PDMS)

Polydimethylsiloxane (PDMS) is king in the microfluidic world. More devices and microfluidic systems in academia rely on this robust polymer to create a wide array of devices used to trap individual cells [55], perform on chip PCR [52], analyte analysis, gradient generators [56], and biofilm studies[57], to name a few. This ubiquitous polymer gets a lot of use due to its ease of use, which opens up the possibility for rapid device preparation and lowers its overall cost. PDMS is actually a mixture of two different components which come together to form the polymer. Typical PDMS applications call for a ratio of base to activator of 10:1. This yields a relatively robust PDMS piece that is perfect for producing microfluidic channels. Increasing and decreasing the amount of base results in PDMS that has a decreased and increased stiffness respectively [58]. Varying the ratio of base to curing agent to create PDMS microfluidics with different stiffness’s led to the creation of microfluidic valve layers, which have been shown to be very effective at trapping small volumes of samples for analysis’s such as PCR [55]. PDMS is also very permeable to oxygen which makes its use in aerobic cell culture studies very appealing [59].

While PDMS is very easy to use, there are several problems associated with its use. First and foremost PDMS is a very viscous fluid prior to curing which makes it unsuited for scale up in manufacturing. PDMS has several physical properties that make
it detrimental for use with biological samples. PDMS tends to be hydrophobic which makes it harder to fill with water. Adding to this complexity is the fact that proteins and cells tend to adhere to the surfaces, which leads to biofouling which is undesired in some situations. Finally PDMS is permeable to hydrophobic small molecules [60]. While PDMS’s permeability in some cases is desirable, when the application requires the detection of small molecules at low concentrations this permeability becomes a detriment. While coatings have been applied to prevent leaching of target analytes into the PDMS, the above mentioned problems hamper the use of PDMS on a larger scale [61]. Nonetheless PDMS remains one of the most used materials of construction for microfluidic devices.

2.3.1.2 Polycarbonate (PC)

Polycarbonate (PC) molds have also been used in the fabrication of microfluidic devices. PC is capable of rapid fabrication seen in PDMS microfluidics. Liu et al. (2001) used PC to construct a microfluidic chip to perform capillary electrophoresis. PC molds were thermally bonded to PC wafers to finish device fabrication [62]. PC is a better choice in systems that will be exposed to higher temperatures since it has a lower thermal coefficient of expansion compared to PDMS [62, 63]. While PC has several advantages over PDMS it suffers from its increased rigidity (depending on application) [63]. Whereas PDMS microfluidics have benefited from the materials flexibility, allowing it to be used in valving applications, PC is too rigid for these applications. PC also has a lower O₂ permeability compared to PDMS (20x10⁻⁹ versus 60x10⁻⁹ cm*cm³/(cm²(cmHg))) which could make it harder to dead fill channels which is one of the benefits of PDMS [64]. Furthermore decreased O₂ permeability could affect the growth of aerobic cell cultures within microfluidic channels.
2.3.1.3 **Polymethylmethacrylate (PMMA)**

Polymethylmethacrylate (PMMA) is a material that has seen use in almost every facet of the world. PMMA has been used as shatter resistant glass (Plexiglass), used as a sacrificial layer in microfabrication applications [27], and has even been used to produce several different types of microfluidic devices [65, 66]. One major advantage of PMMA devices over PDMS is that PMMA is one of the least hydrophobic plastics allowing for easier filling compared to PDMS [67]. An interesting use of PMMA in microfluidics was shown by Le *et al.* (2010). The authors used a PDMS mold as the substrate for the construction of an enclosed PMMA microfluidic channel. A PDMS mold was placed onto a PMMA solution sitting on a glass substrate. The PMMA solvent absorbs onto the PDMS surface creating a 3D channel. When the PDMS is removed the PMMA remains on the glass substrate, leaving a hollow chamber in the middle. The chamber can then be filled via capillary action or pressure driven flow. The authors used this device in the monitoring of a culture of calf pulmonary artery endothelial cells. This application of PMMA was shown to be a good way to mimic the in-vivo vascular architecture that these cells would normally be exposed to.

2.3.1.4 **Polyethylene glycol (PEG)**

Polyethylene glycol (PEG) has a plethora of uses in our society. It has been shown to stabilize different biopharmaceutical products increasing their half-life in the body [68]. PEG has been used in hydrogel applications as a scaffold that other molecules can attach to [69]. Finally it is beginning to be used in microfluidic applications. One of the major benefits of using PEG for microfluidic channel construction is that it is hydrophilic helping to prevent a lot of proteins from sticking to it causing unwanted bio-fouling [70, 71]. Han *et al.* (2009) reported the construction of a microfluidic device
made out of PEG that could resist protein fouling up to concentrations of 1 mg/ml. Their constructed device showed advantages over a similar PDMS constructed device in that it could be used 10 times compared to 4 times for an oxygen-plasma-treated PDMS device [71].

2.4 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* (PA) is a gram negative bacterium that is found throughout nature [72]. PA does not harm healthy individuals, however in immune compromised patients (cystic fibrosis) and wound victims this ubiquitous bug has been found to cause all kinds of problems. In patients who have developed ventilator associated pneumonia mortalities as high as 76% have been found [1]. Due to its prevalence in nature many studies have focused on PA infections and detection strategies [1, 2]. While PA infections are widespread and well documented, little is known about what factors lead to its virulence and why under normal conditions the cell causes little to no harm to humans [1, 2]. It has been hypothesized that infections from PA are due to mutations within PA [73], chemotaxis [54, 74], neutrophil death [75-77], and flagella motility [78, 79].

2.4.1 *P. aeruginosa Infections*

Bragonzi et al. (2009) hypothesized that prolonged lung infections by PA led to the development of mutants that would lead to reduced virulence. The authors tested this hypodissertation in mouse lung models using mice with different genetic dispositions. Over a 16.3 years period PA clones were isolated from 6 mice with cystic fibrosis. The authors found that while the isolates collected later in the mouse's life were more likely to cause acute mortality they did not differ from early and intermediate isolates in their ability to create chronic infections.
Chemotaxis, or the movement of cells due to a chemical gradient, has been thought to cause PA to become virulent in certain hosts. This makes sense given that most cells respond to chemical gradients in their environments and finding out what chemicals lead to this motion could help identify treatments to prevent PA infections. Craven et al. (1981) studied three strains of PA, one that was highly virulent in the burned mouse model and two that were not virulent at all. Using soft agar migration the authors saw that the non-virulent strains were defective in motility and chemotaxis whereas the virulent strain was extremely motile and chemotactic. Jeong et al. (2010) utilized a microfluidic concentration generator to expose PA strains to chemo-attractants and chemo-repellants. Along those lines it is thought that the flagella motion of certain PA strains is linked to virulence.

One potential cause of PA infections could be from low neutrophil counts found in immune compromised patients [75]. Fergie et al. (1994) compared 96 children with cancer that had been treated for PA bacteremia (PA in the blood stream) over a 27 year period. The authors found that 76 cases developed among children that had a low absolute neutrophil count with mortality being higher among those children that had a lower count [76]. Allen et al. (2005) found that PA was able to cause neutrophil apoptosis due to the excretion of pyocyanin, a virulence factor, leading to an impairment of the host’s defenses. The authors found that neutrophil counts increased steadily for 48 hr in mice infected with pyocyanin deficient PA whereas neutrophil levels declined after 18 hr of exposure to wild type PA [77]. Both studies point to the importance of neutrophil death in order for PA to thrive in its host.
Another factor in PA infections is the ability of the bacterium to produce biofilm within the host. The biofilm is one of the most important defenses that PA has against clearance from the host. Cells deficient in biofilm production would have a harder time surviving in vivo versus those cells that can. Caiazza et al. (2007) studied the SadB protein produced by PA and determined that it was responsible for inversely regulating biofilm formation and swarming motility [78]. Being unable to produce biofilm is a large detriment to PA infections and many studies looking at biofilm deficient strains have been performed in the hopes of finding new antibiotics to combat this infection.

One final factor in PA infections is the excretion of molecules from PA that negatively impact the host environment. For instance, pyocyanin is excreted by all PA found in the wild. Allen et al. (2005) found that pyocyanin excreted by PA was capable of causing neutrophil death which in turn prevented the host from being able to evacuate the infection from its environment. Other virulence factors have been noted, so it is worth recognizing that the molecules being emitted from PA play an equal role in the virulence of PA cultures.

### 2.4.2 Quorum Sensing Molecules

In order for bacteria to make decisions that benefit the community as a whole they need a means of communicating with one another. “Talking” between bacteria is accomplished through the use of quorum sensing molecules. A quorum is a decision making body, so in order for the body of cells to make a decision they need a stimulus that tells them what is happening in their environment. In the case of bacteria encased in biofilms this could be a response to a certain chemical attractant, or an antibiotic that is dangerous to the community. QSMs lead to the up or down regulation of specific proteins whose production benefits that biofilm as a whole. Since it might not always be
beneficial for the cells to be producing proteins geared to specific situations, a threshold concentration of QSMs is often needed to create a response. This threshold concentration can be obtained when cell concentrations reach $10^7$-$10^8$ colony forming units/mL [80]. These relate to large cell concentrations needed to produce a specific response to stimuli. Quorum sensing has even been hypothesized as the means by which single cells come together to form complex tissues. Much research has gone into elucidating the quorum sensing pathways of PA in hopes of finding ways to combat this dangerous species [81, 82]. Quorum sensing molecules can also be detected between species and kingdoms, so understanding their production could lead to understanding what signals drive normally benign bacteria to virulence [83].

The major quorum sensing molecules found to be used by PA are acyl-homoserine lactones (AHSLs) and pseudomonas quinolone signal (PQS). These molecules have been shown to affect the las and phl operons of PA. Wagner et al. (2007) studied PA mutants that operated via las or phl QS systems. The author’s findings helped to elucidate the contribution of each of these systems to PA virulence [84].

While a large cell density has been thought to be the key contributor to bacteria responding to QSMs, the advent of microfluidics has led to the discovery that confined volumes can cause low concentrations of cells to respond to their QSMs in a quorum sensing manner [4, 85]. Carnes et al. (2010) showed that single cells confined in small volumes could undergo a quorum event. Using staphylococcus the authors were able to show that quorum sensing and genetic regulation could be obtained in small volumes. Furthermore the authors found that bacteria capable of detecting their own QSMs had a higher viability than bacteria that could not produce QSMs. Hagen et al. (2010) studied
the luxI/luxR QS circuit in Vibrio fischeri (VF) in confined spaces. The authors optically detected the production of QSMs from VF and showed that the fluorescence detected was caused by VF cells detecting their own QSMs.

QSMs have rightly garnered a large amount of focus due to their effect on the viability of bacterial communities to survive. Emphasis on the detection of these molecules has been made, and researchers have looked at new antibiotics that could be used to disrupt the QSM network that different bacteria employ. Understanding of QSM production in confined volumes could help lead to an understanding of how cells cause infection at certain locations in hosts.

2.4.3 Biological Phenazine Production

It was highlighted briefly in section 2.4.1 that PA is capable of excreting a vast array of molecules that can potentially cause harm to the host. Of great interest to researchers are the phenazine pigments that are produced by PA [86]. These phenazines tend to be redox active which allows PA to use them as reducing agents when needed, and as a means of defending PA from the host’s immune system. A number of phenazines have been found to be produced by PA cultures with the blue-green molecule pyocyanin being the prominent phenazine present in stationary cell cultures [86]. Phenazines are produced from the shikimic acid pathway as outlined in the following reaction mechanism.
Figure 1: Shikimic Acid Metabolic Pathway. From Pierson et al. (2010) [87]. Chorismic acid is the branching point for phenazine production, ending in the production of phenazine-1-carboxylic acid (PCA), the precursor of pyocyanin.

Since phenazines are thought to play such a crucial role in PA’s survival within the host researchers have focused on determining the biochemical pathways for phenazine production. This was done with the hopes that being able to manipulate
phenazine production could stop the negative side effects of phenazines or utilize the redox capability of phenazines for beneficial applications such as in fuel cells.

Mavrodi et al. (2001) studied many different mutants of PA that had different phenazine gene sequences removed to determine what effects they had on the production of pyocyanin specifically. The authors proposed the following reaction mechanism for pyocyanin production and also suggested that several other phenazines could be produced when PA was deficient in downstream proteins responsible for pyocyanin production.

![Figure 2: Proposed reaction mechanism for the production of pyocyanin proposed by Mavrodi et al. (2001). Only phenazine genes phzM and phzS are required to convert PCA to pyocyanin [86].](image)

Two studies in particular refined the above work by looking at the crystal structures of the proteins phzS and phzM [88, 89]. phzS is a flavin dependent hydroxylase that adds the hydroxyl group to pyocyanin. phzM is an adenosylmethionine dependent methyltransferase which adds the methyl group seen in pyocyanin. Prior research suggested that phzM added a methyl group to phenazine-1-carboxylic acid, before phzS adds the hydroxyl group to produce pyocyanin. The authors found that without phzS little to no pyocyanin was produced by the proteins, but upon the addition of phzS to phzM
solutions pyocyanin was readily produced. This lead to the hypodissertation that *phzM* and *phzS* work to form a complex that converts phenazine-1-carboxylic acid to pyocyanin [88]. This complex was thought to prevent a detrimental intermediate from being produced within PA cultures [89].

### 2.4.4 Pyocyanin

The following is an overview of pyocyanin produced from *P. aeruginosa*. Highlighted are reports on pyocyanin’s toxicity, its function as a quorum sensing molecule (QSM), its potential uses, and finally how it can be measured. Importantly this section emphasizes how pyocyanin detection, as pursued in this dissertation, can be utilized in a number of potentially beneficial ways.

#### 2.4.4.1 Toxicity

Pyocyanin is one of the most widely studied phenazines due to the fact that it is the most readily found molecule excreted only from PA [90].

![Figure 3: Genes required for the production of phenazines in different bacteria. Note that the Pseudomonad family is not the only one that makes phenazines [90]. However PA is the only bacteria that can produce pyocyanin.](image)

28
Of interest in Figure 3 is that *Pseudomonas aeruginosa* is the only bacterium that can naturally synthesize the production of pyocyanin due to the presence of the *phzS* and *phzM* genes [88, 89]. Concentrations of pyocyanin from the sputum of patients infected with PA have been found in the 1-100 µM range. Pyocyanin has been linked to several severe adverse effects that could explain how PA infects its hosts so readily. Usher et al. (2002) studied the effect of pyocyanin on neutrophil death. The authors found that at a concentration of 50 µM pyocyanin was capable of causing a 10 fold increase in neutrophil death after just five hours of exposure [91]. O’Malley et al. (2004) found that pyocyanin directly oxidizes glutathione in airway cells. Reduced glutathione is an important cellular antioxidant. By spiking cell cultures with pyocyanin it was found that glutathione levels in bronchial cells decreased while in alveolar epithelial cells it did not, indicating that bronchial cells would potentially be unable to cope with oxidative stress [92]. Finally Wilson et al. (1987) found that pyocyanin leads to a decrease in the ciliary beating of human respiratory cells [93]. This was thought to enable PA to remain within the host because the host cilia were no longer able to effectively move the mucoid layers out of the lungs, leading to PA proliferation.

### 2.4.4.2 Pyocyanin as a Quorum Sensing Molecule

*Pseudomonas aeruginosa* (PA) utilizes a number of biochemicals that act as signals for gene regulation. While the most common signals are acyl-homoserine lactones (AHSL) and the pseudomonas quinolone signal (PQS); the virulence factor pyocyanin has also been shown to have an effect on gene regulation [94]. Dietrich et al. (2006) utilized DNA microarrays and quantitative RT-PCR to show that pyocyanin upregulates genes responsible for transport and redox control, while it down regulates genes involved
in ferric iron acquisition. The authors found that phenazines were used as signaling molecules in the wild type strains PA14 and PAO1.

2.4.4.3 Potential uses

Due to pyocyanin’s ability to act as a redox agent for cultures of PA, much interest has been placed on growing PA in biological fuel cells and using pyocyanin to produce energy [95, 96]. Venkataraman et al. (2010) studied the effects of quorum sensing on pyocyanin production in microbial fuel cells. By deleting a gene responsible for the regulation of the GacS/GacA QS system a higher anodic current was observed with respect to the wild type when cells were grown under anaerobic conditions. The increase in current was attributed to the increase in phenazine production, highlighting the importance of phenazines, such as pyocyanin, in power generation potential within fuel cells.

2.4.4.4 Pyocyanin Detection

Pyocyanin can be detected by several different means. Two popular methods are based on electrochemical and optical detection methods [3, 93, 94]. Optical methods utilize measurements of UV absorbance at a set wavelength usually in a high performance liquid chromatography (HPLC) set up. In these systems pyocyanin must be removed from the broth solution using a chloroform extraction/evaporation process. The purified pyocyanin can then be flowed through a HPLC, where a UV detector measures the UV absorbance in the sample as it passes through the column. This results in a peak that the user can read off. Large concentrations of pyocyanin lead to higher levels of UV absorbance, which make it possible to create a concentration versus absorbance curve to monitor the production of pyocyanin from bacterial cultures.
While HPLC systems are robust the need to remove the analyte from the biologically relevant medium prior to testing slows analysis time, and makes its use unwieldy as a point of care diagnostics platform. Since pyocyanin is a redox active molecule researchers have shown that it is possible to detect pyocyanin electrochemically [3, 97]. Bukelman et al. (2009) showed that it was possible to electrochemically detect pyocyanin production via a large scale electrochemical cell that utilized an Ag/AgCl reference electrode. By using an electrochemical system the authors were able to detect the presence of pyocyanin and monitor its production versus time without having to perform a purification step. Sharp et al. (2010) expanded on this idea by fabricating a carbon fibre working electrode which achieved a limit of detection 0.03 µM pyocyanin and a linear range of 1 to 100 µM which covers the appropriate concentrations of pyocyanin that have been found in biologically relevant samples such as patient sputum [91]. The ability to perform sensitive, selective detection of an analyte of interest without purifying the test sample is of great importance as it decreases the amount of sample prep for diagnosis and would allow any user to operate the device with little training.

2.5 Single Cell Bacterial Studies

At the heart of any biofilm is a collection of individual cells that interact to ensure the survival of the community. While most biological studies focus on the effects that chemical stimuli have on the biofilm or bulk culture the net response tends to be averaged over the entire population [53]. With the realization that biofilms are heterogeneous entities, the desire to understand what is happening at the single cell level has gained interest [98]. In fact when single cells are confined in small volumes it has been seen that they can sense their own QSMs [80, 99, 100]. Due to the small size scale of single
bacterial cells (1-10 µm) microfluidics readily lend themselves to the transport, trapping, and monitoring of single cells.

2.5.1 Trapping

There are several well documented techniques for the transport and trapping of single cells within microfluidic channels. All of the techniques mentioned below require the transport of the cell to specific location within the microfluidic platform where the cell is then held during the analysis.

Possibly the simplest approach to trapping individual cells is to flow them into a target area and let them settle out on their own. Boedicker et al. (2009) successfully captured individual cells by creating 100 fL microwells into which cells would settle. A cell solution was flowed into the detection area and the cells were allowed to settle on the microwell array. After settling air was pumped through the system effectively removing all the liquid in the channel, except what was captured in the microwells. Due to the small size of the microwells the authors were able to capture 0-10 cells per droplet depending on the initial loading concentration.

Along the same lines of trapping single cells into microwells it has been shown that it is possible to trap single cells within microbubble emulsions in microfluidic channels. Microbubble emulsions take advantage of solutions with different hydrophobicities. By flowing a sheathing fluid of high hydrophobicity (oil) over another fluid with low hydrophobicity (water) it is possible to make microbubbles of various diameters within microfluidics [101]. Zeng et al. (2010) used this approach to trap single cells with DNA coated magnetic beads flowing through microfluidic channels [102]. The emulsions can contain reagents for PCR and lysing cells, thus opening this method up for biological analysis.
One approach to trapping single cells is the use of a microfluidic channel and valve layer. The microfluidic valve and channel construction is easy to fabricate using standard photolithography techniques [55]. In this approach a dilute cell suspension is flowed through the microfluidic channel to a trapping area. When it is visually seen that a cell has entered the trapping area the microfluidic valves are actuated with air, closing the microfluidic channel and effectively trapping the cell [103]. Once trapped the cell can be exposed to a range of reagents or monitored optically [104].

Single cells can also be trapped by constructing microscale pillars that allow fluid to pass through, but are too small to allow the cell through [99, 105]. Wlodkowic et al. (2009) utilized this principle and created a microfluidic array of microfabricated posts out of the photo resist SU-8. The posts were large enough for a single cell to fit into as they flowed by. When the cells landed between the pillars they became trapped due to the surrounding hydrodynamic forces. The authors were able to show that this system could effectively be used to not only trap cells, but also could be used to screen the effectiveness of different drugs [105]. Connell et al. (2010) constructed bacterial lobster traps to capture single Pseudomonas aeruginosa cells in a microfluidic environment. Using a "multiphotonlithography" approach the authors were able to construct 3-D structures out of bovine serum albumin (BSA). These traps were unique in that they were made completely from a biological component, which was permeable to both the nutrients that the cell required for growth as well as the wastes that the cell was making. The traps worked by loading a single cell at room temperature (18-22 °C). Once a single cell was captured the entire device was warmed to 37 °C at which point the BSA swells...
closing the access to the trap. The cell was then exposed to different growth conditions and its response was monitored optically.

Finally single cells can be captured using optical tweezers. Optical tweezers work by focusing a laser which exerts a small force on dielectric particles such as DNA, proteins, and cells. Once caught in the beam the tweezers can be used to selectively move the cell to a detection platform. Erickson et al. (2010) used this approach to capture cells flowing through a microfluidic device and position them within a detection area. With the trapped cell being held in place they were then able to expose the single cell to a variety of chemical stimulants and optically observe the results [106].

2.5.2 Monitoring

Once a single cell has been trapped how is meaningful information about what is occurring monitored? The different analysis that can be performed on single cells ranges in complexity from simplest to most complex [98, 107, 108]. Analysis can be accomplished by lysing the cells or monitoring whole cells to glean valuable information about molecule release [109], protein production [110, 111], and genetic analysis [112-114].

Once the cell has been trapped within a microfluidic channel the next step is often to lyse the cell and purify the analyte of interest for detection. Marcus et al. (2006) created a PDMS chip which allowed the isolation of sub pigtogram mRNA samples which was then used to synthesize cDNA. This procedure allowed the authors to monitor the gene expression of single cells with the possibility of parallelization [114]. Zhong et al. (2008) accomplished single cell analysis of mRNA from 20 individual cells that was then converted to cDNA. With this set-up a limit of detection 2 mRNA copies per cell
could theoretically be detected [112]. DNA analysis of single cell lysates has also been accomplished [115]. Prince et al. (2002) used osmotic pressure differences to rupture *Escherichia coli* (*E.coli*) cells releasing their DNA and dielectrophoretic trapping to capture the released DNA. Analysis of individual cell DNA has led to the discovery of unique relationships that would otherwise be missed in bulk DNA analysis [116]. As an example Kleparnik et al. (2003) found that the analysis of DNA from a single cardiac myocyte exposed to doxorubin lead to an increased cardiotoxicity.

While single cell monitoring via cellular lysis has many advantages, information can also be gleaned from cells without destroying them. One of the simplest nondestructive approaches to analyzing what is happening to single cells is to optically monitor the trapped cells as they are exposed to different environmental conditions. This approach requires the use of molecules that fluoresce on their own, or tagging the analyte of interest with a fluorescent molecule such as GFP [117]. Wheeler et al. (2003) constructed a PDMS microfluidic system that passively removed single cells from bulk cultures. Using microfluidic valves the authors were able to expose the cells to nanoliter volumes of reagents. Jurkat T-cells were incubated with Fluo3 at room temperature to measure calcium ions. This set up made possible the observation of cell viability, calcium ion flux, and receptor mediated calcium ion measurements [100].

Through advances in microscopy new super-resolution techniques have emerged which allow for the monitoring of processes occurring within cells. Resolution has improved so much that it is possible to detect single proteins within cells [118]. Biteen et al. (2008) combined super-resolution imaging along with enhanced yellow fluorescent protein (eYFP) to detect the bacteria actin protein MreB in single *Caulobacter crescentus*
cells. It was shown using these methods that the motion of single protein molecules could be activated within the cell [119]. Importantly all measurements were made within a living cell.

While optical measurements utilizing fluorescence are well-known and widely used. They suffer from their reliance on inserting a fluorophore or dye into the cell in order to measure a response from a single cell. Another approach to monitoring single cell phenomenon relies on the use of electrochemical detection. There are several different electrochemical techniques that can be used. The most popular are impedance based measurements [120], amperometry [121], voltammetry [122], and capillary electrophoresis [116]. These methods have one major advantage over their optical measurement counterparts in that they do not require the incorporation of a fluorescent tag in order to measure a response from single cells. James et al (2008) interrogated individual macrophages using electrochemical detection [123].
3.0 Dissertation Goals

- **Goal 1:** Detecting pyocyanin in medically relevant samples using disposable screen printed electrodes.
  - Spike pyocyanin in pooled human samples of blood, sputum, bronchial lavages, and urine covering the medically relevant concentrations present.
  - Detect culture of *P. aeruginosa* spiked into human biofluids.
  - Screen other pathogens in the biological window that pyocyanin can be detected to measure for interfering signals.

- **Goal 2:** Utilize miniature electrochemical sensors combined with microfluidic channels to monitor the production of pyocyanin in biofilms.
  - Connect sensors to a polypotentiostat and characterize the response when detecting pyocyanin in TSB.
  - Create microfluidic channels to lay over the fabricated NEAs. Grow biofilms over the NEAs and monitor the production of pyocyanin from healthy *P. aeruginosa* cultures.
  - Flow antibiotic solutions over the healthy biofilms to determine whether pyocyanin can be used as a means of quantitatively monitoring the health of a biofilm in real time.

- **Goal 3:** Utilize disposable electrodes embedded in growth agar to electrochemically monitor the growth of *P. aeruginosa*.
  - Fabricate disposable embedded electrodes in agar plates.
  - Culture *P. aeruginosa* at different bacterial loads.
Measure the electrochemical signal compared to visual inspection of the plates.

- **Goal 4**: Microfabricate a Nanofluidic Electrode Assembly that Contains a Miniaturized Reference Electrode and Working Electrode, to be used for Real Time Monitoring of Pyocyanin Production from PA Strains.
  
  - Test dilution series of Pyocyanin in buffered solutions to determine device sensitivity.
  
  - *P. aeruginosa* cultures will be grown in test tubes to determine whether the microfabricated devices can detect the presence of pyocyanin in the supernatants of bulk cultures.
  
  - Characterize the response of the palladium reference electrode.

- **Goal 5**: Use fabricated NEA to detect the production of pyocyanin from single PA cells (<5) trapped within detection area of exposed to different growth conditions.
  
  - Capture a single *P. aeruginosa* cell over the microfabricated NEA. See if any pyocyanin can be detected.
  
  - Optically monitor the growth of the cell and see if cell division corresponds to the production of more pyocyanin.
4.0 Experimental

4.1 Goal 1: Detecting Pyocyanin in Medically Relevant Samples using Disposable Screen Printed electrodes

The following describes the experimental techniques utilized to accomplish Goal 1 of this dissertation.

4.1.1 Materials

All solutions for this study were prepared using de-ionized (DI) water from a Milli-Q Direct 8 filtration system (Millipore Cat.# ZR0Q00800). Purified pyocyanin (N-methylphenazin-1-one) was purchased from Cayman Chemicals (Cat.# 10009594) and dissolved in DI water to make a 500 µM stock solution. This stock was refrigerated and used to make dilutions. Cell cultures were grown in both trypticase soy broth (TSB) (BD Biosciences Cat.# 211825) and lysogeny broth (LB) (BD Biosciences Cat.# 241410) at 37 °C. Prepared media were autoclaved to sterilize the solutions prior to use. Urine (Lot# BRH723768), bronchial lavages (Lot# BRH723769), sputum (Lot# BRH723767), and heparinized blood (Lot# BRH724097) collected from 20 healthy donors were purchased from Bioreclamation. Electrochemical measurements were made in a faraday cage using the carbon working and counter electrode on Zensor TE100 (EDAQ Cat.# ET077) disposable sensor strips and a 1 M KCl Ag/AgCl reference electrode (CH Instruments Cat.# CHI111). The fabricated reference electrode on the Zensor does not contain a salt bridge separating it from the test solution which could lead to an unstable reference potential. To limit this affect a commercial reference electrode (CH Instruments 1 M KCl Ag/AgCl) with a stable reference potential was used. All electrochemical measurements were controlled and recorded with a potentiostat (CH Instruments Cat.# CHI842C).
Stock cell cultures were prepared in 3 mL of TSB and LB growth media. Three 150 µL aliquots were removed after overnight growth at 37 °C and placed into 6 mL of growth media. Three cultures per species were prepared to obtain an average electrical response for each species. 300 µL of supernatant was removed each day from cell cultures for a period of three days. The supernatants were filtered through 0.2 micrometer nylon filters (Fisher Scientific Cat.# 14-555-329). 150 µL of filtered supernatant was placed onto a Zensor and scanned from -0.5 to 0 volts using SWV at a frequency of 15 Hz and an amplitude voltage of 50 mV to screen for the presence of electrochemical molecules. Three measurements per supernatant were obtained. Three consecutive SWV measurements were performed in less than 5 minutes. Strains of *P. aeruginosa* PAO1, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Bacillus cereus* were used in cell culture experiments.

4.1.3 Electrochemical Testing of Human Samples

Human samples of urine, sputum, bronchial lavage, and whole blood with heparin were purchased in 1 mL aliquots from Bioreclamation. With the exception of the whole blood samples, all biological fluids were stored at -20 °C. Samples were thawed in a water bath prior to experiments. Whole blood samples were stored at 4 °C refrigerator to avoid cellular lysis. A dilution series from 1 to 100 µM was prepared by adding the appropriate volume of stock pyocyanin solution (500 µM) to 500 µL of each human biofluid sample.

*P. aeruginosa* PA14 were grown in 3 mL of TSB media for 24 hours at 37 °C until they reached stationary growth phase (~5 x 10⁷ cells/mL). The cells were spun down in a centrifuge and resuspended in 3 mL of fresh TSB media just before being spiked into biological fluids. 500 µL of each biological fluid was spiked with 150 µL of the *P.*
*aeruginosa* PA14 media and cultured at 37 °C. 150 µL of each spiked biological fluid was removed daily and placed onto a Zensor. The samples were scanned from -0.5 to 0 volts using SWV at a frequency of 15 Hz and an amplitude voltage of 50 mV. Each sample was measured three times with three different sensors for a total of nine measurements. See Appendix C for a graphic and description of general three electrode set up used in this dissertation. See Appendix D for the sensors response to pyocyanin detection when SWV parameters of frequency, amplitude, and incremental voltage were changed.

4.2 Goal 2: Utilize Miniature Electrochemical Sensors Combined with Microfluidic Channels to Monitor the Production of Pyocyanin in Biofilms.

The following describes the experimental techniques utilized to accomplish Goal 2 of this dissertation.

4.2.1 Microfluidic Fabrication From Tape Molds

When a fast microfluidic scaffold is required a simplified approach to making a master mold, for PDMS devices fabrication, is to simply use scotch tape. Scotch tape has found several use niches in microfabrication such as in the production of sheets of graphene from pencil

![Figure 4: A) Glass slide with 9 x 9 x 0.05 mm³ scotch tape masters for PDMS microfluidic channel fabrication. B) Bonded devices for antibiotic susceptibility testing. Devices bonded using 18 sccm oxygen plasma at approximately 100 W for 7 seconds.](image)
Lead [124]. Also due to its small thickness (≈50 μm) it is ideal for the creation of microfluidic master molds for PDMS. This is accomplished by first attaching the tape to a solid substrate such as a microscope slide.

Once attached tape patterns can be made by carefully cutting and removing the desired pattern from the bulk tape (Figure 4A). Once finished the master can be used to produce PDMS microfluidics with a well-defined z dimension. Inlet and outlet holes are drilled through the PDMS to the channel layer. Finished devices are cleaned with acetone and then permanently bonded to disposable electrodes using oxygen plasma (Figure 4B).

4.2.2 Electrochemical Detection of *P. aeruginosa* Antibiotic Susceptibility

Disposable miniature electrochemical sensors were used to accomplish Goal 1. The miniature sensors used for this goal were complete three electrode cells containing carbon working and counter electrodes and an Ag/AgCl paste reference electrode (Zensors Cat.# TE100s). These were chosen for their cost (≈$2/electrochemical cell). The sensors were coupled with a microfluidic chamber (≈4.05 mm$^3$) to perform all experiments in this goal.

The disposable setup was connected to a potentiostat (CH Instruments Cat.# 1040C) and controlled via CHI software. For squarewave voltammetry measurements the working electrode was scanned from -0.5 to 0.2 volts at a frequency and amplitude voltage of 15 Hz and 0.05V respectively. For all cell experiments performed the working electrode was scanned every thirty minutes after bacterial cells were loaded into the chambers.

4.2.3 Materials Used

All solutions were prepared as stock solutions unless otherwise specified. They were purchased and used as received without further purification.
*P. aeruginosa* wild-type strain PA14 was received from the Dr. Pier at Brigham and Women’s Hospital in Boston, Massachusetts. Bacterial cell stocks were prepared in 10% glycerol (Sigma Cat.# G5516-500ML) trypsinase soy broth (TSB, BD Diagnostics Cat.# 211768) solutions and stored at -80 °C in Dr. Asthagiri's lab at Northeastern University. For on demand use PA14 was cultured on a trypsinase soy agar (TSA, BD Diagnostics Cat.# 281230) plate at 37 °C incubator overnight and then stored in a refrigerator at 4 °C. Samples were removed from this stock plate and grown in 3 mL of TSB in a 37 °C incubator overnight.

*Escherichia coli* from Dr. Epstein's lab at Northeastern University were grown under the same conditions as PA14. *E. coli* is known to not produce any electrochemical molecules in the same range as *P. aeruginosa* and as such is an effective control to show that this approach works at monitoring antibiotic susceptibility of *P. aeruginosa*.

Pyocyanin (Cayman Chemicals Cat.# 10009594) was dissolved in dimethylsulfoxide (DMSO, Fisher Scientific Cat.# D128-1) to a final concentration of 5 mg/mL. This sample was used as a stock and stored in a -20 °C freezer. Serial dilutions of pyocyanin were prepared in TSB ranging from 1 to 100 μM to cover the biomedically relevant ranges. These dilutions were scanned using predetermined parameters to determine the response of pyocyanin in TSB with the electrochemical cell used.

Colistin sulphate (Adipogen Cat.# AG-CN2-0065-G001) was chosen as the antibiotic of choice for susceptibility testing with PA14 and *E. coli*. Colistin sulphate is known to negatively impact *P. aeruginosa* while have little to no effect on *E. coli* cells. As a control ampicillin and (Fisher Scientific Cat.# BP1760) was used against PA14.

### 4.2.4 Filling, Loading and Monitoring Bacterial Cells in Microfluidic Chambers
Chambers were filled with TSB via a syringe pump (Chemyx Fusion 200 Cat.# 211097). TSB was pumped at a volumetric flow rate of 10 μL/min through 0.03 inch (inner diameter) medical grade polyvinyl chloride (PVC) tubing (Amazon Supply Cat.# B001GMWZMM) through 19 gauge luer lock syringe tips (Amazon Supply Cat.# NE192PL-25). 0.2 micron regenerated cellulose luer lock membranes (Minisart RC4 Cat.# 17821K) were connected to the inlet and outlet of the disposable chamber. This was done to prevent bacteria from leaving the chamber. Luer lock fittings for microfluidic connections were purchased from Value Plastic (Cat.# MTLL230). Chambers were filled by pulling vacuum on the outlet while pumping TSB at 10 μL/min. By pulling and releasing vacuum the channels were observed to fill (confirmed by optical microscopy).

![Figure 5: Assembled disposable electrochemical cell coupled with microfluidic chamber for *P. aeruginosa* monitoring. 0.2 micron regenerated cellulose filters prevented *P. aeruginosa* from leaving the chamber.](image)

Bacterial cells were loaded into the fabricated disposable devices at a flow rate of 10 μL/min. This was accomplished by removing the filter from the feed line and connecting freshly reconstituted bacteria to the inlet of the PDMS chamber. After cells
were loaded, the TSB feed line was replaced and electrochemical measurements were taken approximately every 30 minutes.

Samples were scanned from -0.5 to 0.2 volts versus the internal Ag/AgCl reference electrode on the disposable electrochemical cell (Zensor). Square wave voltammetry (SWV) was used at an amplitude voltage of 50 mV and a frequency of 15 Hz. SWV was chosen due to its increased sensitivity and its ability to monitor the electrochemical peak of PYO compared to other voltammetric and amperometric techniques [31, 36]. After loading the PDMS chambers with TSB, the sample was scanned 10 times and the average taken to get the mean response of the TSB. All subsequent measurements were then compared to this response. Three measurements were taken during the loading of the cells, with additional measurements taken every 30 minutes during the remainder of the tests. For each concentration of antibiotic tested, three different microfluidic setups were used. Electrochemical measurements were processed by subtracting the baseline signal. One way analysis of variation (ANOVA) was used to determine the statistical significance of resulting measurements. See Appendix C for a graphic and description of general three electrode set up used in this dissertation. See Appendix D for the sensors response to pyocyanin detection when SWV parameters of frequency, amplitude, and incremental voltage were changed.

4.2.5 Scanning Electron Microscopy (SEM) Sample Preparation

Samples were prepared for SEM imaging by fixing in a 2.5% glutaraldehyde (EMSDIASUM Cat.# 16120) in a 0.1M sodium cacodylate buffer (EMSDIASUM Cat.# 11654). After fixing, samples were washed in cacodylate buffer, and then dehydrated in increasing concentrations of ethanol (Fisher Cat.# BP2818-4 30-100%). After
dehydration, ethanol was removed via critical point drying (Samdri-PVT-3D) using liquid CO₂. The final step in SEM preparation was plasma sputtering (Cressington Sputter Coater 208HR) 5 nm of palladium metal onto the samples making them conductive. Once prepared for imaging, samples were loaded into a Field Emission SEM (Hitachi S-4800) and probed at an acceleration voltage and emission current of 3 kV and 10 mA, respectively. A detailed operating procedure for the SEM can be found in Appendix A.

4.3 Goal 3: Utilize Disposable Electrodes Embedded in Growth Agar to Electrochemically Monitor the Growth of P. aeruginosa

The following describes the experimental techniques utilized to accomplish Goal 3 of this dissertation.

4.3.1 Bacterial Culture

Bacterial cultures of P. aeruginosa strain PA14 were grown overnight in 3 mL of trypticase soy broth (TSB, BD Diagnostics Cat.# B11768), centrifuged at 10,000 rpm for 3 minutes, then reconstituted in fresh TSB. Cell counts of the stock solution were performed using an INCYTO C-chip disposable hemocytometer (Cat.# DHC-N01). Stock solutions were diluted to desired concentrations of cells. 5 µL drops were pipetted onto the prepared plates directly above the working electrode to produce bacterial loads of approximately 10²-10⁸ P. aeruginosa cells and incubated at 23, 37, or 42 °C.

4.3.2 Device Fabrication

Disposable electrodes were embedded into culture plates by creating a small slit on the side of the plate approximately 2 mm from the bottom. Disposable carbon electrodes with miniaturized silver chloride pellet references (Zensors Cat.# TE100s) were then inserted into the slits. The slits were sealed with silicone glue and allowed to
cure overnight. Sterilized King’s A agar (BD Diagnostics Cat.# DF0449-17-0) was poured onto the plates covering the electrodes with approximately 3-5 mm of broth. Once the agar solidified, plates were kept at 4 °C until use. Polydimethylsiloxane (PDMS Dow Corning Cat.# 50-366-794) wells were used as molds for agar pouring in some tests performed. Finished devices can be seen in Figure 6.

4.3.3 Optical Monitoring
Bacterial growth was optically monitored by taking images using a Canon PowerShot (12.1 megapixels). Images were generally taken every hour to confirm the presence of bacterial growth. These were then compared to the electrochemical measurements. Images of plates were cropped at the end of these tests.

4.3.4 Electrochemical Monitoring
Samples were removed from incubation and connected to a multipotentiostat (CHI 1040C) to perform voltammetric measurements. Square wave voltammetric (SWV) scans at a frequency of 15 Hz and amplitude voltage of 50 mV in the potential window from -0.5 to 0 volts were taken. This voltage window was chosen because it covers the redox potentials for many biological molecules. Voltammetric scans were analyzed using OriginPro 8.5 to create baselines and determine the maximum current produced by redox active molecules present on the plates. For bacterial studies, samples were run in triplicate. Pyocyanin (Cayman Chemicals Cat.# 10009594) was dissolved in dimethyl sulfoxide (DMSO Cat.# D128-1) to a concentration of approximately 5 mg/mL and used as stock. When not in use, it was stored at -20 °C and allowed to warm to room temperature before use. COMSOL 4.4 was used to model the three dimensional diffusion of dilute pyocyanin concentrations through agar using the transport of dilute species module. See Appendix C for a graphic and description of general three electrode set up used in this dissertation. See Appendix D for the sensors response to pyocyanin detection when SWV parameters of frequency, amplitude, and incremental voltage were changed.

4.3.5 Scanning Electron Microscopy Preparation

After testing samples were prepared for scanning electron microscopy (SEM) imaging. First the agar surrounding the disposable electrode was cut free. Both the agar and the electrode were fixed in a 2.5% glutaraldehyde (EMSDIASUM Cat.# 16120) solution containing 0.1 M cacodylate buffer (EMSDIASUM Cat.# 11654). After dehydrating in ethanol (Fisher Cat.# BP2818-4 30-100%), the samples were further dried with hexamethyldisilizane (EMSDIASUM Cat.# 16700), sputtered (Cressington Sputter Coater 208HR) with platinum or palladium metal (to provide a conductive surface) and
then visually inspected using a field emission SEM (Hitachi S-4800). A detailed operating procedure for the SEM can be found in Appendix A.

Figure 7: Hitachi S-4800 SEM used for imaging bacterial cells and biofilms formed during these goal.

4.4 Goal 4: Microfabricate a Nanofluidic Electrode Assembly that Contains a Miniaturized Reference Electrode and Working Electrode, to be used for Real Time Monitoring of Pyocyanin Production from *P. aeruginosa* Strains.

The following describes the experimental techniques utilized to accomplish Goal 4 of this dissertation.

4.4.1 Materials Used

All chemicals were used as received without further purification. All solutions used in this section were prepared from ultra-purified water from a Milli-Q system
Phosphate buffer was used as a supporting electrolyte and was prepared as a 1M stock using sodium phosphate dibasic heptahydrate (Fisher Scientific Cat.# BP331-1) and sodium phosphate monobasic anhydrous (Fisher Scientific Cat.# BP329-1) at a pH of approximately 6.8. Pyocyanin (Cayman Chemicals Cat.# 10009594) was purchased as a solid and dissolved in Milli-Q water to a concentration of approximately 150 μM and used as a stock for all dilutions. Ferrocene dimethanol (Sigma-Aldrich Cat.# 372625) was used as a standard to ensure that all devices were working properly at concentrations ranging from 1-1000 μM in 1 M potassium chloride (Fisher Cat.# P330-500). 37% Hydrochloric acid (Fisher Cat.# AC42379-5000) was diluted to 2 N HCl for palladium hydride reference electrode charging and testing.

*P. aeruginosa* wild-type strain PA14, pelA, *phzM*, *phzS* was received from the Dr. Pier at Brigham and Women’s Hospital in Boston, Massachusetts. Bacterial cell stocks were prepared in 10% glycerol (Sigma Cat.# G5516-500ML) trypticase soy broth (TSB, BD Diagnostics Cat.# B11768) solutions and stored at -80 °C in Dr. Asthagiri's lab at Northeastern University. For on demand use PA14 was cultured on a trypticase soy agar (TSA, BD Diagnostics Cat.# 281230) plate at 37 °C incubator overnight and then stored in a refrigerator at 4 °C. Samples were removed from this stock plate and grown in 3 mL of TSB in a 37 °C incubator overnight.

3 inch single-side polished (SSP) silicon wafers (University Wafers Cat.# 557) were used to fabricate devices for this goal. Gold, palladium and platinum were purchased from Kamis. All other metals were supplied by the Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University. Chromium
etchant (Transene Cat.# 1020AC ) was used to remove the sacrificial chrome layer in fabricated devices.

4.4.2 Nanofluidic Electrode Assembly Fabrication

Nanofluidic electrodes were fabricated using well known microfabrication techniques. These steps involved thermal oxidation photolithography, metal deposition via electron beam deposition and plasma sputtering, chemical vapor deposition (CVD), reactive ion etching (RIE), and sacrificial etching. The processes are described below.

4.4.2.1 Thermal Oxidation

SSP silicon wafers were insulated via wet thermal oxidation in a Bruce Furnace 7355B. Since silicon by itself is a semi-conductor placing a layer of silicon dioxide helps to lower the electrical noise of the microfabricated electrodes. Wafers were cleaned prior to oxidation using a three step acid regimen consisting of piranha, hydrofluoric acid, and sulfuric acid. After each acid step the wafers were washed in de-ionized water and then dried. Dried wafers were then loaded into the furnace for oxidation. The detailed cleaning procedure can be found in Appendix A for this dissertation.
Conversion of silicon to silicon dioxide was achieved via wet oxidation at approximately 1000 °C. The entire process from the start of loading to unloading the wafers took approximately 40 minutes, with oxide formation taking on average 21 minutes.

### 4.4.2.2 Photolithography

All metal layer patterning in this dissertation was accomplished via photolithography. Photolithography steps can be broken down into three sections: resist spinning and baking, UV exposure, and developing. All photolithography steps were performed in the photo bay in the Kostas Center at Northeastern University.

For all metal patterning steps, the negative photoresist AZ2020 was used. Photoresist was spun using a laurell spinner. This resist was spun at 3000 rpm for one
minute to a thickness of approximately 2 μm. The wafer was then baked for 2 minutes at 115 °C on a hot plate. After baking the wafer was aligned with a mask containing the desired pattern on a quintel aligner and then exposed to UV light for 7 seconds. When negative resist is hit with UV light the exposed

![Figure 9](image)

**Figure 9:** A) Laurell Spinner for applying photoresist B) Quintel aligner used for aligning wafers. C) Optical micrograph of developed wafer prior to metal deposition.

photoresist polymerizes and becomes resistant to the applied developer. To increase the degree of polymerization the wafer was baked for 2 min at 115 °C after UV exposure. 100% MIF 300 developer was used to remove unexposed photoresist on the wafer. The developed wafer was then rinsed with de-ionized water and then blown dry with filtered
nitrogen gas. Developing times varied, but usually were under one minute to achieve good development (Figure 7).

Wafers were inspected after development to ensure that the desired pattern was achieved. Developed wafers could then be stored safely under ambient light until metal patterning. An in depth photolithography standard operating procedure and spin curves can be found in Appendix A and B respectively.

4.4.2.3 Metal Deposition

Figure 10: Top Left: Electron beam evaporator. Top Right: Materials Research Corporation plasma sputtering system. Bottom Left: Optical Micrograph
Metal deposition for this part of the dissertation was accomplished by one of two possible approaches: electron beam deposition (E-beam) or Plasma Sputtering. In E-beam deposition, a beam of electrons is used to heat the metal of choice until it evaporates. A stable beam of electrons is produced under low vacuum (<10^{-6} Torr). Heated metal then evaporates out of the crucible into the chamber and onto the substrate. E-beam deposition yields tightly packed metal layers, but usually only obtains low deposition rates (<0.2 nm/s). As such, it was used for metal deposition for layers under 150 nm.

In plasma sputtering, inert argon gas is converted into a stream of ions. This plasma then interacts with a metal target. By hitting the target with the charged plasma, metal atoms come off of the target and deposit onto the substrate. Plasma sputtering allows faster deposition times and was used for depositing the contact pads (≥150 nm).

After metal deposition, the remaining photoresist was removed using 100% acetone. The acetone attacks the photoresist which is lifted off of the wafer. Only the metal that has adhered to the surface of the wafer is protected from the acetone. Once the photoresist has been removed, the remaining metal on the wafer will be in the form of the pattern from the lithography step (Figure 8). The wafer is then ready to go through another round of photolithography/metal deposition until the desired number of metal layers has been patterned onto the wafer. In this goal, a total of 4 metal deposition steps were performed. An in-depth photolithography standard operating procedure can be found in Appendix A.

4.4.2.4 Chemical Vapor Deposition

Once all metal deposition steps were finished, the wafer was insulated with silicon dioxide at thickness of approximately 500-550 nm. Thermal wet oxidation of silicon
dioxide could not be used due to the high temperatures required for this method. At these temperatures the metals could diffuse within one another, potentially alloying, making further microfabrication impossible. Additionally this method would require first depositing silicon onto the wafer and which could then be converted in silicon dioxide.

Figure 11: A) PECVD system at Harvards Center for Nanoscale Research. B) Wafers, one before and after PECVD treatment (Right and Left respectively).

To avoid metal alloying a lower temperature option was needed. The chosen method was plasma enhanced chemical vapor deposition (PECVD). PECVD works similarly to the metal sputtering. Under vacuum, argon plasma interacts with oxygen and silane gases causing them to react with one another in the following reaction:

$$\text{SiH}_4 (g) + \text{O}_2 (g) \rightarrow \text{SiO}_2 (s) + 2\text{H}_2(g)$$  \hspace{1cm} (6)

The resulting reaction leads to the formation of silicon dioxide which is deposited onto the wafer (Figure 9). PECVD was performed at Harvards Center for Nanoscale Research. An in depth photolithography standard operating procedure can be found in Appendix A.

4.4.2.5 Access Hole Formation

To reach the sacrificial chrome layers used to make the nanofluidic channels for these electrode systems, access holes had to be created. Access holes were patterned using photolithography. Once patterned the access holes were created by reactive ion etching or a wet etch using hydrofluoric acid (HF).
Reactive ion etching (RIE) is a dry process that uses plasma to create reactive gas species that selectively attack the material to be etched without attacking the patterned photoresist. RIE is a well-developed process that is capable of anisotropic etching in a variety of materials. RIE etching of silicon dioxide was achieved using a mix of argon (for the plasma) and sulfur hexafluoride (SF$_6$). The argon plasma reacts with SF$_6$ to produce fluorine radicals that then collide with the wafer surface. Fluorine radicals contacting silicon dioxide react with the silicon creating silicon tetrafluoride that desorbs from the surface of the wafer, creating an access hole. Controlling the exposure time of the wafer to the SF$_6$ argon plasma controls the etch rate and allowed the exposure of the sacrificial chrome layer for etching. The RIE set up used can be seen in Figure 12.

![Figure 12: Left: Unaxis ICP Etch (PlasmaTherm 790) Right: Wet etch set up for selectively removing SiO$_2$ using HF. Samples were removed directly from HF and rinsed in DI H$_2$O.](image)

Wet etching has several advantages of RIE that make it an attractive alternative. These are ease of use and relatively quick etch times (sometimes <10 seconds). To selectively etch SiO$_2$ a 10:1 mixture of DI water and 49% HF was used. This mixture was
found to achieve high etch rates (≈10 seconds) for single chips with little to no loss of the protecting photoresist. HF removes SiO$_2$ via the following reaction:

$$\text{SiO}_2(\text{s}) + 6 \text{HF(aq)} \rightarrow \text{H}_2\text{SiF}_6(\text{aq}) + 2\text{H}_2\text{O(l)}$$  \hspace{1cm} (7)

The wet etch apparatus can be seen in Figure 12. A more in detail standard operating procedure for both RIE and wet etching using HF can be found in Appendix A.

4.4.2.6 Wafer Dicing

After etching the access holes the wafer was ready to be cut into individual devices. This was accomplished using a Micro Automation 1006 dicing saw. 90 ° cuts were made to create separately addressable electrodes for testing (Figure 13). An in depth standard operating procedure for the dicing saw can be found in Appendix A.

Figure 13: Left: Micro Automation 1006 Dicing Saw. Right: Wafer diced into individual devices.
4.4.2.7 Wire Bonding

Devices were connected to the external potentiostat via Ethernet cables using wirebonding at Harvard's center for nanoscale systems (CNS). Wire bonds were accomplished with aluminum wire that was bonded using sonication at 15 ms with a power of 240 watts. Wire bonding allowed reliable connections between devices and Ethernet cables (Figure 14). Prior to wirebonding the devices were glued to glass slides to be raised to the same level as the Ethernet cables. This allowed for easier wirebonding between the cables and the devices. Wire bonded devices could then be used for testing.

4.4.2.8 Sacrificial Etching

Formation of nanofluidic channels was accomplished using chromium etchant from Transene to selectively remove the chrome layer deposited between the top and bottom working electrodes which can be measured electrochemically (Figure 15). The chromium etchant used contains ceric ammonium nitrate and removes chrome via the following reaction:

$$3 \text{Ce(NH}_4\text{)}_2\text{(NO}_3\text{)}_6 + \text{Cr} \rightarrow 3 \text{Cr(NO}_3\text{)}_3 + 3 \text{Ce(NH}_4\text{)}_2\text{(NO}_3\text{)}_5$$  \hspace{1cm} (8)

Removal of chrome leaves behind a spacing between the working electrodes that is approximately 60 nm tall (Figure 16).
Figure 15: Representative current vs. time traces for the sacrificial etching of chrome from the nanofluidic channels. Pure Transene 1020AC chrome etchant was used to remove the chrome. Electrodes were set to a voltage difference of 5 mV.

Figure 16: Left: Electrode assembly with unetched sacrificial chrome layer. Center: Electrode assembly after chrome etchant exposure for 90 minutes at room temperature. Right: Electrode assembly after chrome etchant exposure for 165 minute at room temperature.

4.4.2.9 Electrochemical Measurements

Devices were reversibly bonded to the bottom of a PDMS well, which contained a 500 µm diameter hole in the bottom, forming a water-tight seal. The
sample volume of the wells was approximately 200 µL. An Ag/AgCl RE was placed in the solution through the top of the well during the PdH RE characterization experiments.

All electrochemical measurements were performed using the fabricated devices mentioned above. To control the potentials applied to the NEA a bipotentiostat (CH Instruments Cat.# CHI842C) was utilized. For palladium reference electrode characterization an Ag/AgCl electrode (1 M KCl) (CH Instruments Cat.# CHI111P) was used. To complete the electrochemical cell a platinum (Pt) wire was employed. To ensure that devices were working as expected, cyclic voltammograms of 1-1000 µM ferrocene dimethanol vs. Ag/AgCl RE were obtained. All devices were cleaned and stored in deionized water in between electrochemical measurements.

Square wave and differential pulse voltammetry are more sensitive measurement techniques than cyclic voltammetry and were used for all pyocyanin measurements in this paper [125]. For the purposes of this study a counter electrode was not included in the microfabricated NEA, but the construction of microfabricated Au and Pt electrodes has been well documented and could be added with one extra photolithography step [23, 31, 126]. See Appendix C for graphic and description of general three electrode set up used in this dissertation.

4.5 **Goal 5: Use Fabricated NEA to Detect the Production of Pyocyanin from Single*\textit{P. aeruginosa} Cells Trapped Within Detection Area.**

Nanofluidic electrodes for this goal were prepared using similar methods to **Goal 4** and as such the reader should refer to that section for the general fabrication approaches used in that method. The fabrication of nanofluidic electrodes used in this section differs
only in the number of photolithography/metal deposition fabrication steps and that devices were made from double side polished (DSP) glass wafers. Additionally devices were not wire bonded to an external connection. Instead they were designed to have contact pads that matched with a female USB adapter. This design allowed for a shorter fabrication time since wire bonding was no longer required and allowed for the optical viewing of the bacteria in the detection area.

4.5.1 Materials
All chemicals were used as received without further purification. All solutions used in this section were prepared from ultra-purified water from a Milli-Q system (Millipore, resistance >18 Mohms). Ferrocene dimethanol (Sigma-Aldrich Cat.# 372625) was used as a standard to ensure that all devices were working properly at a concentration of 400 µM in 1 M potassium chloride (Fisher Cat.# P330-500).

*P. aeruginosa* wild-type strain PA14 was received from the Dr. Pier at Brigham and Women's Hospital in Boston, Massachusetts. Bacterial cell stocks were prepared in 10% glycerol (Sigma Cat.# G5516-500ML) trypticase soy broth (TSB, BD Diagnostics Cat.# B11768) solutions and stored at -80 °C in Dr. Asthagiri's lab at Northeastern University. For on demand use PA14 was cultured on a trypticase soy agar (TSA, BD Diagnostics Cat.# 281230) plate at 37 °C incubator overnight and then stored in a refrigerator at 4 °C. Samples were removed from this stock plate and grown in 3 mL of TSB in a 37 °C incubator overnight.

4.5.2 Device Fabrication for Trapping *P. aeruginosa* Cells
To separate individual *P. aeruginosa* cells, a variety of approaches were employed using different approaches in PDMS. PDMS devices were fabricated from master molds prepared using photolithography approaches. This allowed for a variety of
different sized patterns to be developed. Two approaches required the production of microfluidic valves, either always open or always closed, while the third approach utilized constrictions to single out bacteria. Figure 16 highlights the three fabrication approaches used to attempt to separate small populations of cells to monitor pyocyanin production.

Valves were made in a three-step process requiring two separate masks. The first mask contained the channel layer where fluid would eventually be flowed. The second mask contained the valve layer that was used to control flow through the channel layer. The channel layer was always manipulated via the valve layer to control fluid flow. In order to accomplish a 20:1 PDMS mixture was spun using a Laurell Spinner onto the channel mold. This created a thin PDMS layer. The valve layer was typically made from a 5:1 PDMS mixture that was irreversibly bonded onto the channel layer via oxygen plasma after access holes had been punched into it. Access holes to the channel layer were then created and the devices could then be bonded to microfabricated electrode devices.

Always open valves required an external pressure source be applied to the valve layer. The applied pressure causes the channel layer to deform, which would close the channel at high enough pressures. Always closed valves work on the opposite principal. In this system the channel layer dead ends at the valve layer. When a vacuum is applied to the valve layer the PDMS dead ends were lifted from the device surface allowing fluid to fill the channels.
The final approach used was a size based separation techniques. By constructing parallel PDMS channels connected by constrictions slightly smaller than *P. aeruginosa* it was possible to separate small amounts of cells from bulk cultures. This method benefits from passive separation, simply add cells to the main channel, and as they swim and divide they eventually enter the constriction, by tuning the size of the constriction only a single cell can enter and propagate into the next PDMS channel. Constrictions were made via e-beam lithography and chrome was deposited to the desired channel constriction thickness (normally less between 0.75 to 1 μm).

### 4.5.3 Electrochemical Measurements and Optical Monitoring

Electrochemical measurements were made by connecting the microfabricated devices to a female USB junction connected to the potentiostat (CH Instruments CHI 842C). Squarewave voltammetry measurements were taken from -0.5 to 0.2 volts at a frequency, amplitude voltage and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively. Electrochemical measurements were taken approximately every 30 minutes when possible. Samples were imaged using a Zeiss Axio Imager A2 fluorescence...
microscope at 40X after every electrochemical measurement. This was done to determine whether measured signals were from changes in the number of bacterial cells within a growth chamber. Images and their corresponding electrochemical outputs can be found in section 5.5 **Goal 5: Use Fabricated NEA to Detect the Production of Pyocyanin from Single PA Cells Trapped Within Detection Area.**

### 5.0 Results and Discussion

#### 5.1 Goal 1: Detecting Pyocyanin in Medically Relevant Samples using Disposable Screen Printed electrodes

The results presented in this section are from “Electrochemical detection of *P. aeruginosa* in human fluid samples via pyocyanin” in Biosensors and Bioelectronics volume 60, 2014. The results are presented with permission from Biosensors and Bioelectronics.

#### 5.1.1 Detection of Spiked Pyocyanin in Human Fluids

Pooled biological fluid samples from 20 healthy patients were spiked with pyocyanin from 1 to 100 µM to determine the sensitivity and limit of detection (LOD) at an unmodified disposable carbon electrode. SWV scans were performed three times per sample per electrode with a total of three electrodes used. A new sensor was used for each concentration and sample to avoid cross-contamination. Pyocyanin at micromolar concentrations has been found previously in the selected biofluids when obtained from patients infected with *P. aeruginosa* [3, 127, 128]. However, pyocyanin concentrations in healthy individuals and patients at various stages on infection have not been measured and, therefore, the threshold concentration for diagnosis is not yet available for any human fluid sample.
Figure 18: Baseline subtracted SWV scans of Top Left: bronchial lavage, Top Right: sputum, Bottom Left: urine, and Bottom Right: blood with sodium heparin. Biomedical samples were spiked with pyocyanin to a concentration of 1 to 100 μM. Inset is the pyocyanin reaction with the working electrode releasing two electrons. SWV scans performed from -0.5 to 0 V at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.

Raw data from SWV scans was analyzed using Origin Pro 8.5. Baselines were created for each data set using spline interpolation with 8 base points. Created baselines were subtracted from the raw data with Origin Pro 8.5. The resulting current, after subtracting the baseline, was then plotted versus potential for each concentration of pyocyanin and biofluid. Shown in Figure 18 contains the baseline subtracted currents for
human bronchial lavage samples, sputum, urine, and blood spiked with pyocyanin ranging from 0-100 μM.

Figure 19: Unprocessed data of SWV of 5 μM pyocyanin in a 150 μL sample A) bronchial lavage (BR), B) sputum (S), C) urine (U), and D) whole blood with sodium heparin (BH). Scans were performed from -0.5 to 0 volts vs. 1 M KCl Ag/AgCl reference at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.

A dynamic range that covers the pertinent concentrations found in clinical samples is necessary for this approach to be useful. Pyocyanin at concentrations from 10-100μM causes inflammation of the lungs, and has been found in the sputum of infected patients (as high as 128.5 μM) [127, 129]. As seen in Figure 18 pyocyanin could be detected in all samples via SWV scans. Figure 19 shows raw SWV scans for each tested biofluid with and without 5 μM pyocyanin present. An obvious increase in measured
current is observed for the spiked samples at the half wave potential of pyocyanin. This is important as it is possible to measure levels of pyocyanin that do not cause inflammatory responses. The ability to distinguish between harmful levels of pyocyanin exposure could be used as a potential diagnostic for patients and lead to the proper treatment of *P. aeruginosa* infections.

A peak in the measured current is expected at -0.25 volts versus a 1 M KCl Ag/AgCl reference for samples containing pyocyanin [97]. This result is observed for all samples with the exception of bronchial lavages, where the peak was consistently shifted to more negative potentials (Figure 19). It is unknown why this occurred only with bronchial lavage samples as the normal causes of voltage drift (pH and temperature) were ruled out based on the certificates of analysis from Bioreclamation. One possible explanation is that reactive, complexing, or chelating compounds are uniquely present in the bronchial lavage samples that alter the redox potential of pyocyanin [130, 131].

Of significant importance is the complete lack of peaks and low baseline current in the blank raw data bronchial lavage SWV scans indicating that there are no reversible electro-active molecules naturally present in these samples (Figure 19A). This result was also observed in in the blood samples that had heparin added (Figure 19D), which is quite surprising, given the inherent complexity of blood. Small peaks were observed in the sputum and urine measurements (Figures 19B & C). The small peak observed in sputum could potentially be removed by shifting the potential window that is measured to more positive values. For instance, increasing the starting voltage to -0.35 volts could remove the response of this molecule, while still allowing for the detection of pyocyanin. Unfortunately, the same approach cannot be applied to urine samples, as the observed
peak voltage is similar to that of pyocyanin. These peaks indicate the presence of unknown native electro-active molecules at low concentrations, which could potentially affect the ability to detect sub-micromolar concentrations of pyocyanin, thus limiting the early diagnosis of *P. aeruginosa* infections when analyzing these fluids without additional sample processing steps.

The maximum peak currents were found to increase linearly with increasing pyocyanin concentration in each biological fluid (Figure 20). The sensitivity and the LOD were calculated for each biofluid and are reported in Table 1. The sensitivity is the slope of the line calculated from the plot of the maximum current versus concentration while the LOD was calculated as

![Figure 20: Maximum current versus pyocyanin concentration in human fluid samples. Varying concentrations of pyocyanin were added to lysogeny broth (black squares), urine (red circles), sputum (blue triangles), bronchial lavages (pink downward pointing triangles), and blood with sodium heparin (green](image-url)
diamonds). The samples were analyzed using SWV and the baseline adjusted maximum currents are shown. The inset shows an expanded view of the average currents measured at the lower pyocyanin concentrations for each human biofluid.

3σ/sensitivity, where σ is the standard deviation of the blank solution current at the pyocyanin oxidation potential. The average coefficient of variation across three different electrodes was approximately 3.2 and 3.7 % for 50 and 100 µM pyocyanin in growth media respectively (Table 2). The linearity of the fits (R$^2$–values) are very good for all medical samples with added pyocyanin.

<table>
<thead>
<tr>
<th>Biological Sample</th>
<th>Sensitivity (nA/µM)</th>
<th>LOD (µM)</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysogeny Broth</td>
<td>267.0±8.0</td>
<td>0.18</td>
<td>0.993</td>
</tr>
<tr>
<td>Urine</td>
<td>225.0±69.0</td>
<td>1.81</td>
<td>0.989</td>
</tr>
<tr>
<td>Sputum</td>
<td>108.0±2.0</td>
<td>0.13</td>
<td>0.997</td>
</tr>
<tr>
<td>Bronchial Lavage</td>
<td>61.1±13.0</td>
<td>0.14</td>
<td>0.985</td>
</tr>
<tr>
<td>Blood-heparin</td>
<td>19.1±0.8</td>
<td>0.16</td>
<td>0.987</td>
</tr>
</tbody>
</table>
Table 2. Reproducibility of disposable Zensor strip in detecting pyocyanin at 50 μM in trypticase soy broth. Three separate pyocyanin samples were measured per electrode. Electrodes were rinsed with DI water in-between samples.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1.25E-05</td>
<td>1.24E-05</td>
<td>1.24E-05</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>4.34E-07</td>
<td>4.21E-07</td>
<td>5.39E-07</td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td>3.48E-02</td>
<td>3.40E-02</td>
<td>4.34E-02</td>
</tr>
</tbody>
</table>

Table 3. Reproducibility of disposable Zensor strip in detecting pyocyanin at 100 μM in trypticase soy broth. Three separate pyocyanin samples were measured per electrode. Electrodes were rinsed with DI water in-between samples.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>2.43E-05</td>
<td>2.45E-05</td>
<td>2.44E-05</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>7.9883E-07</td>
<td>8.13E-07</td>
<td>7.11E-07</td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td>3.28E-02</td>
<td>3.32E-02</td>
<td>2.91E-02</td>
</tr>
</tbody>
</table>

The data from Figure 18 and Figure 19 was also analyzed using a second method by calculating the first derivative of each measurement. All of the SWV scans had a negative slope for the current with increasing potentials, therefore any peak in the data is caused by the presence of an electro-active molecule and results in a positive slope in the scan. The maximum derivative peaks are reported in Figure 21 and the sensitivities and limits of detection reported in Table 4. This method of analysis provided nearly identical results (see Figure 20 and Table 1), however better limits of detections were obtained for the previous data analysis approach. As such future tests with pyocyanin did not rely on this second method of measurement testing.
Figure 21: Maximum 1st derivative versus pyocyanin concentration. Lysogeny broth (black squares), urine (red circles), sputum (blue upward facing triangles), bronchial lavages (pink downward facing triangles), blood with sodium heparin (green diamonds). I=Current, C=concentration.

Table 4: Pyocyanin Sensitivity and LOD for each Biological Sample Tested after taking the 1st derivative of the raw SWV data.

<table>
<thead>
<tr>
<th>Biological Sample</th>
<th>Sensitivity (µA/µM)</th>
<th>LOD (µM)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial Lavage</td>
<td>0.76±0.03</td>
<td>1.619</td>
<td>0.987</td>
</tr>
<tr>
<td>Urine</td>
<td>3.80±0.13</td>
<td>1.938</td>
<td>0.990</td>
</tr>
<tr>
<td>Sputum</td>
<td>2.30±0.03</td>
<td>0.098</td>
<td>0.999</td>
</tr>
<tr>
<td>Blood-heparin</td>
<td>0.33±0.01</td>
<td>0.187</td>
<td>0.990</td>
</tr>
<tr>
<td>Lysogeny Broth</td>
<td>4.52±0.14</td>
<td>0.513</td>
<td>0.992</td>
</tr>
</tbody>
</table>

The decreased pyocyanin detection sensitivity compared to simple buffer samples is likely the result of compounds present in the biofluids that affect the diffusion of the molecule to the electrode surface [132, 133]. Since the measured current in SWV is directly proportional to the diffusion coefficient of the molecule being detected, the
composition of the media in which the molecule is measured will significantly affect sensitivity. Blood is one of the most complex biofluids tested in this study. As such, it comes as no surprise that it had the lowest electrochemical response for all pyocyanin concentrations (Figure 20). Each electrode was only used once per sample therefore, biofouling is not suspected as a cause of the decreased sensitivity in complex human samples.

In an attempt to explain the differences in the reported electrochemical responses of pyocyanin, 100 μM pyocyanin in trypticase soy broth was scanned using cyclic voltammetry at different scan rates. By measuring the forward and reverse peak currents at different scan rates it is possible to elucidate whether or not the reaction mechanism is diffusion controlled or reaction controlled. As the scan rate was increased it was observed that the peak currents for the forward and reverse currents increased as well (Figure 22).

![Figure 22: Overlay of cyclic voltammograms of 100 μM pyocyanin trypticase soy broth in at different scan rates. Three scans from -0.5 to 0 V were repeated per sample.](image-url)
Plotting the forward and reverse peak currents from Figure 22 versus the square root of the scan rate yielded a linear line for all applied scan rates indicating the measurement of pyocyanin at the surface of the electrode is mass transfer limited via diffusion (Figure 23). This is important since the hindering of diffusion via either viscosity changes or chelating complexes would lead to a lowered electrochemical signal as reported in Figure 20 [132]. From Figure 22 the voltage difference from the forward and reverse currents was calculated to be approximately 56 mV indicating a quasi reversible reaction of pyocyanin at the electrode surface. Completely reversible electrochemical reactions have a peak to peak separation voltage of 59 mV. This could help to explain why the reverse peak currents were smaller than the forward currents.

\[ I = 9.28 \times 10^{-7} V^{1/2} - 3.13 \times 10^{-6} \]
\[ I = -3.43 \times 10^{-7} V^{1/2} - 3.99 \times 10^{-6} \]

Figure 23: Comparing the peak forward and reverse currents for 100 μM pyocyanin in trypticase soy broth.

While the sensitivity for detecting pyocyanin is diminished in complex human samples, the overall results show that biologically relevant concentrations of pyocyanin can still be detected reliably. The clear distinction between the response of the biological
samples from healthy individuals and after adding 5 µM pyocyanin (Figure 19) indicates that simple electrochemical detection can be used to rapidly determine if patients are infected with *Pseudomonas aeruginosa*.

### 5.1.2 Scanning Biological Samples Spiked with *Pseudomonas aeruginosa*

To determine the ability to detect the presence of *Pseudomonas aeruginosa* infection in biological fluids, the wild type strain PA14 was spiked into human samples and scanned using SWV. PA14 was chosen as it is known to be more virulent compared to other strains of *P. aeruginosa* [128]. Overnight cultures of PA14, grown to stationary phase, were pelleted at 10,000 rpm and the supernatant discarded. The PA14 pellets were reconstituted in fresh trypticase soy broth and 50 µL of reconstituted PA14 was loaded into 500 µL of biological fluid in triplicate samples. Scans of the samples immediately after PA14 was inoculated were taken to show that no pyocyanin was present in the initial samples. The average current from three separate samples of each biofluid, after one day of growth in the biofluid at 37 °C, is shown in Figure 24.
Biological solutions, spiked with *P. aeruginosa*, all show the presence of a pyocyanin peak when scanned using SWV after one day of growth (Figure 24). Measured pyocyanin levels in *P. aeruginosa* spiked samples were between 2 and 20 µM (calculated from Figure 24). Blood containing sodium heparin had the largest measured pyocyanin concentration even though the overall response of urine samples at the same time point is larger (Figure 24). This is a consequence of urine giving an overall higher signal level for the same pyocyanin concentration (Figures 18 and 24).

To ensure that the observed response was not due to any residual pyocyanin from the overnight cultures, measurements were taken immediately after PA14 loading (Figure
24). No peaks were observed, indicating that the discernible peaks after one day of incubation were caused by bacteria producing pyocyanin. Differences in the measured current between blank measurements (no PA14 present) and measurements taken immediately after PA14 loading are most likely due to inconsistencies between the disposable electrodes used in this study. Nonetheless, a peak is clearly detectable within 24 hours in all of the biological fluids, demonstrating the successful detection of PA14 via the measurement of pyocyanin (Figure 24).

While the proposed approach does indeed allow for rapid, selective detection of *P. aeruginosa* in the tested biofluids, several factors must be addressed. Literature suggests that pyocyanin is the main electrochemical molecule being produced in this voltage window; however, several published studies have shown that mutants of *P. aeruginosa* deficient in pyocyanin production can produce other phenazines that react in this potential window [86, 134]. Although these other phenazines can affect the ability to accurately measure the concentration of pyocyanin, they should not impede the direct detection of *P. aeruginosa* in clinical samples as it is the only clinically relevant bacterium producing these molecules [97]. At the time of this study, the authors were unable to obtain samples from patients suffering from *P. aeruginosa* infections.

### 5.1.3 Culture Supernatant Scanning

While the direct detection of pyocyanin in biological fluids is pertinent, bodily fluids are exposed to a plethora of bacterial species that can potentially interfere with detection of *P. aeruginosa*. Five different bacterial species were cultured in TSB and LB, two common bacterial culture media, to ensure that only *P. aeruginosa* produced electrochemical molecules in the tested voltage window. Cultures of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) modified *P. aeruginosa* (PAO1),
YFP and green fluorescent protein (GFP) modified *E. coli, S. aureus, S. epidermis,* and *B. cereus* were scanned using SWV to determine whether the other bacterial species would produce and release electro-active molecules in the region between -0.5 and 0.0 volts versus a Ag/AgCl reference. Supernatants from two mutant strains of PAO1 were used to confirm the production of pyocyanin by *P. aeruginosa* [3, 94]. Two mutants of *E. coli* were used to validate previous studies claiming that no electro-active molecules are produced in this potential window by this common bacterial species [3]. The remaining species were selected for analysis because they are commonly found in the environment, are opportunistic pathogens, and do not have electrochemical information available.

Figure 25: SWV of different bacterial cells after A) 1 day, B) 2 days, and C) 3 days of growth in tryptase soy broth. Scans were performed from -0.5 to 0 volts vs. 1 M KCl Ag/AgCl reference at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.

Three batch cultures of each species were grown in TSB and LB and three separate supernatant samples were removed from each culture and analyzed each day using SWV from -0.5 to 0 volts at 15 Hz and an amplitude voltage of 50 mV. The measurements from each day were averaged together and the results are shown in Figure 25. Over the course of three days, it was observed that the cultures of PAO1 produced an electro-active molecule with an oxidation potential similar to pyocyanin (Figure 25).
Of interest is that the SWV scanned supernatants for the other tested bacteria showed no significant peaks (Figure 25). The lack of a detectable signal from the other species in the scanned potential window further confirms that *P. aeruginosa* is the only bacterium producing measurable amounts of electro-active molecules among the species tested and that the possibility of a false positive identification of *P. aeruginosa* using this method is unlikely (Figure 25). While beyond the scope of this study it should be noted that low levels of *P. aeruginosa* present in the infected fluid would produce little to no detectable pyocyanin based on the proposed method. This could potentially lead to undesired false negative results. Nonetheless these findings support the possibility of electrochemically detecting *P. aeruginosa* cultures using pyocyanin.

5.2 **Goal 2: Utilize Miniature Electrochemical Sensors Combined with Microfluidic Channels to Monitor the Production of Pyocyanin in Biofilms.**

5.2.1 **Monitoring Cellular Health in the Chamber**

SWV were collected every 30 minutes from overnight cultures of PA14 in TSB, starting from the point at which they were loaded into the PDMS chambers, to determine whether electro-active molecules were being produced. *P. aeruginosa* continuously produces pyocyanin as it grows, in both planktonic and biofilm phenotypes, which can be monitored electrochemically during the experiments [3]. The utility of this approach is highlighted in Figure 26 where the electrochemical response of PA14 grown in TSB is monitored over time.

The lack of observable peaks during loading indicated that no detectable pyocyanin was present initially in the fresh TSB cell suspension (Figure 26A). As the biofilm formed under stagnant conditions, the oxidation peak height increased over time (Figure 26A & B). SEM images of the PDMS growth chamber and the working electrode substrate showed bacteria carpeting both surfaces (see Figure 27 and 28) after overnight
growth under stagnant conditions. Initiating the flow of fresh TSB into the channels after overnight growth allowed the biofilm to thrive.

**Figure 26:** SWV’s of PA14 and *E. coli* (solid and dashed lines respectively) cultured in trypticase soy broth after loading 24 µL of overnight culture after A) 0 h, B) 12 h, C) 22 h, D) 35 h, E) 40 h, and F) 45 h. Flow of fresh TSB at 100 nL/min was initiated at 22 h. SWV’s performed from -0.5 to 0.2 V at a frequency of 15 Hz and an amplitude voltage of 50 mV.

**Figure 27:** SEM Images of *P. aeruginosa* grown on the working electrode of a three electrode cell. From left to right, SEM images were taken at a magnification of 9,000 X, 15,000 X, and 30,000 X at 3 kV. Note the presence of a large number of cells in all three images interlocked together.
Indeed, the electrical signal increased after TSB flow was initiated (Figure 26D-F), indicating the increased production rate of pyocyanin. The presence of a second peak at later time points was observed. The main peak is due to the oxidation of pyocyanin, while the appearance of a second peak is ascribed to the oxidation of a different phenazine derivative [135, 136]. The change in the oxidation potential, after the initiation of flow, where the peak current was measured can be attributed to the internal Ag/AgCl pellet used as the reference for these studies. Drift due to fluid flow is an unavoidable consequence of having the reference in direct contact with the test fluid [24, 36]. However, the measured peak potential stabilized over time and the peak current at this point was used.

While the overall electrical signal increased over time, a decrease was observed consistently at the initiation of fluid flow. There are two possibilities for the observed result. First, it can be an indicator of how firmly the biofilm has adhered to the surface of the microfluidic channel. The role of shear stress on cell adhesion has been studied previously, showing that at high shear stresses cells can be removed from surfaces [137, 138]. As growth media flows through the channel it may remove bacteria if the biofilm is
not firmly attached [139]. The removal of bacteria in turn would lead to reduced production of pyocyanin in the vicinity of the sensor, lowering the electrical signal. This is unlikely as the applied flow rates in this study are similar to those used by other groups and should be slow enough to avoid significant removal of the bacterial biofilm [140, 141].

Second, it is possible that the decrease in signal is due to pyocyanin in solution being removed during flow, and it is only when a sufficiently large concentration of pyocyanin is produced, to overcome convective transport, that the signal rebounds. Koley et al. (2011) demonstrated the presence of a pyocyanin electrocline/gradient in biofilms of *P. aeruginosa* using scanning electro-chemical microscopy [142]. The authors showed that this electrocline extended hundreds of microns above the biofilm's surface. The change in the pyocyanin electrocline due to fluid flow is likely responsible for the initial drop in signal when bulk fluid flow starts. Regardless, it is clear that even after the initiation of flow within the microfluidic chamber, the peak current rebounds indicating the cells are indeed growing within the chamber (Figure 26).

*E. coli* in TSB was used as a control since it is not expected to produce molecules that are redox-active in this voltage window [3, 143]. The lack of any discernible peak confirms that there are no electrochemical molecules produced by *E. coli* and that there is no contamination of the chambers by *P. aeruginosa* from the environment over the course of the experiment (Figure 26). The absence of oxidation peaks from *E. coli* cells highlights the limitations of the proposed approach to electrochemically monitor the antibiotic susceptibility of other bacterial species. Alternatively, the ability to electrochemically measure the viability of PA14 by the production of pyocyanin can be a
useful selective marker of *P. aeruginosa* in patient samples [143]. Furthermore the transparent nature of the PDMS used to fabricate growth chambers facilitates the use of fluorescent bacterial species and markers as reported in the literature [140, 141].

5.2.2 Effect of Colistin Sulphate on PYO Signal

After overnight growth of *P. aeruginosa*, 0.100 μL/min flow of colistin sulphate at 4, 16, and 100 mg/L in TSB was initiated. These concentrations were chosen to cover the range of colistin sulphate MIC values that are reported in literature [144]. SWV measurements were taken to determine what effect the reported MIC concentrations of colistin sulphate (4 and 16 mg/L) have on pyocyanin production. This, in turn, can be an indicator of *P. aeruginosa* biofilm susceptibility to colistin sulphate. No oxidation peaks were observed for *E. coli* exposed to colistin sulphate signifying a lack of electrochemically active molecules (Figures 29-31) [3, 145].

![Figure 29: SWVs of *E. coli* and *P. aeruginosa* (dashed and solid lines respectively) grown in TSB at A) 0 hr, B) 12 hrs, then exposed to flowing 4 mg/L colistin sulfate TSB after approximately C) 22 hrs, D) 35 hrs, E) 40 hrs, F) 45 Hrs. Baseline subtracted from resulting scans. SWV performed from -0.5 to 0.2](image-url)
volts at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.

Three devices per concentration of colistin sulphate were used and the average peak current reported (Figure 33). Error bars represent the standard deviation of the mean for three separate measurements at that time point, unless otherwise indicated. As a control *E. coli* biofilms were exposed to the same concentrations of colistin sulphate. One replicate per

Figure 30: SWVs of *E. coli* and *P. aeruginosa* (dashed and solid lines respectively) grown in TSB at A) 0 hr, B) 12 hrs, then exposed to flowing 16 mg/L colistin sulfate TSB after approximately C) 22 hrs, D) 35 hrs, E) 40 hrs, F) 45 Hrs. Baseline subtracted from resulting scans. SWV performed from -0.5 to 0.2 volts at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.
Figure 31: SWVs of *E. coli* and *P. aeruginosa* (dashed and solid lines respectively) grown in TSB at A) 0 h, B) 12 h, then exposed to flowing 100 mg/L colistin sulphate TSB after approximately C) 22 h, D) 35 h, E) 40 h F), 45 h. Baseline subtracted from resulting scans. SWV performed from -0.5 to 0.2 volts at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively.

Figure 32: Maximum current from SWVs of pyocyanin in 30 g/L TSB from 0 to 50 μM. SWVs were performed from -0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV.
concentration was performed for these tests. The average peak current was converted to approximate pyocyanin concentrations via a calibration curve. The calibration curve was constructed by measuring the SWV response of pyocyanin from 1- 50 μM in trypticase soy broth. Three separate samples per concentration were measured and the average max current was reported (Figure 32).

After the initiation of antibiotic flow, the average peak current significantly decreased for PA14 exposed to 16 and 100 mg/L concentrations (P<0.05). The average percent decrease in the maximum peak current at the end of testing for PA14 exposed to 16 and 100 mg/L colistin sulfate

![Image Description](https://via.placeholder.com/150)

**Figure 33:** Response of PA14 biofilms at selected time points during the 48 hour experiments. Left axis: average peak current (blank subtracted) measured over time in PA14 cultures exposed to colistin sulphate at 0, 4 (low MIC), 16 (High MIC), and 100 mg/L colistin sulphate. Right axis: Approximate pyocyanin concentration based on calibration curve. * indicates time points where only two replicates were used. ** indicates P<0.05 from ANOVA analysis.
was 68% and 82% respectively. The average percent decrease in the measured current compared to the control cells was calculated by \( \% \text{Decrease} = 100 \times \frac{(I_t - I_c)}{I_c} \) where \( I_t \) equals the average peak current at time \( t \) and \( I_c \) is the average peak current of the control \( P. \) aeruginosa \) cells. The decreased current response is directly related to a decrease in the measured pyocyanin, indicating a correlation between the colistin sulphate concentration and pyocyanin production. In contrast, the average response for cells treated with 4 mg/L colistin sulphate showed no significant difference when compared to biofilms exposed to only TSB, indicating that the lower MIC value was not significantly affecting the production of pyocyanin. Importantly, Figure 33 and Figure 34 showcases that continuous electrochemical monitoring allows the researcher to view the efficacy of an anti-pseudomonas antibiotic via a reduction in pyocyanin production. By reducing the amount of pyocyanin produced by the bacteria, the host’s body may be able to effectively fight off the infection [146].
Figure 34: SWVs of *P. aeruginosa* grown in TSB without flow for A) 12 h. The cells were then exposed to flowing 100 mg/L ampicillin or colistin sulphate (red and black lines respectively) in TSB for approximately B) 22 h, C) 35 h, and D) 40 h of growth. Baseline subtracted from resulting scans. SWV performed from -0.5 to 0.2 volts at a frequency, amplitude voltage and incremental voltage of 15 Hz, 50 mV, and 4 mV.
Figure 35: Images of PA14 colonies after 20 hours of growth at 37 °C on cetrimide agar mixed with different concentrations of colistin sulphate.

The inherent resistance of PA14 to the lowest MIC value used in this study could explain why the pyocyanin response did not significantly differ from blank measurements. Liquid samples of PA14 cultured on 4 mg/L colistin sulphate agar plates were able to grow indicating that this concentration had no effect on planktonic cell attachment and growth (Figure 35). As such it makes sense that biofilms of PA14 exposed to this concentration would not be affected and should produce similar levels of pyocyanin.

The results in Figure 33 suggest that pyocyanin can be a useful marker of antibiotic effect colistin sulphate has on cells grown within the microfluidic chambers. Of interest is whether *P. aeruginosa* cells exposed to an antibiotic it is not susceptible to would have any decrease in pyocyanin production. To answer this question *P. aeruginosa* cells were exposed to 100 mg/L ampicillin in trypticase soy broth. Ampicillin is known to have little to no effect on *P. aeruginosa* cells. As such *P. aeruginosa* cells exposed to it should still produce relatively normal levels of pyocyanin. SWV voltammograms of PA14 cells exposed to 100 mg/L ampicillin can be seen in Figure 34. As the flow of
ampicillin is initiated a clear increase in the electrical signal is observed. When compared with *P. aeruginosa* exposed to 100 mg/L colistin sulphate it is clear that this concentration of ampicillin had little to no effect on the production of pyocyanin and therefore the growth of *P. aeruginosa* within the chambers.

5.2.3 Effect of Colistin Sulphate on Post Exposure Growth

After samples were exposed to 4 and 100 mg/L colistin sulphate, the PDMS chambers were peeled off for post exposure culturing to probe whether the decrease in pyocyanin production was related to cell death. Samples were removed from the electrode surface by introducing 100 µL of fresh TSB onto the disposable Zensor surface. The removed sample was then divided into separate drops on TSB agar and incubated at 37 °C (Figure 36).

![Figure 36: PA14 exposed to 100 mg/L and 4 mg/L colistin sulphate for 20 hours within PDMS chambers then spotted onto TSB plates. Photographs of plates after incubation at 37 °C for A) 4.33 h, B) 6.5 h, and C) 74.33 h (removed from the incubator after 24 h to avoid drying and grown at room temperature (≈ 23 °C)). Arrows added to highlight the locations of the first PA14 colonies seen. Vertical line divides PA14 exposed to 100 mg/L (left) and 4 mg/L (right) colistin sulphate.](image)

Figure 36 shows the culture results at three different time points. Growth was observed in samples exposed to 4 mg/L colistin sulphate after only 4.3 h of incubation implying that this concentration had little effect on the cells’ viability. No growth was observed for cells exposed to 100 mg/L colistin sulphate after 6.5 h of incubation.
Growth was also eventually observed for samples collected from chambers exposed to 100 mg/L colistin sulphate, indicating that the complete elimination of viable bacteria from inside the chamber was not achieved. These results support the findings in the literature, drawing attention to the lower efficacy of reported MICs against microbial biofilms [141]. Nonetheless, it is clear from the results in Figures 33 and 36 that a reduction in pyocyanin production, under exposure to colistin sulphate, is correlated with a reduction in the viability of PA14.

5.3 Goal 3: Utilize Disposable Electrodes Embedded in Growth Agar to Electrochemically Monitor the Growth of *P. aeruginosa*

Probing whether or not pyocyanin detection on growth agar could speed the detection of *P. aeruginosa* was driven by proof of concept experiments performed. Lysogeny broth agar plates were embedded with disposable electrodes as described in section 4.3.2. Overnight cultures of *P. aeruginosa* strain PAO1, *E.coli*, and *S. aureus* were streaked onto the plates (Figure 37). SWV scans were taken and the resulting peak currents reported (Figure 38).

While optically little difference was observed between the different bacteria grown (Figure 37), measured differences were observed from the maximum peak currents between the different bacteria after 25 hours of growth at room temperature (≈23 °C). While the current response was small, lysogeny broth is not the optimal media for producing pyocyanin. Therefore the goal of this objective was to determine whether the use of King's A agar, which is known to up-regulate pyocyanin production, would lead to earlier detection of *P. aeruginosa*. 
Figure 37: Embedded electrode culture plates containing 1) LB after 25 h, 2) LB after 48 h, 3) PAO1 after 25 h, 4) PAO1 after 48 h, 5) *E. coli* after 25 h, 6) *E. coli* after 48 h, 7) *S. aureus* after 25 h, and 8) *S. aureus* after 48 h growth at room temperature ≈23 °C.

Figure 38: Average peak currents after baseline subtraction for voltages between -0.4 to -0.2 V for embedded electrodes with no cells (red circles), PAO1 (black squares), *S. aureus* (blue triangles), and *E. coli* (pink triangles)

5.3.1 Modeling Pyocyanin Diffusion
Fabricated devices were tested to determine whether diffusing molecules of pyocyanin through thicker agar could be measured at the electrode surface. For these
tests, PDMS wells were plasma bonded to disposable electrodes and King’s A agar was poured onto the wells to three different thicknesses. This was done to cover the range of thickness obtained when embedded agar plates were fabricated. After solidification, 200 µL drops of pyocyanin at concentrations of 100 µM were pipetted directly onto the agar. Measurements were taken every minute, from -0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV. The resulting peak current after baseline subtraction was used to calculate the approximate pyocyanin concentration (Figure 39) and was plotted versus time (Figure 40). Samples were run in triplicate and the error bars are the standard error of the mean.

![Figure 39: Pyocyanin concentration curve fit. Each data point represents three scans at three different disposable electrodes, with the exception of the 100 µM concentration. Additionally two replicates per concentration were performed at each electrode with the exception of the 100 µM sample.]

The diffusion of small electrochemical molecules through the agar should lead to an increase in the measured concentration (Figure 40). It was found that the measured
concentrations never reached the initial concentration of pyocyanin (Figure 40). The most plausible reason for this is due to the large water content of the agar even after solidification. Based on the measured volumes of agar (approximately 585 μL for a 5 mm thick slab), it is expected that at equilibrium the final concentration of pyocyanin throughout the agar should be approximately ¼ the initial value. This agrees with the simulated COMSOL results when a constant pyocyanin concentration of 25 μM was used (Figure 40 A).

Figure 40: A) Max peak currents from SWVs of diffusing pyocyanin through agar to the embedded electrode. 200 μL of a 100 μM pyocyanin solution was pipetted onto 2.72, 3.77, and 5 mm agar slabs respectively and allowed to diffuse. Error bars are the standard error of the mean from 3 separate runs per concentration. B) COMSOL simulation of the diffusion of a constant concentration of pyocyanin (25 μM) at the top of a 5 mm agar block after 120 min of diffusion. Diffusion constant 0.75x10^{-9} m^2/s.

Prior studies in the lab have shown that the diffusion coefficient of pyocyanin in TSB is approximately 2.5x10^{-9} m^2/s [143]. For a diffusion coefficient of 0.75x10^{-9} m^2/s the simulated results follow a similar trend to the experimentally measured values for a 5 mm agar slab, suggesting an order of magnitude reduction in the diffusion of pyocyanin through the agar. Similar reductions in diffusion have been reported [136]. Importantly,
the results highlight that the fabricated system can measure the diffusion of pyocyanin down to the electrode surface.

5.3.2 Electrochemically Monitoring *P. aeruginosa* Growth

After confirming that diffusing pyocyanin was electrochemically detected, the specific detection of *P. aeruginosa* on these plates was investigated. Wild type strain PA14 was chosen for these tests due to its increased virulence and associated pyocyanin production [147]. Samples of PA14 were grown overnight in TSB, centrifuged, and reconstituted in fresh TSB. The bacterial concentration in the stock solution was determined via cell counting. The stock solution was diluted to the appropriate concentration to achieve the desired bacterial load in a 5 μL drop. This inoculation volume was found to reliably hold its shape upon inversion of the agar plate. Larger inoculation drops tended to spread around the plate when inverted. 5 μL drops of PA14 were pipetted onto agar plates above the working electrode and incubated at 23, 37, and 42 °C to determine what effect temperature would have on the electrochemical detection of *P. aeruginosa*. The temperatures were chosen to encompass the range that would typically be used for the culture of *P. aeruginosa* [148].

The average peak current from square wave voltammetric scans of the samples from -0.5 to 0 volts after baseline subtraction were recorded and plotted versus time (Figure 41A). The reported current is the average from 3 separate embedded electrode devices at that time point with the error bars equal to the standard error of the mean. Measurements were taken until the peak current reached a maximum indicating the highest level of electrochemical molecule production.

It was found that the current increased over time for all samples inoculated with different bacterial doses (Figure 41A). Not surprisingly, the samples loaded with more
bacteria showed an earlier current response, indicating that pyocyanin was produced earlier in these samples. Pyocyanin has been shown to be regulated by quorum sensing, which requires a large enough concentration of bacteria to produce a response, explaining the bacterial dose dependency on electrochemical detection [94].

Figure 41: A) Maximum peak currents versus time for different initial concentrations of PA14 grown on King’s Agar. Max currents come from SWVs taken every hour at a frequency of 15 Hz and an amplitude voltage of 50 mV. B) Maximum average production rate of pyocyanin per hour between temperatures. * P<0.05.

Temperature is known to affect the growth rate of cellular organisms, as well as strongly influencing the production rate of metabolites [124, 149]. Figure 41A and B clearly show that temperature positively impacted electrochemical molecule production to a point. Bacteria grown at 23 °C showed little measurable electrochemical molecule production compared to cells grown at higher temperatures. Furthermore cells cultured at higher temperatures had an overall higher maximum production rate of pyocyanin (Figure 41B).

Cells grown at 23 °C did eventually produce a measureable electrochemical signal at later time points (Figure 42A). Not only did it take longer for an appreciable peak
current change to occur for cells grown at 23 °C, but the effect it had on different bacterial loads (10^6 versus. 10^8 PA14 cells) was more pronounced (Figure 42B). A linear relationship for the difference in the amount of time required to achieve similar peak currents for different bacterial loads versus temperature was observed. While for this range of temperatures chosen a linear fit was obtained, it can be expected that at higher temperatures the time difference for different bacterial loads would increase. This is a result of the cells being unable to grow at these temperatures lowering the detectable electrochemical signal. The same would be expected for colder temperatures as cell growth becomes stunted at lower temperatures [150].

Figure 42: A) Baseline subtracted peak currents vs. time for cells cultured on King’s A Agar, directly above the embedded working electrode, at, 23, 37, and 42 °C. B) Average difference in time for similar peak currents to be obtained compared for different initial bacterial loads. Red boxes highlight peak currents used to generate B)

5.3.3 Comparing Electrochemical Detection to Optical Detection

Another benefit of the proposed system is the reduced detection time compared to normal visual monitoring. It was observed that the measured rise in electrical signal increased earlier than the optical positive detection of *P. aeruginosa* (Figure 48). Even after the
formation of a visible colony, it was not possible to positively identify *P. aeruginosa* via the blue coloration associated with pyocyanin production on King’s A agar (Figures 43-46). This could indicate that further incubation was required. The electrochemical detection of pyocyanin in this potential window also allows for the specific detection of *P. aeruginosa* from other clinically relevant pathogens Figure 47. The earlier time to detection offered by this method could promote the appropriate antibiotic regimen compared to the current approaches [151]. This in turn could benefit patients suffering from infections as well as helping to curb the increase in antibiotic resistant bacteria [152].
Figure 43: Time lapse images of embedded electrodes exposed to $10^3$ Pa14 cells in a 5 µL drop after A) 0, B) 6, C) 8, D) 9, E) 12, F) 13, G) 14, H) 17 Hr of incubation at 37 °C. Arrows point to bacterial growth above the electrodes.
Figure 44: Time lapse images of embedded electrodes exposed to $10^5$ Pa14 cells in a 5 µL drop after A) 0, B) 6, C) 8, D) 9, E) 12, F) 13, G) 14, H) 17 Hr of incubation at 37 °C. Arrows point to bacterial growth above the electrodes.
Figure 45: Time lapse images of embedded electrodes exposed to $10^8$ Pa14 cells in a 5 µL drop after A) 0, B) 6, C) 8, D) 9, E) 12, F) 13, G) 14, H) 17 Hr of incubation at 37 °C. Arrows point to bacterial growth above the electrodes.

Figure 46: Comparing PA14 growth after 20 Hr of growth at 37 °C for bacterial loads of $10^3$, $10^5$, and $10^8$ cells. Arrows point to bacterial growth above the electrodes.
Figure 47: A) Baseline subtracted maximum peak currents for $10^6$ PA14 and $10^6$ S. aureus cultured at 37 ºC. B) Embedded agar electrode with S. aureus colony.

A significant difference in the time required for the production of a measureable signal between PA14 grown at 23 and 37 ºC, while an insignificant difference was observed between PA14 grown at 37 and 42 ºC for the same time periods. Subsequently embedded electrodes were loaded with PA14 ranging from approximately $10^2$-$10^8$ cells cultured at 37 ºC (Figure 49A). The time to detection for cellular load was determined as the point where the first measureable peak in the voltage range of -0.5 to 0 appeared. The time to detection was plotted versus the cell load (Figure 49B) and a linear response was obtained. This approach could potentially be used to determine whether or not a sample from a patient correlates to an infectious load [153]. More importantly, the positive identification of PA14 by electrochemical detection for all bacterial loads took under 20 hours to complete when cultured at these conditions whereas their optical confirmation took longer (Figure 48C & D).
Figure 48: Maximum peak currents versus time for an initial PA14 load of $10^8$ cells grown on King’s Agar after A) 6, B) 8, C) 14, and D) 19 h AT 37 °C. SWVs were taken every hour at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively. Circles in C) and D) highlight the growth of a visible bacterial culture.
Since PA14 is well known for its mobility and agar is a porous medium, the possibility that the increased electrochemical signal was due to the movement and growth of cells on the surface of the electrodes (biofouling) was considered [154]. After the completion of a test, the agar block containing the electrode was removed and prepared for imaging via scanning electron microscopy. If the cells were indeed growing on the surface of the electrode, then the resulting images of the electrodes and the agar surface in contact with the electrode would contain visible bacteria. The resulting images (Figures 50 & 51) show that there was little to no growth on the surface of the working electrode or the agar in contact with the electrode surface. By comparison, images of the agar surface with the colony had large fields of PA14 confirming that the measured signal was due to the diffusion of pyocyanin and that biofouling from the bacteria being studied was minimized using this approach. This is a great advantage over systems that rely on the removal of sample to test which may change the growth conditions or require complicated solvent extraction [3, 143, 155].
Figure 50: Scanning electron microscopy images of A) $10^8$ PA14 cell exposed Agar cultured at 42 °C, B) 10 times magnified of previous image, C) Surface of agar in contact with embedded electrode, D) magnified view of previous image. Scans performed at an acceleration voltage and emission current of 3 kV and 10 µA respectively.
Figure 51: Scanning electron microscopy images of embedded electrodes after growth of PA14 at $10^6$ and $10^8$ cells on Kings A Agar. A) $10^6$ cells grown at RT, B) $10^6$ cells grown at 42 °C, C) $10^8$ cells grown at RT, and D) $10^8$ cells grown at 42 °C. Scans performed at an acceleration voltage and emission current of 3 kV and 10 µA respectively.

Another prospective use for this system that was not explored, is examining the antibiotic susceptibility of *P. aeruginosa* via the electrochemical detection of pyocyanin. Agar plates mixed with antibiotic cocktails of choice have been used for many years by clinicians to successfully create an antibiotic regimen for infected patients [156]. As shown in Figure 41 and 48, the electrochemical detection of *P. aeruginosa* via pyocyanin occurs much faster compared to waiting to visually observe a change. The proposed method could allow researchers to continuously monitor the effect of applied antibiotics allowing for the smarter application of antibiotics.
5.4 Goal 4: Microfabricate a Nanofluidic Electrode Assembly that Contains a Miniaturized Reference Electrode and Working Electrode, to be used for Real Time Monitoring of Pyocyanin Production from \textit{P. aeruginosa} Strains.

The results presented in this section are from “Electrochemical Detection of pyocyanin in nanochannels with integrated palladium hydride reference electrodes” in Lab on a Chip volume 12, 2012. The results are presented with permission from Lab on a Chip.

5.4.1 Testing of Microfabricated Devices and PdH RE

Once all of the chromium had been removed, the Pd electrode was used as a working electrode and charged at -0.375 V versus an Ag/AgCl RE in 2 N HCl for 20 minutes. This was done to load the Pd electrode with hydrogen to stabilize its potential allowing it to be used as a reference electrode. After charging, the open circuit potential (OCP) versus time was monitored to determine the stability of the PdH RE in 2 N HCl. As can be seen in Figure 52, after charging the OCP increases initially and then obtains a constant value of approximately -0.160 V versus an Ag/AgCl reference electrode for almost 1.0 hour. The potential for palladium hydride reference electrodes is approximately 0.050 V versus a standard hydrogen electrode. When the potential contributed by the Ag/AgCl RE is taken into account the measured potential seen in Figure 52 agrees with literature values. Furthermore, it closely matches what Chassaniol \textit{et al.} (2011) showed for their commercially available palladium reference electrodes at a similar pH [39]. It should be noted that all palladium hydride electrodes are affected by pH so it is important to know what operational pH is being used for experimentation.
In Figure 52 the potential is only constant for about an hour after it reaches -0.160 V, which means that the potential can potentially drift for experiments after an hour. This level of stability was expected as all experiments were performed in an oxygenated solution of 2 N HCl. Oxygenated solutions have been known to cause decreased stability in palladium hydride systems [46]. For many single use devices, such as the one described in this section, the cyclic, square wave, and differential pulse voltammetry measurements take much less than an hour to complete, indicating that the PdH RE performance is adequate.
Figure 53: 20 differential pulse voltammetry scans of 500 μM ferrocene dimethanol in 0.5 M KCl solutions. Scans were measured from -0.4 to 0.3 volts at a frequency of 15 Hz and an amplitude voltage of 0.05 volts.

As can be seen in Figure 52 neither the uncharged nor the charged palladium holds a very steady open circuit potential with respect to time. As such one would expect drift to occur less than a few hours after charging. However it was observed that scans of 500 μM ferrocene dimethanol remained stable over a series of scans Figure 53. In fact Figure 53 highlights that some charging is occurring at the palladium electrode surface that more or less stabilizes after the first couple of scans.

To understand the palladium electrodes response to changes in pH and salt concentration open circuit potential (OCP) measurements were only performed after scanning a 500 μM ferrocene dimethanol solution and rinsing with DI water. Immediately after DI rinsing samples of different pH were loaded into the PDMS well over the fabricated NEA. OCPs were measured for 400 seconds and the average value at
steady state was recorded. In these tests the palladium electrode was used as the working electrode and the OCP was measured versus a commercial silver/silver chloride electrode. For these measurements steady state was assumed to be the plateau potential that was approached during OCP measurements. After completing one pH series the electrode was "recharged" as mentioned above, rinsed with DI water and the pH series was measured again. Each sample was measured in triplicate and reported in Figure 54A.

![Graph A: OCP vs. pH for semi-charged palladium reference electrode.](image)

**Figure 54:** A) OCP vs. pH for semi-charged palladium reference electrode. B) OCP vs. NaCl concentration in 100 mM phosphate buffer (pH 7) for semi-charged palladium reference electrode.

The pH response for fabricated devices was found to be represented by the following linear relationship: OCP (V) = -0.0275pH + 0.37798 for acidic conditions up to pH of 10 (R²=0.93). At higher pH the reference electrodes response was found to deviate significantly. The slope of -0.028 V is just over half the expected value from the ideal value of -0.059V expected from Nernstian reactions. This is not an unexpected result as the hydrogen-palladium complex is known to change and only exhibit ideal pH shifts for very short time periods [39]. Additionally extensive cleaning and pretreatment is often required for the ideal case to hold true [157, 158]. While the electrode itself was found to be dependent of pH changes within
the solution, a negligible impact from changes in salt concentration was observed (Figure 54B). This is to be expected as the measured half-reaction for the palladium system is only supposed to be dependent on pH.

5.4.2 Testing nanofluidic electrode response

Testing of the finished devices was performed using ferrocene dimethanol in concentrations ranging from 1-1000 µM dissolved in 1 M KCl solution (Figure 55A, 1000 µM data removed for clarity). The test solutions were scanned from 0 to 0.5 V versus an Ag/AgCl (1 M KCl) reference electrode using cyclic voltammetry. The PDMS well was washed three times with blank KCl solution between each test to ensure no cross contamination. For these tests, only the gold working electrode was utilized as a means of establishing that the device was functioning. Ferrocene dimethanol was chosen because its electrochemistry has been widely studied. The half wave potential or the point where the current is half of the limiting current was measured to be about 250 mV which matches values reported in literature [159].

Figure 55: A) Cyclic voltammograms of 1-100 µM ferrocene dimethanol in 1 M KCl solution. Scan rate of 0.050 V/s from 0 to 0.750 V using a Ag/AgCl reference electrode. B) CV of 50 mM H₂SO₄ at gold working electrode vs. Pd Re
Device reproducibility was measured by scanning 50 mM H$_2$SO$_4$ solutions using cyclic voltammetry (Figure 55B). The forward scans show the oxidation of the gold working electrode, while the reverse scan show a reproducible reduction peak at approximately 300 mV. The observed response has been reported for gold electrodes exposed to sulfuric acid versus different reference electrode materials [160].

5.4.3 Pyocyanin Testing using an Integrated PdH RE

First, the NEA was used to detect pyocyanin in phosphate buffer. Figure 56 compares square wave voltammograms for three concentrations of pyocyanin in 100 mM phosphate buffer using a commercial reference in Figure 56A and a microfabricated PdH RE in Figure 56B. The bottom gold electrode was used as the working electrode for all experiments. All square wave scans were performed at an amplitude voltage of 0.025 V and a frequency of 15 Hz. The discrepancy in maximum current between the Ag/AgCl and PdH REs is the result of using a different device for the two sets of measurements. Typically, a dilution series calibration is carried out for each device before sensing measurements are started. Variability in maximum currents between batches of fabricated devices is less than one order of magnitude.

Different potential ranges were used in the two sets of experiments to account for the potential difference between the two reference's half-reactions. Figure 56 shows that the pyocyanin oxidation peak versus Ag/AgCl is approximately -0.260 V and approximately -0.480 V versus. PdH. The expected difference between the two peaks for the given reference electrodes at room temperature is -0.220 V. This deviation is negligible for the given experiments and
is caused in part by the buffer solution. The important characteristics are the single oxidation peak and stability of the potential at which the peaks occur.

Figure 56: A) Square wave voltammograms of 0 - 100 µM pyocyanin in 100 mM PB from -0.350 to -0.100 V vs. Ag/AgCl reference electrode. B) Square wave voltammograms of 0 - 100 µM pyocyanin in 100 mM PB from -0.800 to -0.100 V vs. PdH reference electrode. All scans were performed at an amplitude voltage of 0.025 volts and a frequency of 15 Hz.

Differential pulse voltammetry was used to scan pyocyanin solutions (1-100 µM) from -0.7 to -0.1 V at an amplitude voltage of 0.05 V and a frequency of 15 Hz. Three samples were measured at each concentration. The blank 100 mM phosphate buffer background current was subtracted out from each scan. The average maximum current and standard deviation for the three measurements at each concentration are reported in Figure 57. A linear fit of the data was also obtained and is reported in Figure 57. The PDMS wells were rinsed with PB buffer between samples. A limit of detection of 0.597 µM pyocyanin was obtained for this system. The limit of detection was calculated as 3σ/sensitivity, where σ is the
average of the standard deviations from 3 separate blank 100 mM phosphate buffer measurements.

![Image](image.png)

**Figure 57:** Plot of maximum current detected from solutions containing from 0 to 100 µM pyocyanin in 100 mM PB. The current from the blank has been subtracted out so that at a solution containing no pyocyanin has a current of 0 V. Inset: The oxidized and reduced forms of pyocyanin.

### 5.4.4 Detection of Pyocyanin in *P. aeruginosa* Cell Cultures

The microfabricated NEA's were used to detect pyocyanin produced from *Pseudomonas aeruginosa* (PA), PA14 wild type, pelA, *phz* S, and *phz* M. The strains were all grown at 37 °C for a period of 8 days in trypticase soy broth. A single NEA was charged prior to the start of these tests. For measurements 400 µL of solution was removed from each of the liquid cultures and filtered using a 0.2 µm sterile nylon filter (Fisher Cat. 09-719C). The filtered samples were scanned from -0.5 to -0.1 V using differential pulsed voltammetry to detect the presence of
pyocyanin. The measured potential range for these experiments was changed because it was observed that the pyocyanin oxidation peak shifted to more positive potentials in trypticase soy broth compared to phosphate buffer (Figure 58).

![Differential pulse voltammograms](image)

**Figure 58: Differential pulse voltammograms of 100 µM pyocyanin in Phosphate Buffer (Red Line) and in 4 g/L (Blue Dashed Line) and 13 g/L (Pink Dotted Line) Trypticase Soy Broth. Scanned at an amplitude voltage of 0.05 V and a frequency of 15 Hz.**

It is hypothesized that a component in the trypticase soy broth (most likely increased salt concentration) interacts with the PdH RE leading to this shift as no shift in oxidation potential was seen when experiments were performed with respect to a commercially available Ag/AgCl RE. The currents measured in Figure 59 were compared against the measured currents from the supernatants of the PA strain samples to determine pyocyanin concentration. As a control, trypticase soy broth was also scanned. For each sample, seven scans were performed and the
peak value from the final scan was recorded. Each sample was measured 3 times and the average values are plotted in Figure 59.

In Figure 59, as the cells grow the current at the pyocyanin oxidation potential increases. This indicates that pyocyanin is being produced by the bacteria being studied. Of importance is that over the range of potentials being scanned no other peaks were detected, indicating that there are no other electrochemically active molecules within the growth media. This is important because anything that contributes to the background noise of the solution could impact the sensitivity of the device.

Figure 59: Monitoring the pyocyanin production of *Pseudomonas aeruginosa* strains PA14 wild type, pelA, *phz*\textsubscript{S}, *phz*\textsubscript{M} over 8 days of culture at 37 °C. pelA has a gene removed to prevent it from forming robust biofilms, *phz*\textsubscript{S} and *phz*\textsubscript{M} have had the genes for pyocyanin production removed, but can still synthesize precursor molecules.
To confirm that pyocyanin was responsible for the increase in current, four strains of PA were studied, three of which had pyocyanin production inhibited. Strain pelA is capable of producing pyocyanin but does not contain the genes necessary to produce a robust biofilm. It is hypothesized that a biofilm deficient strain would reach a lower cell density than the wild type thus leading to a lower concentration of pyocyanin. The data in Figure 59 shows that this is indeed the case. During the first few days, pelA produced little pyocyanin compared to the wild type. After eight days, the pyocyanin concentration detected for pelA had increased (≈17µM), but was still less than the wild type (≈52 µM) as expected.

Figure 60: Differential pulse voltammogram of wild type PA14 and phzS after 6 days of growth at 37 °C. Scans were performed from -0.5 to -0.1 V at an amplitude voltage of 0.05 V and a frequency of 15 Hz.

In the phzS and phzM strains the necessary genes required to produce pyocyanin have been removed. These gene deletions should ensure that little to no
current is detected from the supernatant of these samples. However, this is not the case. It is hypothesized that the most likely cause of current increase in these strains is from another molecule that is produced by these strains that has a redox potential close to pyocyanin. Several pyocyanin precursor molecules can still be produced by these strains. If the pyocyanin deficient strains produced another phenazine with a very similar redox potential compared to pyocyanin extra steps would be required in the analysis to distinguish between the molecules [97, 136]. It was observed that the peak produced by $phzS$ is at a different potential than the peak generated by the wild type strain, suggesting that an electroactive molecule other than pyocyanin was being produced by $phzS$ (Figure 60).

![Figure 61: Differential pulse voltammograms of PA14 wild type supernatants over a 6 day period. Scans were performed from -0.5 to -0.1 V, at an amplitude voltage of 0.05 volts and a frequency of 15 Hz.](image)
Stability tests for the PdH RE showed that it is not stable for longer than an hour of continuous measurement. Therefore, after an initial charge, measurements, lasting only a few minutes each, were made with the NEA at discrete times during experiments without recharging the electrode. From scans of the wild type samples on different days the oxidation peak potential shifted only approximately 0.020 V (Figure 61). This small shift could have been caused by a number of factors. One possibility is that the pH of the solution changed over time, which is known to affect the redox potential of pyocyanin [97]. It is expected that as PA consumed nutrients in the growth media a more acidic environment would ensue. However pH measurements taken during the experiment did not change from day to day indicating that this is not the cause. A shift in the oxidation potential could also be caused by changes in the chemical composition of the solution as the bacteria grow consuming compounds and producing waste similar to changes observed between phosphate buffer and tryppticase broth (Figure 58). The small shift in voltage observed over the course of the experiment is most likely due to oxidative damage that has been reported to occur in oxygenated solutions. As it is small compared to the shift caused by the increasing salt concentration in tryppticase soy broth it is negligible.
Similarly over a 4 day period of measurements the measured oxidation potential of ferrocene dimethanol versus the Pd Re was found to be approximately 28.3 mV with a standard error between the measurements of only 2.21 mV (Figure 62). The expected oxidation potential is approximately 240 mV versus a silver-silver chloride reference electrode. The measured potential difference is approximately 210 mV which agrees with the measured potential difference seen in pyocyanin measurements. The reproducibility of ferrocene dimethanol measurements highlight the utility of this miniaturized detection platform for potentially monitoring individual \textit{P. aeruginosa} cells.

5.5 \textbf{Goal 5: Use Fabricated NEA to Detect the Production of Pyocyanin from \textit{P. aeruginosa} Cells Trapped Within Detection Area.}

5.5.1 Approaches to trapping small numbers of \textit{P. aeruginosa} for electrochemical monitoring.
Several approaches to separating *P. aeruginosa* cells from bulk samples were employed in this goal of the dissertation. The first approach employed by the author was the use of normally open PDMS valving architecture originally made popular by Stephen Quake’s group in 2000 [55]. The channel layer was constructed from a 20:1 PDMS mixture that was spin coated over the master mold at a thickness of approximately between 2000 to 3000 rpm to obtain PDMS thicknesses of approximately between 25 and 30 microns. To this was oxygen plasma bonded a 5:1 PDMS mixture that had been fabricated from the valve control layer. The bonded PDMS layers were incubated at 80 °C to make the bond permanent. Finally the completed device was plasma bonded to a microfabricated electrode assembly for testing (Figure 63).

The normally open PDMS valve approach was operated by applying air pressure to a liquid filled valve layer. When pressure was applied the channel layer below the valve layer would deform due to its increased elasticity. The deformed layer blocks fluid flow through the rest of channel creating an isolated chamber. This approach was shown to separate single cells from the rest of the fluid, but several drawbacks became abundantly clear (Figure 64).
Figure 63: Left: unfilled PDMS always open valve. Note valves are two parallel lines intersecting perpendicular line underneath. Right: Filled PDMS valve layer. Valve control layer was filled with water before applying pressure to close the valves. Always open valve used to trap concentrated Eosin Y solutions during isotachophoresis.

Figure 64: Left: Zoomed in image of always open PDMS valve layer trapping a single bead simulating the possibility of trapping single cells by this method. Right: Microfabricated electrode assembly beneath always open PDMS valve layer. Channel has same dimensions as left image.

Originally it was attempted to trap single *P. aeruginosa* cells using these valves. In order to accomplish this it was necessary to dilute stock cell solutions increasing the sample handling. Additionally in order to keep the valves closed it was necessary to have
a constant pressure applied, requiring either the author or an air pump to be continually applying pressure. As such an alternative to normally open valves was sought.

The first alternative applied was normally closed PDMS valves. This valving system is similar to normally open systems in that they both consisted of two PDMS layers bonded together and that an applied pressure was required to actuate the valves. The main difference with normally closed valve systems is that an external force (in this case applied vacuum) is only required when the valve needs to be opened. As such this system would seem ideal for manipulating single cells (Figure 65).

![Figure 65: Left: Normally closed PDMS valve being opened (Circle) Right: Zoomed in view of normally closed valve after being loaded with *P. aeruginosa*. Note that the valve did not completely close as indicated by the appearance of a circle. The still opened valve will allow the bacteria to move freely between the chambers.](image)

While promising this approach suffered from a few setbacks. First in the process of permanently bonding the normally closed systems to the fabricated electrodes a vacuum had to be applied to the valves. This was done so that the portion of the valve that was normally closed would never permanently bond to the substrate. Unfortunately this was harder to control in practice and several bonded systems would fail because the normally closed valve remained closed even under the application of vacuum.
A second drawback to this system was that often when the valve did open fluid would flow through the valve as well as around the valve (Figure 65). When vacuum was released the fluid that went through the valve would be pushed out, while fluid that went around the valve would remain providing a gateway for bacteria and media to flow through the chamber that was supposed to be isolated. As such it was desired to find a solution that required no active valving, but would still provide a means of separating a few cells from the bulk solution.

Tandogan et al. (2014) showed that a few bacteria could be separated from the bulk of the bacteria through the addition of a submicron constriction [161]. An added bonus is that this system does not require multiple PDMS layers for fabrication, making it easier to align critical features. This size based separation approach provided a reliable passive means of separating a few cells from the bulk of the solution (Figure 66).

While easier to implement compared to the two valve based approaches, this sized based approach did come with a few problems. First the small size scale of the submicron constriction meant that channels sometimes collapsed during the permanent bonding process. These collapsed channels made it impossible for cells to fit into the adjacent growth chambers, rendering the chip useless. Secondly this set up requires the use of electron beam lithography to fabricate the small feature sizes needed to make this system. While these factors may inhibit this approaches acceptance for mass scale up, its reliability compared to the other proposed approaches made it the preferred choice for this goal of the dissertation.
5.5.2 Monitoring *P. aeruginosa* cells separated from the bulk media for the measurement of pyocyanin using nanofluidic electrode assembly.

Once the microfluidic approach for separating *P. aeruginosa* was identified the next step was combining the chamber with a microfluidic electrode assembly. This required rethinking the design of the previous goals electrode assembly. While the microfabricated nanofluidic assembly utilized in the previous goal was capable of measuring pyocyanin produced from bulk cultures of *P. aeruginosa* it was only composed of two electrodes within the microfluidic chamber. As such it was desired to combine all three electrodes within the nanocavity.

At first designs were created with this concept in mind. However the predisposition of these devices to failure when all three electrodes where present in the nanocavity necessitated a change in design. To this end a fabrication process was created to take advantage of the benefits of nanofluidic channels, namely that the inherent small
size prevents bacteria from interacting with the electrodes directly. To ease the stress on the nanocavity all the electrode were fabricated in the same plane. Figure 67 compares the old approach with the newer design.

Figure 67: Top: Old device design requiring wire bonds to make electrical connection to potentiostat. Bottom: New device design with all metal layers fabricated in the same plane. The larger contact pads allow easy connection to USB connector allowing for a more robust sensor.

Another reason for a new device design was due to the previous design’s reliance on wirebonding to make electrical connections to the potentiostat. While wirebonding provides a permanent means of connection to the potentiostat compared to other approaches using drop down needles; the fragile nature of the wires make them prone to
breaking, rendering them useless [23]. Additionally the PDMS chips employed to make microfluidic chips do not work well with the small working distance employed by the wire bonding tip. To avoid these issues and make a simple means of connecting to the potentiostats, the electrical connections on the chips were designed to fit with a female USB connector (Figure 68). The new design allowed a fabricated device to be plugged directly into a USB connected to the potentiostat avoiding the pitfalls of the previous goals chip.

The final change in the microfabricated electrodes used in this step was the decision to fabricate the devices out of glass wafers compared to silicon with thermally grown \( \text{SiO}_2 \). This was done so that images could be taken directly from the bottom of the glass device, instead of through the PDMS. While PDMS is transparent making it ideal for optical monitoring, the thickness of the constructed devices and the small working distance of the microscopes used to achieve optical resolution of the bacterial cells it was necessary to image from the bottom of the glass electrode. Better resolution was achieved with this approach due to the thickness and superior optical properties of glass compared to PDMS (Figure 68).
Electrodes were tested before permanently bonding with the fabricated PDMS channels to ensure that an electrochemical signal could be measured. Figure 69 shows the general response of the fabricated devices to ferrocene dimethanol and then the response of the device to both ferrocene dimethanol and pyocyanin. Figure 70 shows the responses from electrodes that were not working properly. A strong response at positive voltages correlating to ferrocene dimethanol was observed for fabricated electrodes indicating that the constructed system was functioning. The lowered response when pyocyanin was added in Figure 69B is due to the dilution of ferrocene dimethanol at the electrode surface. Importantly it is possible to discern the presence of pyocyanin in a background of ferrocene dimethanol. The ferrocene family is often used to create a reliable reference for systems employing quasi reference electrodes. The reported results indicate that if the
reliability of the system is in question then ferrocene dimethanol could be added to the system to monitor electrode drift. Finally as is clear from Figure 70A & B it is possible to discern whether or not the fabricated devices are functioning. The reduced electrochemical response and lack of a peak when pyocyanin was added indicate that something is wrong with this fabricated device. This allows for the selection of working devices prior to PDMS bonding saving time with future testing.

Fabricated nanofluidic electrode devices bonded with PDMS channels were filled with TSB. The parallel growth chambers were inspected visually to ensure that they were filled prior to bacteria loading. Overnight grown PA14 were loaded into the inlet of the main channel. Squarewave voltammograms were taken immediately after loading from -0.5 to 0.2 V (pyocyanin peak is normally observed in this voltage window) at a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV. Samples were incubated at 37 °C and removed periodically for measurements. After each electrochemical measurement the growth chamber housing the electrode was optically imaged at 40x magnification to relate cellular presence within the chamber to the observed electrochemical signal.

Figure 71 highlights the response from a nanofluidic assembly with PA14 cells growing within the microfluidic chamber. As can be seen there are a small number of cells growing within the chamber as expected compared to the main channel. This is a result of the nanofluidic PDMS channel connecting the two larger chambers. Of interest is the lack of any discernible peak detected in this solution at the applied voltages. The lack of any detectable peak indicates that the cells within the microfluidic chamber are not producing any pyocyanin or any redox active molecules for that matter. The large
number of *P. aeruginosa* present in the main chamber potentially indicates that the nanofluidic channel itself hinders the diffusion of molecules into and out of the chamber. This could not be confirmed at the time of testing, but previous tests with different microfabricated device geometries within microfluidic channels has shown that *P. aeruginosa* growth leads to a measureable signal, indicating that perhaps the nanochannels provide a large barrier to diffusion within the chambers. While the lack of measurable signal is troubling, the possibility that the nanochannels provide a strong barrier to electrochemical diffusion indicates that if any signal is detected at the electrodes, it will be from redox active molecules produced by cells within the growth chamber.

![Figure 71: A) Squarewave voltammogram of PA14 culture in growth chamber B) 20X optical micrograph of growth chamber with fabricated electrode. To the right is the main chamber containing the majority of the bacteria. Measurement taken approximately hours after inoculation.](image)

Larger access holes were etched over the microfabricated devices and the sacrificial chrome layer was removed in an effort to determine whether the nanofluidic channel over the fabricated electrodes was interfering with the measured signal. The larger access holes were etched into the silicon dioxide with HF etching to a depth of
approximately 500 nm. Devices were then plasma bonded irreversibly with PDMS microfluidic channels. Overnight grown PA14 was then spiked into the chambers and the electrochemical response measured.

Immediately after loading PA14 there was no measurable signal, as no *P. aeruginosa* had reached the growth chamber (Figure 72A & B). The sample was allowed to incubate for approximately 6 hours at 37 °C. Measurements after six hours of growth showed an electrochemical peak indicative of pyocyanin production (Figure 72C). Optical imaging showed that there were indeed cells growing within the chamber. However growth was not constricted to
Figure 72: A) Squarewave voltammogram (SWV) taken immediately after loading PA14 into the inlet of the growth chamber. B) 20x magnification image of nanofluidic electrode assembly (NEA) in growth chamber next to the main chamber. C) SWV after 6 hours of incubation at 37 °C. D) 20x magnification image of NEA with PA14 growth throughout the chamber. E) SWV after 6.5 hours of incubation at 37 °C. F) 20x magnification image of NEA with PA14 throughout the chamber.
Figure 73: A) Squarewave voltammogram (SWV) taken immediately after loading PA14 into the inlet of the growth chamber. B) 20x magnification image of nanofluidic electrode assembly (NEA) in growth chamber next to the main chamber. C) SWV after 23 hours of incubation at 37 °C. D) 20x magnification image of NEA with PA14 growth throughout the chamber. E) SWV after 25.7 hours of
incubation at 37 °C. F) 20x magnification image of NEA with PA14 throughout the chamber.

the chamber. Figure 72D shows that there was a significant amount of growth present not only in the chamber but also in the enlarged access hole itself. An electrochemical measurement taken 30 minutes later retained the electrochemical peak showing that pyocyanin was still present in the solution.

This trend did not hold for repeated tests. Figure 73 highlights another run with loaded PA14 in the growth chamber with a similarly etched device. Initially no electrochemical response was observed in the chamber. A few cells are present, but no single was initially measured. The cells were incubated at 37 °C for approximately 11.5 hours and while a few more cells appear within the image there is no change in the signal indicating that pyocyanin is being produced yet (Figure 72C & D). Finally after 15 hours more cells have grown in the chamber there is no significant growth of present.

The lack of growth and discernible peak is concerning as the aim of this study was to monitor the production of pyocyanin produced from small concentrations of *P. aeruginosa*. It has been hypothesized that phenazines and pyocyanin specifically are only produced under quorum sensing control schemes when a large enough number of bacteria are present in solution. Carnes *et al.* (2010) had challenged this assumption when they captured single *S. aureus* cells in 2 µL drops and showed the *S. aureus* were capable of sensing their own quorum sensing molecules. It should be noted that the volumes worked with in this study were approximately 100 times larger indicating that a 100 fold increase in the number of cells present would be necessary to begin approaching the same level of quorum sensing seen by others [4]. From Figure 73 it becomes clear that this requirement
was not met as the maximum number of cells present never got above 30 cells/200 μL. It is possible that a signal could be measured as the number of bacteria, within the chamber, approaches this reported threshold similar to the results in Figure 72. However in the present study this could not be ascertained.
6.0 Conclusions and future work

6.1 Goal 1: Utilize Miniature Electrochemical Sensors Combined with Microfluidic Channels to Monitor the Production of Pyocyanin in Biofilms.

Human samples, typically associated with bacterial infection, were spiked with increasing concentrations of pyocyanin using commercially available disposable screen printed electrodes. Samples were scanned with squarewave voltammetry from -0.5 to 0 V with a frequency, amplitude voltage, and incremental voltage of 15 Hz, 50 mV, and 4 mV respectively. Of interest was the lack of interfering redox active molecules in the range of voltages scanned.

Importantly low baseline currents allowed the sensitivity and limit of detection of pyocyanin in blood with sodium heparin, urine, sputum, and bronchial lavages to be determined. Indeed all human biofluids had a limit of detection at or below 1 µM indicating the potential to identify the presence of *P. aeruginosa* before dangerous levels of pyocyanin are present [127]. Additionally a pyocyanin signal could be measured after incubating *P. aeruginosa* in each biofluid for only one day at 37 °C. This, coupled with the fact no other bacteria normally associated with infection produced any detectable/interfering electrochemical molecules in this voltage window, highlights the prospective use of this approach to quickly scan patient samples to determine *P. aeruginosa* infection.

Future avenues of this research include using this approach to scan the above mentioned biofluids from patients suffering from bacterial infections, from *P. aeruginosa* or otherwise. The biofluids utilized in this section were pooled from 20 healthy patients, to ensure that any interfering redox molecules would be low. To flesh out this approach it is necessary to gather data from patients suffering from various stages of bacterial infection. One key question that this approach could help answer is does pyocyanin
concentration in the measured biofluid correlate to a patient showing signs of illness versus a patient that has be colonized by *P. aeruginosa*, but is otherwise a healthy individual. If a person is suffering from a *P. aeruginosa* infection then monitoring the presence of pyocyanin may prove to be an effective means of observing whether or not the applied antibiotic regiment is having an effect. Testing samples from different patients will also help to establish robust false positive and false negative data one of the pitfalls that the current approach is unable to address.

6.2 **Goal 2: Microfabricate a nanofluidic electrode assembly that contains the entire electrochemical cell within a microfluidic channel and be used for real time monitoring of pyocyanin production from PA strains.**

The virulent PA14 strain was exposed to varying concentrations of colistin sulphate after being allowed to grow and form a biofilm overnight in a microfluidic chamber coupled with a disposable screen printed electrochemical cell. SWV scans of the cells growing in the chamber showed increasing electrochemical response due to the production of PYO. Cells exposed to 16 and 100 mg/L colistin sulphate experienced a reduction of electrochemical signal by 68 and 82% respectively. Cells cultured on TSB plates after antibiotic exposure showed reduced growth at higher concentrations of colistin sulphate confirming a correlation between *P. aeruginosa* health and PYO production. The ability to electrochemically monitor the health of the cells in biofilms grown in microfluidic channels allows the possibility to cheaply and easily monitor the susceptibility of PA14 exposed to different antibiotics.

Future work for this approach is needed to address the inability of the sensors to determine the effect of applied antibiotics to bacterial cells not
producing any redox molecules. While the system worked well for monitoring *P. aeruginosa* cells *E. coli* cultures grown in the chamber showed little to no change in measured signal. While this was expected it highlights a major obstacle to the current approach. Specific coatings and antibodies for different bacteria of interest may solve the current limitations of this system and should be explored [162].

Another avenue of research that may prove fruitful is finding ways of decreasing the time required to produce an electrochemical signal from *P. aeruginosa* biofilms grown within the microfluidic channels. Sismaet *et al.* have shown that it is possible to upregulate the production of pyocyanin in liquid cultures by using varying concentrations of amino acids [149]. The amino acids used upregulated the production of pyocyanin as well as biofilm formation [163, 164]. Additionally the use of low phosphate and iron containing media may help to improve the production of pyocyanin, leading to measurable signals earlier than seen in this section. Currently there are several commercially available agar based media that can be used for this purpose, and recreating it in liquid form may prove beneficial for this approach.

### 6.3 Goal 3: Utilize Disposable Electrodes Embedded in Growth Agar to Electrochemically Monitor the Growth of *P. aeruginosa*

Disposable screen printed electrochemical cells were embedded within an agar matrix to monitor the production of pyocyanin from the virulent PA14 strain. Diffusion of pyocyanin through various agar thicknesses was measured and compared to theoretical COMSOL simulations for molecular diffusion in a rectilinear coordinate system. Embedded agar plate electrodes exposed to PA14 loads of $10^2$-$10^8$ cells were found to exhibit a concentration dependent time to reach a measurable pyocyanin signal.
Furthermore the electrochemical time to detection was found to be reduced compared to optical observations. The reduced time to detection coupled with the ability to measure and report the presence of pyocyanin, and therefore *P. aeruginosa* presence, at a variety of different temperatures increases the usefulness of the proposed device.

The results of this goal provide some exciting new avenues of research to explore. First and foremost it opens the possibility of continuously monitoring patient samples using a simple electrochemical format that does not require the technician to visually observe the plate for a positive identification. Since *P. aeruginosa* is the only clinically relevant bacterium that is producing an electrochemical molecule in the voltage window scanned a signal generation is indicative of its presence within a patient’s sample [86, 90]. The positive identification of *P. aeruginosa* in an infected individual allows for the application of antibiotics specific to this bacterium instead of the current shotgun approach.

While admittedly the proposed approach is only a viable approach for detecting the presence of *P. aeruginosa*, there are a plethora of agar that could be used to achieve faster selective detection of bacterial species. For instance the popular MacConkey agar is currently used to distinguish between Gram-negative enteric bacteria from non-lactose fermenting bacteria. Crystal violet present in the media hinders the growth of Gram-positive bacteria, while bacteria capable of fermenting lactose turn red due to neutral red present in the agar. Neutral red becomes visible as the local pH changes. Literature suggest that neutral red can be electrochemically measured at -0.5 volts [165]. If the conversion of neutral red can be measured electrochemically faster than optical monitoring then an embedded electrochemical sensor could not only identify the presence
of Gram-negative bacteria, but could also be used to detect the presence of *P. aeruginosa* as it is unable to ferment lactose, meaning no peak at -0.5v would be measured, while presumably pyocyanin at more positive potentials could be measured.

Finally this approach could be used to complement current approaches to antibiotic susceptibility testing for *P. aeruginosa* samples [166]. The embedded electrode approach readily ameliorates itself to use with disk and gradient based diffusion antibiotic approaches as multiple electrodes can in theory be placed onto a single plate at different positions to monitor the production of pyocyanin [167, 168]. By coupling the embedded electrodes with the above mentioned antibiotic susceptibility tests a relationship between pyocyanin production and bacterial fitness could potentially be established.

### 6.4 Goal 4: Microfabricate a Nanofluidic Electrode Assembly that Contains a Miniaturized Reference Electrode and Working Electrode, to be used for Real Time Monitoring of Pyocyanin Production from *P. aeruginosa* Strains

A nanofluidic electrode assembly (NEA) was fabricated to detect the production of pyocyanin from *P. aeruginosa* growing in liquid culture media. The nanocavity contained both a gold working electrode and a palladium reference electrode. To complete the cell a platinum wire was dipped into the test solutions to act as a counter electrode. While the palladium reference electrode was novel, testing showed that it suffered from similar pitfalls to other miniaturized reference electrode materials when exposed to test solutions. Drift was a common problem with this system, potentially hindering its use in many practical applications. However the fabricated system was shown to be sensitive to changes in pH as well as capable of detecting low concentrations of pyocyanin produced from *P. aeruginosa* cells growing in culture media. The system was also able to monitor the production of pyocyanin or lack thereof from different
mutant strains of *P. aeruginosa*. Strains that were deficient in genes to produce pyocyanin showed lowered electrical signal compared to those with intact genomes in the range of applied voltages. The lower electrochemical response measured for these systems was determined to be due to other phenazine molecules in the metabolic pathway for pyocyanin.

While the use of palladium as a reference material did not turn out to provide a vast improvement over other metal options the fact that a pseudo-reference electrode could be inserted into nanofluidic cavity is promising that full three electrode cells can be microfabricated and used for disposable applications. Future research in this area could look at fabricating the entire three electrode cell within a nanofluidic cavity, potentially using the same metal material (i.e. all gold or all platinum electrodes) thus reducing the total number of fabrication steps normally required for nanofluidic electrode design. This approach could find applications in CMOS systems a well-known fabrication approach used by several researchers to produce arrays of working electrodes to monitor different biological samples p[169, 170]. By including multiple complete three electrode cells on a single chip it would be possible to not only monitor changes in pyocyanin gradients, as has been shown in agar cultures, but also perform parallel tests in microfluidic channels similar to optical methods employed currently [57, 136].

6.5 **Use Fabricated NEA to Detect the Production of Pyocyanin from PA Cells Trapped Within Detection Area.**

The final goal of this dissertation was to fabricate a complete three electrode cell within a nanofluidic cavity that could be coupled with a microfluidic platform to monitor pyocyanin production from single bacterial cells. Many approaches were implemented to
achieve this end goal. Both normally open and normally closed PDMS microfluidic valves were initially implemented as means of separating diluted cellular suspensions into single cell volumes. Unfortunately effectively controlling the valves proved to be challenging and was abandoned early on in this aim.

The author instead opted to utilize a size based PDMS separation platform pioneered by Nil Tandogan et al. (2014) that allowed for small numbers of bacterial cells to enter a detection chamber containing a microfabricated electrode [161]. Using this approach low numbers of \textit{P. aeruginosa} were collected and monitored over time. Results from devices with large access holes seemed to indicate that pyocyanin could be detected at earlier time points ($<6$ h) due to the smaller volume within the microfluidic chamber. However any relationship between pyocyanin production and cell number could not be ascertained.

Future work in this area should focus on reducing the size of the microfluidic chamber to reach similar volumes found in literature [4]. This will help to eliminate the possibility that the \textit{P. aeruginosa} cultured within the chambers was unable to sense its own quorum sensing molecules. If the growth chambers can be made smaller and a measureable electrochemical signal from \textit{P. aeruginosa} can be detected then it will be possible to use this system for single cell monitoring. Another hurdle to overcome will be the difficulty that comes from aligning smaller microfluidic devices with the fabricated electrodes.
7.0 References


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8.0 Appendices
The following appendices attempt to summarize all of the standard operating procedures used in this dissertation (Appendix A), common photoresist curves (Appendix B), standard three electrochemical cell operation (Appendix C), the effects of changing squarewave voltammetry parameters on current response (Appendix D), and the authors current curriculum vitae (Appendix E).
Appendix A
The following are standard operating procedures that were used in the fabrication of the electrodes used in part of this dissertation. They serve as a starting point for other Goluch Group members and/or other interested parties who want to replicate the fabrication steps performed in this dissertation.

**Standard operating procedure- wafer cleaning**

1. Gown up for the working with acids
   
   a. Put acid apron on first, then face shield, and finally acid gloves

2. Pour 2:1 sulfuric acid (96%)/hydrogen peroxide (30%) solution into piranha bath
   
   a. Prepare by adding hydrogen peroxide slowly to sulfuric acid not the other way around

3. Turn on piranha acid heater.

4. While piranha bath is heating up transfer wafers to wafer boat

5. When piranha bath reaches 90 °C the piranha acid is ready

6. Shake piranha acid cover slightly and carefully remove cover

7. Place wafer boat into piranha acid bath and start timer (10 minute etch)
   
   a. This etch removes organics and some metallic’s

8. Rinse hands with water after handling any acids solutions in the wet bench

9. While piranha acid etch is taking place ensure that the deionized (DI) water rinser has water in it

10. After 10 minutes remove wafer boat out and place into DI water rinser

11. Run DI water rinser (runs for 5 minutes until resistivity is greater than 1 MOhm*cm)

12. Carefully open the cover of the hydrogen fluoride (HF 49%) bath (50:1 dilution of DI water: HF)

13. Transfer wafer boat to HF bath for 15 seconds
   
   a. removes native oxides from silicon surfaces
14. Immediately remove from HF bath and place into DI water rinser (runs for 5 minutes or until resistivity is greater than 1 MOhm*cm)

15. Rinse gloves with DI water while wafers are being rinsed

16. Place wafer boat into 6:1:1 DI Water: hydrogen chloride (33-40%):hydrogen peroxide (30%) for 10 minutes at approximately 90 °C
   
   a. Removes trace metallic contaminants from previous steps

17. After 10 minutes remove wafer boat out and place into DI water rinser

18. Run DI water rinser (runs for 5 minutes until resistivity is greater than 1 MOhm*cm)

19. Remove wafer boat from DI water rinser and place into the Rinser Dryer

20. Press start
   
   a. System rinses wafer with DI water (2.5 minutes) and then dries under nitrogen (5 minutes)

21. Wafers in wafer boat are now ready for thermal oxidation

**Standard operating procedure- thermal oxidation**

1. Transfer clean wafers from wafer boat to glass wafer holders

2. Check the water bubbler in back chase
   
   a. Ensure that it is filled with deionized (DI) water 1 inch above the drawn line

3. Turn on bubbler power strip (in the back chase)

4. Turn on temperature controller (will heat the bubbler to 103.5 °C)

5. Hit main menu to set desired oxidation recipe

6. Press system options
7. Press sequence limit (default reads 1 and 6)

8. Press enter

9. Check the wet oxidation settings by pressing menu then system options

10. Press interval entry to choose the desired interval to change
    a. Interval 1 is the wafer boat loading stage
    b. Default times for interval 1 is 12 minutes to open the boat loader

11. Press enter when the desired interval has been set for each process step
    a. The default time for interval 2 is 12 minutes to close the boat loader
    b. Default time for interval 3 is 10 minutes, which brings the furnace up to temperature
    c. Default time for the interval 4 the oxidation step is 20 minutes
        (approximately grows 500 nm of Silicon Dioxide)
    d. Default time for interval 5 the temperature ramp down is 10 minutes
    e. Default time for interval 6 the boat unloading is 20 minutes

12. Once all process intervals have been set press start to begin wet oxidation procedure
    a. Total wet oxidation process time is 84 minutes

13. Place quartz boat with wafers into the boat loader between two white paddings
    a. Ensure that polished side is facing forward into the furnace

14. After interval two finishes turn on water bubble in back chase to begin wet oxidation

15. After 40 minutes remove quartz boat from boat loader

16. Allow quartz boat to cool for 15 minutes
17. Turn off power strip in back chase and turn off water bubbler

18. Inspect oxidized wafers using the nanometrics machine to measure silicon dioxide thickness

19. Wafers are now ready for metal patterning via photolithography

**Standard operating procedure- photoresist spinning, photo-alignment, and pattern development**

1. Prior to spinning of photoresist rinse wafer with acetone, then isopropanol, and then drying with nitrogen

2. Clean photomask with acetone, then isopropanol, and then drying with nitrogen

3. Place cleaned wafer onto Laurel Spinner in the photobay

4. Ensure that the wafer it is centered on the spinner chuck

5. Select desired spinning procedure from menu

   a. Standard procedure for metal deposition is to spin AZ2020 at 3000 rpm for 1 minute*

6. Once satisfied that the wafer is centered press vacuum (>20 mmHg)

7. Using a pipette drop desired photoresist onto the wafer (covering as much of the wafer as possible)

8. Press run to start spinning procedure

9. After the spinning procedure ends, press vacuum to release the wafer from the chuck

10. Place wafer into oven at 115 °C for 2 minutes to drive off any excess solvent (known as pre exposure bake)

11. While wafer is baking turn on nitrogen line to Quintel Aligner, turn on the UV power supply, and turn on power to the Quintel Aligner
12. Place photomask with silver side facing up onto the Quintel Aligner

13. Press mask vacuum to lock into place

14. Select exposure time

   a. Standard procedure for AZ2020 is a 7 second exposure time

15. Turn on monitor and microscope lights

16. Use microscope to focus on features on the photomask

17. Place wafer onto Quintel Aligner chuck and load into the aligner

18. Use the joystick to move wafer underneath the photo mask

19. Use the theta knob to change the angle of the wafer under the photomask

20. If satisfied with the alignment of the wafer press contact

21. Once wafer is in contact press expose to expose the wafer to UV light

22. Remove wafer from Quintel Aligner chuck

23. If negative photoresist was used perform a 2 minute post exposure bake at 115 °C (this helps crosslink exposed photoresist making it resistant to development/removal)

   a. Skip this step if positive photoresist is used

24. Fill beaker with desired developer

   a. Standard procedure for AZ2020 is a 45 second development in MIF 300 developer

25. Develop exposed wafer until patterns appear

26. Rinse wafer with DI water and then dry with nitrogen

27. Inspect a test feature using light microscope to determine if development is complete
a. If development has not finished wafer features will appear cloudy

b. It is important to inspect nonessential features as underdeveloped photoresist will be ruined after exposure to light microscope and further development will further damage the features

28. If test feature is developed rinse wafer with DI water and dry with nitrogen

a. It is crucial to thoroughly dry wafer under nitrogen otherwise excess water may impact metal deposition

29. Wafer is now ready for metal deposition

* See Appendix 8.2 for spin curves for various photoresists used in the Kostas Clean Room

**Standard operating procedure- electron beam deposition**

1. Turn off ion gauge

2. Go to back chase turn high vacuum control valve from neutral to close then return to neutral position

3. Repeat step 2, two more times to ensure that the high vacuum valve has closed

4. Unscrew bolts holding chamber door closed

5. Open nitrogen purge valve completely

6. Chamber door will swing slowly open when atmospheric pressure has been reached

7. Shut off nitrogen purge valve

8. Quickly place patterned wafers face down in wafer holder

   a. Electron Beam system can handle up to five 3 inch wafers
b. Allowing chamber to remain open for excess time increases the amount of time required to pump down due to additional water added to the system

9. Place desired metals for sputtering into the crucible

10. Close chamber door and tighten bolts until chamber lock is engaged

11. In the back chase turn on the rough pump

12. Open rough valve underneath electron beam chamber
   a. Use caution as the high voltage source is also under the chamber

13. Allow chamber to pump down to cross over pressure approximately $1.5 \times 10^{-1}$ Torr (takes about 2 minutes and 20 seconds)
   a. If the system takes longer to pump down stop the rough pump and contact clean room technicians

14. Once crossover pressure is reached open high vacuum by turning the high vacuum control valve from neutral to open and then back to neutral

15. Pressure should drop rapidly in the system and the ion gauge will turn on

16. Suitable pressure for electron beam deposition are usually reached within 1 hour of high vacuum pumping ($10^{-6}$ Torr)

17. Once suitable vacuum has been reached turn on cooling water
   a. System will not run otherwise

18. Turn on beam controller

19. Turn on source controller

20. Set rotation to 5

21. Turn on high voltage source to 8.7 kV
22. Rotate crucibles until desired metal is present (use flashlight to see into electron beam chamber)

23. Select desired metal from list of programs and set desired thickness in kA
   a. Make sure you know the conversion factor between kA and nm

24. Adjust the applied current to 10 mA

25. Slowly adjust emission current until metal begins to glow

26. Inspect metal for spitting, if spitting occurs decrease emission current and use a smaller increment

27. Once metal is glowing open the shutter

28. Metal will be deposited on rotating wafers until desired thickness is obtained

29. Immediately decrease emission current to 0 mA and shut off the high voltage

30. Allow metal to cool before proceeding

31. Repeat steps 21 thru 30 for as many metals as desired

32. Once final metal deposition has been performed allow chamber to cool for 10 minutes

33. Shut off cooling water

34. Repeat steps 1 thru 7 to vent the chamber

35. Remove wafers and metals from the chamber

36. Repeat steps 10 thru 14 to return the system back to high vacuum

37. Place wafer into acetone to lift off excess metal sitting on photoresist

38. Rinse wafer in acetone, isopropanol, and dry with nitrogen

39. Inspect patterned wafers with microscope to ensure that small features have been retained
40. Wafer is now ready for next patterning step

Important never vent the system with the high vacuum valve open. This will dump the cryo pump which then needs to be regenerated (Regen). The Regen process takes about a day so not only will you not be able to run the system, but you have no potentially ruined runs for others.

**Standard operating procedure- materials research corporation (MRC)**

1. Turn off CH CRYO GATE (1 click)
   a. Wait for the gate to close (approximately 1 minute)

2. Press GTG/MECH UP (at same time)

3. Press CH N2 Vent (1 click)
   a. Line starts black
   b. Line turns yellow
   c. Turns back to black

4. Press both Hoist Up Switches
   a. Use ruler to push both switches up at the same time
   b. Use one arm to push chamber, prevents the chamber from getting stuck

5. Load Samples

6. Press both Hoist Down Switches
   a. Use ruler to push both switches down at the same time
   b. Use one arm to push chamber, prevents the chamber from getting stuck

7. Turn on rough pump and open rough valve in back chase
8. Press Rough On (1 click)

9. Rough until $1.5 \times 10^{-1}$ Torr (about 60 seconds)

10. Press Rough Off (1 click)

11. GTG/MECH DOWN (at same time)

12. MECH UP only

13. When MECH UP light stops flashing it is safe to open the CRYO GATE (1 click)

14. Turn off rough pump in back chase

15. Wait for chamber to reach vacuum

16. Select desired program to deposit desired metals
   a. MRC can handle deposition of three different metals at a time
   b. Ask technician to change metal targets prior to pumping the system back down

17. Wait for process to complete

18. Repeat steps 1 thru 4

19. Unload samples

20. Repeat steps 6 thru 14

21. Place samples in acetone to liftoff excess metals*

22. After liftoff samples are ready for further processing

* Note samples deposited with metal using the MRC required longer periods of soaking in acetone to liftoff excess metal. This is due to the fact that in Plasma Sputtering the metal coats the entire surface of the wafer. Contrasted with electron beam deposition, which only covers the exposed portions of the wafer to metal, the
MRC process requires longer processing times; additionally certain metals cost more money when using the MRC versus the electron beam deposition system.

**Standard operating procedure- NEXX PECVD system for silicon dioxide deposition**

This recipe is performed at Harvard’s Clean Room.

1. Run burn in and pre-clean recipe to clean the CVD chamber
2. After cleaning the chamber vent the load lock to atmosphere
3. Place wafer onto chuck
4. Select SIO2HR program
5. Change deposition time to obtain appropriate thickness
   a. SIO2HR recipe deposits silicon dioxide at a rate of approximately 20.1 nm/min
   b. For 500 nm thick SiO$_2$ layer set time to 1500 seconds/25 minutes
6. Run SIO2HR recipe
7. Vent load lock to atmosphere
8. Remove sample
9. Repeat step 6 for remaining wafers
10. Pump down system to exchange pressure
11. Inspect thickness using nanospec at Kostas Clean Room Facility

**Standard operating procedure- microautomaton dicing saw**

1. Turn on power strip
2. Turn stop button to power on
3. Turn on city water
4. Turn on vacuum
5. Turn on compressed air

6. Turn on DI water (two full turns only)

7. Check blade
   a. If not cutting silicon wafers blade must be changed

8. Check screw holes for vacuum

9. Check water level in beaker next to the dicing saw, empty if full

10. Hit RESET (wait)

11. Hit SPINDLE (wait for beep)

12. Mount sample using blue film

13. Hit PROGRAM

14. Hit 1st INDEX (enter in mm the width of the cuts in first direction) Hit enter

15. Hit 2nd INDEX (enter in mm the width of the cuts in second direction) Hit enter

16. Hit HEIGHT (for Si the height is a constant of 0.175 mm) Hit enter

17. Hit THICKNESS (measure the total thickness of the sample and blue film) Hit enter

18. Hit ANGLE (90 degrees normally) Hit enter

19. Hit SPEED
   a. Si 3.0-5.0
   b. Glass (change blade) 1.5
   c. SiC (change blade) 2.5

20. Hit DIA (75 mm for 3 inch wafers) Hit Enter

21. Hit PROGRAM

22. Place CHUCK PLATE in center of the chuck
23. Hit CHUCK LOCK (check vacuum > 15 mmHg)
24. Hit CHUCK ZERO
25. Hit CHUCK UNLOCK
26. Center sample on chuck hit CHUCK LOCK (check vacuum is > 15 mmHg)
27. Using the Align and index buttons bring the microscope to the bottom flat side of the wafer. Use the theta and left/right buttons to align the sample. Hitting fast align will move the sample much faster
28. Make a practice cut away from key features
29. Using UP/DOWN buttons align with your patterns
30. Hit single cut to make first cut
31. Repeat steps 29 and 30 until first cuts are finished
32. Press Index and theta button to rotate wafer 90 degrees
33. Repeat steps 29 and 30 until second cuts are finished
34. When dicing is completed press STANDBY
35. Turn off DI water
36. Turn off compressed air
37. Turn off vacuum
38. Turn off city water
39. Hit stop button
40. Turn off power strip
41. Sample has been successfully diced

**Standard operating procedure- inductively coupled plasma (ICP)**

1. Log in to system
2. Click Vent system
3. When system reaches atmosphere carefully open ICP chamber
4. Remove ceramic plate by removing holding screws
5. Remove oxygen clean wafer and replace with sample
6. Replace ceramic plate by screwing it back into place
7. Carefully close ICP chamber
8. Click Pump Chamber (Turbo)
9. When system has reached the appropriate vacuum load desired recipe
   a. To etch silicon and silicon dioxide use TW-Sietch
10. Run loaded process
11. After process has run repeat steps 1 thru 3
12. If more samples need to be etched place new samples and repeat 5 thru 9
13. If done replace oxygen clean wafer
14. Repeat steps 5 thru 7
15. Load and run recipe O2CLEAN1
16. Leave system under vacuum
17. Log out of system

**Standard operating procedure- hydrofluoric (HF) acid etch**

1. Put own acid apron
2. Put on face shield
3. Remove 49% HF from acid cabinet in back chase
4. Put on acid gloves before opening HF bottle
5. Make 10:1 Deionized (DI) water: HF solution
a. No more than 50 mL

6. Using tweezers dip chip into HF solution

7. Immediately start timer for 10 second etch

8. Immediately move chip into DI water to quench HF reaction
   a. Important to not delay this process as excess HF will eat away/undercut SiO$_2$

9. Inspect etched chip using light microscope to ensure etching is complete

**Standard operating procedure- ANATECH O$_2$ PLASMA ASHER**

1. Turn on the oxygen cylinder in the back chase by opening the valve prior operating the ANATECH chamber

2. Turn on ANATECH

3. Turn on PUMP

4. Clean chamber by running the system for 3 minutes
   a. During this operation check that the chamber reaches the desired pressure (about 0.35 mTorr)
   b. Check that the O$_2$ plasma turns on

5. After chamber is cleaned set the system to run for the desired time period
   a. Most applications in the Goluch Group are less than 10 seconds

6. Wait for system to beep before opening the door after a run

7. Put system in standby after finishing runs

8. Wait for system to pump down

9. Switch back into neutral

10. Turn off PUMP
11. Turn off ANATECH

12. Close oxygen cylinder in the back chase

**Standard operating procedure- biological sample preparation for SEM**

1. Immerse sample in 2.5% glutaraldehyde in 0.1 M cacodylate
2. Leave sample in 4 °C for 2 hours
3. Wash 3 times in 0.1 M cacodylate to remove excess glutaraldehyde
4. Place sample into 30 volume % ETOH solution for 10 minutes
5. Place sample into 50 volume % ETOH solution for 10 minutes
6. Place sample into 75 volume % ETOH solution for 10 minutes
7. Place sample into 80 volume % ETOH solution for 10 minutes
8. Place sample into 95 volume % ETOH solution for 10 minutes
9. Place sample into 100 volume % ETOH solution for 10 minutes
10. Place sample into 100 volume % ETOH solution for 10 minutes
11. Place sample into 100 volume % ETOH solution for 10 minutes
12. Leave sample in 100 volume % ETOH until ready for drying

**Standard operating procedure- freeze point drying**

1. Check that the CO$_2$ tank has enough to perform drying run
2. Unscrew top to critical point dryer
3. Place sample into mesh boat and place into drying chamber
4. Fill chamber with 100 volume % ethanol (pure)
5. Rescrew top to critical point dryer
6. Cool chamber to below -10 °C
7. Fill chamber with liquid CO$_2$ until air bubble appears
8. Hold for 2 minutes

9. Purge system until the chamber is 2/3 full

10. Repeat 6 thru 9  8-10 more times
    a. If chamber temperature rise above -10 °C cool the chamber again

11. Turn on heater

12. Wait until the pressure rise to 1400 PSI

13. The heater will shut off

14. Open bleed valve so pressure drops at about 50 PSI/min

15. Unscrew top to critical point dryer

16. Sample is now dry and ready for metal deposition

**Standard operating procedure- HMDS drying**

1. Remove sample from 100 volume % ethanol (pure)

2. Place sample into pure HMDS solution for 10 minutes

3. Remove sample and place into fresh pure HMDS solution

4. Remove samples from HMDS solution and allow to air dry*

5. Drying can be increased by pulling slight vacuum over system

6. Once dried system is ready for metal deposition

* HMDS must evaporate otherwise metal deposition will not be possible due to outgassing that occurs during pump down

**Standard operating procedure- metal deposition for SEM imaging**

1. Place samples into Metal Sputtering system

2. Start rotation at setting 2

3. Ensure that samples don’t hit sensor
4. Pull vacuum on the system until 0.02 mbar is obtained
5. Flush the system for 5 seconds with argon gas
6. Press leak
7. Leak light will stay on for about 4 minutes
8. During this time the system pumps back down to 0.02 mbar
9. Repeat steps 5 thru 8 2 more times
10. Set desired thickness using controller
11. Reset sensor to 0 nm
12. Press terminator so t=1 appears
13. Press start
14. Plasma is created and the desired metal is deposited on the samples
   a. Usually <10 nm is enough for biological samples
15. Power off pump and system
16. Wait for the deposition chamber to vent to atmosphere
17. Samples are now ready for SEM imaging

**Standard operating procedure- operation of scanning electron microscope (SEM)**

1. Press AIR to vent loading chamber
2. Place sample onto rod in loading chamber
3. Close loading chamber and press EVAC
4. Once loading chamber has reached the exchange pressure press OPEN
5. When SEM chamber is open load sample on rod into the chamber
6. UNLOCK rod from the sample and pull the rod back out of the chamber
7. Press CLOSE
8. Sample is now ready for SEM imaging
9. Open SEM software
10. Click Home to bring sample under the SEM beam
11. Click stage and set the size and height of sample being imaged
12. Make sure EDX has been selected
13. Click setup column
14. Click normal for short focal length or high for long focal lengths
15. Choose desired focus mode for most applications high resolution (HR) is good enough
16. Select 8 which is usually good for most applications
17. Select condenser 1, 5.0 is usually good for most applications
18. Select focus depth, 2.0 allows you to have two surfaces in focus at the same time
19. Setup Imaging rates
20. TV1=4, TV2= 8, Fast1= 16, Fast2= 32 TV1 and Fast1 imaged at 32 frames, while TV2 and Fast2 imaged at 64 frames
21. Set accelerating voltage and emission current, 3.0 kV and 10 µA are usually good for most applications
22. Before turning the beam on flash the system if it hasn’t been flashed in the last 45 minutes. Note time of flashing
23. Turn beam on
24. Using control knobs focus on your sample under TV scan rates
25. Focus on sample under high magnification
26. Under high magnification adjust alignment and x/y stigmation to get better images

27. When satisfied with the image switch to slow scan rate
   a. Slower scan rate image capture yields better images

28. Capture image

29. Save image

30. When done with imaging turn off beam

31. Click EXH to return sample to exchange position

32. Press OPEN

33. Lock sample to rod and remove from SEM chamber

34. Press CLOSE

35. Once SEM chamber has closed press AIR to vent loading chamber

36. Remove sample from the rod

37. Close loading chamber and press EVAC

38. Save sample for later imaging if desired
The following is a list of the spin curves used for common photoresists utilized in this dissertation as well as other projects worked on in the Kostas Center Clean Room at Northeastern University. The interested reader should talk with the clean room technicians for any specific questions regarding choices of photoresist.

Sylgard 184 PDMS 10:1 spin curve
Az 4620 Spin Curve (purple squares)

Az 2020 Spin Curve
S1818 Spin Curve

950 PMMA 9 and 11 % Anisole
Appendix C
The following is a description of the basic three electrode cell used for electrochemical measurements in this dissertation.

**Benchtop three electrode cell used in the majority of electrochemical experiments.**

Typical bench scale three electrode system. The potentiostat (black box) controls the potentials applied to the working electrode which are measured versus the reference electrode. To prevent any current from passing through the reference electrode the counter electrode is used to complete the circuit. Supporting electrolyte prevents extensions of the electrical field from the electrodes so that the electrochemical molecule of interest only interacts at the surface.
Left: Application and principle of SWV measurements. Right Expected SWV result if an electrochemical molecule is present.

The above figure shows the principles of squarewave voltammetry (SWV). SWV is a voltammetric scanning technique that is employed in this dissertation to detection the presence of pyocyanin and by extension *P. aeruginosa*. This technique scans a range of potentials by incrementally changing the voltage. In addition to each voltage increment, an oscillating squarewave voltage is applied to incremental voltage. This means that after the incremental step the squarewave value is subtracted, then upon the next incremental step up the squarewave value is added to the step. This allow the current before and after the step to be measured. By subtracting the forward current from the reverse current the effects of charging that hinder other step voltammetry methods can be avoided. If an electrochemically active molecule is present in solution then its reduction or oxidation will appear as a peak in the scan. The height of the peak can be related to the concentration of electrochemical molecule present in solution via a calibration curve.
Appendix D
The following figures represent the changes in peak height and peak voltage when the SWV parameters of scanning frequency, amplitude voltage, and incremental voltage were varied while holding the other two parameters constant. While not exhaustive the results highlight why the parameters 15 Hz, 50 mV, and 4 mV were chosen for frequency, amplitude voltage, and incremental voltage respectively in all SWV experiments.

The derived relationship between peak current and squarewave parameters can be found below:

\[ I = nFAD^{1/2}f^{1/2}C\Psi \]  \hspace{1cm} (A1)

Where \( I \) is the current, \( n \) is the number of electrons transferred per molecule, \( F \) is the faraday constant, \( A \) is the electrode area, \( D \) is the diffusion coefficient of the molecule, \( f \) is the scanning frequency, \( C \) is the concentration, and \( \Psi \) is the dimensionless squarewave peak current which depends on the applied amplitude voltage and incremental voltage.\textsuperscript{171,172}
and incremental voltages held at 50 and 4 mV respectively.

The above figure shows that as the scanning frequency increases, the resulting peak current increases. While higher scanning frequencies lead to higher currents they do so at the expense of increased background noise. Additionally lower scanning frequencies resulted in a broadened peak, which could potentially hinder the detection of pyocyanin at low concentrations. As such a scanning frequency of 15 Hz was chosen for measurements taken in this dissertation.

Changing amplitude voltage applied to Zensor to detect pyocyanin. Frequency and incremental voltage held at 15 Hz and 4 mV respectively.

Changes in amplitude voltage had differing effects on the measured pyocyanin response. At low amplitude voltage, a subdued peak was measured, whereas at higher voltages a larger current was measured. From the standpoint of measured peak current there appears to be little difference between an applied squarewave voltage of 50 and 100 mV. However, higher amplitude voltages lead to a broader peak. This could potentially
obscure peaks caused by other redox molecules present. As such an amplitude voltage of 50 mv was chosen for measurements taken in this dissertation.

![Graph showing the effect of incremental voltage on the measured response of pyocyanin. Amplitude voltage and frequency held at 50 mV and 15 Hz respectively.](image)

Changing the incremental voltage’s effect on the measured response of pyocyanin. Amplitude voltage and frequency held at 50 mV and 15 Hz respectively.

When the incremental voltage was changed to larger values a broader peak with rougher features was obtained. Since the incremental voltage controls how many measurements are taken during a scan at higher incremental voltages the measured response would be expected to get rougher, potentially obscuring results. Low incremental voltages have been suggested for better data acquisition.\textsuperscript{172} As such an incremental voltage of 4 mV was chosen for the measurements performed in this dissertation.
Appendix E
Thaddaeus A. Webster

50 Brookside Dr L5 Exeter, NH 03833  (207)-641-5811  thaddaeus.webster@gmail.com

PhD CHEMICAL ENGINEERING CANDIDATE

KEY SKILLS AND EXPERIENCE:

Analytical: Spectrophotometry, High Performance Liquid Chromatography, Enzyme-linked Immunosorbent Assay, Squarewave Voltammetry, Cyclic Voltammetry, Amperometry, X-ray Diffraction, Thermogravimetric Analysis, Viscometry, Refractometry, Gas Chromatography, Surface Plasmon Resonance Imaging


Computer Programs: Statistical Analysis System, COMSOL, Microsoft Office, MATLAB

Equipment Operations: Tangential Flow Filtration, Normal Flow Filtration, Lyophilization, Plasma Enhanced Chemical Vapor Deposition, Electron Beam Deposition, Photolithography, Microfabrication, Distillation, Reverse Osmosis, Class 10,1000, and 10000 Clean Room Usage

EMPLOYMENT HISTORY

Research Assistant/Lab Safety Officer, Northeastern University  Fall 2010-Present
Goluch Group, Chemical Engineering Dept.
Boston, Massachusetts
• Lead the design, fabrication, and characterization of biosensors for bacterial toxins
• Prepared media and maintained bacterial cells for testing
• Operated machinery required to produce microfabricated biosensors
• Managed and trained undergraduates in lab safety and experimental techniques
• Lead author on three peer-reviewed journal articles and presented results at national conferences
• Operation in Class10 to 10000 clean rooms
• Taught undergraduate students the use/theory of a variety of different unit operations

Intern, Biogen Idec  Summer 2009 & Summer 2010
Protein Pharmaceutical Development
Cambridge, Massachusetts
• Designed and successfully executed experiments to determine the effect of needle gauge & viscosity on syringeability measurements of protein solutions
• Concentrated and measured bulk drug product via normal flow filtration and refractive index respectively
• Collaborated with multiple departments to achieve target goals
• Performed mixing studies & product compatibility testing with disposable technologies
• Authored protocols and reports detailing the implications of the experimental work on future formulations of drug product
• Involved in failure mode and effects analysis

Lab Researcher, University of New Hampshire  Fall 2007 - Fall 2009
Biodiesel Program, Chemical Engineering Dept.
Durham, New Hampshire
• Prepared media, monitored cultured algae growth, and extracted lipids for biodiesel production
• Tested design of new disposable biodiesel reactor on algae growth
Intern, Environmental Protection Agency
Summer 2007
Pollution Prevention Program
Southern New Hampshire & Maine
- Performed energy audits and reported findings on buildings of communities participating in EPAs Community Energy Challenge

EDUCATION HISTORY
Northeastern University
Doctorate of Philosophy in Chemical Engineering, GPA 3.94
Boston, Massachusetts
Expected January 2015

University of New Hampshire
Bachelors of Science in Chemical Engineering, GPA 3.88
Durham, New Hampshire
Completed May 2010

SELECTED PUBLICATIONS
  Miniaturized and integrated components for electrochemical detection in micro- and nano-fluidic devices are of great interest as they directly yield an electrical signal and promise sensitive, label-free, real-time detection. One of the challenges facing electrochemical sensing is the lack of reliable reference electrode options. This paper describes the fabrication and characterization of a microscale palladium hydride reference electrode in a single microfabrication step. The reference electrode was integrated inside of a nanoscale constriction along with a gold working electrode to create a complete electrochemical sensor. After charging the palladium electrode with hydrogen, the device was used to detect pyocyanin concentrations from 1-100 μM, with a 0.597 micromolar detection limit. This is the first time that a palladium hydride reference electrode has been integrated with a microfabricated electrochemical sensor in a nanofluidic setup. The device was then used over the course of 8 days to measure pyocyanin produced by four different Pseudomonas aeruginosa strains in growth media. By utilizing square wave and differential pulse voltammetry, the redox active molecule, pyocyanin, was selectively detected in a complex solution without the use of any electrode surface modification.

  Surface Plasmon Resonance imaging (SPRi) was used for real-time detection of bacterial growth inside microfluidic channels. To do this, *Escherichia coli* was loaded into polydimethylsiloxane (PDMS), and the PDMS was placed into SPRi for imaging. The sample was also observed under stereo microscope. Both images showed bacterial growth at the same spot of the prism. The same procedure was also repeated with 50 micron beads directly loaded on the prism surface and both SPRi and microscope detected the beads at the same spot. Thus, we can conclude that bacterial growth can be directly monitored in real-time by SPRi.

  Microalgae lipids/oils are a promising feedstock for biodiesel production. The desired lipids are triacylglycerols. These can either be transesterified to biodiesel or decarboxylated to “green diesel”. Increasing microalgae lipids production by thermal stressing is important in improving the economics
of biodiesel production, but its effectiveness needs to be determined. This paper focuses on the effect of cooling stressing lipid triggering on the microalgae production and lipids yield. Two microalgae species were studied, Chlorella sp. and Dunaliella. In each case, microalgae were grown in two identical 2 L PBRs (photobioreactors) at room temperature. At the end of the exponential growth phase, one PBR was placed in a cold environment while the other PBR was left at room temperature. Microalgae was harvested, freeze dried and the algae oil was extracted. Measurements show that cooling stressing slightly increased the biomass of algae (11% for C2 and 13% for Dunaliella), but it decreased the lipids content of the microalgae, 62% for Chlorella sp. and 13% for Dunaliella. The net effect is a decrease in the lipid production rate (mg lipid/L-day) 58% in case of Chlorella sp., and 2% in case of Dunaliella.


Microfabricated nanofluidic electrode assemblies (NEAs) with integrated palladium references were used to amperometrically monitor changes in pyocyanin concentration. Pyocyanin is an electro-active molecule that is produced by the opportunist pathogen *Pseudomonas aeruginosa* and is directly linked to cellular processes that increase both robustness and virulence in this bacterium. This is the first time that pyocyanin has been measured in real time using microfabricated sensors. A linear response in faradaic current ($R^2 = 0.96$) was observed over a biomedically relevant range of pyocyanin concentrations (0-100 µM) while continuously measuring the current for 2 hours. Measurement of the current that results from the repeated oxidation and reduction of pyocyanin at two closely spaced electrodes inside the device nanochannel yielded a 1.07 µM limit of detection without electrical isolation of the electrochemical cell. Since a reference electrode is integrated inside the nanofluidic channel of these sensors, they can potentially be employed to detect pyocyanin and other redox-active molecules in wide range of medical and environmental settings where space is limited. NEAs were also used with an external Ag/AgCl reference electrode to determine the concentration of pyocyanin in trypticase soy broth samples. This type of analysis is completed in less than two minutes and the detection limit was determined to be 441 nM.


The ability to quickly detect the presence of pathogenic bacteria in patient samples is of the outmost importance to expedient patient care. Here we report the direct, selective, and sensitive detection of the opportunist pathogen *Pseudomonas aeruginosa*, spiked in human whole blood with sodium heparin, urine, sputum, and bronchial lavage samples using unmodified, disposable carbon electrode sensors that detect the presence of pyocyanin, a virulence factor that is unique to this species. Square wave voltammetry scans of biological fluids from healthy individuals spiked with P. aeruginosa showed a clear pyocyanin response within one day of culturing at 37°C. Scans of supernatants taken from cultures of P. aeruginosa, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Bacillus cereus* taken over a span of three days in the potential range from -0.5 to 0 V vs. an Ag/AgCl reference showed no electrochemically detectable molecules with the exception of P. aeruginosa. The results indicate the potential to sensitively and selectively determine the presence of P. aeruginosa in human samples via the electrochemical detection of pyocyanin in less than 5 min, without any sample preparation or separation steps.


This work focuses on developing a faster method for electrochemically detecting a *Pseudomonas aeruginosa* infection through the addition of amino acids to cell culture samples. We performed square-wave voltammetry measurements of pyocyanin produced by P. aeruginosa using commercially available carbon-based electrodes connected to a Ag/AgCl reference. The electrochemical response
resulting from the production of pyocyanin by bacteria was measured in the presence of various amino acids while varying three different culturing parameters: liquid media type (trypticase soy broth vs. M63 minimal media); concentration of amino acids in the solution; and initial concentration of the P. aeruginosa in the solution. Our results demonstrate a faster and stronger electrochemical response in media containing tyrosine and valine at elevated concentrations, lending promise to using amino acids as up-regulatory molecules for faster bacterial detection.

  **SUBMITTED**

Described is the fabrication of a disposable electrochemical assay that is integrated with standard King’s A agar culture plates, for the selective and specific detection of *Pseudomonas aeruginosa*. Cultures of *P. aeruginosa* start from initial cell counts of 10^2-10^8 cells were incubated at 23, 37, and 42 °C and monitored both visually and electrochemically. Squarewave voltammetry scans confirmed the production of a redox species, pyocyanin, over time that was dependent on the initial load of cells. The pyocyanin easily diffuses through the agar to reach the electrode surface. Using this simple and cheap approach, positive identification of *P. aeruginosa* was achieved several hours faster via electrochemical detection compared to visual observation.

**PRESENTATIONS**

**Oral Presentations:**

- Bristol-Myers Squibb Job Interview October 2014
- Toward Electrochemical Screening of Pseudomonas Aeruginosa Antibiotic Susceptibility November 5th, AIChE 2013 Annual Meeting
- Electrochemically Detecting *P. aeruginosa* Toxins in Relevant Biomedical Samples October 2013 Chemical Engineering Seminar Northeastern University
- Electrochemically Detecting *P. aeruginosa* Toxins in Relevant Biomedical Samples September 2012 Chemical Engineering Seminar Northeastern University
- Electrochemically Monitoring Single Cells with Nanofluidic Sensors September 2011 Chemical Engineering Seminar Northeastern University

**Poster Presentations:**

- SCIX 2014 Reno, NV
- NEBEC 2014 Boston, MA
- PITTCON 2014 Chicago, IL
- BMES 2013 Seattle, WA
- BMES 2011 Hartford, CT
- Gordon Research Conference 2011 Waterville Valley, NH